Maize For Biological Research

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BURNHAM BEADLE RHOADES EMERSON McCLINTOCK

ITHACA 1929

This Collection of Articles on Maize is dedicated to

the memory of

R. A. Emerson

and to his four co-workers

George W. Beadle
Charles R. Burnham
Barbara McClintock
Marcus M. Rhoades

Who still share with us the enthusiasm
persistence, and generosity that he exemplified

ROLLINS ADAMS EMERSON

1873-1947

Rollins Adams Emerson, American Geneticist, son of Charles David and Mary Adams Emerson, was born at Pillar Point, New York, May 5, 1873 and died at Ithaca, New York on December 8, 1947. The results of his studies are published in some seventy odd papers—papers that are a permanent record of an active and productive scientific life.

At the age of seven Emerson was taken by his parents to a prairie farm in Kearney County, Nebraska where he grew up and went to grade and high school. He subsequently attended the College of Agriculture of the University of Nebraska at Lincoln and there received his B.Sc. degree in 1897. After spending two years as Assistant Editor of Horticulture of the Office of Experiment Stations of the United States Department of Agriculture in Washington, D.C., Emerson returned to Lincoln Nebraska as Assistant Professor of Horticulture in the College of Agriculture and as Assistant Horticulturist in the Experiment Station. He spent the academic year 1910-11 in graduate work at Harvard University with Professor Edward M. East. Harvard awarded him the D.Sc. degree in 1913. In 1914 he left his professorship at Nebraska and went to Cornell university as head of

the Department of Plant Breeding of the College of Agriculture, a position he held until his retirement in 1942.

EMERSON was married in 1898 to HARRIET HARDIN, a sympathetic and understanding woman who helped greatly in creating a happy home in which four children, THERA, STERLING, EUGENE and MYRA were born and reared. Visits to the EMERSON home were not soon forgotten by the many students, associates and visitors who were privileged to make them.

EMERSON began his scientific career as a horticulturist but his interests soon shifted in the direction of genetics. He was one of America's outstanding pioneers in developing the new field in the years immediately following the rediscovery of MENDEL'S paper. His early work was on garden beans. In growing Indian corn for class material to illustrate Mendelian segregation he ran into a distorted ratio of starchy to sugary endosperm in a cross between white rice popcorn and a variety of sweet corn. His curiosity as to the basis of this unexpected result was undoubtedly influential in his replacing beans with corn as experimental material. In any event he and corn became inseparable.

The contributions of EMERSON to genetics were many and came at a time when the supports for the new science were weak, and the doubters many. His persistence and objectivity were great and he never published until he had extracted the truth from his experimental material and verified it not once but many times in many ways. Predecessors had studied the inheritance of plant and aleurone colors in corn and had been distracted by incidental modifying factors and apparent inconsistencies with Mendelian principles to the point where some of them actually publicly renounced these principles. It was EMERSON'S persistence, clear thinking and hard-headed checking of facts which found the truth and established beyond any doubt that these apparently complex systems of inheritance have in reality a simple genetic basis. His papers on aleurone and plant color inheritance in maize are outstanding for sound experimental work, straight reasoning and clear presentation; they stand as inspiring models to all future generations of students. His investigations of the inheritance of quantitative characters, carried on partly in collaboration with PROFESSOR EAST had an important influence on the thinking of geneticists. His studies on variegated pericarp led to the concept of unstable genes and represent another significant point in the history of genetics.

But important as were his direct scientific contributions, it is EMERSON the man who will be most vividly remembered by the many persons who knew him well. He was physically strong, well built and over six feet tall. He was cordial and warm in his relations with others. The contagious enthusiasm and zest so clearly displayed in his scientific work were extended to other activities. He hunted and bowled with vigor and evident enjoyment. During the corn season he prided himself on being the first to the garden and the last to leave, an example that without doubt increased the productiveness of all the students and research fellows who worked with him. It was the accepted practice to work from dawn to dark seven days a week during the pollinating season. Lunches in the garden house during these periods of intense activity were events of great influence on students. It was on such occasions that the unprinted lore of corn genetics was transmitted and EMERSON became best known to those who worked with him. It was through such informal contacts that EMERSON did his teaching. He gave no formal courses for many years, but he was nevertheless a really great and inspiring teacher—one who taught by example and friendly encouragement, not by lecturing. He was always available to students but he never directed their studies. They were treated as mature individuals and as equals.

EMERSON'S generosity is legendary. All his research materials were freely available to his own students and to other investigators the world over. Often he suggested thesis problems to students on which he himself had worked. But once such a problem was turned over to a student, it was then the student's respon-

sibility. If a paper were published, it was the student's paper, not an EMERSON and DOE or even a DOE and EMERSON paper. This generosity played a very important part in making corn the best known of all plants from a genetic standpoint. It had the effect of interesting many people in many places in the material and in greatly speeding up progress. EMERSON derived as much pleasure from a good piece of research done by a student or a colleague as from one completed through his own efforts. This spirit of unselfishness combined with enthusiasm, fairness and a high order of ability naturally soon made him the intellectual and spiritual leader of the corn group—a group that grew and spread to include workers of many institutions and many countries who were not EMERSON students in the technical sense.

With the growth of the corn group the system of communicating unpublished information through conversation became inadequate. During 1932 at the Genetics Congress at Ithaca a "corn meeting" was held where it was decided that a central clearing house of information and seed stocks would be established at CORNELL. Out of this there evolved a series of mimeographed "corn news letters" edited by MARCUS RHOADES and sent to all interested corn geneticists. Later this became the "Maize Genetics Cooperation," a somewhat more formal organization for the dissemination of unpublished information and for maintaining seed stocks. At least to a certain extent the early corn news letters inspired the establishment by BRIDGES and DEMEREC of the analogous *Drosophila Information Service*, the first issue of which appeared in 1934.

During his life EMERSON received many honors. He was awarded the LL.D. degree by the University of Nebraska. He was a member of the National Academy of Sciences, the American Philosophical Society, and numerous other scientific societies. He served as President of the American Society of Naturalists in 1923 and as President of the Genetics Society of America in 1933. He was Dean of the Graduate School of CORNELL UNIVERSITY from 1925 to 1931. He was a member of the original editorial board of GENETICS at the time of its establishment in 1916 and served on the board until his death. Recognition and honors did not change EMERSON; he continued to the end to lead a simple, unpretentious life.

ROLLINS ADAMS EMERSON earned and had the admiration and respect of all those who came in contact with him. His own life was an effective, full and happy one and he enriched the lives of many others.

G. W. BEADLE
California Institute of Technology

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PHOTO CREDITS

FRONT:

Top Left:	Immature ear segregating for vp1, viviparous kernels are white with germinating embryos. W.F. Sheridan
Top Center:	Tassel of mature plant bilaterally sectored with ramosa (ra) phenotype on the right side. M.M. Johri
Top Right:	Plant regenerated from tissue culture. C.A. Rhodes
Bottom Left:	Seedling heterozygous for wd with sectors of white tissue resulting from loss of normal allele exposing wd (white deficiency) E.H. Coe, Jr.
Bottom Center:	Embryos with prominent scutellar-like structures in regenerable maize cultures. W.F. Sheridan
Bottom Right:	Crossing banding of mutant seedling leaves in response to diurnal temperature fluctuation. M.G. Neuffer

BACK:

Top Left:	Mature ear infected with Diplodia maydis with vivipary exhibited by normal kernels in the area of infection. M.G. Neuffer
Top Center:	Cultured anthers with young embryo on N6 medium containing 6% sucrose. W.F. Sheridan
Top Right:	Same embryo as in adjacent photograph but 48 hours after transfer to medium containg 2% sucrose. W.F. Sheridan
Mid Left:	Mature leaf with lesions produced by the dominant mutant (Les-1). M.G. Neuffer
Mid Center:	DNA sequencing gel analyzing zein genomic subclones. N-T. Hu
Mid Right:	Mature ear showing segregation for anthocyanin factors. M.G. Neuffer
Bottom Left:	Expression of genes for plant color factors, plant on left contains B, Pl, plant on right is b b. E.H. Coe, Jr.
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PREFACE

In January of 1981 while I was visiting the University of Missouri E. H. Coe, Jr. showed me a copy of the Newsletter published by the Plant Molecular Biology Association (PMBA). In it, the editor-in-chief, Maureen Hanson described the goals of the PMBA and noted that issues of the PMB Newsletter had already appeared which were devoted to the individual plant species, petunia and tomato. She stated that additional special issues devoted to other plants would be desirable and invited inquiries in that regard. I discussed with Ed Coe the idea of a special issue of the PMB Newsletter devoted to maize and he suggested that maybe I should contact Maureen Hanson. During February I wrote to her and stated my interest in such an issue. Three days later I received a telephone call from Maureen Hanson and was asked to serve as a special editor for an issue on maize.

The idea of a collection of articles which would bring together information on all aspects of maize, especially genetic and cytogenetic resources, so that it might be shared and more readily accessible to individuals in other areas of research, was an appealing notion. From the first conversation on this topic with Ed Coe I had expressed my hope that such a collection of articles would be another expression of the cooperative nature and spirit that characterizes the maize genetics community.

During the Maize Genetics Conference at Allerton Park, Illinois in March of 1981 the participants were surveyed for suggestions on topics and contributors of articles as well as volunteers for such contributions. In addition, since a major group of biologists to be served by the special newsletter issue on maize was the membership of the Plant Molecular Biology Association, a mail survey was conducted of the members of the Association. The response to the survey at Allerton and by mail was both encouraging and sobering. It was apparent that the spirit of cooperation and sharing was alive and well and that there was much interest in maize as a potential experimental organism. But it was also apparent that, if all of the suggested articles and volunteered articles were to be included, it would be a very large issue of the PMB Newletter.

Nevertheless an original outline was compiled and the authors were requested to prepare and submit their articles by mid-summer, later the target date was extended to early fall. Although I was anxious to receive the articles prior to going off on developmental leave at the University of Minnesota in St. Paul in mid-August only a few articles arrived prior to that time and the remainder arrived in a slow but steady pattern over the next six months so that by the next Allerton Conference in mid-March of this year, essentially all 63 of the articles comprising this collection had been received. I am grateful to the authors who contributed their articles early thereby making possible the completion of the editorial work in a timely fashion, and I am also grateful to those authors who submitted their articles later, including those whom I had given up on and concluded that no article would be forthcoming.

During June 1981, while attending the Gordon Research Conference on Plant Tissue Culture, I discussed the dimensions of the special Newsletter issue with Maureen Hanson. We concluded that, if the final publication was to be anything of the magnitude of the planned collection, it should be published as a special PMBA publication rather than as a Newsletter issue.

This seemed desirable both because the publication would be far too long for a Newsletter issue and also because articles in Newsletters are generally not subject to citation without permission of the author and we wished to avoid that limitation on citation.

Care has been taken in publishing this collection of articles. However, the editor will be grateful to the readers if they will notify him of any errors. In addition, he will appreciate any suggestions which may improve the accuracy and usefulness of future editions of this publication.

I wish to acknowledge the support and patience of Maureen Hanson who has been constant in her faith in this project since its inception. I greatly appreciate the contributions of the maize geneticists and molecular biologists whose suggestions shaped this collection and whose contributions of articles comprise it; I am also grateful for their patience while waiting for it to be published.

I am especially grateful to Susan M. Howard for her careful attention to detail in the typing work and other editorial activities during much of the period of article preparation and to Suzanne M. Poe for assistance in manuscript preparation during the latter part of this period. The responsible and cheerful efforts of Diane L. Johnson in producing the final camera ready copy at the UND Word Processing Center is acknowledged with much appreciation. I thank Ken Ness and his co-workers at the UND Press for their careful attention to details during the printing of this publication. I am also grateful to Janice K. Clark and Yvonne R. Potts, who have helped in numerous ways in maintaining production activities in Grand Forks while I was in St. Paul. I thank Ed Green and the other members of the Department of Agronomy and Plant Genetics at the University of Minnesota for their hospitality and assistance during this academic year.

It is appropriate in this context to express my gratitude to two of my friends and colleagues in maize genetics at the University of Missouri, Ed Coe and Jerry Neuffer. In their generosity and by their example they have provided me with support and encouragement during the seven years since I left Columbia, Missouri and joined the University of North Dakota. I also thank Jerry Neuffer for suggesting the title for this collection of articles. I thank my colleagues here in the Biology Department and the other members of the University community who have made me feel welcomed and who have supported my research activities.

In addition I thank my sons Michael and Kevin for their encouragement. I also acknowledge my mother who in her seventy-sixth year still provides an example of how to live with enthusiasm, generosity and courage; and I acknowledge the memory of my father, who still inspires me with his warmth, his love and his kindness.

William F. Sheridan
Grand Forks, North Dakota
June 1982

1 INTRODUCTION TO MAIZE FOR BIOLOGICAL RESEARCH

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Maize is the flowering plant best suited for biological research, including molecular studies, because of its favorable biological features, its economic importance and the resulting attention focused upon it, as well as the considerable amount of genetic and cytogenetic information currently available.

There is much variation available among the many strains of maize, and it can be grown in many locales and under a wide range of conditions. It bears its male and female flowers on separate structures which facilitates controlled pollinations, and it produces a large ear with 500 or more individual kernels--each containing a prominent endosperm as well as a large embryo.

Maize is the most important crop plant grown in the United States; after wheat and rice, it is the third most important crop plant in the world. It has long been a favorite subject of investigation by plant physiologists and biochemists. Consequently, there is a large amount of information available on the response to plant growth regulators, photosynthesis, nutrient transport, enzymology, and cellular organelles of maize as well as other topics. Although these areas are generally not covered by the articles in this collection, the extensive amount of data available on these topics add considerably to the value of maize as an experimental organism.

Since the origin of maize genetics early in this century, there has been a steady accumulation of data, so that today maize is the most extensively characterized flowering plant from a genetic and cytogenetic point of view. Recently, considerable attention has been addressed to the molecular analysis of the maize genome using restriction endonuclease, cloning, and sequencing techniques. These studies, although at an early stage of development, promise to extend the present understanding of the structure and function of the genome to an even more detailed and molecular level.

This collection of articles was developed with the goal of bringing together the current state of knowledge on several aspects of maize and to make this information available to all interested individuals. But, it was especially developed for those persons seeking a flowering plant that is best suited for molecular biology studies. The collection is organized to provide an introduction to maize as an organism, to describe how to grow and handle it for genetic analysis, and then to consider several areas of current knowledge and methods of utilization for future research.

The Plant, Its Cultivation and Genetic Categories

R. S. Poethig introduces the maize plant as an organism composed of various parts. With the aid of figures, he describes and names the parts and provides a working vocabulary that is of value in reading the other

articles in the collection. This is followed by M. G. Neuffer's paper describing how to grow maize, how to pollinate it, store it, and maintain genetic records (pedigrees). This article also includes a list of sources of the various supplies needed for growing maize for genetic (or other) purposes. This third article in this section is by S. A. Modena and coauthors. It describes the different genetic categories of maize (varieties, hybrids, etc.) and describes several factors that should be considered when one is selecting experimental material.

Genetics and Cytogenetics

This section provides a summary of the present extent of genetic and cytogenetic data and a description of procedures on how these data are obtained and can be used. The first article by W. F. Sheridan briefly describes the role of the group of investigators at Cornell, led by R. A. Emerson, in developing the early linkage maps. These are reprinted in the article as well as the most recently published map. A reference to an up-dated, soon-to-be-published map, and a cytological map are included. In addition, it contains a photograph of pachytene stage maize chromosomes and a listing of the gene marker stocks that are currently available, as well as a list of recommended stocks for use in mapping work.

This is followed by an article by M. M. Goodman, L. W. Stuber, and K. J. Newton, which is a summary of the various isozyme loci, the number of allelic forms, and certain properties of their products. It also includes figures illustrating the linkage groups of isozyme loci on chromosomes 1, 3, 5, and 6. This is followed by an article on mutant induction in maize by M. G. Neuffer. Protocols for the use of ultraviolet light treatment of pollen, X-ray treatment of kernels and pollen, and ethyl methane sulfonate treatment of pollen are included.

Next come several papers describing procedures for locating genes to chromosomes. These include a paper by C. R. Burnham describing the locating of genes to chromosomes by the use of interchange (translocation) stocks (with either the linked endosperm marker method or the all arms marker system) as well as by the use of trisomics. Next is a description of the use of BA translocations to locate genes to chromosomes by J. B. Beckett and then an article by J. A. Birchler describing the use of compound BA translocations to localize genes within a chromosome arm once they have been located to a particular arm.

This is followed by D. F. Weber's article describing the use of monosomics to locate genes to specific chromosomes. This procedure depends on the use of the r-X1 deficiency, which includes the R locus on chromosome 10, to generate monosomics at a high frequency. The monosomics are identified readily by using Mangelsdorf's tester, which carries a recessive marker on each of the ten chromosomes, as the male parent to cross onto a R /r-X1 stock. The following article is by E. B. Patterson. It describes the mapping of genes by the use of chromosome aberrations and multiple marker stocks. It provides an overview of the use of aberrations for gene location and mapping as well as a description of the use of stocks containing reciprocal translocations between the A chromosomes.

Next is a paper by E. H. Coe, which provides some guidelines for two matters of general concern in various experiments: the planning of progeny sizes and tabulation of recombination percentages. He provides a means of calculating the number of progeny that must be obtained in order to be confident that at least one of the desired class expected will, in fact, be present in the progeny. The last article in this section is by C. R. Burnham. In it, he describes his personal recollections of the events leading to the correlation of linkage maps and chromosomes in maize and barley. Much of the article describes Burnham's interaction with R. A. Brink at Wisconsin and with Barbara McClintock at Cornell.

Cytological Techniques

The cytological map of maize and the correlation of the linkage maps with the individual chromosomes was made possible by the development of the procedure for staining pachytene stage chromosomes of meiotic cells with aceotocarmine. The original procedure was worked out by Barbara McClintock as described in C. R. Burnham's article in the previous section. The details of this procedure as well as techniques for collecting and fixing tassel material and other procedures are described in his article on the smear technique in this section. This is followed by P. J. Sallee's protocol for prefixation and staining of somatic chromosomes, wherein root tips are exposed to mono-bromonapthalene in a solution containing DMSO prior to fixation. The next paper, by R. L. Phillips and A. S. Wang describes a procedure for the *in situ* hybridization of RNA to maize meiotic chromosomes and the detection of hybrids by autoradiography with Kodak NTB-2 emulsion.

Genes and Known Protein Products

The study of several gene systems in maize has progressed to the point where the protein products of the genes have been identifed and analyzed. A description of these systems are presented in this section. The first article, by H. K. Dooner, reports on the gene-enzyme relationship in anthocyanin biosynthesis. He lists the nine loci, mapping to seven chromosome arms, that are involved in anthocyanin synthesis. Detailed consideration is given to UDP-glucose: flavonoid *3-O*-glucosyltransferase (UFGT) and flavanone synthase (FS). Evidence is summarized for the conclusion that *Bz1* is the structural gene for UFGT, while it is suggested that *C2* has a structural role and *C1* a regulatory role in FS synthesis in maize.

Starch mutants and their protein products are discussed in the article by P. S. Chourey. He describes four gene systems, *shrunken-1*, *shrunken-2*, *brittle-2*, and *waxy*, that affect starch level or quality in the endosperm and points out that a mutation in each of these systems uncovers a second isozyme that is coded by another gene at a different locus. These observations are especially interesting with regard to their potential value for understanding the genetic regulation of metabolic pathways in maize.

The article by J. C. Sorenson describes studies on catalase and its genetic control, which is proposed as a model system for the study of genetic regulation during development. This is followed by an article on the maize anaerobic response by M. M. Sachs and co-authors. They report that

about 20 polypeptides, including the products of Adh1 and Adh2, are synthesized after about 90 minutes of anaerobic treatment of maize roots. They also report on studies using cell and tissue cultures aimed at developing plant transformation methods using ADH as a model system. The next article is an annotated bibliography of the Adh genes of maize by C. W. Karoly and co-authors with a preface by M. Freeling. The preface to this article considers the question of whether "classical" maize genetics has a further role to play (and provides an affirmative answer) while the bibliography is extensive and comprehensive.

The last article in this section is by F. Salamini and C. Soave. It describes genetic and biochemical studies in their laboratories leading to the localization of two clusters of zein structural genes, those for the 20 Kd polypeptides on chromosome 7 and those for the 22 Kd polypeptides on chromosome 4. Included is a linkage map showing the position of five zein gene loci relative to three markers on the short arm of chromosome 7, a result of combining classical procedures with gel electrophoresis techniques.

Nucleic Acids, Cloning, and Controlling Elements

This section begins with a protocol by C. J. Rivin, E. A. Zimmer, and V. Walbot for isolating DNA from leaf or other tissues of maize. This procedure yields nuclear DNA suitable for restriction endonuclease digestion and cloning. An article from the same laboratory by E. A. Zimmer and K. J. Newton describes a similar protocol that is suitable for isolating high molecular weight DNA from individual maize seedlings, parts of a single scutellum, or the primary root of a seedling. Next, G. H. Kidd and M. E. Davis describe the different RNA polymerases and their role in transcription including RNA polymerases located in the nucleus, chloroplast, and mitochondrion.

This is followed by an article on the cloning of maize zein genes by B. A. Larkins and K. Pedersen. They discuss, among other things, the evidence indicating the absence of intervening sequences in zein genes. Another paper follows, in which R. Langridge and co-authors describe their work with zein genomic clones, and they also present evidence for the absence of introns in zein structural genes. The next article is by I. Rubenstein; he presents evidence showing that the zein genes comprise a multigene family and that this family can be subdivided into subfamilies with closely related nucleotide sequences. This is followed by a paper by E. L. Sheldon describing the development of maize DNA libraries. He has constructed a library containing essentially the complete genome of Black Mexican sweet corn and partial libraries of the inbreds W22 and W23 using Charon phage vectors.

An introduction to maize controlling elements is provided by N. Federoff. She distinguishes the autonomous controlling elements, which are capable of autonomous excision and transposition, from the non-autonomous controlling elements, which are not capable of autonomous transposition. She also discusses how the selective interaction among controlling elements allow six or more families of controlling elements to be distinguished and describes in some detail two of these, the Ac-Ds family and the Spm family. Results on the study of the shrunken-1 locus involving known Ds mutations at that locus are also included in Federoff's article. This topic is also the subject

of the article by Doring and co-authors. They are seeking to isolate and sequence the Ds element. The last article in this section is by N. Shepherd and co-authors. It also concerns controlling elements and briefly reports on efforts to clone the C2 gene as part of their program of studying controlling elements at the molecular level.

Cytoplasmic Male Sterility, Mitochondria, Chloroplasts and Pollen.

The types and availability of male sterile cytoplasms of maize are described by V. E. Gracen. His article includes a table describing the relative degree of pollen sterility and maturity of different sources of cytoplasmic male sterility in different maize inbred backgrounds. A detailed description of nuclear and extranuclear gene interaction of the cytoplasmic male sterile systems in maize and recent approaches to their molecular interpretation are described by J. R. Laughnan and co-authors. In the next article, J. R. Laughnan and S. Gabay-Laughnan describe the nuclear control over reversions to male fertility in cms-S maize. They provide considerable evidence that the nuclear genome has predominant influence over both the frequency of reversion to fertility and the site of reversion. The following article by J. E. Carlson and co-authors provides direct evidence that the nuclear genes control the reversion of cms-S to fertility and that the nuclear genes influence the replication of the mitochondrial DNA S1 and S2 plasmids. A brief review of reversions of cms-T sterile cytoplasm to male fertility is presented by A. Cornu and S. Gabay-Laughnan.

Other aspects of cytoplasmic male sterility are treated in the next three articles in this section. The effects of *Helminthosporium maydis* race T toxin on mitochondria and protoplasts from T cytoplasm maize are described by E. Earle. She points out the potential value of studying the mitochondrial DNA and proteins in callus or plants obtained by protoplast fusion of protoplasts with different cytoplasms. The next article, by B. Gengenbach and D. Pring describes the isolation by tissue culture techniques of revertants from cms-T to a male fertile condition. They also present the results of analyzing the restriction endonuclease digestion patterns of the DNA of these revertants and observe that reversion to male fertility and toxin resistance may be conditioned by changes or rearrangements within a particular fragment of mitochondrial DNA. Detailed analysis of the fate of the S1 and S2 DNA sequences in revertants of cms-S to fertility is described in the article by R. J. Mans. He reports the detection of S1 and S2 sequences in the mitochondrial DNA of normal fertile maize.

The next article in this section presents some speculations on the possible role of the mitochondria and chloroplasts in heterosis (hybrid vigor). The interaction of the polypeptides coded for by the nuclear, chloroplast and mitochondrion genomes may play a role in this phenomenon according to A. Berville and M. Charbonnier. The following article by J. I. Stiles, presents a restriction endonuclease cleavage map of the maize chloroplast genome, which indicates considerable complexity in its structure including tRNA genes with intervening sequences and at least one example of overlapping genes. The next two papers concern maize pollen. The first, by L. S. Bates, describes methods for the bulk collection of pollen and the second, by P. D. Miller, is a review of the composition of maize pollen.

DEVELOPMENT, PIGMENTS, AND PHOTOSYNTHESIS

The first article in this section is by E. H. Coe and R. S. Poethig. They describe the numerous variant forms of genes affecting the development of tassels, ears, leaves, roots, and stems. Some of these genes have pleiotropic effects. This is followed by an article by M. M. Johri and E. H. Coe reporting their development of genetic approaches to analyzing meristem organization. This involves the genetic marking of cells by inducing clones of cells lacking a dominant alelle of a factor required for pigment formation.

The next paper is by P. Langridge and co-authors, briefly describing a procedure whereby developing kernels can be removed throughout the period of maturation of a single ear. They observed that it is feasible to make at least ten different samplings without harming the development of the remaining kernels.

A brief description of the chlorophyll and carotenoid mutants of maize is presented in the article by D. S. Robertson. This group of mutants is of considerable interest, not only because they include mutants defective in various steps of photosynthesis, but also because they are probably the most frequently-observed class of mutants in maize.

A detailed review of photosynthetic mutants of maize is the subject of the next article by K. Leto. He describes the selection of photosynthetically-impaired mutants, which are normally pigmented but exhibit high levels of chlorophyll fluorescence. This article also includes a description of the genetic analysis of these mutants and results of recent efforts at identifying the molecular basis of their defects.

BREEDING, WILD RELATIVES, AND GERMPLASM

Maize breeding and future goals are introduced by J. H. Lonnquist in his brief historical review. In considering the future, he expresses both a concern about maintaining genetic variability in maize, as well as a continued confidence in the effectiveness of contemporary selection techniques for achieving advances in productivity.

In his article on raw materials for maize breeding, W. C. Galinat points out the potential value of utilizing genetic variability present in the wild relatives of maize, teosinte and *Tripsacum*. These wild relatives and their systematics are the subject of the article by G. Wilkes, who also considers their origins and evolutionary relationships.

The systematics of *Zea* are discussed by R. McK. Bird. In addition, he considers the selection of experimental material and provides guidelines for sampling the genetic variation in maize and in teosinte. The accompanying paper by R. McK. Bird, on maize and teosinte germplasm banks, provides a list of sources from which seed samples may be obtained of the recommended strains described in the preceding article.

The problems and promise of utilizing the *opaque-2* form of maize, with its superior protein composition, are discussed by L. S. Bates. He points out the potential for the utilization of modified "hard-endosperm" *opaque-2* maize and describes the progress to date in achieving this goal.

The genetic diversity of maize and disease resistance is reviewed by A. L. Hooker. He discusses the genetic complexity of disease resistance and points out that polygenic inheritance is common, as is gene interaction.

TISSUES AND CELL CULTURE

The papers in this section begin with a review of plant regeneration in maize tissue cultures by C. E. Green and C. A. Rhodes. They describe the procedures for establishing regenerating cultures and the factors currently known to be crucial for successful regeneration including the nature of the tissue source, the composition of the culture medium, especially the 2, 4-D level, and the genotype of the donor plant. They also report on a new type of maize tissue culture which is friable, grows rapidly, and regenerates plants by somatic embryogenesis.

The current status of protoplasts of maize and other cereals is reviewed by C. T. Harms. The serious problems in obtaining protoplast cultures capable of regenerating walls and producing totipotent callus are thoroughly described.

The origin of Black Mexican sweet corn and how it came to be popular for maize tissue cultures, especially suspension cultures, is described in the next article by W. F. Sheridan. This is followed by a review of the present "state-of-the-art" of culturing maize anthers, by the same author. This article includes a protocol for anther culturing and the composition of the N6 medium.

Next is an article on maize endosperm cultures by J. C. Shannon. In addition to a protocol, it contains the formula for the medium of Linsmaier and Skoog. The last article in this section is by G. Gavazzi and co-authors. They provide a brief consideration of the potential that *in vitro* techniques offer for genetic analysis at the cellular level.

2 MAIZE - THE PLANT AND ITS PARTS

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One of the greatest deterrents to an appreciation of plant morphology is the terminology used to describe various plant parts. This problem is compounded in the case of maize because of its relatively unusual structure. We all learn that plants have a vegetative body composed of stems, leaves and roots, and that flowers contain sepals, petals, pistils and stamens. Maize, however, has at least three kinds of leaves, two kinds of stems, two kinds of roots, and two kinds of flowers in which glumes, lemmas and paleas take the place of sepals and petals. Fortunately, these parts are arranged in a relatively simple fashion, so the task of mastering maize morphology is not as difficult as it might seem. In this article we will identify some of the most important parts of the maize plant and describe their organization. More detailed descriptions of the developmental morphology of maize have been provided by a number of investigators. Kiesselbach (1949, reprinted 1980) gives a good general picture of maize structure and development. The external morphology and the histology of the vegetative and reproductive shoots have been studied by Bonnett (1948, 1953), Sharman (1942) and Abbe and co-workers (Abbe and Phinney, 1951; Abbe et al., 1951), while the most comprehensive descriptions of the embryogeny are those of Randolph (1936) and Abbe and Stein (1954). A summary of the histology of the corn plant, written by Sass in 1955, has been reprinted in the recent edition of Corn and Corn Improvement (1976).

The organization of the plant body: Maize is a member of the grass family, the Gramineae, and as in all grasses, most of the plant body is leaf tissue (Fig. 1a). To appreciate the general organization of the maize plant it is helpful, therefore, to see it in a leaf-less state (Fig. 1b). Stripped naked, the maize plant is not very impressive. Its main stem, or culm, is a slender, segmented shaft similar to a stalk of bamboo or sugarcane. The enlarged joints along the stem, the nodes, mark the points of leaf attachment; the stem segment between nodes is called the internode. Each node bears a single leaf in a position opposite that of the neighboring leaf, giving the plant two vertical rows of leaves in a single plane (Fig. 1a; 2). This so-called distichous phyllotaxy is typical of all leaf-like appendages, wherever they occur on the plant.

Maize has unisexual, rather than bisexual flowers. Male (staminate) flowers are located at the apical tip of the main stem in the tassel, a branched inflorescence. Female (pistillate) flowers are found in one to several compact ears, located on the ends of short branches near the middle of the stem (Fig. 1b; 2).

This partitioning of male and female flowers in separate structures distinguishes maize from other cereals and is one of the principal reasons that its genetics has been so conveniently explored. Making controlled pollinations in maize requires little more effort than that involved in placing a bag over the tassel and ear shoot. To perform a controlled pollination in rice, wheat, barley and other cereals, it is necessary to emasculate each

flower used as a female parent, an especially tedious job when each flower yields only one seed.

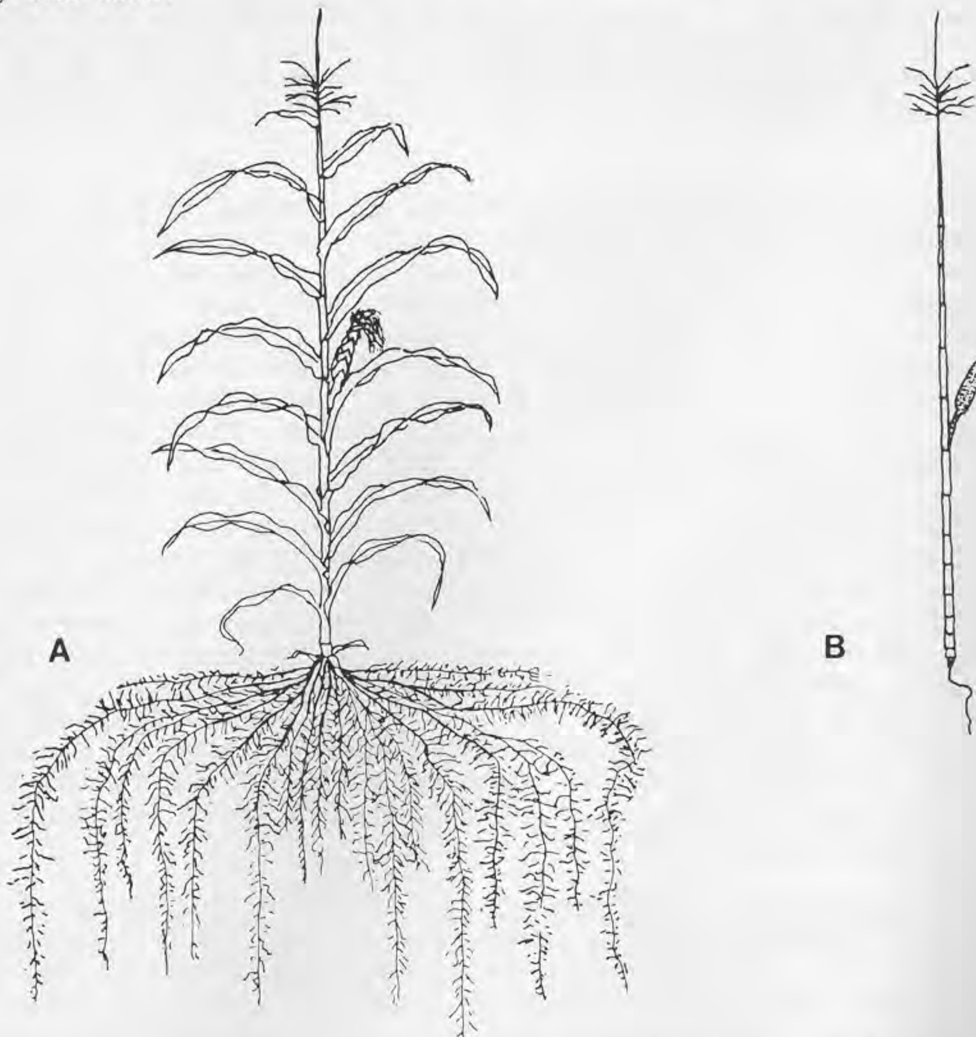

Figure 1. a) Mature maize plant (after Kiesselbach, 1949). b) Mature maize plant drawn without leaves and adventitious roots. The apical end of the main stem (culm) terminates in the tassel, while the basal end terminates in the primary root (radicle). The ear shoot arises from an internode near the center of the culm.

Maize also differs from closely related species in that it has relatively few branches. Only the lower 10 to 12 internodes of the stem produce branch primordia, and most of these remain suppressed. Above-ground primordia develop into ear shoots, while those located at subterranean internodes develop into tillers--branches identical in structure to the main stem. Commercial hybrids (except sweet corns) generally tiller very little, and typically produce a single viable ear shoot. In contrast, some "varieties" may have several large tillers and may produce 2 ears on the main stem and some ears on tillers.

The stem: During the first four weeks after germination, the growing point of the stem lays down all the nodes and internodes of the plant and then differentiates into a tassel. At the time of tassel formation the stem is not more than 3-4 inches tall, even though the plant may be 3-4 feet in

height (Fig. 3). Subsequently, the stem begins to elongate rapidly, with most of the growth occurring at the base of the internodes. The lowermost 6-8 internodes do not participate in this growth, however, and remain below ground where they produce the root system and tillers. These subterranean internodes taper sharply towards the base of the stem, forming a distinctive region, the crown (Fig. 1b). The stem is thickest a few inches above ground, and tapers gradually towards the tassel. All the internodes from the top ear downward have a distinct groove associated with the axillary bud at the base of the internodes; internodes above the ear lack axillary buds and are smoothly cylindrical.

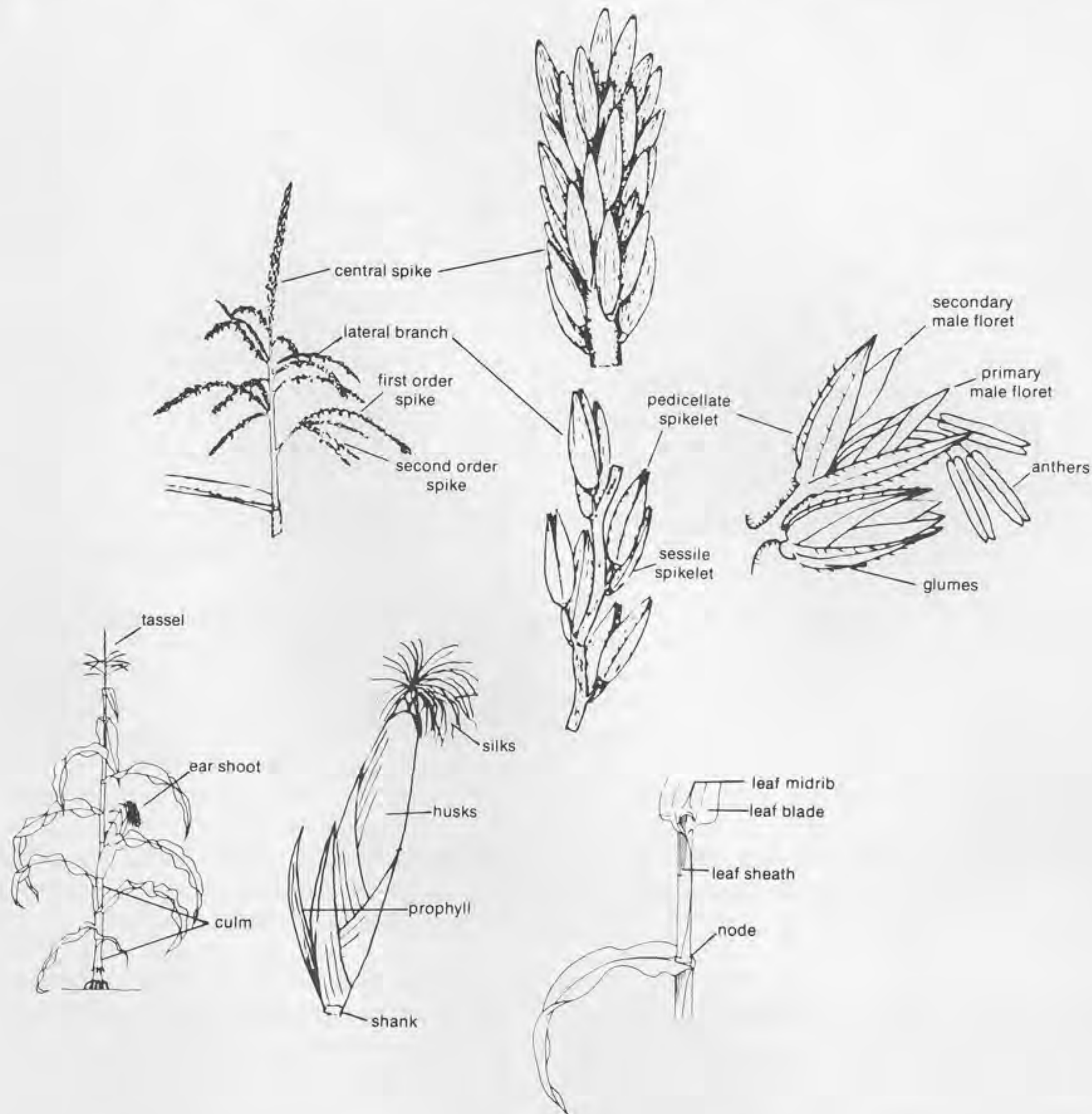

Figure 2. The major parts of the maize plant. Drawings in part from P. Weatherwax in Corn and Corn Improvement, 1955, and E. D. Styles et al. in Can. J. Genet. Cytol. 15:59, 1973; figure assembled by M. M. Johri and E. H. Coe.

The stem of an ear shoot, called the shank (Fig. 2), differs from the main stem in being relatively short in most strains. In addition, the internodes of the shank are variable in number, irregular in shape and size, and tend to have a crinkled rather than smooth surface. Secondary ear shoots commonly occur on the shank of several types of maize, but are rare in most commercial strains unless fertilization of the apical ear is prevented.

Figure 3. A four week old plant (approximately 3 feet tall) in which the stem apex has differentiated into a tassel. As shown on the right, the stem is still relatively short at this stage.

The tassel: The tassel, located at the top of the culm, consists of a series of large branches (spikes) covered with numerous, small flower-bearing branches (spikelets: Fig. 2) Each branch point on a spike bears two spikelets, one on a long stem (pedicellate), the other on a short stem (sessile) (Fig. 4a). Each of these spikelets, in turn, produces two functional florets. Although tassel florets contain both stamens and a pistil, the pistil normally degenerates soon after it is initiated, making the floret functionally male. However, pistils will develop at the base of the tassel under some environmental and physiological conditions, and are quite common on tillers.

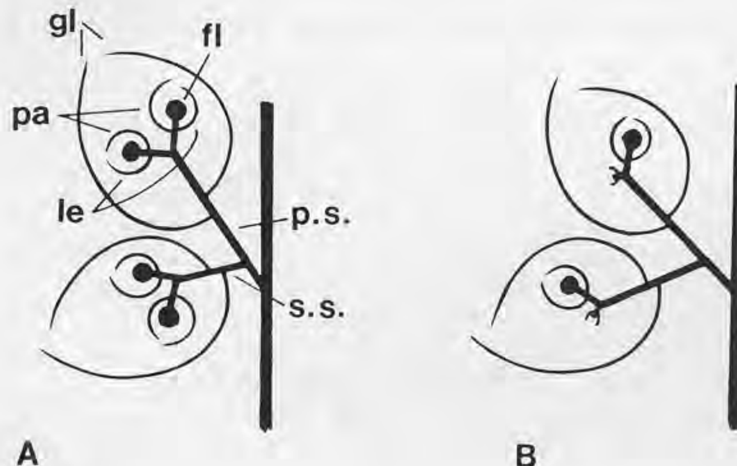

Figure 4. Schematic drawing of a pair of tassel spikelets (A) and a pair of ear spikelets (B). Note that the lower floret in the ear spikelet aborts early in development. p.s. - pedicellate spikelet; s.s. - sessile spikelet; gl - glumes; le - lemma; pa - palea; fl - floret.

Surrounding both florets on a spikelet are 2 leaf-like scales called glumes (Fig. 2; 4a). Within the glumes, each floret is individually enclosed in another pair of scales, one located adjacent to the glume (the lemma), the other located between the two florets (the palea) (Fig. 4a). At anthesis, these scales are forced apart by the swelling of conical structures (lodicules) at the base of the 3 stamens, and the filamentous base of the stamens elongates, forcing the anthers out of the flower (Fig. 2). As they dangle downwards, the anthers shed pollen from openings at their tip.

Pollen grains are the multicellular products of the haploid microspores that result from the meiosis of a microspore mother cell (microsporocyte). Meiosis takes place in the anther before the tassel emerges from the leaf sheaths. After meiosis, the 4 resulting haploid microspores separate from each other, and each forms a thick wall. Shortly before shedding, each microspore undergoes two mitotic divisions. The first division is asymmetric, and produces a relatively large vegetative cell and a smaller generative cell. In the second division, the generative cell divides to form two sperm cells.

The ear: The ear is morphologically similar to the tassel, although this resemblance is obscured by differences in the relative size of their parts. The crucial difference between them is, of course, that the tassel contains male flowers, and the ear bears female ones. This difference is due simply to the fact that during the formation of an ear floret, stamen primordia are arrested at an early stage in their development, while the pistil develops fully. Each functional ear floret has a single ovary, which terminates in an elongated style, or silk (Fig. 5). Within the ovary is a single embryo sac. The embryo sac is the product of one of the four haploid cells resulting from the meiosis of the megaspore mother cell. While its three sister cells degenerate, the nucleus of this cell divides three times to produce 8 haploid nuclei within a common cytoplasm (the embryo sac). Two of these nuclei (polar nuclei) migrate to the center of the embryo sac where they become closely associated. The three nuclei remaining at the base of the embryo sac

subsequently undergo cellularization to form the egg cell and two synergids, while the 3 nuclei at the tip of the embryo sac proliferate to form 24-48 antipodal cells.

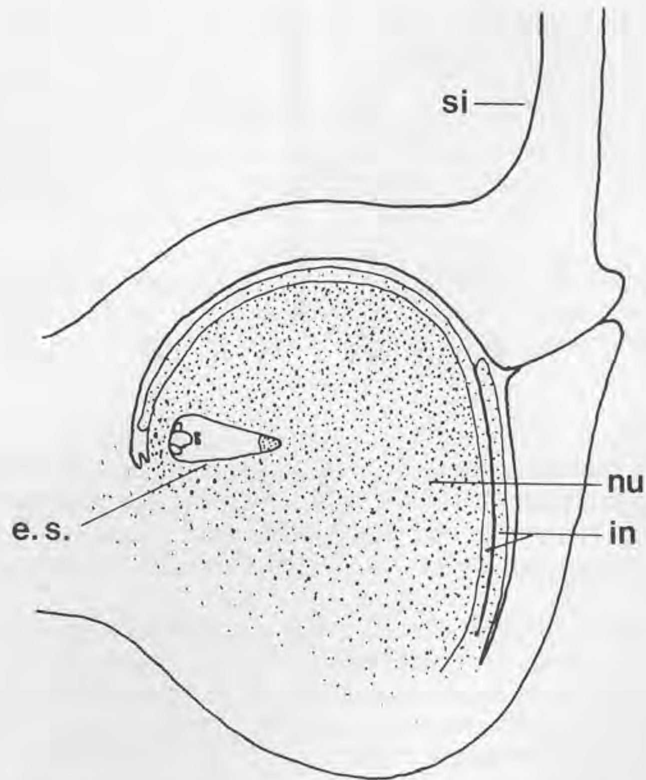

Figure 5. Radial longitudinal section of an ovary with an unfertilized embryo sac (after Randolph, 1936). Upon fertilization, the nucellus is digested by the expanding embryo sac and the tissue surrounding the nucellus is transformed into the pericarp. si - silk; e.s. - embryo sac; nu - nucellus; in - integuments.

The ear also differs from the tassel in that it has no major lateral branches. Its thick, lignified axis, the cob, is homologous to the central spike of the tassel. As in the tassel, ear spikelets come in pairs, but in the ear they are equal in size and only one of the florets in each spikelet is functional (Fig. 4b). An ear therefore has an even number of parallel rows of equally sized kernels equal to the number of spikelets on the cob. The number of rows (or ranks) of kernels ranges from 4 to 30.

The glumes, lemmas and paleas of the ear spikelets are readily visible in an unfertilized ear, but are soon obscured by the enlargement of the ovary after fertilization. In a mature ear these structures are represented by the chaff that adheres to the cob and the base of the kernel after it is shelled.

The leaf: Maize produces three kinds of vegetative leaves: foliar leaves, husk leaves and prophylls. A foliar leaf is located at each of the nodes on the main stem, husk leaves are located on the shank of the ear shoot, and prophylls are found at the base of the shank between the ear shoot and the stem (Fig. 2).

The foliar leaf has two distinct parts--the blade, a flat portion extending away from the stem, and the sheath, a basal part that wraps tightly around the stem (Fig. 2). Internally, the blade consists of a spongy network of cells traversed by a series of parallel, longitudinal veins. This flexible lamina is supported by the midrib, a thickened, translucent structure located in the center of the leaf. The sheath is thicker and more rigid than the blade, possesses fewer longitudinal veins, and lacks a prominent midrib. The sheath completely encircles the internode above the node to which it is attached and may extend the entire length of that internode. During the early development of the plant, the leaf sheaths provide most of the mechanical support necessary to keep the stem upright. At the boundary between the blade and the sheath there is a distinct hinge of translucent tissue. In this region, the leaf blade and leaf sheath narrow sharply, forming an indentation in the leaf margin. The wedge of translucent tissue adjacent to this indentation is known as the auricle. The ligule is the thin collar of filmy tissue located on the inside of the hinge.

The husk leaves surrounding the ear are usually considered modified leaf sheaths, with vestiges of the blade portions occasionally present. In some strains husk leaves develop a prominent ligule and leaf blade. In contrast to the leaf sheath, husk leaves are relatively thin and flat. Each husk leaf is attached to a unique node on the shank, and all but a few upper ones are arranged distichously.

Located between an ear shoot and the stem, the prophyll looks superficially like a husk leaf, but is distinguished by having two keels (midribs) and a split apex. These features suggest that the prophyll arose evolutionarily from the fusion of two foliar leaves. The homology of the prophyll is still controversial, however. Galinat (1959), for example, considers the prophyll one of the basic units of maize morphology, the others being the internode, leaf and axillary bud.

The root: More is known about the growth, cell biology, physiology and anatomy of the primary maize root, or radicle, than perhaps any other organ of the plant. Its histological structure, described by Sass (1976) and Kiesselbach (1949), is typical of roots in general. The apex of the root is sheathed in a loose network of root cap cells. Immediately behind the apex is a zone of cell division and elongation, beyond which root hairs are initiated. Larger lateral roots arise at varying points behind the zone of root hair formation. Cell division is restricted to the apical 3 mm of the root, and occurs at a maximal rate 1.25 mm behind the apex. The zone of elongation extends 8 mm behind the apex, the rate of elongation being maximal 4 mm from the tip (Erickson and Sax, 1956). Those interested in using the root for physiological or cell cycle studies should consult Silk and Erickson (1979; 1980) and Green (1976) for an analysis of the growth parameters that must be taken into consideration in such studies.

The primary root represents the basal end of the plant axis, which in maize and other grasses contributes relatively little to the ultimate root system (compare Fig. 1a and b). Most of the root system consists of adventitious roots produced by the basal-most internodes of the stem. The primordia of a few adventitious roots are normally present in the embryo, and these emerge soon after germination. New root primordia are subsequently initiated at the base of all subterranean internodes, and also appear

at 2 or 3 above-ground internodes after the stem has elongated. Subterranean adventitious roots are sometimes called crown roots, while those initiated above ground are known as brace roots.

Adventitious roots grow horizontally for several feet before turning downwards. As a result, the root system of a single plant often covers a region 6-8 feet in diameter, while the depth of the root system may be as much as 6 feet. As it grows, the root branches profusely in the region behind the apex, forming both secondary roots and unicellular root hairs. The total length of root system of a mature plant has been estimated to be 6 miles.

The kernel: The events surrounding the process of fertilization have been described by Miller (1919), Kiesselbach (1949) and Pfahler (1975); unfortunately, ultrastructural information about this phenomenon is still unavailable.

The silk is receptive to pollen along its entire length. Within 5 minutes after a pollen grain lands on a silk it sends out a tube which penetrates the silk and grows downward towards the ovary. During this process the vegetative nucleus and the two sperm cells migrate to the tip of the pollen tube where they remain throughout its growth. Upon reaching the embryo sac, 12 to 24 hours after germination, the end of the pollen tube bursts, releasing the two sperm. One sperm nucleus fuses with the two polar nuclei in the center of the embryo sac to form a triploid cell that gives rise to the endosperm. The other sperm nucleus fuses with the egg nucleus to form the zygote. As often as 2% of the time the polar nuclei and the egg nucleus are fertilized by sperm from different pollen grains, with the extra sperm nuclei being somehow lost (Sarkar and Coe, 1971). This phenomenon, called heterofertilization, can lead to a non-correspondence between the genotype of the endosperm and embryo when the male parent is heterozygous.

The development of the kernel following fertilization has been described in detail by Randolph (1936). We will only note here that this process takes 40-50 days and is accompanied by a 1400-fold increase in the volume of the embryo sac. The growth of the embryo and the accumulation of food reserves in the endosperm is completed by about day 40, and the remaining 10-20 days is spent maturing and drying.

A mature kernel has three major parts: the pericarp, endosperm and embryo (Fig. 6). The pericarp, the tough transparent outer layer of the kernel, is derived from the ovary wall and is therefore genetically identical to the maternal parent. The endosperm and embryo represent the next generation.

The endosperm makes up about 85% of the weight of the kernel and is the food source for the embryo for several days after it germinates. This food takes the form of intracellular starch grains and protein bodies, and is concentrated to varying degrees in different parts of the endosperm (Duvick, 1961). In flint-type kernels the concentration of starch and protein bodies is higher around the periphery of the endosperm than in the center, giving the endosperm a hard, corneous external layer, and a soft, granular center. In dent kernels, the granular tissue extends to the crown of the endosperm so that it collapses upon drying and produces a distinct indentation. These two traits are polygenic in their inheritance and are

characteristic of specific races of maize. Other common endosperm traits, such as sugary, floury or shrunken, are single gene mutations and can exist in either a flint or dent background.

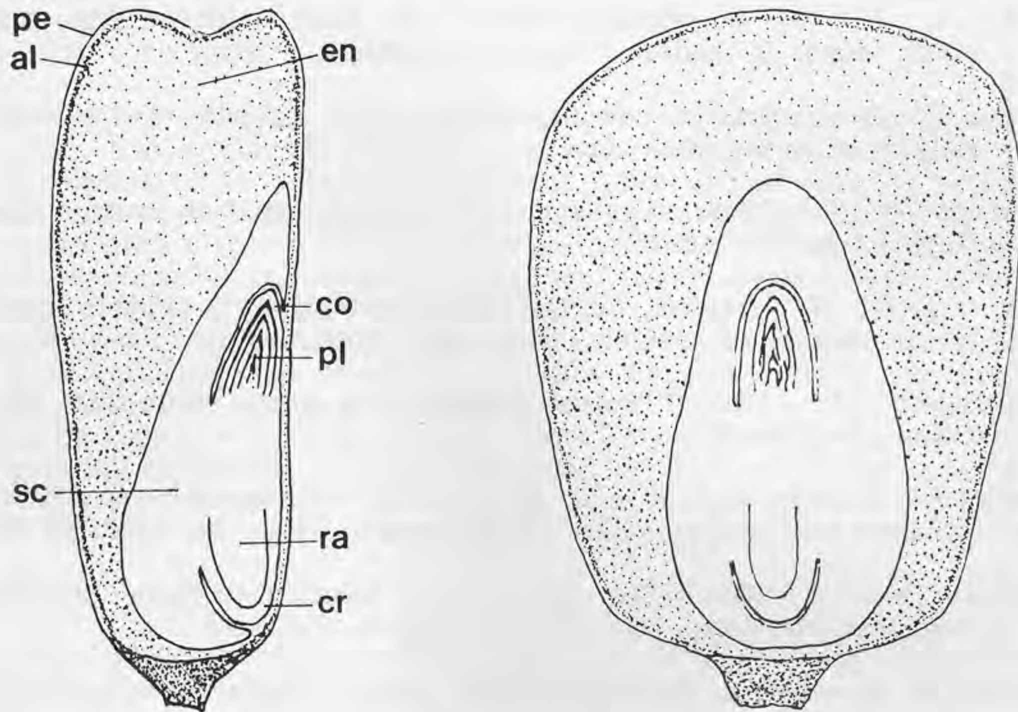

Figure 6. Longitudinal sectors of a mature dent kernel, taken perpendicular (left) and parallel (right) to the upper face of the kernel (after Kiesselbach, 1949). pe - pericarp; en - endosperm; al - aleurone; sc - scutellum; co - coleoptile; pl - plumule; ra - radicle; cr - coleorhiza.

Much of our understanding of gene action in maize is based on the analysis of genes affecting the pigmentation of the external layer of the endosperm, the aleurone. This specialized single cell layer is the only part of the endosperm capable of becoming intensely pigmented. Internal endosperm cells may be either yellow or white.

The embryo is located on the broad side of the kernel facing the upper end of the ear, beneath a thin layer of endosperm cells. Most of the tissue in the embryo is part of the scutellum, a spade-like structure concerned with digesting and transmitting to the germinating seedling the nutrients stored in the endosperm. The shoot and root axis are recessed in the outer face of the scutellum. In a mature kernel, the shoot (plumule) has 5 to 6 leaf primordia that are arrested at successive stages of development (Abbe and Stein, 1954). Surrounding the shoot is a cylindrical structure called the coleoptile. Upon germination, the coleoptile elongates until it is above ground and is then ruptured by the more rapid expansion of the rolled leaves within it. The root is enclosed in a sheath of tissue called the coleorhiza. Unlike the coleoptile, the coleorhiza does not elongate very much, and gives way to the radicle as soon as it emerges from the seed.

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3 GROWING MAIZE FOR GENETIC PURPOSES

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Corn is a large kerneled, highly domesticated vigorous annual plant of tropical origin. Since it is a natural cross pollinator it is highly heterogenous and responsive to selection pressure. Because of this it has been taken by man into all but the harshest of cropping environments. Strains of corn are known which grow as far north as southern Canada while others range to the extremes of the tropical forest and desert oasis. Some grow at sea level and others at 11,000 feet elevation. However, those lines and strains most used for breeding and genetic purposes are much more restricted in adaptation and therefore require narrower limits on growth conditions in order to produce a useful crop. There is no doubt that strains could be found or developed that would be satisfactory for use under most plant growing conditions. For the purpose of this article the conditions of choice will be described while keeping in mind that a great deal of latitude can be obtained by proper manipulation and by choice of strains used.

A. FIELD CULTIVATION

1. Choice of Field. Corn grows best and is most easily handled on fairly flat, well drained, fertile land. The field should be large enough to permit some crop rotation. Continuous cropping with corn is feasible but requires closer attention to the buildup of injurious pathogens and insect pests. Proximity to forests (racoons), marshes (black birds), rodent populations, college residence houses and dense human populations should be avoided. The area should be appropriately fenced; a wandering four-wheel-drive vehicle can mean disaster.

2. Soil Preparation. In most temperate zones fall plowing is best. When soil is dry enough to work in spring it should be tilled to a medium texture with a disc harrow. Avoid working when the soil is too wet as large hard clods which prevent good seed-soil contact or excessive compacting will result. Best germination and weed control are obtained by working the soil the final time just before planting. When final soil preparation is complete mark the rows using a two row corn planter (strings in small plots). The rows should be 36" (91.4 cm) apart or at distances that match and are in synchrony with cultivating equipment that will be used later in the season. At the time of marking a band of starter fertilizer (NPK balanced for area soil) may be applied through the planter. Following the marking a pre-emergence herbicide spray may be applied.

3. Planting. Planting should not begin until the soil temperature has reached 50°F (10°C) but after that any time that allows a 90 day growth period before the first frost is suitable. Early spring plantings usually perform better than late ones because this allows the crop to flower before summer stress periods that may occur and to avoid the diseases and insects. Early planting also allows the plants to make use of long days and relatively warm nights (note, tropical strains will not flower in the long days of the temperate zone). Seed should be prepared in envelopes labeled with family

numbers. Stakes with corresponding numbers should be prepared and placed in the field in the marked rows and the seed planted with an "Allan Jab" hand planter at a spacing of 1 foot (30 cm) apart in 3 feet (91.4 cm) apart rows. Up to 1/3 closer planting is feasible on fertile land and under ideal conditions. Wider spacing is of little advantage except under dry conditions. Wide spacing does allow high tillering lines to spread out and produce several stalks if such are needed. The seed should be planted not more than 1 inch deep and pressed firmly into the moisture zone of the soil. If the moisture is not that high in the soil, irrigation immediately after planting will be required to achieve uniform germination. Some workers prefer to plant two kernels per hill and thin at the 4-5 leaf stage to one plant per hill. This should be done after all seedlings are up and growing and before any tillers are produced. See Wallace and Bressman (1937) and Larson and Hanway (1977) for additional details on cultivation practices.

4. Pest Control. Corn can be grown in most areas without herbicides, insecticides, and fungicides and using only organic fertilizers, but to do so the grower must pay careful attention to many critical agronomic practices such as crop timing, rotation, isolation, residue removal and cultivation. He must be prepared to watch his crop daily and physically remove those invaders which will periodically attack his crop. Barring such dedication a successful crop may be obtained only through the prudent use of whichever of the following practices apply in the locality for the planned planting. See your local county extension agent, farmers group or government agricultural representative for advice.

a. Weed Control. Control may be obtained either by frequent cultivation and hoeing or by combining herbicide treatments and judicious supplementation of hand cultivation. In cultivation avoid cutting roots by refraining from deep, close cultivation. Herbicide control may be attained by using a pre-emergence spray on the soil just before or just after planting. Use a combination of broad leaf and grass herbicides according to the recommendation of the local farm experts and the supplier of the chemicals. Materials used and methods of application vary from time to time and place to place. A combination product labeled "Biceps" produced by Ciba-Geigy and applied at the rate of 1 1/2 quarts (1.42 l) per acre in aqueous solution and sprayed over the soil surface has been successful. For good control the soil must not be disturbed for some weeks after application. Later control may be obtained by hoeing out surviving weeds and/or applying a leaf contact spray of Banzel produced by Velsical and applied at 1/4 lb (113 g) per acre sprayed on the short weeds and soil when the corn is about 3 feet or more tall. Care should be taken to avoid spraying on the corn leaves, or drift of the herbicides to nearby plants of other species.

b. Insect Control. A general insect control can best be attained through the use of a systemic insecticide such as Furadan applied as follows: at the 8 leaf stage sprinkle a few granules of Furadan in the top whorl of leaves. This can be done by taking a small glass jar with a metal screw top lid, punching 5 holes in the middle of the lid and using this as a shaker to dispense the Furadan. In areas infested by cutworm and army worms an application of Lorsban insecticide should be made before or immediately after planting. It should be worked into the top soil at a rate of 2 pounds per acre. Red spider mites and aphids are a serious problem with greenhouse plantings. These can be controlled by a combination of sanitary conditions

and the use of the systemic insecticide Temik applied according to the manufacturer's directions. Note: Extreme care should be taken to follow manufacturer's directions in the use of modern systemic insecticides such as Furadan and Temik. The most serious problems with systemic insecticides are:

(1) They should not be applied to corn that may be eaten
(2) While they are extremely effective against insects they are also dangerous to humans and deadly for the insect eating birds which are our helpers and not the seed eating birds which often are pests. See Dicke (1977) for additional information on insect control.

c. Control of Other Pests. In areas where fungi and bacterial diseases are prevalent fair control can be obtained by regular application to the leaf surface of a spray containing Dithane. Use according to directions given by the supplier. Insect borne diseases are best controlled by clean agronomic practices and by insect control. See Ullstrup (1977) and Shurtleff (1981) for additional information on diseases and their control.

5. Irrigation. In many localities adapted corn strains and hybrids produce a satisfactory crop in most years without irrigation; however the investigator will no doubt want greater assurance of a better than average crop or will be required by the constraints of his experiment to use genetic stocks which are often poorly adapted to his locality. In any case supplemental water is important in most areas of the world. Water is best applied by an overhead sprinkler system or some other means where it can be turned on or off without too much difficulty.

Water should be applied whenever the crop shows wilting or other signs of drought stress. The critical period to avoid drought stress is during the flowering period. Irrigation during the pollination period should be done in the afternoon and evenings so as not to disrupt other operations. Good seed set requires adequate moisture and moderate temperature during this time. Excessive moisture due to irrigation followed by unexpected rains may cause yellowing of plants. This may be overcome by hand application of a nitrogen fertilizer (ammonium nitrate) at the rate of 50 pounds (22.5 kg) per acre spread over the root area around the plant. Excess water may also cause the plants to fall over (root lodging). In case of lodging do not lift plants upright but let them recover their upright position naturally which they will do in a few days without help. Except in the dryest of climates irrigation should cease 10 days to 14 days after the last pollination. This helps to speed maturity.

B. CONTROLLED POLLINATIONS

Controlled pollinations require several essential steps:

1. Ear Shoot Bagging. Bagging is a fundamental requirement and yet, of all the pollinating operations, it is the most difficult to do properly. The ear shoots must be covered before the silks emerge in order to protect the silks from contaminating pollen until the desired pollen can be applied. The bag used (Lawson 217) measures 2 x 1 x 7 inches (5 x 25 x 18cm) and is made of semi-transparent treated 36 lb wet strength paper with waterproof glue. The daily shoot bagging operation should begin when the first tassels appear. At that time the tip of the first ear shoot may be visible in the axil of the 6th or 7th leaf down from the top. This shoot may be covered by

placing the bag over the tip of the shoot with the longer lip next to the culm towards the stem and so that the shorter lip slides between the tip of the ear shoot and the leaf sheath. The edges of the bag should be pulled around to conform to the shape of the culm. The bag should be given a sharp downward pull to firmly attach it between the tissues mentioned. The plants should be examined every day to catch new ears as they are ready. At this time those bags already on the plants may be pulled down again to make them more secure. Bagging too early is not very satisfactory because the tissues will not hold the bag firmly in place. Bagging too late risks exposure of silks and resulting contamination. Ears should not be bagged after silks appear. Complete honesty is required of all helpers in this regard otherwise contamination will get completely out of hand.

Some workers use a larger bag 2 1/2 x 1 x 8 1/2 inches (6.4 x 2.5 x 22 cm) (Lawson 218), bend back the 7th leaf and cover the whole auricule and ligule area at the top of the leaf sheath and staple the bag around the culm even before the first shoot appears. This allows one trip shoot bagging but sometimes the wrong leaf axil is taken and the shoot comes out elsewhere and is exposed. In any shoot bagging practice care must be taken in handling the plant so that the support of the leaf sheath to the culm of the next node is not weakened; otherwise the top of the plant will break out and the whole plant will be lost.

Under natural conditions receptive silks appear over a 4-5 day period. They are pollinated by the pollen shed each of these days and a full set ear results. In the case of hand pollination the pollen is applied only once in a very short period of time. The result would be a partially filled ear unless additional steps are taken. The standard practice to overcome this is called <u>cutting back</u>.

2. <u>Cutting Back</u>. This consists of cutting off with a knife the tip of the husks and silks on an ear having the first day's silks visible and then re-covering the ear to prevent exposure. The cut should be made squarely and cleanly across the ear and as far down the husks as possible without cutting off the tip of the cob inside. Contamination is avoided because the wet ends of freshly cut silks are not receptive to stray pollen grains which may fall on them during the operation. The shoot bag covering the cut silks should be marked by folding the corner to indicate which ears were cut back. The next day the silks will have grown to form a thick brush all the same length and all will be ready for pollination. Pollen applied at this time will reach all silks and result in a full set ear. Failure of unpollinated silks to grow or irregular growth indicates that the silks were already past the most receptive stage or that conditions were not good for pollen germination. Unpollinated silks will grow continually during this period to a length of 6-8 inches (15-20 cm) or more. Pollinated silks stop growing in an hour or so and become darker in color. Under normal conditions silks are usually receptive for about 3 days. After that the chance of successful pollination diminishes rapidly.

3. <u>Collecting Pollen</u>. Pollen is shed from mature anthers beginning on the upper third of the main spike of the tassel usually 1 or 2 days before silks appear on the ear shoots. The shedding is in daily increments that spread downward to the tassel branches toward the base and upward towards the tip of the main spike. The shedding moves along the tassel in two waves about 2 days apart. The first wave begins from the primary anthers

in each floret and the 2nd from the secondary anthers. A healthy tassel should shed pollen for about 1 week. On a warm sunny day fresh anthers begin to extrude from the florets by filament elongation about 7 a.m. These anthers will open up (dehisce) about 30 minutes later and pollen will fall out and be scattered by air currents. As the morning progresses additional anthers will appear until about 10 a.m. After that and under these conditions no new anthers appear until the next morning. If the weather is cool or cloudy and humid the extrusion of anthers will be delayed until later, sometimes until evening. Some workers report successful pollinations made in the evening. The author finds this undependable in most conditions, and impossible in others. As long the humidity is near 100% the anthers will not open. If the weather is hot and dry the process will proceed more rapidly.

4. Pollination. To make controlled pollinations it is necessary to collect viable pollen. This can be done by covering shedding tassels, before the time the pollen is shed, with a brown paper tassel bag and then collecting and carrying the pollen to the desired protected silks. If the plants to be used as male parents are sturdy and heavy winds are not in the forecast, the best time to cover the tassels is in the evening or after pollen has shed on the previous day. If the plants are weak or heavy winds are expected, the tassels may be bagged after the dew is off the tassels but before shedding has ended on the morning of the day of the desired pollinations. Moisture from dew trapped in the tassel bag will prevent the anthers from opening.

Cover the tassel with the bag keeping it as flat as possible, pull it down past the first flag leaf (which may be removed) then fold firmly around the sheath and stem of the tassel and finally secure it in place with a regular paperclip. If tassels were bagged the day before, pollination can begin as soon as nearby unbagged tassels are seen to be shedding. Bees determine this proper time very well and can be a good indicator. If tassels are bagged in the morning sufficient time (at least one-half hour) should be allowed for all stray pollen grains to die and for anthers with fresh pollen to emerge inside the bag.

To collect the pollen, carefully bend the plant so that the open end of the bag is higher than the closed end. Remove the paperclip and shake the bag and tassel sharply. Withdraw the tassel while being careful not to allow the open end of the bag to be low enough so that the pollen falls out. The pollen can be carried in this manner to make a self pollination or to cross on silks of one or two nearby plants. One cannot depend upon pollen remaining viable in the removed tassel bag more than 10-30 minutes depending on conditions. The best procedure for standard pollination is to carry the tassel bag, with open end folded, to the plant with intended silks. Place the stalk and tassel of the female parent under the pollinator's arm thus bending the plant so that the tassel is at the pollinator's back and the ear is immediately in front (this protects the ear from pollen falling from its own tassel). Then raise the shoot bag slightly, tear off the top, and squeeze it so that the shoot bag forms a chimney above the silks (this protects the silks from pollen carried by air currents from the side). Open the tassel bag in such a way that the pollen can be poured out in small amounts and dust a small amount of pollen on the silks; then quickly fold over the open torn end of the shoot bag to protect the silks and close the tassel bag in case there is need to make additional pollinations. After the pollinations

with one pollen source are completed return to the plants carrying the ears pollinated with that source. Use a tassel bag and write on it the family and plant number of the female (ear) and also the family and plant number of the male (pollen), in that order. Other things like notes about the pollination, plant types of the parents and date may also be written on the bag. Then pull the tassel bag over the pollinated ear so that the long lip slides between the stalk and the ear and the short lip hangs loosely on the outside. Pull the two edges on the long lip side of the bag near the bottom around the stalk and staple them together on the side of the stalk opposite the ear, thus securing the bag to the stalk so that the ear is free to enlarge without disrupting the bag.

If a large number of pollinations are to be made on a single day from one pollen source (as many as 100 pollinations may be made from one tassel) the best procedure is as follows: prepare a "Carpenter 3/75" (3 x 7 1/4 in, 7.6 x 18.4 cm) glassine bag by folding up and sharply creasing the bottom inch of the bag. Then pour in the contents of the tassel bag. Sift the pollen past the crease into the bottom fold taking care to keep the anthers in the top where they may be discarded. Turn the bag on its side and tear off the upper of the two bottom corners of the bag. Carry the pollen in this bag to the waiting silks where it may be sifted sparingly through the torn corner of the glassine bag into the top of the torn shoot bag covering each ear. Then fold the shoot bag to protect the pollinated silks and move rapidly to the next ear. Speed is the essence here because the pollen will remain viable only a short time. Fresh pollen appears light yellow and will flow freely from the glassine bag. If the pollen becomes deeper yellow or clumps together these are signs that it is no longer viable (collectively speaking even though there may be some viable grains mixed among the majority of inviable ones). After all pollinations from one source are completed then there is time to go back and write information on tassel bags, to place them over the ears, and staple them on the plants. It is often more efficient to have two people working together - a pollinator and a bagger.

5. Some Precautions. A. Contamination. During the shedding period pollen will be flying about on air currents, will be on leaves, the pollinator's clothing and hands. Most of it will already be inviable, but enough will be viable to make contamination a serious problem. Precautions should be taken to keep receptive silks covered at all times. B. Pollen Viability. The life of a pollen grain is usually less than 20 minutes after it leaves the anther. It is less than that in hot dry weather and much more than that in cool humid weather. (Ways to considerably extend pollen viability are known but the author has not found them to be reliable. They require special knowledge and handling.) There is little hope for fertilization if the temperature goes much above 95°F (35°C) during the shedding period or several hours thereafter. Conversely pollen may appear to be normal but will not grow on the silks unless the temperature during pollination and some hours thereafter goes above 55°F (13°C). Pollen is especially sensitive to drying conditions. Any time pollen grains change in color or begin to collapse irreversible degeneration may have already occurred. High humidity has different but equally important consequences. Anthers will not open in high humidity thereby trapping the pollen within. While this does not kill the pollen immediately it does pose mechanical difficulties in spreading pollen, and causes premature loss of viability. Moisture in bags or other containers for carrying pollen will cause immediate clumping. Pollen is promptly killed when immersed in water and most other liquids. Known exceptions are

paraffin oil and properly buffered aqueous solutions. See Poehlman (1979) for additional information or pollination practices.

C. HARVESTING

Most early strains of corn grown under optimum conditions will be ready for harvesting 6 weeks after pollination. Viable seed may be obtained after only one month. However, for well matured seed with high viability a period of 7-8 weeks may be required. Mid-season or long season lines may take even longer. Kernels of most strains reach physiological maturity in 35-60 days after pollination. Actual maturity time is affected by many factors including temperature, day length, moisture and soil condition. A safe time to harvest is when the husks have become dry and the kernel is hard.

Harvesting may be accomplished by breaking off and husking the ears and tagging them directly in the field, or by husking the ear in the field, placing it in the tassel bag, and bringing the ears into the laboratory where they may be tagged under more comfortable conditions. The ears may be labeled with a 1 x 2 in (2.5 x 5 cm) heavy paper label with a reinforced hole. The label can be attached to the ear by a wire parcel hook. After the label has been written upon and strung on the parcel hook the parcel hook may be inserted in the soft center at the base of the cob. In some stocks the cob is quite hard so a hole needs to be made with a medium size screwdriver. Ears should be tagged prior to drying since the cob is still soft enough to allow easy insertion of the parcel hook or the screwdriver. After tagging, the ears should be put away in some sort of drying facility for a few days to reduce the moisture content to 12% or less. This is important as ears stored at a high moisture content will mold or lose their viability rapidly, and are also more subject to attack by seed storage insects.

After the harvesting, tagging, and drying is completed then the ears should be laid out in consecutive order by female parent and a careful harvest record should be made. The ears may be filed away by placing them tag up in a number 10 flat bottom Kraft paper bag that is cut to 6 inches (15 cm) high. Then place these bags in consecutive order in a waterproof cardboard box (approximate dimensions 13 x 24 x 6 in, 33 x 61 x 15 cm) with close fitting cardboard lid and a label indicating the included consecutive families. To prevent insect damage about 1/4 cup (225 g) of naphthalene flakes should be sprinkled in the box before closing. Good viability may be maintained in storage by keeping the corn at comfortable room temperature as long as the humidity is kept fairly low. For long term storage it is best to keep the corn below 60°F (15°C) and as low a humidity as can be obtained by refrigeration type cooling. Under these latter conditions acceptable germination is still possible after twenty years.

D. MAINTAINING PEDIGREES

One of the most important aspects of conducting genetic research with corn, or with any other species for that matter, is the maintenance of proper pedigrees. This can be done by keeping a system of records in which every individual has an identity and an ancestry and, for selected individuals, a recorded posterity. Every individual should be identified by year, by crop, by family, family subdivision and finally by individual number. All of the information about a particular family or culture planted

can be placed upon a pedigree card which may then be used as a portable source of information and a place upon which to keep notes in the field and which can finally be filed away as a permanent record of the transactions of that culture for future reference. A sample card appears in Figure 1. We use 7 x 4 in (18 x 10 cm) sheets of heavy weight subdued blue paper (colored to reduced glare in the field). The left hand 1" margin of the sheet is punched for insertion in a ring binder notebook. The card is identified by a pedigree number, including series (crop year), family number and family subdivisions. It also contained numerical pedigrees of parents, genotypes of parents, date planted, types of seed and subdivisions planted, and handling directions; an area on the card is reserved for field notes and later notations as they are needed for this family's permanent record. As things are done with this family they can be written on the card in the appropriate places. For example we use blue ink to write the pollinations completed in the same general area as the handling directions. We also use red ink to write in the lower right hand corner of the sheet the subsequent plantings of descendants from this family as they are made in future years. On the back of the card we indicate in pencil the harvest record showing the actual ears obtained in the harvest. When followed systematically the combination of typed information and hand written notations in various colors of ink or pencil allows for a great deal of information to be stored on a single piece of paper. These sheets are eventually cut down to 4 x 6 in by trimming off the 1" margin and are filed in a standard file box. They are kept up to date as additional information about the family arises. The file boxes are kept in consecutive order and individual crops are indexed for easy access.

Following this system one can go directly to the index and then to the file for a particular genotype and can follow its pedigree back to the original source or follow the descent forward to succeeding generations and can find detailed information at any point. Every corn plant and every ear of corn has an individual identity and can be tied into this network.

The crucial step in this system is the preparation of a pedigree card at the time the seed is being prepared. Without exception when seed is being prepared for planting the pertinent information for the pedigree card should be written upon the envelope in which the seed is being placed. After the planting is made the information can be copied off the envelope onto a new pedigree card and a permanent record is initiated.

E. GREENHOUSE CULTIVATION

Corn can be grown in the greenhouse by following good cultural practice that provides conditions that fall within the crop's natural requirements. Corn grows best in a greenhouse ground bed but will produce a satisfactory crop in ten inch diameter clay pots. With careful management, it will even produce an ear in a five inch clay pot. Clay pots are far superior to plastic pots or other non-porous containers. The soil should be fertile, well drained and not too high in organic matter. Fertility can be maintained throughout the growing season in pots by mixing a "time release" fertilizer such as "Osmocote" in the soil before potting. The temperature should not drop below 65°F (19°C) nor above 90°F (32°C). The best temperatures are in the low 70's (20's C) during the night and low 80's (high 20's C) during the daytime. Higher temperatures should be accompanied by higher light intensity.

(Front)

1 P .2-1, .3-6

2 Dom. Les! .2-1

3 29:501.1-3

4 8:364.2-3 X 285-17

5 5/9, 16, 23

6 A Sh/a sh, Pr pr X a sh, pr

7 .1 5 Cl Sh
.2 5 Cl Sh pr
.3 10 cl sh

8 .3-2⊗ seg w

9 Handling directions

Cross.1 and .2 X .3 sib 3 of .2 for stock

11 .1X.3 /// .2-1 on 317
.2X.3 // .2-1⊗
.2 X Sib /// .3⊗ 1

10 6/14 4+5+8 all N sdlg

6/22 4+5+7, 6 lf stage. Several in all three plantings show unusual lesions on older leaves. .2-1 and .3-6 are best.

12 .2-2X.3-6 = 30:211
.3-1⊗ = 31:982
.3-2X.3-1 = 31:1006

(Back)

13 Harvest

.1 3 ears X .3	Cl Sh/cl sh	saved-1
.2-1 ⊗ (Les)	Cl Sh/cl sh	saved
3 ears X .3	" "	saved-2
.3 3 sibs	cl sh	saved all

14

15

Figure 1. Pedigree Card. Use light blue 40# stock printed with lines and punched to make 4" x 7 3/16" field sheets that can be cut to 4" x 6" cards for filing. Information is placed on the sheets as follows:

1. Photo record (black ink)
2. Special attention getting note
3. Family number. Series 29, family 501, subdivisions 1, 2 & 3
4. Numerical pedigree of parents
5. Planting dates
6. Genotype of parents
7. Subdivision and types of seed planted
8. Seedling progeny test (graphite pencil)
9. Handling directions
10. Field notes (pencil)
11. Pollination record (blue ink)
12. Descendants (red ink)
13. Harvest record (pencil)
14. Field Notes continued (pencil)
15. Descendants continued (red ink)

The most critical factor in growing corn in the greenhouse is light intensity. Corn requires high light intensities that are often not available in the winter time in northern latitudes. To grow a suitable crop in those locales requiring greenhouse protection in the winter one should have suitable supplemental lighting. The supplement should be enough to bring the light on the corn plants to 3000 foot candles and if this can be maintained during eight hours of sunlight and extended at 2000 foot candles for another four hours a good healthy crop may be achieved. High pressure sodium vapor lamps are a suitable source of supplemental lighting. Good results are obtained by using 400 watt lamps in rectangular fixtures suspended by adjustable chains or ropes 5 ft (1.5 m) apart in rows 8 ft (2.4 m) apart and held at a distance of 2-3 ft (.76-.91 m) above the canopy of leaves. The lamps should be controlled by automatic timer to give 12 hours of light.

Lines of corn originating in the southern latitudes of the tropics generally have a short day requirement for flowering. Lines originating in northern latitudes and the corn belt of the United States generally will flower under most day length situations.

F. Supplies and equipment-where to get them

1. Envelopes- for storage, seed separation and seed preparation
   Coin envelopes, 20 paper, 3 sizes
   5 1/2" x 3 1/8", 4 1/4" x 2 1/2", and 3 9/16" x 2 1/4"
   St. Regis Paper Co., 601 Cannonball Lane, O'Fallon, MO 63366

2. Field stakes- for planting and marking plants
   Painted white wood stakes, 3 sizes
   1" x 12" for marking families in field
   7/8" x 10" for marking plants in field
   5/8" x 4" for sand bench and pot label
   Dayton Garden Labels, 1215 Ray Street, Dayton, Ohio 45404

3. Planter- Allan Jab hand planter
   Allan Machine Co., P.O. Box 1803, 222 Duff Avenue, Ames, Iowa 50010

4. Plant tags- for marking plants in field
   Plastic wrap around tags
   Hummert Seed Co., 2746 Chouteau Ave., St. Louis, MO 63103

5. Ear shoot bags- to cover ear shoots
   Translucent white water repellent paper bag 2" x 1" x 7"
   Lawson 217- recently priced $8-$12 per thousand
   Lawson Bags, 480 Central Ave., P.O. Box 577, Northfield, IL 60093

6. Tassel bags- for covering tassel and pollinated ear
   Kraft 40 wet strength water repellent paper bag with waterproof glue
   Size 5.75" x 5.25" x 4.25" x 14" Lawson 402
   Recent price $23-$28 per thousand
   Lawson Bags, 480 Central Ave., P.O. Box 577, Northfield, IL 60093

7. Pollinating bags- for collecting and disseminating pollen
   Transparent glassine paper bag 3" x 7 1/2"
   Carpenter shoot-tector 3/75
   Recent price $10-$13 per thousand
   Carpenter Paper Co., 106-110 SW 7th St., P.O. Box 568, Des Moines, IA 50302

8. Cut back knife- for cutting ear shoots
   Old hickory knife 3 1/8" carbon steel blade
   Ontario Knife Co. USA

9. Stapler- for securing bags on plants
   Ace Clipper 702, use 700 staples
   Ace Fastener Co., Chicago, IL

10. Paper clips- for securing bags on tassels
    Acco Monarch 1 non-skid
    Acco International Inc., Chicago, IL 60630

11. Pollinators Apron- for carrying pollinating equipment
    Corn pollinators apron
    Corn States Hybrid Service, Inc., P.O. Box 2706, Des Moines, IA 50135

12. Pencils- for writing on tassel bags
    Mechanical pencil-soft thick graphite lead

13. Harvesting bags- for harvesting ears
    10, 20, and 50 pound onion sacks- misprints can often be purchased cheaply

14. Parcel hooks- for attaching tag to ear
    Parcel hook 18-505
    Dennison Manufacturing Co., Framingham, MA 01701

15. Ear tag- for labeling ears
    1" x 2" cardboard tag with reinforced hole
    Dennison Manufacturing Co., Framingham, MA 01701

16. Storage boxes- for ear storage
    Waxed cardboard (Freezur Board) boxes 12 1/2" x 23 1/2" x 5 1/2"
    Lawrence Paper Co., Lawrence, KS 66044

17. Time release fertilizer- for pots in greenhouse
    Osmocote 14-14-14
    Hummert Seed Co., 2746 Chouteau Ave., St. Louis, MO 63103

18. Lights- for supplementing lighting in greenhouse
    400W high pressure sodium vapor lamp
    Stymulyte ET-400-000
    Energy Technics, P.O. Box 3424, York, PA 17402

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4 INBREDS VS. HYBRIDS VS. RANDOM MATING POPULATIONS: CATEGORIES OF MAIZE (*ZEA MAYS* L.) AND THEIR SUITABILITY FOR CERTAIN EXPERIMENTS

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Maize is a diverse and highly variable species. When a choice of experimental material is to be made, what are the factors that should be weighed? Is there a "best choice" suited for all experimentation? Some of the following considerations ought to be borne in mind. They relate to the genetic makeup of maize strains and their expected performance and biological characteristics.

The genetic categories are, among others, *land varieties*, *composites*, *synthetics*, *inbreds*, *hybrids*, and *pedigreed stocks*. Their performance categories (in the broadest sense) are *homogeneity*, *homozygosity*, *consistency*, and *heterosis*. These two sets of categories will be defined and discussed briefly.

Genetics Categories Defined

Land varieties are naturally occurring open-pollinated populations that are or were indigenous to certain localities or even a particular farm. They were developed, for the most part, by the oldest method of plant breeding: mass selection, which simply is the retention of the best ears of the best plants from this year's crop to be used as seed for the next year's planting.

Composites are populations formed by the deliberate intercrossing and subsequent random mating of several selected composites, land varieties, synthetics, hybrids, or inbreds. Generally the composite is subjected to selection pressure for desired traits. Drift due to environmental factors may also be important. Ideally the composite is maintained by open-pollination in isolated blocks, that is, the mating scheme is determined by the wind and the coincidence of tassel and earshoot maturities, but the group of plants is located away from extraneous sources of wind borne pollen. Logically, all other types of populations had their earliest origins in composites, though naturally formed historical composites may not have been well isolated.

Synthetics are populations formed by intercrossing and random mating inbred lines. A particular synthetic population can be exactly reconstructed, since the synthetic is defined by its initial inbred line composition. Usually six or twelve inbreds are used in a synthetic's formation. Selection of the inbreds is based on the particular characteristics desired in the final synthetic.

Inbreds, or inbred lines, are derived by the self or sib pollination of successive generations of descent. *Released inbreds* may have diverse origins, but all have been subjected to intensive selection for agronomic and

plant traits and also have been critically evaluated for their performance in hybrid crosses. Many released inbreds are related by pedigree, specified in the description given at the time of release.

Hybrids, in the broadest sense, are the progeny of the mating of two non-identical parents. As a matter of commercial practice, there are several categories of hybrids:

Single cross (F1 hybrid): the cross of inbred A with inbred B.
Modified single cross: the cross of an F1 hybrid produced by crossing two related inbreds (AxA') with an unrelated inbred C.
Three-way cross: the cross of an F1 hybrid with an unrelated inbred.
Four-way cross (double cross): the cross of two different F1 hybrids, each having no inbred parent in common with the other [(AxB) X (CxD)].
Variety cross: the cross of a land variety or population with either another land variety population, an inbred line, or an F1 hybrid.

Pedigreed stocks are strains in which information on parentage and genetic constitution is maintained. Clearly, some of the above categories qualify as pedigreed stocks in certain respects. However, we may narrow the meaning to those strains developed by deliberate (hand) pollination so that the strict descent is known and is often purposeful in order to develop or reveal the expression of a specific genetic character.

Performance Categories Defined

Homogeneity is a measure of the genetic likeness of an individual to another within the population. It is the degree to which individuals have the same genotype. It is used in the collective sense of all of the genes of an individual compared to those of another individual.

Homozygosity, in diploid maize, means that at a given genetic locus, both homologs carry the same allele of the gene. Heterozygosity implies (1) that within an individual there are different alleles at the locus; or (2) that within the population there are at least two allelic forms of the gene. These terms will be used in the collective sense in consideration of all the genes of an individual or of a population of individuals.

Consistency is the expectation that one lot of seed will be like the previous lot of the same population of maize.

Heterosis is an operational definition: the ratio of the improved physiological performance of the progeny in comparison to that of the better parent, or alternatively, in comparison to the average, or midparent, performance.

Interpretation of Genetic and Performance Categories

Inbreds display high degrees of homogeneity, homozygosity, and consistency, but lack heterosis. Because of successive generations of sibbing and selfing, coupled with continuance of relatively few individuals to the next generation, a line of descent becomes rapidly fixed (gene frequency = allele frequency = 1.0) for many genes.

The degree to which the whole genome becomes homozygous is related to the effective number of generations of selfing or sibbing of a single seed line of descent during the development of that inbred. Seven generations of selfing, regarded as sufficient inbreeding, theoretically brings the genome to 99.22% homozygosity, on the starting assumption that the original individual was completely heterozygous (gene frequency = 0.5 at every locus) throughout its genome. Since no individual can be completely heterozygous, the above figure is an underestimate. At the molecular level, however, because of residual heterozygosity and ongoing spontaneous mutation, one should mentally calculate the effect and the importance of the residual heterozygosity and the likelihood that it will lead to ambiguous results.

Logically, a high degree of inbred homozygosity dictates a high degree of homogeneity and consistency. Since the basis of heterosis has been hypothesized to be heterozygosity for dominant, favorable genes, the rule of thumb is that highly homozygous maize plants are not heterotic.

The relations of the genetic and performance categories are given in Table 1.

Table 1.

Category	Homogeneity	Homozygosity	Consistency	Heterosis
Inbreds	yes	yes	yes	no
Single cross hybrids (F1)	yes	no	yes	yes
Modified single cross hybrids	no	no	yes	yes
Three-way cross hybrids	no	no	yes	yes
Four-way cross (double cross) hybrids	no	no	yes	yes
Synthetics	no	no	some	yes
Land varieties	no	no	no	yes
Composites	no	no	no	yes
Pedigreed stocks	yes/no	yes/no	yes/no	yes/no

Two notes: (1) Inbred lines vary considerably in their general vigor. Recently released inbreds tend to be vigorous and fertile plants. Hybrids have improved over the last fifty years in part because the inbred parental lines themselves have been significantly improved. Nonetheless, some inbred lines are close to being weaklings and others are difficult to maintain due to their lack of self fertility or to poor synchronization of flowering (male vs.

female). (2) For reasons not understood and mechanisms completely unknown, heterozygosity persists after intensive and lengthy inbreeding. Some inbred lines are simply "unstable" and require continual rogueing (rooting out before sexual maturity of spontaneously appearing off-type plants), in order to maintain phenotypic uniformity.

Single-cross hybrids are not homozygous, but are homogeneous, consistent, and heterotic. One generally considers a hybrid individual to be heterozygous, because different inbred parents differ in being homozygous for different alleles--over the whole genome. The more unrelated the genetic origins of the particular inbred parents, the more the expected heterozygosity that will exist in the hybrid progeny. In any hybrid, however, much of the genome will be homozygous, because of the allelic commonality between the parents. The hybrid population is homogeneous and consistent because all of the individuals are identically heterozygous for the same set of genes, and at the same time identically homozygous for all of the rest of their genes.

Modified single-cross hybrids offer a technical advantage in hybrid seed production while remaining close to the characteristics of single-cross hybrids. Three-way and four-way hybrids probably maintain about the same degree of heterozygosity as a single cross, but definitely introduce more heterogeneity. They are consistent in their lack of homogeneity.

Not every possible combination of inbred lines will produce a significantly heterotic hybrid. However, one can be confident that virtually every commercial hybrid displays impressive heterosis. Three-way and four-way crosses make up less than 15% of current hybrid seed production (Zuber and Darrah, 1980).

Variety cross hybrids are likely to be encountered in breeder's materials or in materials originating in areas of the world that are developing their national breeding programs.

Synthetics are not homogeneous or homozygous but are heterotic. The exceptional notation "some" for consistency in Table 1 emphasizes that synthetics are the next step away from four-way crosses in descending degree of consistency. The limited genetic basis of synthetics delimits the maximum heterozygosity at any locus for the population.

Land varieties and composites are similar in their performance categories. They are not homogenous, homozygous, or consistent, but are heterotic. One ought to think of these two types of populations as differing mainly in degree. Both land varieties and composites will show uniformity for some traits (in the agronomic sense). Any population that has been subjected to selection (pressure) will move toward phenotypic uniformity for a desired trait(s). However, uniformity here refers to appearance to the eye. At the genetic level, uniformity may not necessarily be accounted for by homogeneity, homozygosity, or consistency. This is because most traits or gross phenotypes are the result of an interaction of the plant's genotype with the environment (in the broadest sense). Most specific traits are the result of the quantitative action of many independent and dependent genes. Each gene has a small, usually algebraically additive, contribution toward the level of phenotypic expression of a trait or character. This is further blurred by the vagaries of day to day existence. Therefore it is possible

for many different unique genotypes to have the same net phenotype. (See Kacser and Burns, 1981, for a nice presentation for the biochemically or mathematically oriented.)

Land varieties have been subjected to directional selection for certain agronomic characters more intensively and longer than have composites. Both are a complex mixture of genotypes. The individuals of the population are heterozygous and heterogeneous--genetically. Nonetheless, outstanding land varieties gained their reputations because they were uniform in appearance and quality while also maintaining a high level of productive vigor. Composites are maintained these days to improve the germplasm from which new land varieties and inbred lines will be developed.

Pedigreed stocks are on the face of it unpredictable for any of the performance categories. The value of pedigreed materials is in the control of important variables, explicit or unexpected.

Other Considerations

Supplies of inbred lines and hybrids in quantity include organizations such as Clyde Black and Son, Inc. (Ames, IA), Mike Brayton Seeds, Inc. (Ames, IA), Callahan Enterprises, Inc. (Westfield, IN), Holden Foundation Seed, Inc. (Williamsburg, IA), Illinois Foundation Seeds (Champaign, IL), and the Agricultural Alumni Seed Improvement Association (Romney, IN) [Mention of a supplier does not constitute a guarantee or warranty by the U. S. Department of Agriculture and does not imply approval to the exclusion of other suppliers that also may be suitable]. Seed sold by commercial suppliers normally has the advantage of being more uniform and clean than "home-made" materials.

Many land varieties have been collected and preserved at the USDA Plant Introduction Stations and at the International Maize and Wheat Improvement Center (CIMMYT) in Mexico. A few special exotics and many marker stocks are available from the Maize Genetics Cooperation Stock Center (University of Illinois/Urbana). These are non-commercial sources which supply very small quantities of seed in the expectation that the requester will assume responsibility of increasing the seed himself. Thus, these are really sources of "starter" seed. They do not supply enough seed for laboratory use.

The cost of pedigreed materials is the availability of a "geneticist" to construct them, the facilities to do that, and the length of time to arrive at a satisfactory level of genetic identity. For some research objectives strictly pedigreed materials are altogether unnecessary. Other objectives cannot be achieved without them. Conferring with a geneticist conversant with your specialty is the best way to determine the nature, usefulness, availability, and feasibility of pedigreed stocks to suit your purpose.

Careful pedigree work with maize entails sufficient long term planning, mental effort and oversight so that it is an important part of the design and experimentation input in well planned studies that involve unitary genetic factors and their interactions. For experiments with quantitatively inherited characters, proper design and analysis require highly developed statistical-genetic methods that are the forte of maize breeding laboratories.

A final note: Please use identified materials, faithfully specified in "Materials and Methods", for all published results. This is the starting point of judging the significance of a discrepancy between the results of two workers: were the experimental materials genetically alike, similar, or different? More importantly, the validity of projecting a specific result to the general case is supported if the particular materials are specified and the result can be repeated with other materials.

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5 MAPS, MARKERS AND STOCKS

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It is the extensive genetic and cytogenetic data, including both mapped and unmapped loci, along with the availability of a rich and extensive collection of genetic markers and cytogenetic stocks that, together with its favorable biological traits, results in maize being the flowering plant of choice for molecular studies. These features, of course, also make it well suited for cellular and developmental studies.

Many individuals have contributed to the extensive amount of information presently available on the genetics and cytogenetics of maize. However, one person, R.A. Emerson, stands out in this regard. First at Nebraska and then for the remainder of his career at Cornell, Emerson assiduously searched for and collected variant forms of maize. By his example and through the encouragement of his co-workers he led in the identification of the ten linkage groups of maize and the mapping of individual genes in each group.

Emerson was fortunate to have associated with him at Cornell during the few years spanning the latter 1920's and the early 1930's, four workers who each played a major role in the development of maize genetics. These were George W. Beadle, Charles R. Burnham, Barbara McClintock and Marcus M. Rhoades. The years they spent with Emerson at Cornell marked the "Golden Age of Maize Genetics." The article by Burnham in this volume describes the establishment of the linkage groups of maize in which he and McClintock played a major role at Cornell. By 1935, the mapping had proceeded to such a degree that a landmark report was published as the Cornell University Agricultural Experiment Station Memoir 180, "A summary of linkage studies in maize," by R.A. Emerson, G.W. Beadle, and A.C. Frazer. The recombination values for this report were recalculated by Charles Burnham (personal communication).

A linkage map of maize was prepared by Marcus Rhoades from the data compiled by Emerson, Beadle, and Fraser (1935) and it appears as Figure 1 in their report. (It is reproduced in this article.) There are 59 gene loci on this linkage map; however, centromere locations are not shown. The orientation of all of the chromosomes except for chromosome three is like that of all subsequent maps with the short arm at the top and the long arm at the bottom. This map included five of the loci controlling anthocyanin synthesis in the aleurone layer of the kernel (a1, a2, pr1, C, and R) as well as the B locus, Y, sh and wx.

During the following 15 years additional loci were identified and located so that when Rhoades (1950) published a revised map, 89 loci were located, including bronze 1 (reprinted in this article). The approximate centromere locations for chromosomes three, five and seven were also indicated. Subsequently Neuffer, Jones, and Zuber (1968) published a linkage map containing 159 gene loci, including the two additional loci involved in anthocyanin synthesis in the aleurone, bz2 and c2. This map was revised to

show the approximate location of all of the centromeres and the approximate location of the break points for some of the B-A translocations (Coe and Neuffer, 1977). This map is reproduced in this article and includes the names of the gene loci.

Recently the linkage map has been revised, the gene loci have been deleted for those mutants that are no longer available, and some additional loci have been added; this map containing 170 loci, an accompanying description of each phenotype, and references to the original descriptions and designations will soon be published (Coe, Hoisington, and Neuffer, 1982).

The cytological studies of Barbara McClintock (1959, 1960) as well as those of A.E. Longley and T.A. Kato (1965) led to the development of the cytological map of maize (Neuffer, Jones and Zuber, 1968); (see copy in this article). The distribution of chromosome knobs is characteristic of different races of maize (Brown and Anderson, 1947; McClintock, 1959, 1960, 1978; Longley and Kato, 1965) and the patterns of knob distribution are believed to be of value in the study of the origin and relationships of the various races of maize (for reviews see Brown and Goodman, 1977; McClintock, 1978, and the article on races of maize by Bird in this volume). Recently, a long awaited book has been published by McClintock, Kato, and Blumenschein (1981) describing extensive observations on the chromosome constitution of races of maize and teosinte.

The detailed and extensive studies on maize chromosomes have been possible because of the development by Barbara McClintock in the late 1920's of the technique for staining pachytene chromosomes with acetocarmine (see article by Burnham on personal recollections in this volume). An example of a carmine stained and well spread pachytene preparation with the centromere regions labeled and short and long arms designated is included in this article immediately following the cytological map.

The extensive work by E. G. Anderson et al. (1949) and A. E. Longley (1961) resulted in the accumulation of about 800 cytogenetic stocks of maize consisting of different reciprocal translocations between the A chromosomes. These stocks and their breakpoints are listed in the 1981 Maize Genetics Cooperation News Letter (55:140-145) and their use in the location and mapping of genes is described by Patterson in his article in this volume. These stocks can be obtained from the Maize Genetics Stock Center at the University of Illinois (see below).

For many years genetic marker stocks have been developed by maize geneticists and these have been shared in a spirit of cooperation and generosity that is a legacy of R.A. Emerson. This cooperation has been greatly aided over the years by the existence of the Maize Genetics Stock Center, first at Cornell University and subsequently at the University of Illinois. Presently several thousand stocks are maintained at the Stock Center and small samples are available upon request. Those stocks currently available are listed in the following pages of this article and each year's current list of available stocks is published in that year's volume of the Maize Genetics Cooperation News Letter. Its editor is: Dr. E.H. Coe, Jr., Curtis Hall, University of Missouri, Columbia, MO 65211.

For linkage studies, mapping, and allelism tests, as well as other purposes, these genetic stocks are of considerable value. In the accompanying

lists of stocks the gene symbols are used. The full names are spelled out for most of these symbols on the accompanying linkage map and additional details of the phenotypes and other information on these markers are available in Neuffer, Jones and Zuber (1968), Coe and Neuffer (1977), and Coe, Hoisington and Neuffer (1982).

For mapping work a recommended set of marker stocks for 19 of the 20 chromosome arms (all except 8S) is included following the list of available stocks. This set of marker stocks is an ideal set, based on markers that are known and available. However, the particular combination of markers for a given arm may not be available from the Stock Center, in which case the most preferred set of markers available is also shown. It should be kept in mind that the most convenient markers are those for endosperm traits, and next are seedling traits followed by mature plant traits including pollen; least convenient are maternal traits such as pericarp or cob color.

Small quantities of the genetic marker and cytogenetic stocks are available from the Stock Center. Those individuals requesting a small starter sample are expected to propagate and maintain the stock for their own use and should not expect to repeatedly request a particular stock be sent to them. In addition it will be helpful if samples of new useful combinations and new markers are sent to the Stock Center. Requests for stocks should be addressed to:

Director of Maize Genetics Stock Center
Department of Agronomy
University of Illinois
1102 South Goodwin
Urbana, IL 61801

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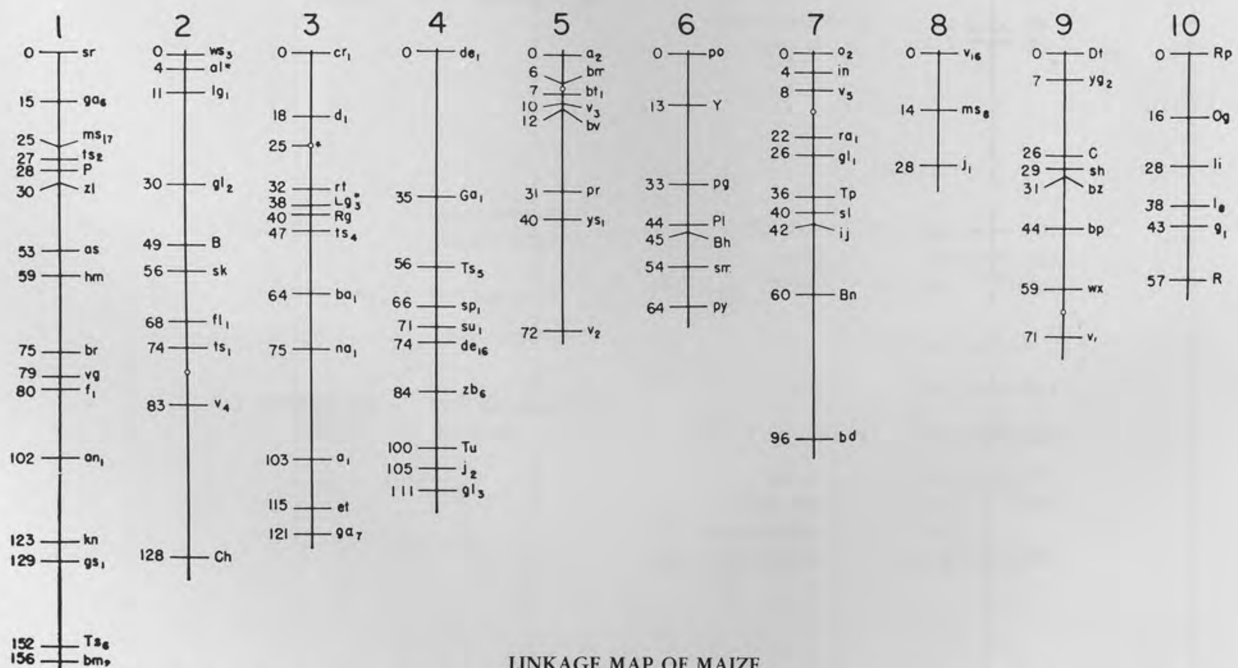
Linkage map of the ten chromosomes of Zea Maps showing the Loci of those genes whose position can be determined with reasonable certainty
(Prepared by Dr. M. M. Rhoades from data presented in this summary)

Reprinted with permission of M. M. Rhoades from Emerson, R. A., G. W. Beadle, and A. C. Fraser. 1935. Cornell Univ. Agr. Exp. Sta. Memoir 180.



LINKAGE MAP OF MAIZE

The genes in linkage group 1 are carried by chromosome 1, etc. The centromere regions whose map location has been established with some certainty are represented by circles. The zero ends of the maps are in the short arms of each chromosome. The location of those genes marked with asterisks is only approximate.

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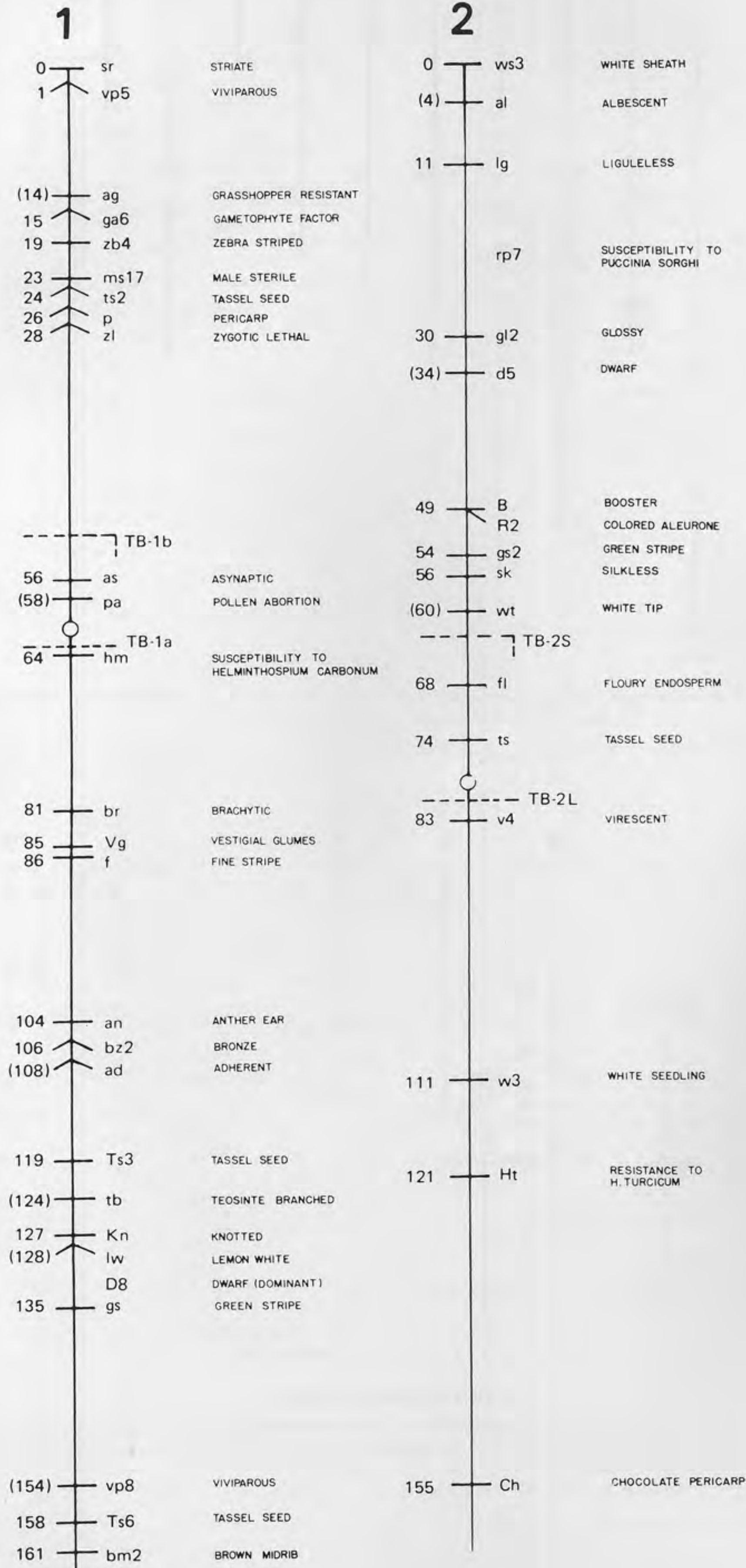
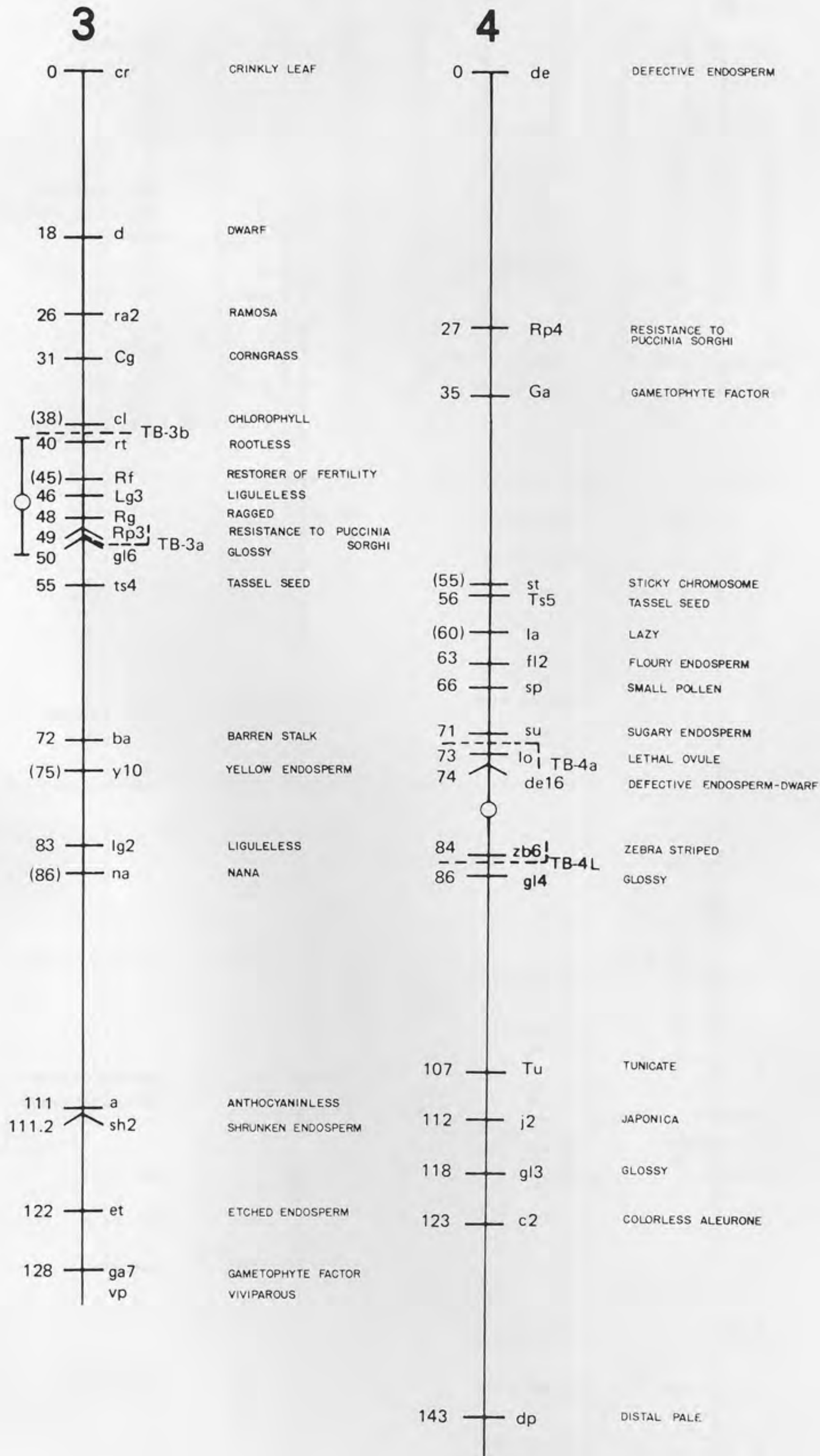
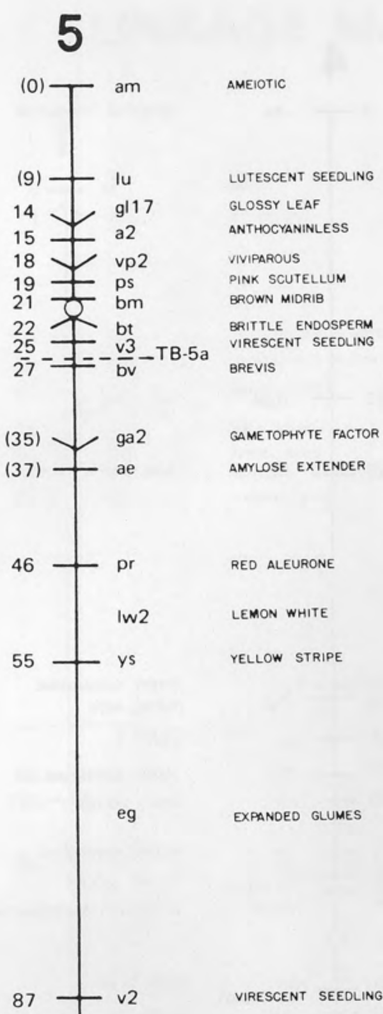
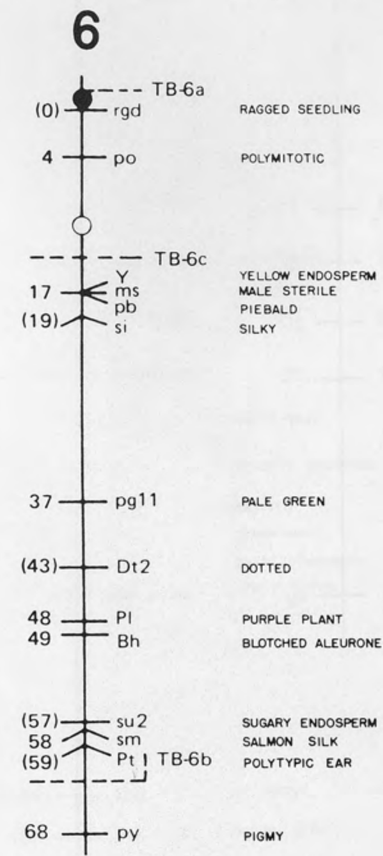
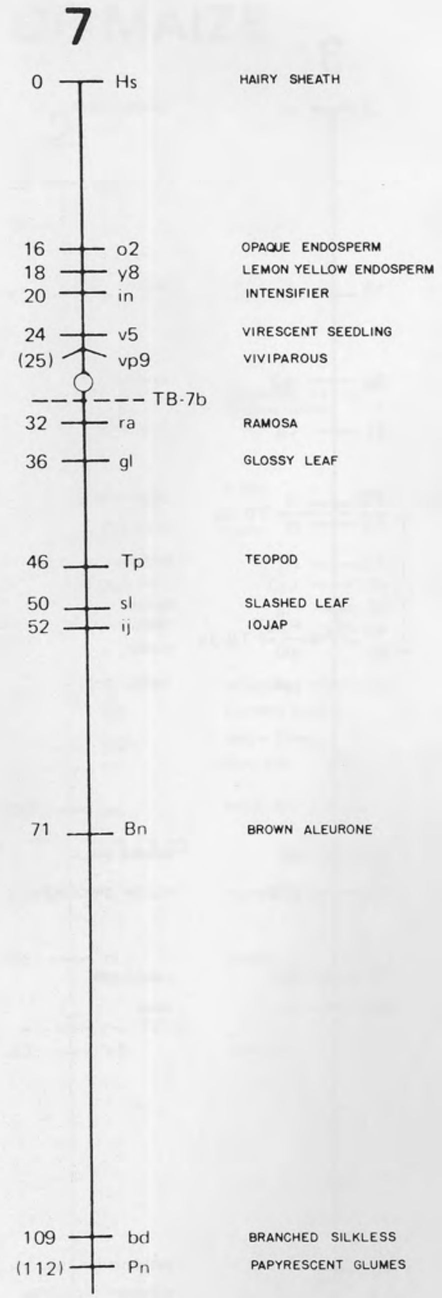
LINKAGE MAP OF MAIZE

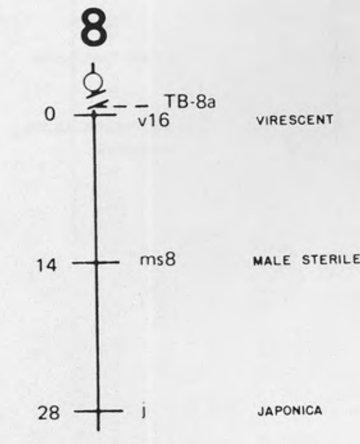


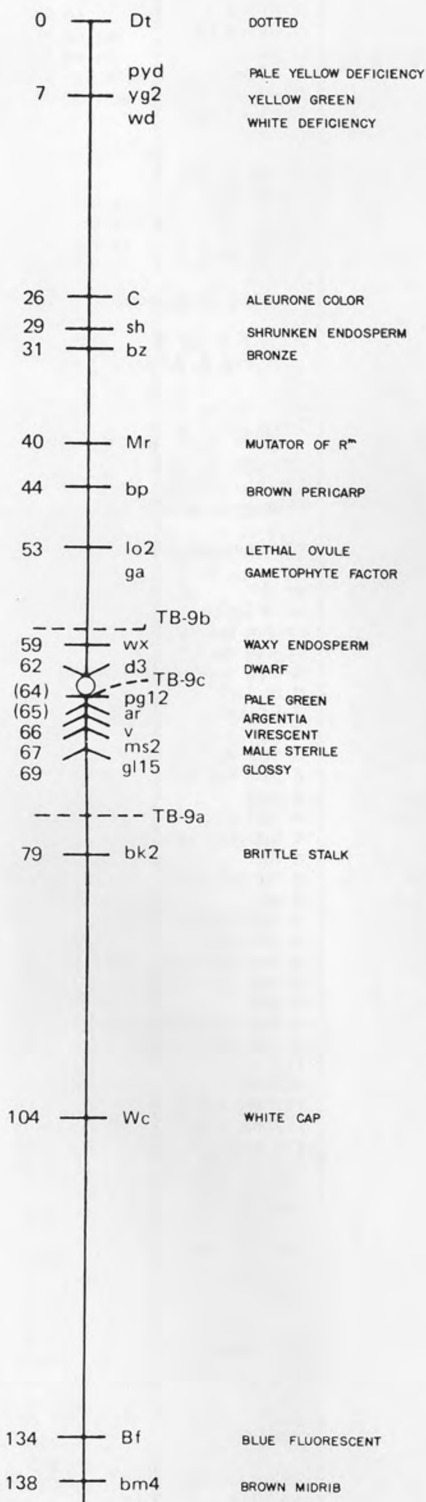

3
0 cr CRINKLY LEAF
18 d DWARF
26 ra2 RAMOSA
31 Cg CORNGRASS
(38) cl CHLOROPHYLL
TB-3b
40 rt ROOTLESS
(45) Rf RESTORER OF FERTILITY
46 Lg3 LIGULELESS
48 Rg RAGGED
49 Rp3 RESISTANCE TO PUCCINIA SORGHI
TB-3a
50 gl6 GLOSSY
55 ts4 TASSEL SEED
72 ba BARREN STALK
(75) y10 YELLOW ENDOSPERM
83 lg2 LIGULELESS
(86) na NANA
111 a ANTHOCYANINLESS
111.2 sh2 SHRUNKEN ENDOSPERM
122 et ETCHED ENDOSPERM
128 ga7 GAMETOPHYTE FACTOR
vp VIVIPAROUS
4
0 de DEFECTIVE ENDOSPERM
27 Rp4 RESISTANCE TO PUCCINIA SORGHI
35 Ga GAMETOPHYTE FACTOR
(55) st STICKY CHROMOSOME
56 Ts5 TASSEL SEED
(60) la LAZY
63 fl2 FLOURY ENDOSPERM
66 sp SMALL POLLEN
71 su SUGARY ENDOSPERM
73 lo LETHAL OVULE
TB-4a
74 de16 DEFECTIVE ENDOSPERM-DWARF
84 zb6 ZEBRA STRIPED
TB-4L
86 gl4 GLOSSY
107 Tu TUNICATE
112 j2 JAPONICA
118 gl3 GLOSSY
123 c2 COLORLESS ALEURONE
143 dp DISTAL PALE

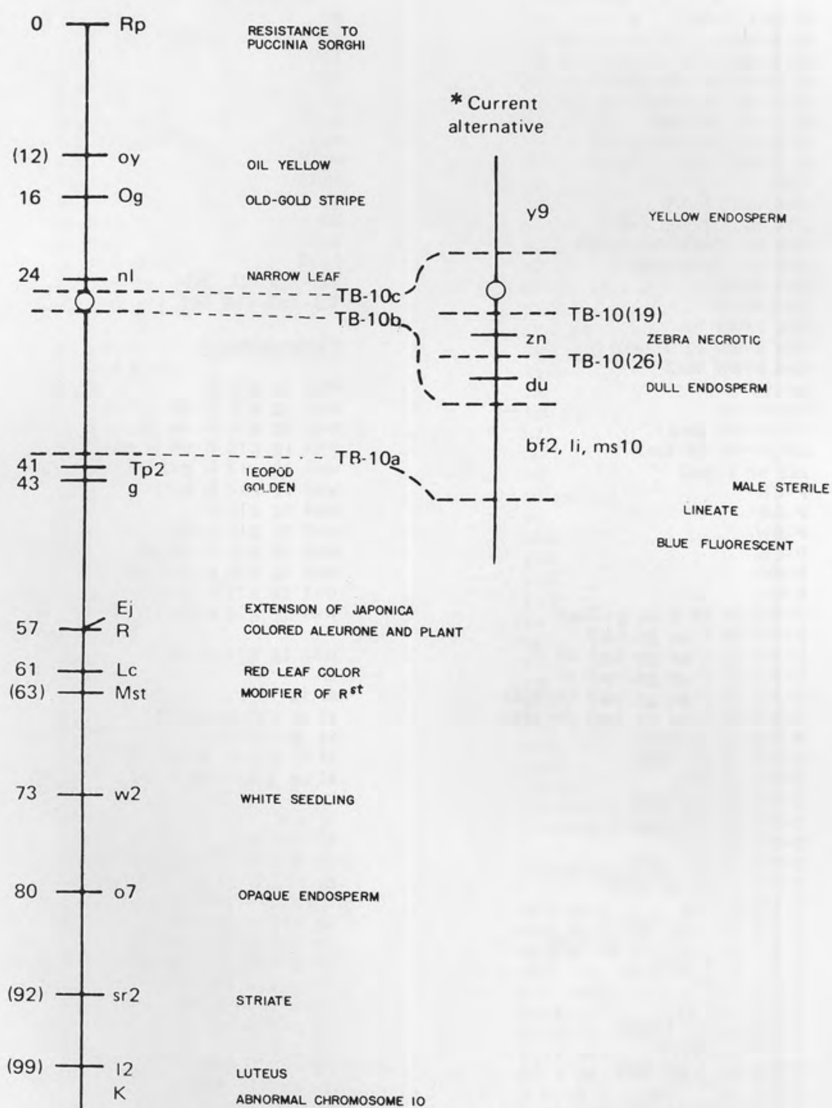

5
(0) am AMEIOTIC
(9) lu LUTESCENT SEEDLING
14 gl17 GLOSSY LEAF
15 a2 ANTHOCYANINLESS
18 vp2 VIVIPAROUS
19 ps PINK SCUTELLUM
21 bm BROWN MIDRIB
22 bt BRITTLE ENDOSPERM
25 v3 VIRESCENT SEEDLING
TB-5a
27 bv BREVIS
(35) ga2 GAMETOPHYTE FACTOR
(37) ae AMYLOSE EXTENDER
46 pr RED ALEURONE
lw2 LEMON WHITE
55 ys YELLOW STRIPE
eg EXPANDED GLUMES
87 v2 VIRESCENT SEEDLING


6
TB-6a
(0) rgd RAGGED SEEDLING
4 po POLYMITOTIC
TB-6c
Y YELLOW ENDOSPERM
17 ms MALE STERILE
pb PIEBALD
(19) si SILKY
37 pg11 PALE GREEN
(43) Dt2 DOTTED
48 Pl PURPLE PLANT
49 Bh BLOTCHED ALEURONE
(57) su2 SUGARY ENDOSPERM
58 sm SALMON SILK
(59) Pt TB-6b POLYTYPIC EAR
68 py PIGMY


7
0 Hs HAIRY SHEATH
16 o2 OPAQUE ENDOSPERM
18 y8 LEMON YELLOW ENDOSPERM
20 in INTENSIFIER
24 v5 VIRESCENT SEEDLING
(25) vp9 VIVIPAROUS
TB-7b
32 ra RAMOSA
36 gl GLOSSY LEAF
46 Tp TEOPOD
50 sl SLASHED LEAF
52 ij IOJAP
71 Bn BROWN ALEURONE
109 bd BRANCHED SILKLESS
(112) Pn PAPYRESCENT GLUMES


8
TB-8a
0 v16 VIRESCENT
14 ms8 MALE STERILE
28 j JAPONICA





( ) Indicates probable position, based on insufficient data

O Indicates centromere position

● Indicates organizer (NOR)

TB Indicates translocation involving A and B chromosomes, with A break point at broken line or in direction indicated. TB-2S, TB-2L and TB-4L are short designations for TB-2S,3L(6270), TB-2L,1S(4464) and TB-4L,9S(6222) from Rakha and Robertson (1970). TB-10(19) and TB-10(26) are as designated by Lin (1974).

* Lin (1974); Beckett (Personal communication)

After Coe and Neuffer (1977)

Chromosome 1

sr zb4 P-WW
sr P-WR
sr P-WW
sr P-WR an gs bm2
sr P-WR an bm2
sr P-RR an bm2
sr P-RR gs bm2
sr P-WR bm2
vp5
zb4 ms17 P-WW
zb4 ms17 P-WW rs2
zb4 ts2 P-WW br f bm2
zb4 ts2 P-WW bm2
zb4 P-WW
zb4 P-WR
zb4 P-WW br
zb4 P-WW br f bm2
zb4 P-WW bm2
ms17
ts2 P-RR
ts2 P-WW bm2
ts2 P-WW br bm2
ts2 br f bm2
P-CR
P-RR
P-RW
P-CW
P-MO
P-VV
P-RR as br f an gs bm2
P-RR br f an gs bm2
P-RR br f an gs bm2 rd
P-RR br f an gs bm2 id
P-RR br f an gs bm2 v*-8983
P-RR br f an gs bm2 v*-8943
P-RR an ad bm2
P-RR an gs bm2
P-RR ad bm2
P-WR an Kn bm2
P-WR an ad bm2
P-WR an bm2
P-WR an br bm2
P-WT = WR an bm2
P-WR br Vg
P-WR br f gs bm2
P-WR br f an lw gs bm2
P-Wr br f bm2 id
P-WW rs2
P-WW rs2 br f
P-WW as br f bm2
P-WW hm br f
P-WW br f ad bm2
P-WW br f bm2
P-WW br f an gs bm2
P-WW br Vg
as
as br2
as rs2
rd Hy
br f
br f bm2 v*-5588
br f Kn
br f Kn Ts6
br f Kn bm2
br bm2
Vg
Vg an bm2
Vg br2 bm2
v22
bz2 m ; A A2 C Pr
bz2 M ; A A2 C R Pr
bz2 ad bm2 ACR
an bm2
an-bz2-6923 (apparent deficiency including an and bz2)
br2
br2 bm2
br2 an bm 2
tb-8963
Kn
Kn Ts6
Kn bm2
lw
Adh1-S
vp8

Chromosome 1 (continued)

gs
gs bm2
Ts6
bm2
id
nec2
ms9
ms12
ms14
mi
D8
L1s
Les2
TB-1La (1L.20)
TB-1SB (1S.05)

Chromosome 2

ws3 lg g12 B
ws3 lg g12 B sk
ws3 lg g12 B sk v4
ws3 lg g12 B sk fl v4
ws3 lg g12 B gs2 v4
ws3 lg g12 B ts
ws3 lg g12 b
ws3 lg g12 b sk
ws3 lg g12 b sk v4
ws3 lg g12 b gs2 v4
ws3 lg g12 b fl v4
ws3 lg g12 b sk fl v4

ws3 lg g12 b v4
al
al lg
al lg g12 B sk v4
al lg g12 b
al lg g12 b sk v4
al lg g12 b sk fl v4
lg
lg g12
lg g12 B
lg g12 B gl11
lg g12 B gs
lg g12 B gs2 v4
lg g12 B gs2 Ch
lg g12 B gs2 sk Ch
lg g12 B gs2 sk v4
lg g12 B sk
lg g12 B sk v4
lg g12 B v4
lg g12 b
lg g12 b gs2
lg g12 b gs2 Ch
lg g12 b gs2 sk Ch
lg g12 b gs2 v4
lg g12 b gs2 v4 Ch
lg g12 b gs2 sk v4 Ch
lg g12 b sk
lg g12 b sk fl
lg g12 b sk fl v4
lg g12 b sk v4
lg g12 b wt v4
lg g12 b fl
lg g12 b fl v4
lg g12 b fl v4 Ch
lg g12 b v4
lg g12 b v4 Ch
lg g12 mn v4
lg g12 wt
lg g12 b gs2 wt
lg g12 w3
lg g12 w3 Ch
lg g12 Ch
lg b gs2 v4
lg Ch
g12
d5 = d*-037-9
B gl11
B ts
gl14
gl11
wt
mn
fl
fl v4 Ch

Chromosome 2 (continued)

fl Ht v4
fl Ht v4 Ch
fl w3
fl v4 w3
fl w3 Ch
fl v4 w3 Ch
ts
v4
v4 w3 Ht Ch
v4 Ht Ch
w3
w3 Ht
w3 Ht Ch
w3 Ch
Ht_2(A & B source)
ba
R2 ; r A A2 C
r2 ; r-g A A2 C
Ch
gs2
Les 2 2^{Trip} Tri^2 /ws3 lg g12
TB-1Sb-2L4464
TB-3La-2S6270
Primary trisomic 2

Chromosome 3

cr
cr d
cr d Lg3
cr pm ts4 lg2
cr ts4 na
d-Tall = d*-6016 (short)
d rt Lg3
d Rf lg2
d ys 3
d ys3 Rg
d ys3 Rg lg2
d Lg3
d Lg3 gl6
d Lg3 ts4 lg2
d Rg
d Rg ts4 lg2
d pm
d yg*-(W23)
d ts4 lg2
d ts4 lg2 a-m ; A2 C R Dt
d ts4
d gl6
d lg2 a-m A2 C R Dt
d a-m A2 C R Dt
ra2
ra2 Rg
ra2 Rg ts4 lg2
ra2 Rg gl6
ra2 ys3 Lg3 Rg
ra2 ys3 Rg
ra2 Rg lg2
ra2 pm lg2
ra2 ts4
ra2 ts4 lg2
ra2 lg2
Cg
cl
cl ; Clm-2
cl ; Clm-3
cl-p ; Clm-4
rt
ys3
ys3 Lg3

ys3 gl6 lg2 a-m et ; A2 C R Dt
ys3 ts4
ys3 ts4 lg2
Lg3
Lg3 Rg pm
gl6
gl6 lg2 A ; A2 C R
gl6 lg2 A-b et ; A2 C R Dt
gl6 lg2 a-m et ; A2 C R Dt

Chromosome 3 (continued)

pm lg2
ts4
ts4 na
ts4 na pm
ts4 ba na
ts4 lg2 a-m ; A2 C R Dt
ts4 na a-m ; A2 C R Dt
ig
ba
y10
lg2
lg2 A-b et ; A2 C R Dt
lg2 a-m sh2 et ; A2 C R Dt
lg2 a-m et ; A2 C R dt
lg2 a-m et ; A2 C R Dt
lg2 a-st sh2 et ; A2 C R Dt
lg2 a-st et ; A2 C R Dt
na
na lg2
A sh2 ; A2 C R B Pl dt
A-d31 ; A2 C R
A-d31 ; A2 C R pr dt
A-d31 ; A2 C R B Pl dt
A-d31 ; A2 C R Dt
A-d31 ; A2 C R pr Dt
A-d31 sh2 ; A2 C R B Pl dt
A-d31 sh2 ; A2 C R Dt
A-d31 sh2 ; A2 C R B Pl Dt
A-d31 et ; A2 C R Dt
a-m ; A2 C R B Pl dt
a-m ; A2 C R Dt
a-m ; A2 C R B Pl Dt
a-m sh2 ; A2 C R B Pl dt
a-m sh2 ; A2 C R B Pl Dt
A-m et ; A2 C R Dt
a-st ; A2 C R Dt
a-st sh2 ; A2 C R Dt
a-st sh2 A2 C R B Pl Dt
a-st sh2 et ; A2 C R dt
a-st et ; A2 C R Dt
a-p sh2 et ; A2 C R B Pl Dt
a-p et ; A2 C R dt
a-p et ; A2 C R B Pl Dt
a-x1
a-x3
a Ga7 ; A2 C R
sh2
vp
Rp3
pg14
a3
g5
te
h
yel*-5787
TB-3La (3L.10)
TB-3Sb (3S.50)
TB-3Lc (distal to 3La (3L.10)
Primary Trisomic 3

Chromosome 4

Rp4
Ga
Ga su
Ga-S
Ga-S ; y
Ga-S ; A A2 C R
st
st Ts5
st fl2
Ts5
Ts5 fl2
Ts5 su
Ts5 la su gl3
Ts5 su zb6
Ts5 su zb6 o
Ts5 su gl3 o
Ts5 Tu
la
la su Tu gl3
la su gl3

Chromosome 4 (continued)

la su gl3 c2 ; A A2 C B
la su gl3 o
la su bt2 gl3
fl2
fl2 su
fl2 bt2
fl2 su bm3
fl2 su gl4 Tu
su
su-am
su bt2 gl4
su bm3
su zb6
su zb6 bt2
su zb6 Tu
su zb6 gl3 dp
su gl4 j2
su gl4 o
su gl4 o Tu
su j2
su gl3
su gl3 o
su o
su gl4
bt2
bm3
gl4
gl4 o
Tu
Tu-1 1st
Tu-1 2nd
Tu-d
Tu-md
Tu gl3
j2
j2 c2 ; A A2 C R
j2 C2 ; A A2 C R
j2 gl3
v8
gl3
gl3 o
gl3 dp
c2 ; A A2 C R
C2 ; A A2 C R
c2-Idf (Active-1) ; A A2 C R
dp
o
v17
v23
ra3
Dt4 su ; a-m A2 C R
TB-4Sa (4S.20)
TB-1La-4L4692
TB9Sb-4L6504 (9S.40-.83: 4L.09)
TB7Lb-4L4698 (7L.30-.74; 4L.08)
Primary Trisomic 4

Chromosome 5

am a2 ; A A2 C R
lu
lu sh4
ms13
gl17
gl17 A2 pr ; A C R
gl17 a2 ; A C R
gl17 a2 bt ; A C R
gl17 a2 bt v2 ; A C R
A2 vp7 pr ; A C R
A2 bm bt pr ys ; A C R
A2 bm pr ; A C R
A2 bm pr ys ; A C R
A2 bm pr ys eg ; A C R
A2 bm pr v2 ; A C R
A2 bt v3 pr ; A C R
A2 bt pr ; A C R
A2 bt pr ys ; in A C R
A2 v3 pr ; A C R
A2 pr ; A C R
A2 pr v2 ; A C R
A2 pr na2 ; A C R
A2 pr ys ; A C R
A2 pr zb3 ; A C R

Chromosome 5 (continued)

A2 pr v12 ; A C R
a2 ; A C R
a2 bm bt bv pr ; A C R
a2 bm bt pr ; A C R
a2 bm bt pr ys ; A C R
A2 bm pr v2 ; A C R
A2 v3 pr ; A C R
a2 bt v3 pr ; A C R
a2 bt v3 PR ; A C R
a2 bt pr ; A C R
a2 bt v2 ; A C R
a2 v3 pr ; A C R
a2 pr ; A C R
a2 pr ; A C R B Pl
a2 pr v2 ; A C R
vp2.
vp2 pr
vp2 gl8
vp7
bm
bm yg
bt
ms5
v3
td ae
ae
sh4
gl8
na2
lw2
ys
eg
v2
yg
ms13
v12
br3
nec3
TB-5La
TB-5Lb
Primary Trisomic 5

Chromosome 6

rgd po y
rgd po Y
rgd y
rgd Y
po = ms6
po y pl
po y Pl
po y wi
po Y pl
y = pb = w-m
y rhm
y l10
y l11
y l12
y W15
y pb4
y pb4 pl
y pb4 Pl
y si
y wi Pl
Y Dt2 ; a-m A2 C R
y pg11 ; Wx pg12
y pg11 wi ; wx pg12
Y pg11 ; Wx pg12
y pg11 ; wx pg12
Y pg11 ; wx pg12
y pg11 su2 ; wx pg12
y pl
y Pl
y Pl Bh ; c sh wx A A2 R
y pl Bh ; c sh wx A A2 R
y su2
Y l10
Y l12
Y pb4
Y wi pl
Y wi Pl
Y su2
wi

Chromosome 6 (continued)

Pl Dt2 ; a-m A2 C R
pl sm ; P-RR
Pl sm ; P-RR
Pl sm py ; P-RR
Pl sm Pt py ; P-RR
Pt
w
w14
ms6
2NOR ; a2 bm pr v2
TB-6Lb
Primary Trisomic 6

Chromosome 7

Hs o2 v5 ra gl
In-D
In-D gl
o2
o2 v5
o2 v5 ra gl
o2 v5 ra gl sl
o2 v5 ra gl Tp
o2 v5 ra gl ij
o2 v5 gl
o2 v5 ms7
o2 ra gl ij
o2 ra gl sl
o2 gl
o2 gl sl
o2 ij
o2 bd
y8 v5 gl
in ; A2 pr A C R
in gl ; A2 pr A C R
v5
vp9
vp9 gl
ra
ra gl ij bd
gl
gl-M
gl Tp
gl o5
gl mn2
Tp
ij
ms7
ms7 gl Tp
Bn
bd
Pn
o5
o5 mn2 gl
va
Dt3 ; a-m A2 C R
V*-8647
yel*-7748
TB-7Lb (7L.30)
Primary trisomic 7

Chromosome 8
gl18
v16
v16 j
v16 ms8 j
v16 ms8 j nec
v16 ms8 j gl18
ms8
nec
v21
f13
f13 j
TB-8La (8L.70)
Primary Trisomic 8

Chromosome 9

yg2 C Bz Wx ; A A2 R
yg2 C sh bz ; A A2 R
yg2 C sh bz wx ; A A2 R
yg2 C-I sh bz wx ; A A2 R

Chromosome 9 (continued)

yg2 C bz wx ; A A2 R
yg2 c sh bz wx ; A A2 R
yg2 c sh wx ; A A2 R
yg2 c sh wx gl15 ; A A2 R
yg2 c sh wx gl15 K-S9; A A2 R-g
yg2 c bz wx ; A A2 R
wd-Ring C-I ; A A2 R (temp. out of seed)
C sh bz ; A A2 R
C sh bz wx ; A A2 R
C sh bz wx bm4 ; A A2 R
C-I sh bz wx ; A A2 R
C sh bz wx gl15 bm4 ; A A2 R
C sh ; A A2 R
C sh wx ; A A2 R
C wx ar ; A A2 R
C sh wx K-L9 ; A A2 R
C sh ms2 ; A A2 R
C bz Wx ; A A2 R
C Ds wx ; A A2 R Pr y
C Ds wx ; A A2 R pr Y
C-I Ds Wx ; A A2 R
C-I ; A A2 R
C ; A A2 R
C ; A A2 R B Pl
C wx ; A A2 R
C wx ; A A2 R B Pl
C wx ; A A2 R b Pl
C wx ; A A2 R B pl
C-I wx ; A A2 R y
C-I wx ; A A2 R y B pl
C wx ar da; A A2 R
C wx v ; A A2 R
C wx v ; A A2 R Pl
C wx gl15 ; A A2 R
C wx gl15 ; A A2 R pr
C wx Bf ; A A2 R
c bz wx ; A A2 R
c sh bz wx ; A A2 R y
c sh wx ; A A2 R
c sh wx v ; A A2 R
c sh wx gl15 ; A A2 R
c sh wx gl15 bk2 ; A A2 R
c sh wx gl15 Bf ; A A2 R
c sh wx bk2 ; A A2 R
c ; A A2 R
c wx ; A A2 R y
c wx v ; A A2 R
c wx gl15 ; A A2 R
c wx Bf ; A A2 R
sh
sh wx v
sh wx d3
sh wx pgl2 gl15 ; y pgl1
lo2
wx*
wx-a
wl1
wx d3
wx d3 wl1
wx d3 v gl15
wx d3 gl15
Wx pgl2 ; y pgl1
wx pgl2 ; y pgl1
Wx pgl2 ; Y pgl1
wx pgl2 ; Y pgl1
wx pgl2 bm4 ; y pgl1
wx v
wx v gl15 bk2 Bf bm4
wx bk2
wx bk2 bm4
wx Bf
wx Bf bm4
v
ms2
gl15
gl15 Bf
gl15 bm4
bk2 Wx
Wc
bm4
bm4 Bf
16

Chromosome 9 (continued)

17
yel*-034-16
w*-4889
w*-8889
w*-8951
w*-8950
w*-9000
Tp9 N9 N3 Df3
TB-9La (9L.40)
TB-9Sb (9S.40)
TB-9Lc
Primary trisomic 9
*Additional waxy alleles available from collection of O. E. Nelson.

Chromosome 10
oy
oy R ; A A2 C
oy bf2
oy ms11
oy bf2 R ; A A2 C
oy bf2 ms10
oy zn R ; A As C
oy du R ; A A2 C
oy du r ; A A2 C
oy sr2
oy zn
sr3
Og
Og B Pl
Og du R ; A A2 C
ms11
ms11 bf2
bf2
bf2 zn
bf2 li g r ; A A2 C
bf2 g r sr2 ; A A2 C
bf2 g r sr2 ; A A2 C
bf2 r sr2 ; A A2 C
nl zn g R ; A A2 C
nl g R ; A A2 C
nl g r ; A A2 C
nl g R sr2 ; A A2 C
y9
y9 v18
nl
li zn g r ; A A2 C
li g R ; A A2 C
li g r ; A A2 C
li g r v18 ; A A2 C
li g r v18 ; A A2 C
ms10
du
du v18
du o7
du g r ; A A2 C
du sr2
zn
zn g
zn g R sr2 ; A A2 C
zn g r ; A A2 C
Tp2 g r ; A A2 C
g R sr2 ; A A2 C
g r ; A A2 C
g r sr2 ; A A2 C
g r sr2 l ; A A2 C
g R-g sr2 ; A A2 C
g R-g sr2 v18 ; A A2 C
g R-g K10 ; A A2 C
g R-g sr2 ; A A2 C
g R-r K10 ; A A2 C
g r-r sr2 ; A A2 C
Ej r-r ; A A2 C
Ej r-r sr2 ; A A2 C
r sr2 l ; A A2 C
R-g ; A A2 C
r-g sr2 ; A A2 C
r K10 ; A A2 C
r-g ; A A2 C
r-r ; A A2 C
r-ch Pl ; A A2 C
R-mb ; A A2 C
R-nj ; A A2 C

Chromosome 10 (continued)

R-r ; A A2 C
R-ch B Pl ; A A2 C
R-lsk ; A A2 C
R-sk-mc.2 ; A A2 C
R-sk ; A A2 C
R-st ; A A2 C
R-st Mst
R-st Mst o7
R-scm2 ; bz2 A A2 C C2
R-scm2 ; a-st A2 C C2
R-scm2 ; c2 A A2 C
R-scm122 ; pr A A2 C C2
R-scm2 ; a2 A C C2
R-scm2 ; c A A2 C2
Lc
w2
w2 1
o7
o7 ; o2
1
v18
mst
1 yel*-5344
vel*-8721
yel*-8454
yel*-8793
cm
TB-10La (10L.35)
TB-10Sc
TB-10L19
Primary trisomic 10

Unplaced Genes

dv
dy
el
14
o9
o10
o11
o13
Rs
v13
ws ws2
ub
zb
zb2
zn2
1*-4923
nec*-8376

Multiple Gene Stocks

A A2 C C2 R-g Pr B Pl
A A2 C C2 R-g Pr B pl
A A2 C C2 R-g b Pl
A A2 C C2 r-g Pr B Pl
A A2 C C2 r-g Pr b pl
A A2 C C2 r-g Pr B pl
A A2 c C2 R-g Pr B pl
A A2 C C2 R-r Pr B Pl
A A2 C C2 R-r Pr B pl
A A2 C C2 R-r Pr b Pl
A A2 C C2 R-r Pr B Pl wx
A A2 C C2 R-r Pr B pl wx
A A2 c C2 R-r Pr B Pl
A A2 C C2 r-r Pr B pl
A A2 C C2 r-r Pr B Pl
A A2 C C2 R Pr
A A2 C C2 R Pr wx
A A2 C C2 R Pr wx gl
A A2 C C2 R pr
A A2 C C2 R pr y wx gl
A A2 c C2 R Pr y wx
A A2 C C2 r Pr Y wx
su pr y gl wx ; A A2 C C2 R
A su pr ; A2 C C2 R
bz2 a c2 a2 pr Y/y c bz wx r
a su A2 C C2 R
bm2 lg a su pr y gl j wx g
colored scutellum
lg gl2 wt ; a Dt A2 C C2 R
a su pr y gl wx A A2 C C2 R
hm hm2
ts2 ; sk
lg gl2 wt ; a-m A2 C C2 R Dt
A A2 C C2 R-nj ; purple embryo S. Chase
Stock 6 ; Hi-haploid R-r B Pl

Popcorns

Amber Pearl
Argentine
Black Beauty
Hulless
Ladyfinger
Ohio Yellow
Red South American
Strawberry
Supergold
Tom Thumb
White Rice

Exotics and Varieties

Black Mexican Sweet Corn (with B-chromosomes)
Black Mexican Sweet Corn (without B-chromosomes)
Knobless Tama Flint
Gaspe Flint
Gourdseed
Maiz Chapolote
Papago Flour Corn
Parker's Flint
Tama Flint
Zapaluta Chica

Tetraploid Stocks

P-RR
P-VV
B Pl
a A2 C R Dt
su
y
gl
Y sh wx
sh bz wx
wx
g A A2 C R
A A2 C R B Pl

Cytoplasmic traits

NCS2
NCS3

Cytoplasmic Steriles and Restorers

WF9-(T)	rf rf2
WF9	rf rf2
R213	Rf rf2
Ky21	Rf Rf2

Waxy Reciprocal Translocations

wx 1-9c (1S.48; 9L.22) * Sx
wx 1-94995 (1L.19; 9S.20) * Sx
wx 1-98389 (1L.74; 9L.13) W23 only
wx 2-9b (2S.18; 9L.22) * Sx
wx 3-9c (3L.09; 9L.12) * Sx
wx 4-9b (4L.90; 9L.20) * Sx
wx 4-95657 (4L.33; 9S.25) * Sx
wx 4-9g (4S.27; 9L.27) W23 only
wx 5-9a (5L.69; 9S.17) * Sx
wx 5-9c (5S.07; 9L.10) W23 only
wx 6-9a (6S.79; 9L.40) * Sx
wx y 6-9b (6L.10; 9S.37) * Sx
wx 7-9a (7L.63; 9S.07) * Sx
wx 7-94363 (7 cent; 9 cent) * Sx
wx 8-9d (8L.09; 9L.16) * Sx
wx 8-96673 (8L.35; 9S.31) * Sx
wx 9-10b (9S.13; 10S.40) * Sx

Non-waxy Reciprocal Translocations

Wx 1-9c (1S.48; 9:.24) * Sx
Wx 1-94995 (1L.19; 9S.20) * Sx
Wx 1-98389 (1L.74; 9L.13) * Sx
Wx 2-9c (2L.49; 9S.33) W23 only
Wx 2-9b (2S.18; 9L.22) * Sx
Wx 3-98447 (3S.44; 9L.14) *
Wx 3-98562 (3L.65; 9L.22) * Sx
Wx 4-9e (4S.53; 9L.26) * Sx
Wx 4-95657 (4L.33; 9S.25) * Sx
Wx 5-9c (5S.07; 9L.10) * Sx
Wx 5-94817 (5L.69; 9S.17) M14 only
Wx 5-98386 (5L.87; 9S.13) * Sx
Wx 6-94778 (6S.80; 9L.30) * Sx
Wx 6-98768 (6L.89; 9S.61) *
Wx 7-94363 (7 cent.; 9 cent.) *
Wx 7-9a (7L.63; 9S.07) W23 only
Wx 8-9d (8L.09; 9L.16) * Sx
Wx 8-96673 (8L.35; 9S.31) * Sx
Wx 9-108630 (9S.28; 10L.27) M14 only
Wx 9-10b (9S.13; 10S.40) * Sx

* = Homozygotes available in both M14 & W23 backgrounds

Sx = Single cross of homozygotes between M14 & W23 versions available

Inversions

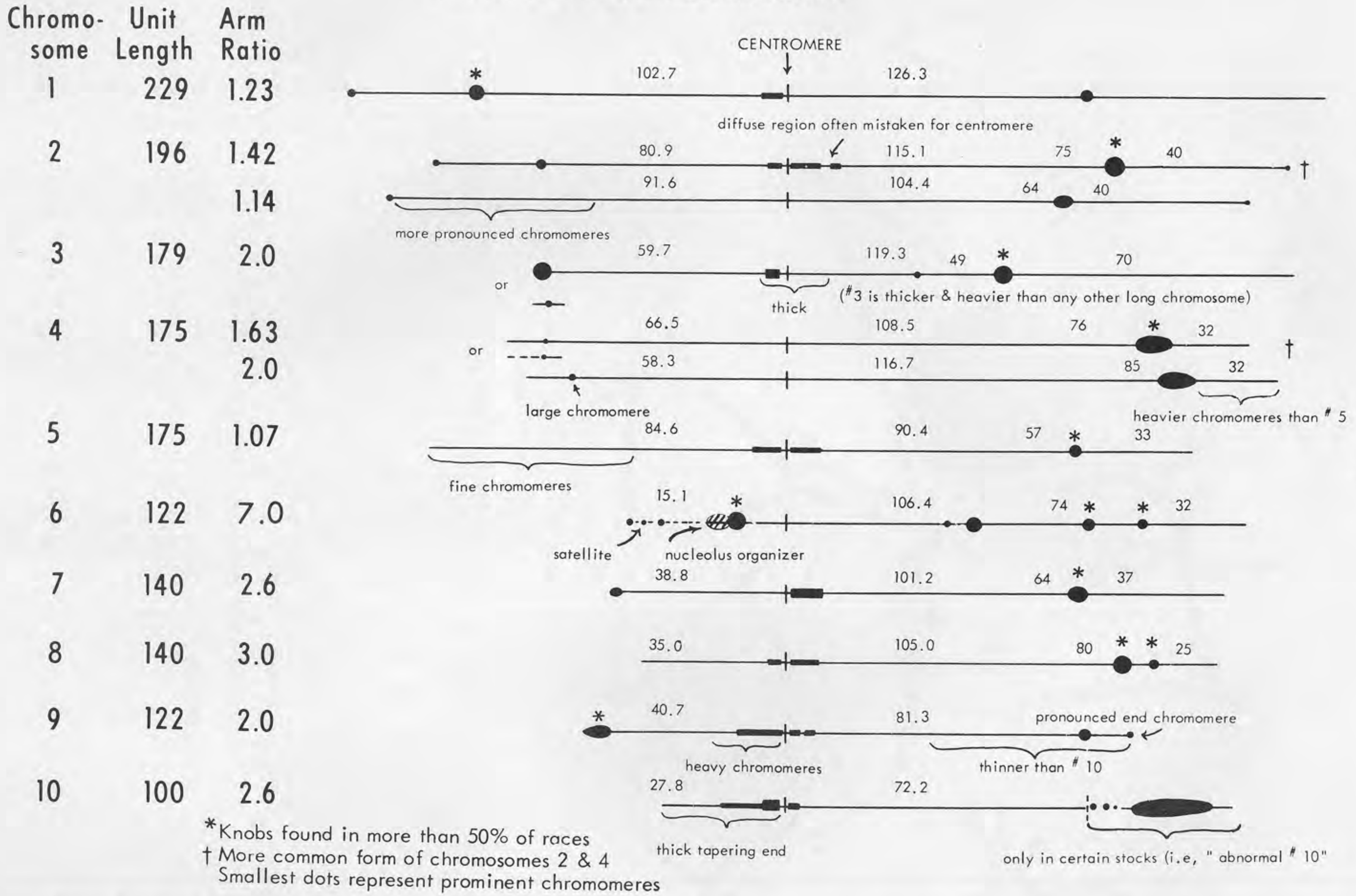
Inv.1a (1S.30-L.50)
Inv.1c (1S.35-L.01)
Inv.1d (1L.55-L.92)
Inv.1L-5131-10 (1L.46-L.82)
Inv.2a (2S.70-L.80)

Inv.2S-L8865 (2S.06-L.05)
Inv.2L-5392-4 (2L.13-L.51)
Inv.3a (3L.38-L.95)
Inv.3L (3L.19-L.72)
Inv.3L-3716 (3L.09-L.81)
Inv.4b (4L.40-L.96)
Inv.4e (4L.16-L.81)
Inv.5-8623 (5S.67-L69)
Inv.6-8452 (6S.77-L.33)
Inv.6-8604 (6S.85-L.32)
Inv.6-3712 (6S.76-L.63)

Inv.7L-5803 (7L.17-L.61)
Inv.7-8540 (7L.12-L.92)
Inv.7-3717 (7S.32-L.30)
Inv.8a (8S.38-S.15)
Inv.9a (9S.70-L.90)
Inv.9b (9S.05-L.87)
Inv.9c (9S.10-L.67)

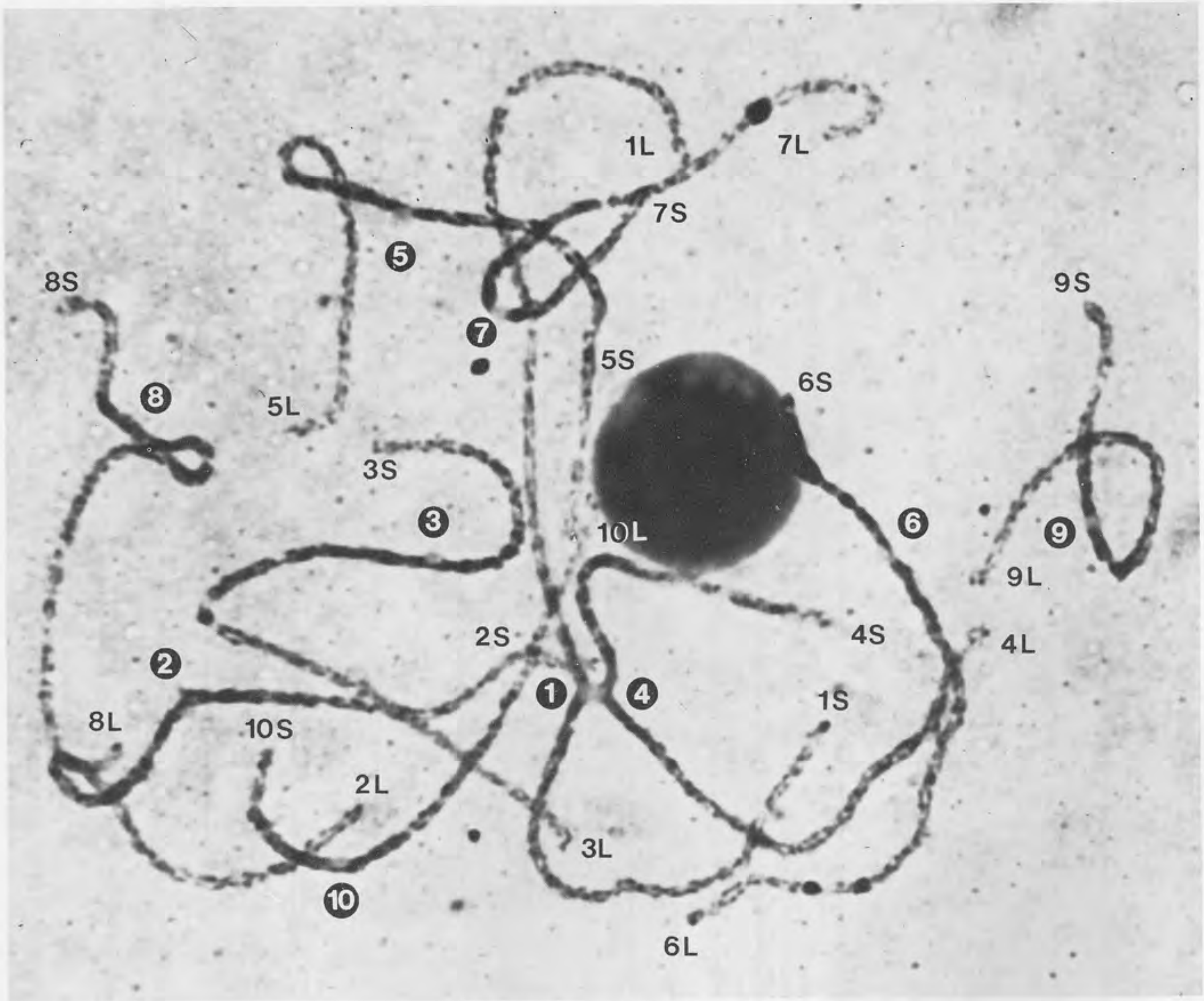
RECOMMENDED LINKAGE MARKER STOCKS

1S	sr1 zb4 P1-WW
1L	br1 bz2 gsl bm2 (not available with bz2)
2S	ws3 lg1 gl2 B1 fl1
2L	v4 w3 (lethal) Ch1
3S	cr1 d1
3L	gl6 lg2 a1 et1
4S	Ts5 su1
4L	gl4 Tu1 c2 (not available) <u>or</u> zb6 gl3 dp1 (available)
5S	gl17 a2 bt1
5L	bt1 pr1 v2 (not available) <u>or</u> a2 bm1 pr1 v2 (available)
6S	rgd1 (seedling semi-lethal) y1
6L	y1 Pl1 py1
7S	o2 v5
7L	ra1 gl1 ij1 Pn1 (not available) <u>or</u> ra1 gl1 ij1 bd1 (available)
8L	v16 ms8 j1
9S	yg2 C1 sh1 bz1 wx1
9L	wx1 gl15 bk2 Bf1 bm4
10S	Oy1-yg zn1 (not available) <u>or</u> oy1 zn1 (available
10L	zn1 g1 R1 sr2



Drawn to scale with major distinguishable characteristics (based on data from McClintock and Longley, also Longley and Kato, 1965)

Reproduced from *Mutants of Maize*, 1968, p. 4, by Neuffer, Jones, and Zuber, by permission of the Crop Science Society of America.



Maize Pachytene Chromosomes

Centromere locations are indicated by the adjacent solid circles with white numbers. The long and short arms of each chromosome are indicated by the chromosome's number and L and S respectively which are arranged near the terminal regions of the chromosome arms. The lengths of the arms and their ratios are listed below. Note that these do not agree in all cases with those shown with the cytological map, this is evident for chromosomes 1, 3, 4, 6 and 8. In the above figure arms 1L, 3L, 4S, and 8L are stretched somewhat and this results in the discrepancy of the ratios of these chromosomes. In measuring the length of arm 6S in the above figure the entire NOR and satellite region was included and this region was not included in the measurements used in preparing ratios listed with the cytological map (see preceding page). The pachytene spread was prepared and photographed by John T. Stout and the negative used to prepare the figure was kindly provided by Ronald L. Phillips.

Chromosome arm lengths in above figure (mm) and ratios

Chromosome	Long arm	Short arm	Ratio
1	135.20	92.53	1.46
2	101.33	70.60	1.43
3	112.23	48.30	2.32
4	89.23	62.63	1.42
5	82.70	72.80	1.14
6	88.73	32.60	2.72
7	92.33	35.50	2.60
8	96.87	29.73	3.26
9	72.53	36.13	2.01
10	74.66	27.66	2.70

6 ISOZYME LOCI IN MAIZE

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This summary briefly describes various isozyme loci, most of which have been used in genetic survey work and for which much information about relative variability is known. Several other loci, mostly esterases and peptidases, have been studied to a lesser extent and are not included herein.

Many of the techniques appropriate for maize are outlined in Cardy et al. (1980), while more general reviews and other recipes are found in Scandalios (1969), Shaw and Prasad (1970), Selander et al. (1971), Schaal and Anderson (1974), and O'Malley et al. (1980). The available variation among standard inbred lines of maize is documented, for many of these loci, in Goodman and Stuber (1980).

Acid phosphatase (ACPH) isozymes are coded for by three loci (Efron, 1970). The products of Acph1 on chromosome 9 are active as dimers (Stuber et al., 1980). Fourteen alleles have been identified for that locus.

Alcohol dehydrogenase (ADH) is coded for by two unlinked loci, Adh1 and Adh2 (Freeling and Schwartz, 1973). The adh1 gene is responsible for most of the ADH activity and is located in the long arm of chromosome 1, approximately 1.5 map units from lw (Schwartz, 1971). Adh2 is linked to su in the short arm of chromosome 4. ADH is active as a dimer and forms ADH1: ADH2 intergenic heterodimers. At least seven alleles have been identified at the Adh1 locus.

Maize catalase (CAT) is a tetrameric enzyme encoded by at least three structural genes with differences in temporal expression. Cat1 and Cat2, located on 5S and 1S, respectively, interact to produce heterotetramers, while Cat3 which may be located on IL does not interact in vivo with Cat1 and Cat2 (Scandalios, Tong and Roupakias, 1980). At least seven alleles have been found at Cat3.

Endopeptidase (EP) is coded for by a single major structural locus localized to chromosome 6 (Nielsen and Scandalios, 1974). Seven alleles have been identified at the Ep locus.

Esterase (EST) isozymes are coded by several loci, five of which have been localized (see Table). Nine alleles have been identified for ES, located on the short arm of chromosome 3.

Beta-glucosidase (b-GLU) isozymes are coded for by a single locus located on chromosome 10L. b-GLU is active as a dimer (Pryor, 1976, 1978; Stuber, Goodman and Johnson, 1977). Twenty-eight alleles have been identified at the Glu locus.

Glutamate-oxaloacetate transaminase (GOT) isozymes occur as dimers and are known to be associated with different subcellular compartments (MacDonald and Brewbaker, 1972; Scandalios, Sorenson and Ott, 1975).

Got1 specifies slowly migrating allozymes which are associated with the glyoxysomes; Got2 specifies forms active in the cytoplasm, while Got3 encodes the rapidly migrating, mitochondrial GOT allozymes (Scandalios et al., 1975). Interallelic heterodimers are produced as the result of interaction between alleles at each locus, but no intergenic heterodimers are formed. Got1 has been localized to chromosome 3, while Got2 and Got3 have been localized to chromosome 5. Eight alleles have been identified at the Got1 locus, seven alleles at the Got2 locus and five alleles at the Got3 locus.

Glutamic dehydrogenase (GDH); the inheritance of GDH is poorly understood, but apparently governed by two loci (Misharin et al., 1979; Sukhorzhevskaya, 1979, 1980), one of which has been localized to 1L (Pryor, 1979; Goodman et al., 1980b) and one of which is linked (12.5±3.2 m.u.) to Glu on chromosome 10 (Goodman and Stuber, unpublished).

Isocitrate dehydrogenase (IDH) is coded for by two unlinked loci, Idh1 and Idh2, on chromosomes 8 and 6, respectively, which interact to produce both interallelic and intergenic heterodimers (Stuber and Goodman, 1980a). Six alleles have been identified at the Idh1 locus and seven alleles at the Idh2 locus.

Malate dehydrogenase (MDH); five major structural loci have been identified in this sytem (Goodman et al., 1980a; Newton and Schwartz, 1980). Duplicate genes, Mdh4 and Mdh5, encode MDH isozymes active in the cytoplasm of the cell. Mdh4 was located proximal to Adh1 in the long arm of chromosome 1 and Mdh5 was located distal to a2 in the short arm of chromosome 5. The MDH isozymes compartmentalized in the mitochondria are specified by three nuclear genes: Mdh1 is on chromosome 8, Mdh3 is in the distal region of the long arm of chromosome 3, and Mdh2 has been located to the distal region of the long arm of chromosome 6. MDH is active as a dimer. Subunits of MDH isozymes which occupy the same subcellular compartment readily interdimerize but heterodimers between soluble and mitochondrial MDH isozymes are not observed. A sixth locus, Mmm, linked closely to Mdh4 on chromosome 1, affects the electrophoretic mobilities of the mitochondrial MDH isozymes. Thus far 15 alleles have been identified at Mdh1, 19 at Mdh2, 9 at Mdh3, 11 at Mdh4, 10 at Mdh5 and 3 at Mmm.

Malic enzyme (ME) was reported by Pupillo and Bossi (1979) to have two different forms in maize, but genetic studies reported have been limited to a single locus on chromosome 3 (Goodman, Stuber and Newton, 1981) which is expressed in the coleoptile of maize and which has at least four alleles, three of which are very rare.

Phosphoglucomutase (PGM) is coded for by two independently segregating loci, Pgm1 and Pgm2, located on chromosomes 1 and 5, respectively. Each allele at each locus appears to produce a pair of isozymes, with no apparent interaction of loci or alleles (Stuber and Goodman, 1979; Goodman et al., 1980). Sixteen alleles have been identified at the Pgm1 locus and 18 alleles at the Pgm2 locus.

6-Phosphogluconate dehydrogenase (6-PGD) is coded for by two unlinked loci, Pgd1 and Pgd2 on chromosomes 6 and 3, respectively. Interallelic and intergenic heterodimers are formed (Goodman et al., 1980; Stuber and Goodman, 1980b). Nine alleles have been identified at the Pgd1 locus and five alleles at the Pgd2 locus.

Summary of the known chromosomal locations of enzyme loci in maize

Symbol	Name	Location	Reference
Acph	acid phosphatase	9	STUBER et al. (1980)
Adh1	alcohol dehydrogenase-1	1L	SCHWARTZ (1971)
Adh2	alcohol dehydrogenase-2	4S	FREELING and CHENG (1978) DLOUHY (1979)
Amp1	aminopeptidase-1	1L	OTT and SCANDALIOS (1978)
Amp2	aminopeptidase-2	1L	OTT and SCANDALIOS (1978)
Amp3	aminopeptidase-3	5S	MCMILLIN and SCANDALIOS (1980) OTT and SCANDALIOS (1978)
Amy2	amylase-2	5S	ROUPAKIAS et al. (1980)
Cat1	catalase-1	5S	ROUPAKIAS et al. (1979)
Cat2	catalase-2	1S	TSAFTARIS et al. (1980)
Cat3	catalase-3	1L?	ROUPAKIAS et al. (1980)
Cx	catechol oxidase	10	PRYOR and SCHWARTZ (1973)
E1	esterase-1	7	SCHWARTZ in BROWN and ALLARD (1969)
E3	esterase-3	3	BROWN and ALLARD (1969)
E4	esterase-4	3S	HARRIS (1968) KLEESE and PHILLIPS (1972)
E8	esterase-8	3S	GOODMAN et al. (1980b, 1981)
E16	esterase-16	7	BROWN and ALLARD (1969)
Ep	endopeptidase	6L	NIELSEN and SCANDALIOS (1974)
Glu	Beta-glucosidase	10L	PRYOR (1978)
Gdh1	glutamic dehydrogenase-1	1L	PRYOR (1979)
Gdh2	glutamic dehydrogenase-2	10	GOODMAN and STUBER (unpublished)
Got1	glutamate-oxaloacetate transaminase-1	3L	GOODMAN et al. (1980b)

Summary - continued

Symbol	Name	Location	Reference
Got2	glutamate-oxaloacetate transaminase-2	5L	GOODMAN et al. (1980b)
Got3	glutamate-oxaloacetate transaminase-3	5S	"
Idh1	isocitrate dehydrogenase-1	8	"
Idh2	isocitrate dehydrogenase-2	6L	"
Mdh1	malate dehydrogenase-1	8	NEWTON and SCHWARTZ (1980)
Mdh2	malate dehydrogenase-2	6L	"
Mdh3	malate dehydrogenase-3	3L	"
Mdh4	malate dehydrogenase-4	1L	"
Mdh5	malate dehydrogenase-5	5S	"
Me	malic enzyme	3L	GOODMAN et al. (1980b)
Mmm	modifier of mitochondrial MDH's	1L	NEWTON and SCHWARTZ (1980)
Pgd1	6-phosphogluconate dehydrogenase-1	6L	GOODMAN et al. (1980b)
Pgd2	6-phosphogluconate dehydrogenase-2	3L	"
Pgm1	phosphoglucomutase-1	1L	"
Pgm2	phosphoglucomutase-2	5S	"
Phi	phosphohexose isomerase	1L	"

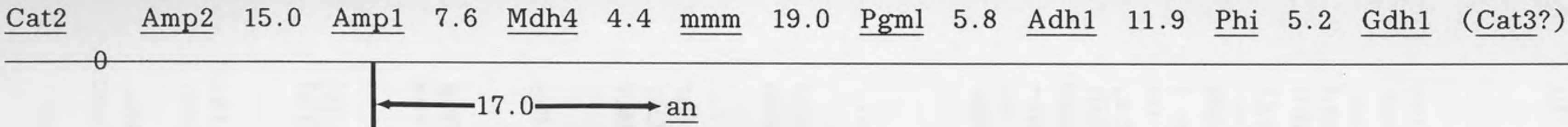

Figure 1. Chromosome 1

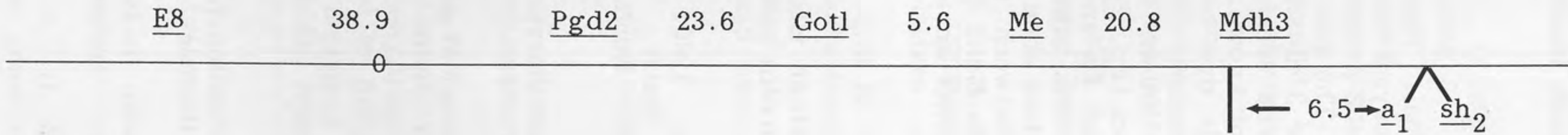

Figure 2. Chromosome 3

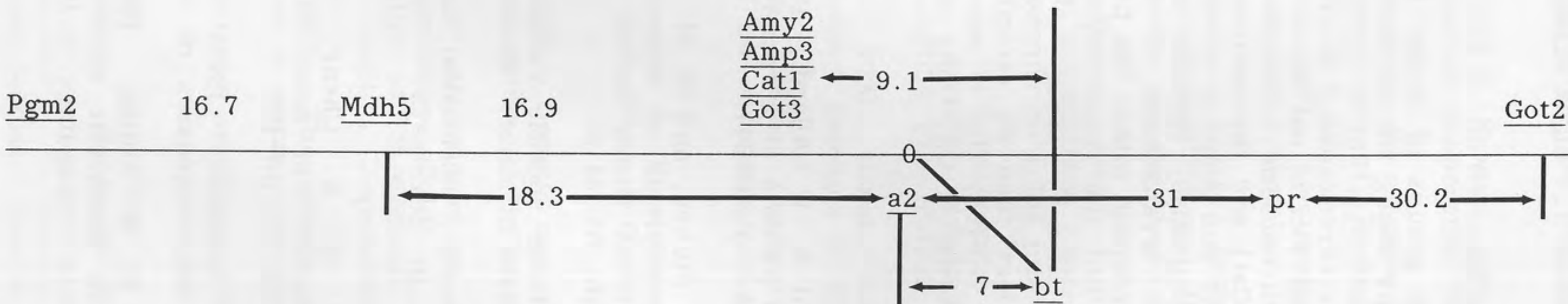

Figure 3. Chromosome 5

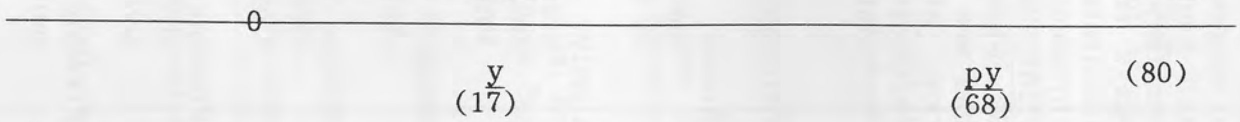

Figure 4. Chromosome 6

Phosphohexose isomerase (PHI) appears to be coded for by a single locus, Phi, on chromosome 1. Eight alleles have been identified at the Phi locus.

Linkage Among Isozyme Loci

There are several groups of linked isozyme loci. Figure 1 illustrates the linkage relationships among the chromosome 1 isozyme loci. Cat3 has not been mapped relative to Adh1, Phi or Gdh1. Figure 2 presents the relationships known among the chromosome 3 isozyme loci. The placement of E3 and E4 relative to the other loci is not known. Figure 3 illustrates the known arrangements of the chromosome 5 isozyme loci. The arrangement of Amy2 and Amp3 relative to Cat1 and the centromere is not known, but the recombination % between Cat1 and Amp3 is about 3% while that between Cat1 and Amy2 is about 5% (Roupakias, McMillin and Scandalios, 1980). Figure 4 illustrates the relative arrangement of the four known isozyme loci on chromosome 6. The ordering within the pairs of loci (Ep, Pgd1) and (Idh2, Mdh2) is not known, but the recombination between Ep and Pgd1 is about 5%, while that between Idh2 and Mdh2 is about 1%. Two other sets of linked loci are known: Idh1 and Mdh1 on chromosome 8 show about 22% recombination, while there is less than 6% recombination between Cx and Glu on chromosome 10 (Pryor, 1979); Glu is also linked to Gdh2 (about 12.5% recombination), but the order of Cx, Gdh1 and Glu is not known.

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7 MUTANT INDUCTION IN MAIZE

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Various sorts of heritable changes in the maize genotype may be induced by a number of mutagenic agents including chemicals, ultraviolet radiation, ionizing radiation and certain internal factors such as unstable chromosome configurations and controlling elements. If chromosome breaks, deficiencies, duplications, translocations and inversions are needed, x-ray or gamma ray treatment of seeds or pollen or ultraviolet treatment of pollen is the most effective approach (Stadler and Roman 1948, Sax 1957, Neuffer 1957, Sparrow 1961). McClintock (1957) showed that several types of change in the genome could produce aberrations that initiated unhealed broken chromosome ends. If on the other hand small deficiencies and point mutations are desired, pollen treatment with the chemical mutagen ethyl methanesulfonate (EMS) (Neuffer and Coe 1977) or with ultraviolet light (Stadler 1939, Neuffer 1957) is best. Finally, if inactive or unstable alleles of a particular locus are desired then the genome should be subjected to the action of one of the controlling elements systems e.g., Ac-Ds (McClintock 1951).

Potential uses, methodology and problems of mutagenesis are too diverse and sophisticated for detailing in this publication, therefore a description will be made of only the most productive methods available. Excellent information about the various agents and methods are found in the references indicated above. Special attention should be paid to Stadler's description of methods for studying mutation (Stadler 1946), to the present author's consideration of the merits of seed versus pollen treatment (Coe & Neuffer 1977, Neuffer 1978) and to the ontogeny of the germ cells (Coe & Neuffer 1978).

X-ray Treatment-For the production of a random assortment of chromosome aberrations the best procedure is to treat dry kernels of a uniform line or hybrid with 12,000 - 15,000 R units of x-rays. Plant the kernels and grow the M1 plants to maturity and self-pollinate to produce a M2 generation which may be grown and observed for partial pollen sterility (an indicator of aberrations) and selfed again. The resulting self ears should include many with partial kernel set also indicating chromosome aberrations, mostly reciprocal translocations. If the purpose is to produce specific aberrations involving certain loci then treatment of male germ cells with subsequent crosses on appropriately marked female stocks is recommended. Treatment may be applied at any stage in the development of the plant provided that the treatment hits the germ line and that an effective dose is used. Premeiotic treatment results in the screening out (by the meiotic process) of most deleterious cases. Treatment during or immediately following meiosis is not very productive as the germ cells are so sensitive that doses below 500 R are required if viable pollen is to be produced.

Microspores near the first microspore division stage will tolerate up to 1200 R units. Treatment at this stage will produce many deficiencies and other aberrations most of which will appear in both the endosperm and the

embryo of a kernel when appropriate crosses are made. Microspores at a stage from between the 1st and 2nd microspore divisions until pollen shedding will tolerate doses above 2000 R and will produce many aberrations that will appear independently in the endosperm and the embryo. The most productive stage for many types of aberrations is at the first microspore division but this requires accurate staging of microspore development by sampling and cytological observation. Treatment of mature pollen is also productive and much easier to achieve. Treatment may be accomplished by taking branches of tassels of the selected male genotype early in the morning before they have shed and placing them in a petri dish with a moist blotter. In this condition they may be treated with 2000 R of x-rays; then, after the anthers come out of the glumes, the dish may be uncovered allowing the anthers to dry out. When pollen begins to shed, shake the branches in a glassine bag and pollinate silks of an appropriate tester genotype.

Ultraviolet Treatment-Because ultraviolet light will not penetrate beyond one cell layer this treatment may be applied only to pollen grains. The procedure consists of collecting pollen as described above for x-ray treatment but shaking it in a single layer on a quartz slide then placing the slide with pollen in a specially constructed box containing two sterilamp tubes located 8 inches apart. The slide is left between the tubes for 30 seconds, then withdrawn and the pollen poured into a porcelain combustion boat and quickly carried to and spread carefully on silks of an appropriate genotype. The resulting kernels will show endosperm losses and mutations if appropriate genotypes are used but these will be independent of other similar changes in the embryo. The kernels may be sown and the resulting plants selfed and the progeny screen for the desired mutants.

Chemical Treatment-The most effective mutagenic agent for maize is the alkylating compound ethyl methanesulfonate. The best method is the paraffin oil technique for treating pollen (Neuffer 1978, Neuffer and Coe 1977). Also see the recent revision of this method (Neuffer 1982). For this procedure one should plant uniform lines or hybrids that are tested and known to be free from spontaneous mutants and that are of the proper genotype for expressing the desired mutants being sought.

For these treatments prepare a 1% stock solution of EMS (Eastman #7830) in a light domestic paraffin oil (Fisher 722268) and stir vigorously for 1 hour. For each treatment prepare a treatment solution of 1 part of this EMS stock solution and 15 parts of the same paraffin oil. Place one volume of fresh pollen and at least 15 volumes of the treatment solution in a 25 ml plastic vial with a cap. Close vial tightly and shake periodically to prevent pollen and EMS from collecting in the bottom of the vial. After 45 minutes apply the pollen-oil mixture to fresh silks using a #10 camel hair brush. Apply only enough to get good distribution of pollen. Stirring with the brush is necessary between each application. Note-extreme-precautions-mixing under hood, protective clothing, sanitary disposal-should be taken to protect all personnel. Chemical mutagens are particularly penetrating in oil. The kernels produced will be M1 material carrying one treated and one untreated genome. If the need is for mutants at particular loci, then appropriately marked male and female stocks should be used, and the results will be found by growing the kernels and testing the M1 plants produced. It is estimated that this treatment will produce one recessive mutant for a particular locus for each 1000 gametes treated. If the need is for mutants in general then the stocks used must be free of spontaneous mutants and as

uniform as possible. The M1 kernels should be planted and the resulting plants noted for dominant cases. Those dominants that survive should be selfed and crossed to another stock to prove that they are dominant. The rest of the M1 plants should be selfed to produce ears that will carry M2 kernels that will segregate 3:1 for recessive kernel mutants and, on testing, 3:1 for seedling and plant mutants (see Neuffer and Sheridan 1980 for expected mutant frequencies).

Controlling Elements-To produce (at a particular locus) a mutable allele at a locus that is subject to the action of a particular controlling element obtain a stock that carries the gene control system and the dominant allele for the locus to be studied. If the Ac-Ds system is chosen, use *bz2-m*, *Ac* stock which carries a modified *Ds* at the bz2 locus on chromosome 1. The stock should have two or more of the Ds elements (homozygous *bz-m*) but carry only one *Ac* element (so that the events will be early and some therefore large enough to be included in the germ line for succeeding progeny). Use this stock as a female and cross the ears by a stock that is recessive for the locus in question, but free of controlling elements. Examine the progeny (kernels or seedlings) for appropriate recessive mutants of that locus. The observed cases might or might not be mutable (show instability) depending on whether the mutant gamete gets an *Ac* element or not. Subsequent tests by selfing (to prove the case), by crosses on a *bz2-m* no *Ac* (to confirm the presence of Ac) and on the recessive marker stock will prove gene supression and response of the regulating element *Ac*. More frequent cases of new *Ds* activity may be expected if the site of the original *Ds* activity and the new locus are on the same chromosome because controlling elements are transposed most frequently to nearby sites on the same chromosome and less frequently to distant sites and to other chromosomes.

The production of a mutable allele at the *A2* locus will be used as an example. The *bz2-m*, *Ac* stock described above is *A2A2* and will be used as a female parent. The male stock will be *a2a2* and lacks both *Ac* and *Ds*. The ears produced will have all purple kernels except for the putative cases. These will be colorless kernels of two types; those having purple spots (mutable) and those lacking spots (apparently stable because no *Ac* is present). These two classes should be planted and tested. All the progeny should be selfed to show heritability; they should all be crossed to an *a2a2* tester stock to demonstrate that it is the A2 locus that is involved and to demonstrate again the mutability of the mutable cases; and they should be crossed to a *bz2-m*, no *Ac*, *A2A2* tester stock to test whether *Ac* is present. In the latter test the presence of *Ac* will be evident because of the presence of bronze kernels with purple sectors.

Alternatively, the parent carrying the controlling element system and the subject locus (*bz2-m* *bz2-m* one *Ac* *A2A2*) may also be used as a male to cross on *a2a2* no *Ac* ears in producing transpositions to *A2* but the investigator should be aware that transpositions occurring before meiosis will appear in the progeny as multiple copies of the same event and may be falsely interpreted as separate occurrences. Also events occurring after the first microspore division and before fertilization will appear in the endosperm but will be associated with a normal embryo. Similarly transpositions at this stage will produce an equal number of cases where the endosperm is normal but the embryo contains a transposed element at the *A2* locus. Transposition events that occur during the stage just following meiosis and prior to the first microspore division are the only ones that will produce single seed

cases that are not a part of a sector or that are not associated with a non-corresponding endosperm or embryo, whichever is the case. See Coe and Neuffer (1977) and the article by Dooner in this publication for details on the role of the bz locus in anthocyanin (purple pigment) formation and the article by Federoff in this publication for further details on controlling elements.

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8 THE LOCATING OF GENES TO CHROMOSOME BY THE USE OF CHROMOSOMAL INTERCHANGES

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The breakpoints in the maize interchange stocks were determined from the + (cross-shaped) configurations seen at pachytene of meiosis in the interchange heterozygote. The center of the cross is at or near the original breakpoints, each of which is measured as the distance from the centromere to the center of the cross relative to the total length of the arm in which the break occurred. For example the first interchange stock in corn happened to be an interchange between chromosomes 1 and 2 and was designated T1-2a (1L.5, 2L.4). The second one involving these two chromosomes was T1-2b (1S.4, 2S.4). (L = long, S = short and .5 and .4 mean 50% and 40% out from the centromere towards the end of the arm, respectively.) For an adequate test of the entire genome a break point located in each chromosome arm at not less than about .5 is needed (probably for all chromosome arms except the short arm of chromosome 6 which is very short).

In maize, heterozygotes for most of the interchanges have about 50% pollen and ovule abortion, i.e. they are semi-sterile. About 50% of the kernels on the ear are missing (random gaps on the ear). If one of the exchanged segments is very short there may be only 25% spore abortion (partial sterility). In crosses with normal stocks, the abortion behaves as a dominant located at the original exchange points in the two chromosomes. Half the progeny will be semisterile, designated S.S., half will have normal fertility. For example if a stock homozygous for an interchange (T) is crossed with a recessive marker stock, e.g. a fertile dwarf, the cross will be TDD x fertile dd, the F_1 will be semisterile (S.S.Dd). If this F_1 is backcrossed to the fertile dd stock, the progeny will be segregating 1:1 for S.S. vs. fertile and D vs. d. If there is no linkage between D vs. d and S.S. vs. fertile the ratio of S.S.:fertile will be 1:1 for the D and for the d plants. If there is a linkage, e.g. 10% recombination, there will be a ratio of 9 parental classes:1 recombinant classes, i.e.: 9 S.S. D:1fertile D:1S.S. d:9fertile d. The d marker may be in either of the two chromosomes involved in the interchange. This example is illustrated below using T1-2a and dwarf for the interchange and recessive marker respectively (see next page).

If an interchange heterozygote is self-pollinated, half the progeny again are semisterile, and half are fertile but the latter are of two types, either standard normal or homozygous for the interchange in equal numbers. The two types of fertiles can be distinguished by crossing on a standard normal stock and classifying the progeny for sterility.

If the S.S.Dd F_1 in the above example is self-pollinated, and D vs. d and S.S. vs. F are independent, there will be a ratio of 3 D:1d in the S.S. and in the F groups of plants. If there is linkage, the % of dd plants in the F class will be higher than 25% and less than 25% in the S.S. class, since the d parent was fertile.

Original cross: $\frac{\text{T1-2a D}}{\text{T1-2a D}}$ x $\frac{\text{+(fertile)d}}{\text{+(fertile)d}}$

F1: $\frac{\text{T1-2a D}}{\text{+ d}}$

Backcross: $\frac{\text{T1-2a D}}{\text{+ d}}$ x $\frac{\text{+ d}}{\text{+ d}}$

Progeny:	Male gametes all + d	Frequency Independent Assortment	vs.	10% Recombination
Female gametes				
Parental: T1-2aD	$\frac{\text{T1-2aD}}{\text{+ d}}$ (S.S. tall)	25%	vs.	45%
Recombinant: T1-2ad	$\frac{\text{T1-2ad}}{\text{+ d}}$ (S.S. dwarf)	25%	vs.	5%
Recombinant: + D	$\frac{\text{+ D}}{\text{+ d}}$ (fertile tall)	25%	vs.	5%
Parental: + d	$\frac{\text{+ d}}{\text{+ d}}$ (fertile dwarf)	25%	vs.	45%

Since an observed linkage between a marker and a single interchange stock means only that the marker is linked with either of the two breakpoints, various schemes have been devised to determine which of the two chromosomes carries the marker. Four methods have been used:

1. The linked endosperm marker system (Anderson 1943, 1956).

2. The all-arms marker system (Burnham and Cartledge, 1939).

3. The duplication-deficiency system (Phillips et al. 1971).

4. The B-A translocation system--translocations involving a B chromosome (Beckett, 1978).

The procedures for the first two will be described.

The linked endosperm marker method

One series uses interchanges closely linked with the waxy (*wx*) endosperm marker gene located in the short arm of chromosome 9. For example, to adequately test for any gene located in chromosome 1, two 1-9 interchanges are needed, one with the breakpoint in 1 in the short arm and one with the breakpoint in the long arm, each breakpoint not closer to the centromere of 1 than about .5. Since all of the stocks are closely linked with *wx*, if the marker being tested shows linkage with only one *wx* marked translocation, the marker must be located not in 9 but in the other chromosome involved in that interchange. If the marker is in 9 close enough to *wx*

to show linkage, it will show linkage with all the interchanges in the wx linked interchange series.

If a complete, ideal series of interchanges very closely linked with wx were used, i.e. one for each arm of the other 9 chromosomes (except the 6S arm which is very short), the only portion of the genome not tested would be a distal segment of 9L so far from wx that a marker in that segment would show no linkage with any interchange in the series.

The steps in the procedure, using a T1-9 stock homozygous for wx, and a stock of the mutant to be tested, e.g., a recessive dwarf (d) are as follows:

1. Cross the two stocks; the genotypes and phenotypes will be:

   (wx wx T1-9) DD (tall) x (Wx Wx N) dd (dwarf)

   The $F_1 = \frac{\text{wx T1-9}}{\text{Wx N}} \frac{D}{d}$ (semisterile, tall)

2. Self the F_1 plants, the ears will segregate 3 Wx:1 wx.

3. Classify and separate the Wx and wx seeds, using the KI = I_2 solution: Wx seeds stain blue, wx seeds stain brick red.

Plant the seeds in separate rows. Compare the ratios of D:d in the two rows. If the ratio of D:d is the same (3:1) in both rows, segregation is independent of the interchange. If there is linkage, the wx class will have fewer dwarfs than the Wx class (the wx parent was D). The gene is in chromosome 1. The gene might be in chromosome 9, linked with the break in 9 and wx, but in that case it would be linked with all the wx interchange stocks being used. If a wxwx dd stock were available, the F_1 might be backcrossed to that stock.

If the character being studied is complex in inheritance, e.g. a quantitative character, the test may be made in the field, Wx and wx being planted in adjacent rows (replicated). Comparisons can be made at all stages of growth.

The efficiency of the test using selfs of the S.S. F_1's can be increased by using equal numbers of Wx and wx seeds. For variations of the procedure, see Burnham (1966).

The wx-linked series is not complete, but can be supplemented by a similar series with one breakpoint in each closely linked with su in chromosome 4. One disadvantage of this series is that su seeds often germinate poorly under field conditions. This problem can be avoided by backcrossing the F_1 to the non-sugary (Su) stock that has the marker being tested. Half of the progeny will be SuSu and half will be Susu. If these are grown in an isolated plot, and allowed to open pollinate, the ears on Susu plants will be segregating for su and should be semisterile also because of the close linkage of su with the interchange. The ears on SuSu plants will not be segregating for su and should also be fertile. The test is completed by classifying the plants also for the marker being tested.

The all-arms marker system

In this system, a series of interchanges is used in which each chromosome arm is marked by a breakpoint in two different interchanges, e.g., a T1-9 with breakpoints in 1 L and 9 L might be checked by a T1-2 with a breakpoint in 1L and by a 4-9 with a breakpoint in 9 L. For example, if the 1-9 stock showed linkage and the 1-2 did not, the gene is located in 9 L. If so, there should also be a linkage with 4-9. If both 1-9 and 1-2 showed linkage the gene would probably be in 1 L. If so, there should be no linkage with the 4-9.

The complete series should test the entire genome. A series of 24 interchanges backcrossed to Minnesota inbred A188 at least 6 times has been established. The procedure is:

1. Cross the interchange stocks with the stock carrying the contrasting character to be studied.

2. Backcross the partially sterile F_1 plants to the parental stock that is recessive for the character. For a quantitative character, backcrosses to both parents may be needed.

3. Grow the backcross progeny and classify each plant for partial sterility and for the character being studied. The parents and F_1 should be included in the test and similar notes taken on each plant for the character being studied.

4. Compare the grades of expression for the character and their frequencies among the partially sterile and fertile plants. A significant difference in frequency indicates linkage. Parental classes should be in excess.

The use of trisomics to locate genes to chromosome

Trisomic corn plants have 21 chromosomes. At meiosis they have 9 bivalents plus one trivalent (three homologous chromosomes). The three chromosomes may be associated or they may form a bivalent plus a univalent. The extra chromosome may lag and not be included in the microspores or megaspores. The frequency of trisomics in the progeny from self-pollination of a 21-chromosome corn plant or from crossing it as a female with normal plants varies from about 25% to about 40%, the longer chromosomes having the higher frequencies. In corn the extra chromosome usually is not transmitted through the pollen. A trisomic plant heterozygous for a recessive genetic marker, e.g. a in that chromosome may be AAa or Aaa. Selfs of those plants or reciprocal crosses with aa will give ratios that differ from normal, disomic ratios. The ratios are shown in Table 1.

Hence if a new (unplaced) genetic marker is crossed with the 10 different trisomic stocks, 9 of the tests will give disomic ratios, and only one will give trisomic ratios.

The steps in the procedure are:

1. Cross a trisomic plant as the female plant with the mutant to be located.

Table 1. Ratios from selfing and reciprocal backcrosses of trisomic and diploid heterozygotes in corn; assuming 25% and 50% transmission of n + 1 through the female, none through the pollen.

Genotype	Ratio of n:n+1 gametes	Selfs A:a	% a	Backcrosses as ♀ x aa A:a	% a	aa x as ♂ A:a	% a
AAa	3:1*	11:1	8.3	3:1	25.0	2:1	33.3
AAa	1:1+	17:1	5.5	5:1	16.7	2:1	33.3
Aaa	3:1*	11:7	38.9	5:7	58.3	1:2	66.6
Aaa	1:1+	2:1	33.3	5:3	37.5	1:2	66.6
Aa	-	3:1	25.0	1:1	50.0	1:1	50.0

*The ratio of female gametes containing n chromosomes versus those containing n + 1 chromosomes assuming 25% transmission of n + 1 through the female.

+The ratio of female gametes containing n chromosomes versus those containing n + 1 chromosomes assuming 50% transmission of n + 1 through the female.

A represents the dominant phenotype and a the recessive.

2. The trisomic F_1 plants may be self-pollinated and/or crossed reciprocally with the mutant (if recessive).

3. Do the same for 2n sibs as checks.

4. Grow the progeny, compare the segregation ratios from trisomic (2n + 1) vs. the 2n sibs.

Trisomic plants must be identified at least in step 1. The smaller kernels on the main portion of the ear on a trisomic plant, excluding tip and butt kernels, are more likely to be trisomic than are the larger seeds. Positive identification of trisomic plants can be made by root tip smears, examination of sporocytes at meiosis, or first post-meiotic divisions in microspores. Plants trisomic for chromosome 5 usually have short, broad leaves, those for 7 usually have narrow, stiff leaves. In crosses with certain inbreds these trisomics have not been identifiable phenotypically.

Occasionally there may be non-disjunction for one of the bivalent chromosomes in a trisomic plant. A trisomic plant resulting from that n + 1 cell will not be the parent trisomic. A linkage test between markers known to be in the chromosome and a marker placed in error in that chromosome will detect such an error.

A trisomic stock homozygous for a marker in that chromosome might be used to avoid the need for cytological identification of trisomic plants. Identification of the trisomic plants for use in step 1 would still be desirable.

Telotrisomics (2n + telocentric for one chromosome arm) can be used to locate a gene to chromosome arm. These stocks, one for each chromosome, have not been established in maize.

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9 LOCATING GENES TO CHROMOSOMES: B-A TRANSLOCATIONS

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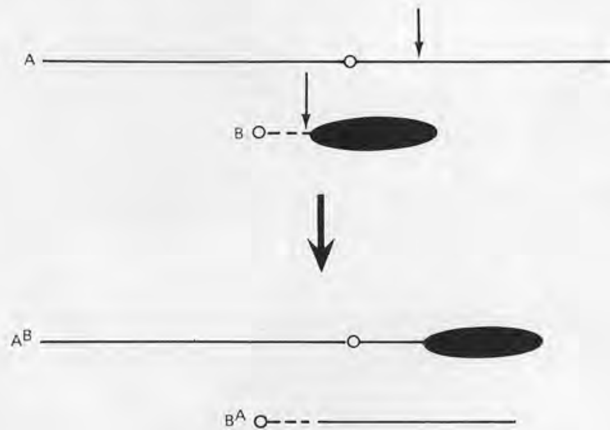
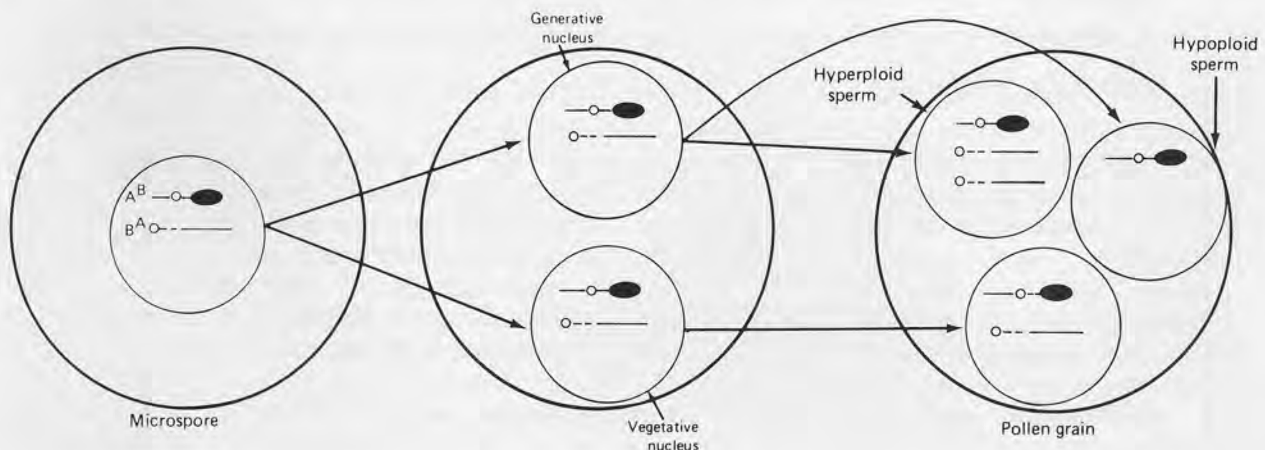
The B-A translocations of maize comprise an efficient system for determining, in the F1, the chromosome arm on which recessive endosperm and plant factors are located (Roman and Ullstrup, 1951). Translocations are now available on 18 or perhaps 19 of the 20 chromosome arms of maize.

B-A translocations are formed by breakage of a supernumerary (B) chromosome and an arm of one of the basic genomes, followed by reciprocal rejoining of broken ends, as illustrated:

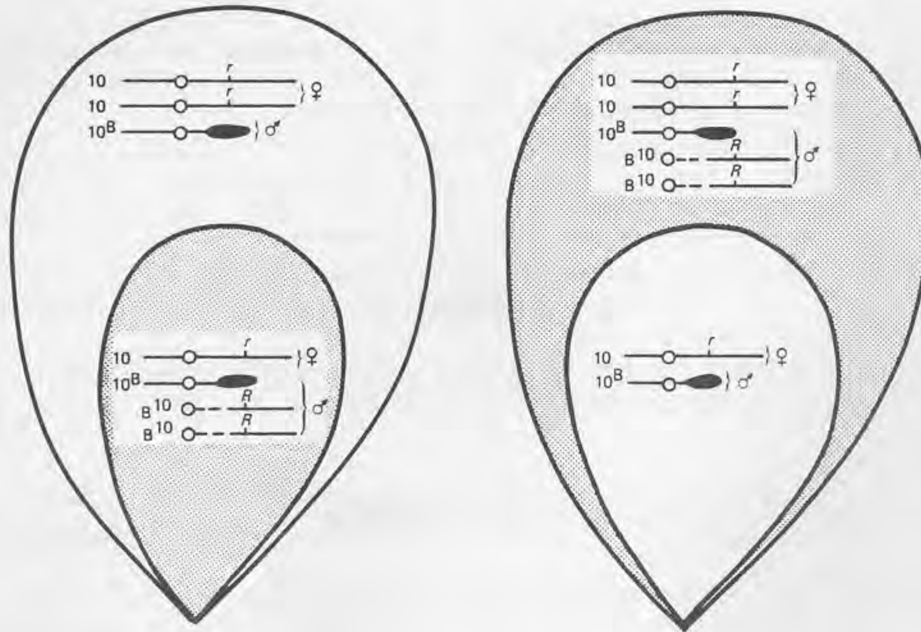


The B^A (like the B) frequently undergoes nondisjunction at the second pollen division; in other words, instead of dividing and separating, the B^A divides, but both products go to one pole, thus producing one sperm with 2 B^A's and the other with none, as shown below:



Both sperms are functional because the vegetative nucleus is balanced in constitution.

One sperm from a pollen grain fertilizes the haploid egg to give the diploid embryo, and the other sperm fertilizes the two polar nuclei to produce the triploid endosperm. When one sperm lacks a B^A and the other has two, then either the embryo or the endosperm will fail to carry the B^A, depending on whether the deficient sperm fertilizes the egg or the polar nuclei. Note that when the endosperm lacks the B^A, the embryo has two B^A's; when the endosperm has two B^A's, the embryo has none (see figure below):



Egg fertilized by hyperploid ($10^B B^{10R} B^{10R}$) gamete.

Egg fertilized by hypoploid (10^B) gamete.

Alternative results of pollinating colorless (rr) with a pollen grain bearing gametes of the constitution $10^B B^{10R} B^{10R}$ and 10^B, respectively. Male (♂) and female (♀) contributions to embryo and endosperm are indicated. Presence of anthocyanin color is indicated by stippling.

The ability of B-A translocations to locate genes to chromosome arm results from their ability to produce F1 kernels lacking a male contribution for a particular chromosome arm in <u>either</u> the embryo or the endosperm. Therefore, any recessive endosperm or plant trait carried by the female parent will be expressed in a certain percentage of the endosperms or seedlings when the ear is pollinated by the critical translocation, i.e., one with the breakpoint between the gene of interest and the centromere.

To locate recessive genes to chromosome arm, plants homozygous or heterozygous for the gene of interest are crossed by stocks carrying the

basic set of translocations listed by Beckett (1978). For endosperm factors, all ears should express the normal phenotype except for those crossed by the critical translocation, in which case the mutant phenotype will appear to segregate on that ear. To locate seedling traits, seeds are best tested in a sandbench. Mature plant traits usually require field planting.

Genes located between the centromere and the breakpoint of a B-A translocation, or on an arm lacking a translocation, will fail to segregate when tested by the basic set of translocations. Such genes can often be located by self-pollinating hypoploids (plants monosomic for part of a chromosome arm). The critical hypoploid, with breakpoint distal to the gene, will produce wholly or mainly mutant progeny because the A^B chromosome will fail to be transmitted. Even when the breakpoint is in the opposite arm, greatly distorted ratios are often produced.

The compound B-A translocations, originally described by Rakha and Robertson (1970), are unusual in that they uncover portions of two chromosome arms, so care must be taken to determine which arm is involved [see Rakha and Robertson (1970) or Beckett (1978) for details].

Using B-A translocations, Neuffer and Beckett (unpublished) have recently located to chromosome arm approximately 484 kernel and plant mutants produced by chemical mutagens. I have estimated (Beckett, 1978) that 85 percent of the genome is uncovered by the basic set of translocations, but experience indicates that the estimate may be too high.

B-A translocations are useful for purposes other than location of mutant genes. Dosages of portions of chromosome arms can be varied from one to three with ease, and higher numbers are not difficult to obtain. Enzyme activity may increase linearly or remain constant as the number of gene copies increases. Birchler (1981) has demonstrated that, for one gene, the latter behavior is due to negative modifiers on the same chromosome arm.

This brief description of B-A translocations will serve to acquaint the reader with the B-A translocation system in maize. A more complete discussion is found in Beckett (1978). Translocations are listed and described; suitable tester genes are listed, and sources of stocks are given.

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The compound B-A translocations, originally described by Rakha and Robertson (1970), are unusual in that they uncover portions of two chromosome arms, so care must be taken to determine which arm is involved [see Rakha and Robertson (1970) or Beckett (1978) for details].

Using B-A translocations, Stadler and Beckett (unpublished) have recently located to chromosome arm approximately 400 kernel and plant mutants produced by chemical mutagens. I have estimated (Beckett, 1978) that 65 percent of the genome is uncovered by the basic set of translocations. Our experience indicates that the estimate may be too high.

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10 THE MAPPING OF GENES BY THE USE OF SIMPLE AND COMPOUND TRANSLOCATIONS

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Once a locus has been determined to the respective chromosome, several means can be employed to more accurately define its position. In this article the use of translocations for more precise interstitial placement will be described; in particular, the use of overlapping translocations for the cytogenetic localization of genes with alleles encoding electrophoretic variants will be discussed. The procedure relies on the testing for the presence of the locus in a duplication formed by segregation in a heterozygote of two translocations involving the same chromosomes but that have displaced breakpoints or by altered segregation ratios in the same. The protocol has been described in detail for the localization of the alcohol dehydrogenase gene (Birchler, 1980).

The procedure for producing interstitial deficiencies and duplications was described by Gopinath and Burnham (1956). The relative positions of the breakpoints will determine whether the types of aneuploid gametes produced are deficient-deficient, duplicate-deficient or duplicate-duplicate for the regions present between the respective breakpoints.

Since the gamete constitution must support the development of the haploid gametophyte generation, those that are deficient for substantial chromosomal regions almost always abort. For this reason, the type of heterozygote that is useful in these analyses are those that produce double duplication gametes. For this, one of the translocations (A) has a breakpoint in one chromosome that is proximal and in the other that is distal; in the second translocation (B), the relative position of the breaks must be reversed. Therefore, the heterozygote of A and B will produce four types of gametes when it undergoes meiosis: 1) balanced translocation A; 2) balanced translocation B; 3) deficiencies for the regions between the breakpoints; 4) duplications for the regions between the breakpoints. The deficiency class will abort, resulting in approximately 25 percent pollen and ovule sterility. Thus, the progeny in crosses of heterozygous females by normal males contain 33 percent heterozygotes for translocation A, 33 percent heterozygous for B and 33 percent that are segmentally trisomic for all regions between the translocation breakpoints.

The production of the duplicated progeny in the above cross provides the basis for the ability to place genes in relationship to the translocation breakpoints. If the translocation heterozygote is crossed by a stock that carries an allele that encodes an electrophoretically distinct product and this gene lies between the two translocations, the maternal and paternal forms of the allozyme or protein will be skewed toward the maternal type in one third of the F1 because two doses of this allele are inherited in the duplication. The remaining two thirds of the progeny will have the normal heterozygous pattern.

In isolated cases, one translocation might be linked to one electrophoretic form and the other is linked to a second. If this heterozygote is crossed

by yet a third variant, then the segmentally trisomic individuals (inheriting the duplication from the female parent and a normal chromosome from the male) will have all three variants if the gene is present between the breaks. If, however, the progeny exhibit no indication of duplicated gametes, then the gene in question must lie outside the boundaries of the region spanned by the translocations. However, because the two variants are linked to the respective interchanges, it is equally valuable for cytogenetic localizations because the two alleles will not be recovered in a 1:1 ratio. This is the case because the two variants will more often be linked to the lethal deficiency produced in the cross. The variant linked to the most distally broken translocation will be recovered more often if the locus is proximal to both breakpoints. If the variant linked to the more proximally broken translocation is recovered more frequently, then the locus is distal to both translocation breakpoints. If the gene lies some distance from the two breaks, recombination between the locus and the nearest translocation will tend to randomize the skewed frequency but since the translocations _per se_ reduce crossing over in their vicinity, this produces little or no complication to the analysis. By comparing the results of cases of exclusion vs. inclusion in the duplication, the gene can be localized with considerable precision. This is due to the fact that over 1,000 translocations are available in maize (Longley, 1961). For the long arm of chromosome one, for example, there are 128 combinations of translocations that have the appropriate breakpoints to segregate double duplication gametes. These duplications span the regions of 1L from 0.04 to 0.95 (expressed as the fraction of the arm from the centromere to the telomere).

To confirm the positioning of molecular variants relative to the translocation breakpoints, one might construct compound B-A translocations and test for inclusion of the gene in question. Such translocations were first constructed by Rakha and Robertson (1970) and have portions of two chromosome arms linked to the B centromere. To produce such a chromosome, a hyperploid simple TB-A($AA^B B^A B^A$) is crossed as a female by the A-A translocation of interest. Among the progeny of such a cross will be heterozygotes of the B-A and A-A translocations. If recombination occurs between the B-A and A-A breakpoints, a chromosome of B^A-A constitution is formed. If it segregates with the complementary portions of both the B-A and A-A translocations, it can be recovered by outcrossing as a male to a tester stock that has an endosperm mutant of some sort that is located in one of the two A regions translocated to the B. This will allow the detection of the translocation as well as its recovery as a hyperploid heterozygote. Once constructed the translocation can be crossed as a male to the mutant to be cytogenetically placed. Due to the nondisjunction of the B centromere at the second microspore division, which gives rise to the two maize sperm, a substantial fraction of the male gametes will be deficient for both chromosomal regions attached to the B centromere. Thus, when crossed to female stocks carrying the mutant electrophoretic form, the gene will be uncovered if present in these regions. If the locus is present elsewhere in the genome, all progeny will exhibit a heterozygous pattern. Whether the variants are uncovered by the compound TB-As will confirm the localization established by the overlapping translocation method.

Recently a procedure has been proposed that should allow an even greater precision of localization than is possible with the present set of reciprocal translocations (Birchler, 1981). The method involves the recognition of newly induced translocations in pre-selected regions of the maize

genome. The basis relies on the induction of the appropriate translocations to produce duplicated gametes as described above that include the alcohol dehydrogenase-1 gene as a marker. The first requirement is the previous existence of one reciprocal translocation broken in 1L and in the other chromosome near the region of interest. If such exists, the second translocation that satisfies the necessary conditions to segregate duplication gametes can be induced and selected.

The pre-existing translocation would be used as a female for X-irradiated pollen from males homozygous for the Adh-Cm allele. The enzyme produced by this allele is relatively inactive, having only about 5 percent of normal activity, and also has a unique electrophoretic mobility. In the *in situ* staining reaction of pollen (Freeling, 1976) this mutant is weak and allows a ready classification. If the irradiation induces a translocation that will allow the production of segmental duplications including Adh, the ratio of ADH positive to negative unaborted pollen from the F1 plants will be 2:1, rather than the 1:1 ratio that would normally be found. Skewed ratios might also be produced if a linked translocation is formed that segregates duplications adjacent to but excluding Adh. This, however, is inconsequential since the two types of duplications, *i.e.*, including or excluding Adh, can be distinguished by subjecting an extract of the pollen to electrophoresis. This will allow a distinction since heterodimers are not usually observed in pollen (Schwartz, 1971) but when Adh is present in duplicated form, they will be. If a duplicated pollen class is produced, the Cm subunit will dimerize with the product of the other allele present in the same gametophyte. The other type of skewed ratio of pollen phenotypes would not have heterodimers. In this way the appropriate breakpoints can be recognized. In subsequent generations the Cm allele can serve as a genetic marker for the duplication. This procedure should be useful in that a precise genetic recognition of translocations involving pre-selected regions can be performed in the F1.

Finally, one might locate genes for which no variation can be found by testing chromosomal dosage series for a dosage effect. This approach is considerably less desirable since regulatory responses (Birchler, 1979) might complicate the analysis and result in dosage compensation (Birchler, 1981b). A fair number of the B-A translocations have phenotypic or allozyme markers that will allow discrimination of 1-3 doses of the respective chromosome arm (Birchler, 1982). Allozyme markers can be used to mark interstitial trisomics (Birchler, 1980) and can be extended to produce segmental tetrasomics (Birchler, Alleman and Freeling, 1981) or to form interstitial deficiencies (Birchler, 1982). With the appropriate allozyme markers and translocations, a rather substantial fraction of the genome could be tested in 1-4 doses.

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11 USING MAIZE MONOSOMICS TO LOCATE GENES TO SPECIFIC CHROMOSOMES

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Introduction

Maize is a diploid organism, and in diploids, two copies of each chromosome are present in every somatic cell. A monosomic is an individual in which one of the chromosomes is missing; therefore, a monosomic maize plant contains one copy of one chromosome and two copies of the other nine chromosomes.

Monosomics are perhaps the most interesting of the aneuploid types because a chromosome lacks a pairing partner in each meiotic cell and because genes on an entire chromosome are present singly in each somatic cell. However, monosomics in diploid organisms have seldom been utilized in studies because they are rarely produced, and once produced, the monosomic condition is not transmissible to progeny of monosomics. For this reason, they must be produced each time they are needed.

Recently, a system has been found which produces a high frequency of monosomics in maize (Weber, 1970, 1973). With this system, monosomics are available in a diploid organism in relatively large numbers for the first time. I have been working with this system for over a decade and have produced several thousand monosomic maize plants with it. Plants monosomic for nine, and probably all ten, of the maize chromosomes have been recovered utilizing this system. This is the only series of its type that has been produced in any diploid form. The monosomics are an extremely powerful tool for assigning genes to specific chromosomes.

Procedures for Producing and Selecting Monosomics

Maize monosomics are generated in high frequencies by the r-X1 deficiency, a sub-microscopic deficiency including the R locus on chromosome 10. The dominant allele of this locus is necessary for anthocyanin production in the aleurone layer of the endosperm of kernels. If R/r-X1 plants (which are heterozygous for the deficiency) are testcrossed as female parents, about 55-60% of the kernels produced are colored (R/r) while the remaining 40-45% are colorless (r/r-X1) deficiency-bearing kernels. Thus, the deficiency is transmitted through the female parent with a high efficiency; however, it is not transmitted through the pollen. Plants generated from R/r kernels are invariably diploid while those generated from r/r-X1 kernels include 10-18% monosomics and 10-18% trisomics (individuals with an extra chromosome). The remaining plants are diploid except for an occasional multiply aneuploid individual.

To select for plants monosomic for a specific chromosome, one can cross a male bearing a recessive sporophyte-expressed mutation onto a female parent carrying the dominant allele of this locus and the r-X1 deficiency. Plants expressing the recessive phenotype of this gene are almost invariably

monosomic for the chromosome bearing the gene; however, some cases of a partial loss of this chromosome has been found.

We typically cross a male which is r/r and which also carries a recessive mutation on each of its ten chromosomes (Mangelsdorf's tester) to a R/r-X1 female parent which bears the corresponding dominant alleles. The colorless deficiency bearing kernels are selected and planted directly into our research nursery. This cross is given below:

Female parent (R/r-X1)	Male parent (r/r)	Chromosome number	Gene
Bm2	bm2	1	brown midrib
Lg	lg	2	liguleless
A	a	3	anthocyaninless
Su	su	4	sugary endosperm
Pr	pr	5	red aleurone
Y	y	6	yellow endosperm
Gl	gl	7	glossy seedlings
J	j	8	japonica striping
Wx	wx	9	waxy endosperm
G	g	10	golden plant

Five of the mutations are for traits expressed in the sporophyte (bm2; lg; gl; j; and g on chromosomes 1, 2, 7, 8, and 10 respectively), and plants expressing these recessive traits are identified. These plants are almost invariably monosomic.

The other five mutations are for traits expressed in the endosperm of kernels, and plants monosomic for the chromosomes bearing these genes cannot immediately be detected. However, plants monosomic for these chromosomes are detected by crossing plants of sub-normal stature which have 50% or greater pollen abortion (presumptive monosomics) with a line that is a; su; pr; y; wx; and R. If a plant is monosomic for chromosome 3, 4, 5, 6, or 9, this testcross will only produce kernels of the recessive phenotype for the chromosome bearing that gene. Diploids and all other monosomic types will give a 1:1 ratio for that gene. For example, plants monosomic for chromosome 4 will give only wrinkled (su) kernels in this testcross while all other monosomic types and diploids will give a 1:1 ratio of wrinkled (su) to round (Su) kernels. Monosmics for chromosomes 3, 4, 6, and 9 can also be recognized on the basis of their distinctive plant morphologies.

Methods of Locating Genes to Chromosomes with Monosomics

1) One way that this system can be utilized to assign genes to specific chromosomes is immediately evident. Plants bearing a dominant allele of a gene plus the r-X1 deficiency can be crossed as female parents by plants bearing the recessive allele of this unplaced gene. Almost all plants expressing the mutant phenotype (pseudodominants) are monosomic for the chromosome bearing this gene, and identification of the monosomic chromosome is identification of the chromosome bearing this gene. Simcox and Weber (unpublished) have utilized this approach to tentatively assign the bx

locus (which in its recessive condition produces no cyclic hydroximide, alias DIMBOA) to chromosome 5 in maize. This procedure requires cytological identification of the monosomic chromosome.

2) A genetic approach utilizing maize monosomics to assign genes to specific chromosomes has been described (Weber, 1974). For this purpose, a Mangelsdorf's tester (carrying the ten recessive marker genes) which also carries the r-X1 deficiency is crossed as a female by a plant bearing a recessive allele of an unplaced gene expressed in the sporophyte. Progeny recessive for the unplaced mutation can be identified and backcrossed to the regular Mangelsdorf's tester (which carries the dominant allele of the unplaced gene and does not carry the r-X1 deficiency). The exceptional plants expressing the recessive phenotype of the unplaced gene received the chromosome bearing the dominant marker allele from the male parent but none from the Mangelsdorf's tester parent. In these plants the single chromosome bearing the unplaced gene carries the dominant marker allele while gene markers on all other chromosomes are heterozygous. A testcross of the exceptional plants onto the regular Mangelsdorf's tester would produce only plants of the dominant phenotype for the marker gene carried on the monosomic chromosome, and the other nine marker genes would segregate in the progeny in a 1:1 ratio. This procedure could be utilized to efficiently map sporophyte-expressed mutations in maize and requires no cytological analysis.

3) Monosomics generated by the r-X1 deficiency are especially useful in determining the chromosomal location of genes whose gene products have different electrophoretic mobilities. For example, Stout and Kermicle (1979) crossed R/r-X1 maize plants bearing a rapidly migrating histone H1 band (which is coded for by a single locus designated Hla) by a male parent which carries the brown midrib-2 gene on chromosome 1 and a slower migrating allele of the Hla locus. A monosomic brown-midrib plant was recovered and it contained only the slower migrating band which was present in this male parent. Monosomics 2, 4, 5, 6, 7, 8, 9, and 10 each contained both bands. Clearly, the locus coding for this histone is located on chromosome 1 in maize. Similarly, R/r-X1 plants bearing a slowly migrating allele of the peroxidase-3 locus were crossed by Mangelsdorf's tester which carried a rapidly migrating allele. Plants monosomic for chromosome 7 carried only the rapidly migrating allele while other monosomic types as well as their diploid siblings contained both alleles (Weber and Brewbaker, unpublished). Clearly, this gene is carried on chromosome 7.

4) Monosomics can also be used to alter the number of copies of known genetic loci. For example, Morgan (1956) found a maize plant that was monosomic for an unidentified chromosome, and this plant was also asynaptic. Because the gene, asynaptic, is located on chromosome 1 in maize, they hypothesized that the monosomic plant may have been monosomic for chromosome 1, and that asynapsis was due to hemizygosity of the dominant allele of this locus. To test this hypothesis, Baker and Morgan (1969) produced three monosomic 1 maize plants, and all three were indeed asynaptic. Monosomic 1 plants generated by the r-X1 deficiency (Weber, 1973) are also asynaptic.

5) When a monosomic plant of a diploid species undergoes meiosis, two haploid (n) cells and two cells nullisomic (n-1) for the chromosome which was

monosomic are produced'. In this way, a population of cells nullisomic (completely lacking) for a specific chromosome can be produced. By comparing these nullisomic and haploid cells, one compares the presence and absence of all genetic loci on a specific chromosome. One example utilizing this approach is given below. Chromosome 6 in maize contains the nucleolar organizing region at which the 18S and 28S rRNA templates are located. Cells nullisomic for this chromosome lack nucleoli as expected. Chromsome 2 carries the 5S rRNA templates, and cells nullisomic for chromosome 2 contain a normal appearing nucleolus; therefore, the 5S rRNA template is not a nucleolar organizing region (Weber, 1978).

6) Monosomics can also be used to explore the genome on the basis of gene dosage. By comparing a plant monosomic for a specific chromosome with its disomic siblings, one compares the effects of one and two copies of all genes on a given chromosome. If a gene present on the monosomic chromosome exhibits dosage effects, then a difference will be found between these two plant types. In this way, one screens all genetic loci on a given chromosome simultaneously by gene dosage comparisons. With this method of analyzing the genome, one analyzes each genetic locus on a given chromosome without inducing gene mutations. This powerful methodology is being utilized to explore the maize genome for genetic determinants of several types. For example, Plewa and Weber (1975) determined the effects of monosomy of specific chromosomes on the relative amounts of the different fatty acids in maize embryos. Monosomic 2 embryos had significantly more oleic acid and significantly less linoleic acid than diploid control embryos. Because the conversion of oleic acid to linoleic acid is a single-enzyme-mediated reaction, they suggested that a gene involved in this conversion is located on chromosome 2. Other alterations were found in other monosomic types.

This same approach has also been used to study the acid-extractable amino acids (free amino acid pool) in maize leaves. The free amino acid pools in specific monosomic types were compared with those in diploid control plants (Cook and Weber, 1976, Cook, 1977), and it was found that the profiles in the six monosomic types tested were each unique and each was different from the profile found in diploid plants. In many cases, there were increased levels of a precursor and depressed levels of an end product in an amino acid biosynthetic pathway, and this suggested that a structural or regulatory gene resides on the monosomic chromosome which was responsible for this biosynthetic step. For example, monosomic 2 plants had significantly higher levels of glutamic acid (117.7% of diploid levels) and significantly lower levels of glutamine (46.7% of diploid levels). Because glutamine is derived from glutamic acid in plants, a factor on chromosome 2 might be involved in this conversion. In other cases several products in the same biosynthetic pathway were all depressed or increased suggesting genetic control of a precursor of that pathway. This study may give a clue to the chromosomes which carry certain structural or regulatory loci involved in amino acid biosynthesis in the maize genome.

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12 THE MAPPING OF GENES BY THE USE OF CHROMOSOME ABERRATIONS AND MULTIPLE MARKER STOCKS

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Assignment of map position to a newly-discovered mutant gene that is not allelic to a known locus is ordinarily a three-step procedure: location to chromosome, localization within the chromosome and, finally, determination of position relative to immediately-flanking marker loci. Three principal kinds of stocks are employed in one or more of these steps. Multiple marker stocks may incorporate marker genes on each of several chromosomes or, alternatively, may consist of gene combinations selected to provide marking at spaced intervals over an extended region of a single chromosome. Chromosome aberrations (chiefly reciprocal translocations and inversions) represent a second category of stocks useful in locating or localizing genes. A third approach to locating genes to chromosome is provided by monosomics and trisomics. Comments here will be restricted to the first two of these categories, the multiple marker gene stocks and chromosome aberrations.

Maize geneticists in the past have developed numerous multiple gene stocks for specific purposes. Many of these combinations have included marker loci on each of several chromosomes and are commonly referred to by the generic term "multiple gene testers". Perhaps the most extensive of these, the "Mangelsdorf Tester", carries a marker allele on each of the ten chromosomes. This tester was frequently used in past years in a screening method to locate genes to chromosomes, but was only marginally effective. Location of dominant genes is reasonably successful since the Mangelsdorf Tester may be used as a parent in testcrosses. Most mutant alleles employed in genetic studies, however, are recessive to wild-type alleles. Linkage data from the use of multiple gene testers are, therefore, usually sought in F_2 progenies. In such progenies, the presence of only a single marker locus per chromosome is not very effective in yielding unambiguous evidence of linkage.

Maize geneticists have developed a large number of stocks carrying multiple combinations of genes located on the same chromosome. Stocks in which the marker genes are distributed over a long chromosome region are commonly referred to as "chromosome testers". These are no longer widely used in the initial step of assigning new genes to chromosomes, since more efficient general screening techniques are available. However, they are frequently used in a second stage of mapping to obtain preliminary information on the approximate position or sequence of a gene in the standard linkage map, once location to a specific chromosome has been established.

Assignment of map position should ideally be accomplished with a three-point linkage testcross using immediately-adjacent flanking markers if the nature of the traits makes this approach feasible. Usually, however, the requisite three-locus tester combination would need to be extracted following recombination with the flanking markers. In practice, then, map positions are frequently assigned by a two-generation mapping procedure in which the

first generation is a two-point testcross. In the subsequent generation allelic constitution at the third locus in a sample of progeny plants may be determined by testcross or in an F_2 generation. The ideal three-point tester combination, then, even in those cases in which the combination can be perpetuated in homozygous condition, is usually a product of mapping rather than a tool for mapping.

Three principal kinds of chromosome aberrations in maize are used in gene location and mapping: inversions, B-A translocations and reciprocal translocations between chromosomes of the standard (A) complement.

Relatively few inversions have been identified and perpetuated in maize. In large part this neglect is explained by the large number of cytologically characterized A-A and B-A translocations that are available for gene location and for use as tools in other cytogenetic studies. A set of gene-marked chromosome aberrations that is widely used for location of genes to chromosome includes as components two inversions, Inv 2a and Inv 9a.

The usefulness of heterozygous inversions in locating genes to chromosome results from the phenomenon commonly referred to as "crossover suppression". In heterozygous inversions, recombination between the interchange points of an inversion results in unbalanced, duplicate-deficient chromatids. Meiotic products containing such unbalanced genomes are commonly abortive or noncompetitive, particularly in male transmission. In long inversions the apparent crossover suppression is more strongly attributable to non-transmission of recombinants than to actual reduction in synapsis and crossing over. The usefulness of inversions in locating genes by virtue of linkage results from the increased proportions of gene combinations carried on the parental homologs that are transmitted intact.

B-A translocations result from reciprocal interchanges between a chromosome of the standard (A) complement and a supernumerary (B-type) chromosome present in some strains of maize. The uses and transmission characteristics of these interchanges are discussed elsewhere in this publication (see article by J. B. Beckett). B-A translocations provide a particularly efficient tool for location of genes to chromosome. In the first instance, assignment of a gene to a specific segment of a particular chromosome may be apparent by the expression of the hemizygous recessive mutant phenotype among hypoploid individuals in the immediate F_1 progeny generation. In the event that a mutant locus is not situated in the hemizygous segment in hypoploid plants, the same F_1 hypoploid plants may be testcrossed. The frequency of transmission of an allele carried on the deficient chromosome is a direct measure of recombination between that locus and the point of deficiency. A comparison of this recombination frequency with recombination frequencies between the same point of deficiency and other mapped loci in the chromosome permits an estimate of the probable sequence in the linkage map of the mutant locus under test. For a detailed report on the use of B-A translocations in locating genes, see Beckett, 1978.

About 865 reciprocal translocations between the standard (A complement) chromosomes of maize have been identified and perpetuated. The cytological positions of the interchange points of this large collection are listed by Longley, 1961. These stocks are available from the Maize Cooperation Genetics Stock Center (see below) and are listed in the 1981 issue of the Maize Genetics Cooperation News Letter, Vol. 55 pp. 140-145.

The only chromosomally balanced spores produced by plants heterozygous for a reciprocal translocation contain either both interchanged chromosomes or both normally arranged homologs. In crosses in which unbalanced chromosome complements are not transmitted, a complete artificial linkage is established between the two interchange points and between the corresponding points on their normal homologs. This complete linkage between points on nonhomologous chromosomes is a direct consequence of non-transmission of other combinations of these points. Because of their complete association in transmission, the two interchange points may be considered as one in inheritance. In plants heterozygous for a reciprocal translocation, typically about half the products of meiosis carry chromosome imbalance that is expressed as spore abortion. The semisterile phenotype resulting from pollen or ovule abortion is the usual basis for identifying heterozygotes in segregating progenies. In crosses of standard maize by plants heterozygous for a reciprocal translocation, the translocation may be followed as if it were a dominant gene for semisterility.

Since each reciprocal interchange involves two chromosomes, semi-sterility maps to two chromosomes and the linkage relations so derived may be graphed as a cross-shaped linkage map in which the two interchange points are simultaneously represented as the center of the cross configuration. Since interchange points in the physical chromosomes can be correlated with positions in linkage maps, it is possible to infer positions of gene loci in the physical chromosomes.

Our current collection of reciprocal translocations permits complete artificial linkage of some 865 pairs of points in the maize genome. Alternatively, they represent about 1730 genetic loci, all classified by the same character, semisterility.

Reciprocal translocations have been widely used to locate genes for both qualitative and quantitative traits. Many stocks have been developed and maintained for special purposes. Examples of some of their applications and proposed uses have been presented by Anderson (1956) and Burnham (1966).

Duplicate-deficient chromosome complements that are egg-transmissible are produced by plants heterozygous for some reciprocal translocations. With respect to a particular rearrangement, these duplicate-deficient complements carry a translocated and a normal chromosome, rather than the usual transmissible combinations of both translocated or both normal chromosomes. Descriptions of the inheritance and uses of duplicate-deficient complements, together with proposed further applications, are presented by Phillips, et al (1971) and Patterson (1978). Beyond their uses in mapping, these stocks and other chromosome aberrations have broad potential as tools in several areas of biological research.

A comprehensive collection of genetic stocks is maintained by the Maize Cooperation Genetic Stock Center, Agronomy Department, University of Illinois, Urbana. The collection includes seed stocks of mutant alleles, multiple gene combinations, chromosome aberrations and numerous other strains of interest to geneticists and breeders. Listings of available stocks appear in the annual Maize Genetics Cooperation News Letter. Small seed samples are provided without charge upon request. The Center also serves as a clearinghouse for information in maize genetics and is able to provide assistance and advice on choice of stocks and on their classification and use.

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13 PLANNING PROGENY SIZES AND ESTIMATING RECOMBINATION PERCENTAGES

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In order to plan for efficient and economical experiments, and to make estimates of recombination frequencies with standard errors, what standards are available? What are the rule-of-thumb sizes for progenies?

The size of progeny needed to include at least one of the recessive class in a family segregating in a ratio of p:q (e.g., 3/4:1/4) can be calculated from the first term of the binomial, p^n. This expression represents the frequency with which all n members of the progeny will be dominant (p class). That frequency is desired to be small, e.g., 1%. Thus, if p^n = 0.01 and p = 3/4, n is 16, which means that a progeny of 16 will contain one or more of the recessive class in 99% of such progenies. Similar calculations for a 1:1 ratio yield n = 7. The accompanying Table gives progeny sizes for several frequencies and probabilities.

A valuable table developed by W. L. Stevens (1942; also in Fisher and Yates' tables, 1963) provides for estimation of limits and fit for binomial and Poisson distributions, for low expectations in small to infinite progeny sizes; this table can be used also (in reverse) to help in planning progeny sizes. For progeny sizes to distinguish between two different proportions (e.g., 3:1 vs. 9:7), and for several other planning calculations, see Hanson (1959) or Mather (1957).

For recombination data from a testcross (e.g., + +/a b X a b/a b), p is calculated by adding the two recombinant classes and dividing by the total observed. The standard error of p is the square root of pq/n. The progeny size, n, required for a certain precision can be calculated for an assumed p and its standard error. Thus, for p = 10 ± 1%, n = 900; for p = 10 ± 5%, n = 36; for p = 20 ± 5%, n = 64; for p = 40 ± 5%, n = 96, all for testcrosses in coupling (cis) or repulsion (trans).

Recombination data from an F2 progeny or other sources are only treated with precision, accounting for all the proportions observed, by the mathematically exact method of maximum likelihood. Tables, formulas, examples and evaluations for a wide range of data sources are given in Allard (1956). A simple, more easily applied method that has been shown to be equally exact mathematically for normal purposes is the product method. Immer (1930) and Immer and Henderson (1943) give tables and formulas for the product method in F2 for 3:1 A:a with 3:1 B:b, several interaction ratios (e.g., 9:7 with 3:1), and partial backcross (3:1 A:a with 1:1 B:b, from A a B b X A a b b). Stevens (1939) gives a more convenient table for the 3:1 with 3:1. Standard errors of p values for the F2 and other ratios, second derivatives of the appropriate function, can be calculated with the aid of the same tables, or from formulas and tables in Allard (1956).

In a coupling F2, for p = 10 ± 5%, n = 41; for p = 20 ± 5%, n = 83; for p = 40 ± 5%, n = 176. In a repulsion F2, larger numbers are required: for p = 10 ± 5%, n = 390; for p = 20 ± 5%, n = 363; for p = 40 ± 5%, n = 275.

Tabulations of recombination data should be presented as follows:

For two-point data:

	Progeny					
	A B	A b	a B	a b	T	Rec
A b / a B F2	#	#	#	#	#	%

For a three-point testcross: A b C / a B c x a b c / a b c

	Parentals		Recombinants						Total
			Region 1		Region 2		Reg. 1 & 2		
	AbC	aBc	ABc	aBC	Abc	aBC	ABC	abc	
A b C/a B c	#	#	#	#	#	#	#	#	#
Subtotals	#		#		#		#		#
Percentages		%		%		%		%	

The complete, systematic tabulation of all classes (as above) is essential to evaluation of the data by others, and to re-analysis as new information develops.

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Minimum progeny size needed in order to include at least one of the desired class, with a chosen probability level. After Mather (1957) and Hanson (1959).

Class desired	Probability Level 90%	95%	99%	99.9%
1/2	3.3	4.3	6.6	10.0
1/3	5.7	7.4	11.4	17.0
1/4	8.0	10.4	16.0	24.0
1/7	14.9	19.4	29.9	44.8
1/8	17.2	22.4	34.5	51.7
1/9	19.5	25.4	39.1	58.6
1/16	35.7	46.4	71.4	107.0
1/27	61.0	79.4	122.0	183.0
1/32	72.5	94.4	145.1	217.6
1/64	146.2	190.2	292.4	438.6
1/100	229.1	298.1	458.2	687.3
1/1000	2301.4	2994.2	4602.9	6904.3

2/3	2.1	2.7	4.2	6.3
3/4	1.7	2.2	3.3	5.0

Example: Suppose that, in a case where the segregation is 3:1, a progeny large enough to include at least one of the recessive (a a) class is to be obtained. The class desired occurs with a frequency of 1/4 (3d row of the table), and we choose a probability level of 99%. In the body of the table we find that 16.0 individuals are sufficient--i.e., that about 99% of progenies of 16 individuals will contain at least one of the recessive class. While the progeny size on purely mathematical grounds should be rounded upwards, these numbers are suitable to use simply as approximate guides to adequate progeny sizes.

14 PERSONAL RECOLLECTIONS OF EVENTS LEADING TO A CORRELATION OF LINKAGE MAPS AND CHROMOSOMES IN MAIZE AND BARLEY

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This discussion is based on my personal recollections of the genetical and cytological studies that resulted in the identification of each linkage group with its particular chromosome in corn and in barley.

The first example is corn with ten chromosome pairs, a cross pollinated crop for which large numbers can be obtained by applying pollen to ears that have been bagged before the silks emerge. Backcrosses to multiple recessives can be produced in large numbers for linkage tests. Increases by self pollination require as much work as crossing.

The second example is barley with seven chromosome pairs, a self-pollinated crop in which emasculation must precede crossing (unless male sterile plants are used). Backcrosses are not feasible for linkage tests. Increases to produce F_2s from F_1s, etc., are easy and can be used for linkage tests.

When corn breeders began self pollinating plants in open-pollinated varieties, the progeny from many plants segregated for one or more mutants that had accumulated over the years. Many of these were saved and tested first against the different endosperm characters: yellow vs. white, the factors complementary for aleurone color, and purple vs. red aleurone color or against other markers. Emerson, an early worker doing such tests in Nebraska, also instigated a "freak corn" class at state and county fairs. That is the source of a number of current genetic markers. The purple plant color type came from a single ear at one of those fairs.

In farmers' fields of open-pollinated varieties, there were yellow or white seedlings which died. A common statement by farmers was that these were a sign of a high-yielding crop. The fine-striped character in chromosome 1 was seen by Emerson in a farmer's field and he obtained permission to transplant it to his own plot. The emphasis in the corn shows held at that time, "show corn," was on ear characters that had little or no relation to yield.

Since the characters were tested first against the endosperm markers, the genes in the first linkage maps were likely to be clustered around those markers, e.g., c in the short arm of 9, and pr in the long arm of 5. The long arm of 9 and the short arm of 5 were without markers for many years. Markers for those arms were found when other methods became available, e.g., trisomics and interchanges.

In certain cases the map began with two genes that were not endosperm markers. By 1929, corn geneticists believed they had established the 10 linkage groups and had numbered them from I to X (Lindstrom, 1928). In 1926 a triploid maize plant was reported (Randolph and McClintock, 1926; McClintock, 1929, her Ph.D. thesis). The cross of 3n X 2n had, among its

progeny, plants with one additional chromsome (2n + 1), trisomics that had 9II + 1III at meiosis.

In normal diploid corn and also barley, each microspore (with n chromosomes) divides once to form the tube nucleus and the generative nucleus. The latter then divides once to produce two sperm nuclei. The mature pollen grain therefore has three nuclei. At the proper prophase stage of the first postmeiotic division of the microspore McClintock was able to identify the different chromosomes based on differences in total length and in the position of the centromere. She published an idiogram of the ten chromosomes in 1929. Since one chromosome was represented twice in an n + 1 microspore, she knew which chromosome was trisomic in the plant from which she had taken the tassel sample for cytology. That plant came from a cross of 2n + 1 plant as a female with a diploid that carried genetic markers, some of which were endosperm markers. By selfing those trisomic plants, she could determine if any of the markers segregated for trisomic ratios. She knew the marker constitutions of most of the plants she had crossed--stocks for the different linkage groups. Those segregating for yellow endosperm (chromosome 6) or Pr vs pr (red) (chromosome 5) could be examined on the ear. She couldn't wait for the ears to fully mature, but peeled back the husks to count the ratios - I remember seeing an ear on a plant she had identified as 2n + 1 for chromosome 5. She checked them every few days. As the aleurone color began to develop, she had counted the kernels on enough of the ear to know that Pr:pr was a trisomic ratio, about 5:1, not a diploid 3:1. This linkage group, therefore, was in chromosome 5.

During the early 1920's, Dr. L. J. Cole (Genetics Department, University of Wisconsin, Madison) encouraged inbreeding work with corn. A number of characters appeared in the various lines. Dr. R. A. Brink directed some of the work after he joined the Department. One of the new characters in one of the lines was brown midrib. William H. Eyster had described a number of new characters, one of which was a brown midrib, bm1, another a deficient type which he said could be corrected with insulin. R. A. Emerson told me later that he was very much impressed by Eyster's work, but there was no later report. I wrote to Eyster for seed of his brown midrib. Plants from a cross between the stocks were brown midrib. They were not different genetically. One of the crosses I happened to make involved brittle endosperm and Pr-pr (purple vs. red) aleurone color. I was very much surprised to find the two characters were linked. It was exciting, since it was my first finding of a linkage.

The cytological information on corn revealed that usually there were 10 pairs of chromosomes but certain stocks had higher numbers (Fisk, 1927; Kuwada, 1919). The meiotic figures (diakinesis and metaphase I) showed little or no promise of any distinguishing features of the different chromosomes.

While that was happening, Brink, working on the biochemistry of the waxy gene, identified WxWx and Wxwx plants in segregating populations by microscopic examination of pollen in a drop of I-KI solution (Wx starch is blue, wx starch is brick red). Pollen grains of Wx Wx plants are all Wx (blue-staining), those of Wx wx are 50% Wx (blue):50% wx (brick red). He discovered a case of semisterility (S.S.), 50% of the pollen and ovules was aborted. Studying its breeding behavior he concluded it was due to a chromosome change (Brink, 1927; Brink and Burnham, 1929). Progeny from

selfing a S.S. plant included semisterile and fertile plants in a 1:1 ratio; but half the latter were homozygous for the chromosome change. These, when crossed on a standard normal stock produced F_1s that were 50% sterile. The other class of normals produced F_1s that were fertile, and therefore were standard normal.

The explanation proposed first was that a terminal segment of one chromsome became attached to the end of a non-homologue (a "simple" translocation). To explain the observed 50% spore abortion it was assumed that pollen with a duplication and pollen with a deficiency aborted. The observations at pachytene later showed that an exchange of segments had occurred and that the aborted spores had a chromosome deficiency. The standard normal stock used for the tests was pr wx. Tassel samples from the standard plants used in crosses had been preserved in alcohol (70% + formalin - later the formalin was omitted - a great improvement!). When the tassels were examined later, three of the standard plants used in the tests had been partially sterile. Two of them had been crossed. Those crosses were given to me by Dr. Brink and I grew them the next summer at Wisconsin. They were segregating for one semisterile and one new, high sterile (about 75% sterile) class. The fertile plants from Brink's S.S. (semisterile designated S.S.-1) crossed on the two S.S. plants in the standard pr wx stock must have been homozygous S.S.-1. In both crosses, plants with high sterility were self-pollinated and also crossed with standard normal plants. The two new semisteriles were designated as semisteriles -2 and -3. The various crosses involving these stocks were what I had proposed to study on a National Research Council Fellowship at Cornell University. Samples of the various crosses were sent to Cornell for planting in 1929. I believe George Beadle was one of those who helped in the planting.

At Wisconsin, we had always written at the time of planting the culture numbers on lath stakes that had been planed smooth on one surface. At Cornell they used tagboard tags that could be numbered in advance and then tacked on the stakes (they asked why I hadn't sent the tags for the stakes!). My material had been planted in what was known as "The Hole," a low area of possibly 2 or 3 acres, protected on all sides by a high bank, an area not subject to the early fall frosts common in outlying fields. This was a short distance from the Plant Breeding Department, and was used for material to be crossed. A small building at the edge of the field was used for supplies and shelter.

When I arrived in early June and went out to the field, McClintock was in the field and helped to identify my cultures and how they were arranged in the field. The next few weeks and the rest of the year were really exciting. Here was my material segregating for different degrees of pollen and ovule sterility. Belling had proposed a 2-factor explanation for a case of semisterility in the Florida velvet bean, Stizolobium; later changed to a chromosomal interchange explanation. McClintock had found a stock which in crosses with normals formed what she called quadrivalents at meiosis: the stock, a quadrivalent-former. When her plants started shedding pollen she found they were semisterile. The next thing was for me to do cytology on my material for which there had been data on pollen sterility but no cytology. I had taken all the courses in Botany at Wisconsin except taxonomy and the laboratory in Cytology (C.E. Allen). The laboratory portion probably would not have helped me with the cytology I was about to do, since McClintock was in the process of developing the smear technique for corn sporocytes.

McClintock's lab was on the second floor of Stone Hall. She had a table where she worked with her microscope. Beadle had a table where he was doing cytology on male sterile lines. When another persòn started work, another table was set up. Sponge rubber was placed under the table legs and under the microscopes to minimize the vibrations caused by people walking across the wood floor. The person who is now Mrs. Ernst Abbe had a desk at the opposite side of the room from McClintock. I had a small table opposite McClintock's. For several days I had no success in locating the stages. She would show me what she was finding on her slides - clearly showing the chromosomes. That kept me trying. Finally I found that by reducing the light intensity I could see the chromosomes, and then began to get results. I was beginning to get pollen classifications on some of the progenies.

Since semisteriles -2 and -3 occurred in the standard normal pr wx stock, the high sterile F_1's were heterozygous for wx. Some of the high sterile F_1's had been crossed with standard normal non-waxy (Wx). When the numbers in the various classes for degree of sterility and for waxy in the pollen for the crosses involving semisterile-2 were summarized there was a ratio of about 1 high sterile:1 fertile:2 S.S. plus a few 25 to 30% sterile and a few intermediate in sterility between S.S. and high sterile. The segregations for waxy (as shown by pollen classification) were about 1 Wx Wx:1 Wx wx from the cross Wx Wx normal x Wx wx high sterile. Considering both sterility and the Wx vs. wx segregation the numbers were:

Fertile		High sterile		Semisterile	
Wx Wx	Wx wx	Wx Wx	Wx wx	Wx Wx	Wx wx
74	10	18	61	86	80

The ratios for Wx Wx:Wx wx are 1:1 only in the S.S. class. The numbers in the other classes suggest a linkage between wx and sterility (28/163 = 17.2%). I had found that high sterile plants at diakinesis had 2 ⊙ 4 + 6II (two rings of four plus six pairs). Hence semisterile -2 involved two chromosome pairs that were different from those in S.S. -1 (Burnham, 1930). The 1 Fertile:1 H.S.:2 S.S. ratio from H.S. x N is: 1 with neither ring:1 with both rings:1 S.S. #1:1 S.S.#2. The linkage was between wx and S.S.#2.

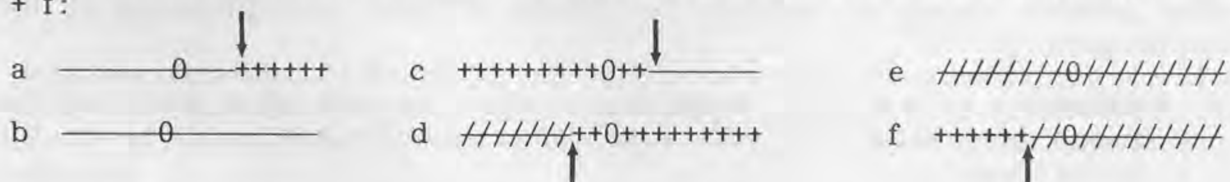
McClintock up to this time had not used the pachytene stage for studying chromosome morphology. She had discovered that applying heat to fresh smears of corn sporocytes that had been killed in acetic acid and alcohol and then stained with aceto-carmine spread better and also gave better contrast between stained cytoplasm and chromosomes. While examining sporocytes of semisterile -2 she found that at pachytene one of the chromosomes had a very large terminal knob. This pair of chromosomes could be recognized in every cell. She could distinguish individual chromosomes, locate the positions of centromeres and sub-terminal or terminal knobs, trace them with a camera lucida, then measure total lengths and arm lengths. In semisterile -2 heterozygotes she found a cross-shaped configuration, interpreted as indicating that an exchange of segments had occurred to produce semisterile -2. The second and third smallest chromosomes were involved (McClintock, 1930), later identified as chromosomes 8 and 9 respectively, the

terminal knob being on the short arm of 9. Semisterile -2 is now T8-9a. In many interchange heterozygotes, the position of the "cross" may vary so that either axis may be longer or shorter. Since this brings non-homologous parts together (or there may be asynapsis), recombination may be reduced in regions adjacent to the breakpoints. This has been found in many interchange heterozygotes in corn. Subsequent studies of T8-9a showed very little variation in position of the "cross" (Burnham, 1934). Recombination in regions close to the breakpoints in this interchange was not tested adequately.

Brink had found a single plant in the sweet corn breeding plot that had leaves with torn and split leaves which he named Ragged. This was a dominant character which proved to be linked with d in one linkage group and also with ts4 which was supposed to be in a different linkage group. Therefore both were in the same group. For a few years only 9 linkage groups were known.

The other chromosome involved in semisterile #2 was 8. The japonica striping character (j) had been tested with many markers but no linkage had been found. My tests showed a loose linkage between j and semisterile -2. A trisomic test with chromosome 8 showed j was in this chromosome. This was the missing linkage group (Burnham, 1934).

Semisterile -3 plants crossed with homozygous S.S.-1 produced high steriles and semisteriles in 1:1 ratio. The cross of those high sterile with normals produced progeny, most of which were semisterile. A few were low sterile. How could that happen? Thinking about it on the way home and during one night it occurred to me that the probable answer was that one chromosome involved in semisterile -3 was the same as one involved in semisterile -1, i.e., only 3 chromosome pairs were involved in these two semisteriles: In the following diagram for the F_1 between the two interchanges, S.S.#1 contributed the a + c + e chromosomes and S.S.#3 contributed b + d + f:



Except for possible crossovers between the c and d chromosomes in the region between the exchange points, indicated by arrows, the only viable chromosome combinations would be the parental ones: a + c + e and b + d + e. When this H.S. was crossed with normal, 1/2 of the progeny would be S.S.-1 from a + c + e, the other half S.S.-3 from b + d + f. The prediction would be a ⊙6 (ring of 6) at diakinesis in H.S. plants assuming an association of homologous ends. The next morning a ⊙6 + 7II was found. What were the low steriles and the intermediates between S.S. and high steriles? McClintock suggested they might be 2n + 1 from 3-1 disjunction from ⊙4, or 4-2 from a ⊙6, the extra chromosome covering some of the otherwise deficient combinations. This was found to be true, they were trisomics. Root tips were obtained by pulling away the top soil at the base of the plant, watering it well and covering the ground with wet sacks. Roots grew to the top after a few days and were taken for chromosome counts (in those days done by paraffin embedding, microtome sectioning and staining, Randolph's schedule). Some of the low steriles from crosses involving S.S.-3 had narrow, stiff leaves; characteristic of the trisomic for

chromosome 7. Therefore S.S.-3 involved chromosome 7 and either chromosome 1 or 2 that were involved in S.S.-1. Semisterile-3 was linked with factors in 1 and is now T1-7a.

Meanwhile Brink had made tests for linkage with S.S.-1. In his first tests there was no linkage with P in chromosome 1 or lg in chromosome 2. In the next year's tests he found linkage with br and f in chromosome 1 and v4 in chromosome 2 (Brink and Cooper, 1931). The first year's tests had been with a marker in each of these chromosomes.

A trisomic stock for each of 9 of the 10 chromosomes had been established by McClintock and for many of them she had determined the linkage group each carried. The original plant that was trisomic for chromosome 1 succumbed to smut and this trisomic stock was not available for many years. Information from the trisomic tests, linkages with interchange stocks, and the results of cytological analysis of intercrosses between interchange stocks was coordinated to complete the identifications. In a few cases, identified trisomics were crossed with an interchange stock to identify one of the chromosomes in the interchange. In the trisomic F_1 plants from such a cross the extra chromosome might be one of those in the ring or it might not be. If the plant had a III + ⊙4 + 7II the trisomic chromosome was not involved in the ring. If the plant had a chain of 5 + 8II, the trisomic chromosome was involved. To summarize, linkage groups were placed to chromosome in corn by:

1. Use of trisomics, each identified cytologically and by trisomic ratios for genes carried in the trisome.
2. Use of chromosomal interchanges, by linkage of marker genes with semisterility and identification of chromosomes involved and breakpoints by cytology at pachytene.

One problem remaining was the orientation of each linkage group in the chromosome.

Method 1: Cytological examination of two interchanges involving the same chromosome but with breakpoints at different positions in the linkage map.

Example 1.

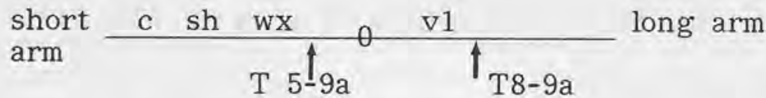
short arm lg 10 gl 19 B 0 21 v4 long arm

↑ T2-3a ↑ T1-2a

The break in chromosome 2 in T2-3a was found by pachytene cytology to be in the short arm and the break in 2 in T1-2a was in the long arm. Only the above orientation of the linkage group in the chromosome will fit that information with the observed linkages between the genes shown in the linkage maps and the close linkage between lg and T2-3a and 13% recombination between v4 and T1-2a.

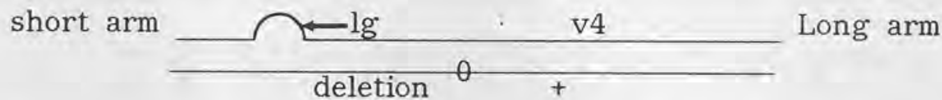
Example 2. In semisterile -2 (T8-9a) the break in 9 was in the long arm. The gene order could be short arm c shwx vl T, or it could be: short arm _ _ _ T vl wx sh c long arm. In T5-9a the break in 9 was

in the short arm and the order was c sh wx T v1. Hence the c sh wx genes are in the short arm. Also in the homozygote for T5-9a v1 and wx were no longer linked. Therefore the map orientation in 9 is:

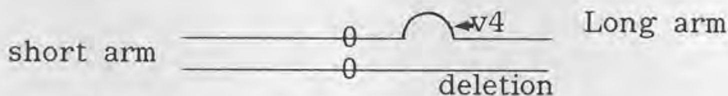


Method 2: The use of pachytene observations on plants with deletions for genes with known positions in the linkage map.

X-raying of pollen produces deletions in the sperm cells which can bring about fertilization if the tube cell carrying that sperm is normal (no deficiency) to produce plants hemizygous for the deleted region. For example: a lg v4 stock pollinated with x-ray pollen of the normal Lg Lg V4 V4 stock may produce some plants that are lg if the deleted segment carried Lg; and others that are v4 if the deleted segment carried V4. Cytological examination at pachytene of those plants will show a loop (buckle) in the chromosome without the deletion: McClintock (1931) did this for lg and several other genes. Since lg was near one end of the short arm of 2 and at one end of the long linkage map known at that time this was sufficient to orient the map in 2:



Pachytene observations in a plant with a deletion for V4 would have clinched it without question:



Deletions will vary in length. The shortest one that uncovers the locus of a gene will furnish the most precise location (assuming the position of the buckle is not variable - there is evidence it may vary (Weinheimer, unpublished).

Progress in corn cytogenetics was rapid from 1929 through the next few years. In 1929, Lindstrom presented a paper on the status of corn genetics in which he showed the ten linkage groups as known at that time. There was no cytological information. In 1932, Emerson presented a paper at the Genetics Congress at Ithaca on the same topic. The chromosomes had been numbered according to length from 1 to 10, 1 being the longest; the linkage group carried by each chromosome was known, the orientation of the linkage group in each chromosome was known and the positions of several genes were known. (Many years later large numbers of interchange stocks were found in material that had been exposed to radiation in the Bikini and Eniwetok bomb tests. These were studied by Randolph, Clokey, Anderson, and Longley at Cal. Tech.)

Emerson gave me a number of stocks segregating for partially filled ears. Several were interchanges that I identified as to the chromosomes involved, and are now in the collection maintained by the Coop. None proved to be the lethal ovule type of semisterile ears found by Singleton.

As information accumulated and more interchanges were identified as to the chromosomes involved, a tester set of identified lines was selected with which the chromosomes in any new interchange could be identified by crossing with the testers and examining the F_1's for the kinds and number of configurations. This tester set (the one still in use) is:

T1-2a and T2-4d will identify 1, 2, 4

T3-7c and T5-7c will identify 3, 5, 7

T8-9a and T8-10b will identify 8, 9, 10

Chromosome 6, the satellite chromosome is not included, since any interchange involving it is clearly associated with the nucleolus at diakinesis. There are three possible results that can be observed at diakinesis of meiosis in the F_1 of a cross between a stock homozygous for a single interchange whose chromosomes are to be identified, and the conclusions to be drawn are as follows:

1. If 1-2a X unknown has 2 ⊙ 4, the unknown does not involve 1 or 2.

2. If 2-4d X the same unknown has a ⊙6, the unknown involves either 2 or 4; but the first cross eliminated 2, therefore this unknown involves 4 + ?

3. If 1-2a X unknown has 10II or a ⊙4, the unknown and tester involve the same two chromosomes, i.e., 1 and 2. When I went to the California Institute of Technology in 1930, Anderson was attempting to identify the interchanged chromosomes by linkages. He had worked with Drosophila in which linkage between markers in different chromosomes was used to identify interchanges. Since there was no crossing over in the male, backcrosses using as males heterozygotes for the interchange and also for a single marker in each of the four chromosomes would give complete linkage between the two markers in the interchanged chromosomes. When he saw how testers involving known chromosomes could be used, he adopted that method using his own set of testers. He didn't use ones that involved two short chromosomes as a tester, but ones between long or medium and short. He was concerned that a ring of short chromosomes might not be recognized as a ring.

The presence of supernumerary 'B' chromosomes may be confusing; but they have a distinctive appearance (darkly stained), and also lag at late metaphase.

Barley

The second example is barley with seven chromosome pairs. Until the interchanges were discovered, linkage maps were built up by running linkage tests between genetic marker stocks. By 1941 what were thought to be the seven linkage groups had been established (Robertson et al., 1941). There are several dominant markers in barley varieties, e.g., 6-row vs. 2-row, Rough vs. smooth awns, Black vs. colorless glumes, blue vs. colorless aleurone, and normal vs. waxy endosperm. These had been used extensively in establishing the linkage groups. Ideas changed over the years as to the extent of each linkage map and what constituted a test for

locating a gene in any portion of the map. I remember Dr. Immer (Minnesota) stating that by using a multiple marker stock with one marker in each group, a new gene in the map could be adequately tested by using that stock in linkage tests. Actually, only about 30 map units on either side of the marker are covered adequately by an F_2 test. Also it took time and the development of formulas for using F_2 and F_3 data to realize that such data were satisfactory for linkage tests. *Drosophila* geneticists depended on backcrosses almost exclusively.

With the discovery that mutations could be produced by X-rays (1928-1930), some attempts were made to look for mutations that might be of agronomic value. Immer and Henderson had X-rayed seed of the barley variety, Mars. Plants from the treated seed had been grown in the greenhouse and the progeny from each plant were growing in a single row in the field (plants space-planted so they were well-tillered). One group came from heads that were fertile, the other from heads that were partially sterile. This was ideal material also as a source for possible chromosomal changes that might cause pollen abortion, e.g., interchanges, inversions, etc. When the plants were at the flowering stage, a few florets were taken (as early as possible) from a single head each from three to four plants in each row, placed in 70% alcohol and checked for pollen aboriton. Plants were found with about 25% abortion. Most of them had tillers young enough to get sporocytes, usually several heads were taken to be more certain of getting at least one at the proper meiotic stages. Of a total of 143 rows sampled, 56 had plants with pollen abortion. All but four of these had sporocytes at the proper stage (Burnham et al., 1954).

35 had one or more plants with a ⊙4 + 5II, about 25% pollen abortion*
1 had a ⊙6 + 4II,
1 had 2 ⊙4 + 3II, and
1 had a long-chromosome mutant.

Step 1 was to establish the homozygote for the chromosome change. Fertile progeny from a partially sterile (P.S.) heterozygote were increased and test crossed on a normal stock. Thirty-four different homozygous lines were established. One line that had an occasional ⊙4 but mostly pairs was lost. A test supposedly identified the line, but subsequent tests showed the line was standard normal. None of the progeny descended from the original plant had the interchange. Frank White took over the material for a thesis problem. He proceeded to make intercrosses between the lines and also to cross with genetic marker stocks for the different linkage groups. We had furnished H. H. Kramer at Purdue University with seed of the stocks we were using in our tests. He had additional interchange lines and used them plus ours for linkage tests as a thesis problem for H. H. Hanson. They obtained linkage data and published the results. Frank White, meanwhile had interrupted his graduate work to take a job in Canada, tobacco breeding, and did not complete his thesis.

Information was gradually accumulated, linkage data and cytological information which finally led to a tester set of interchanges to identify the chromosomes involved in other interchanges. For example, an intercross that had 2 ⊙4 in F_1 showed that the two parents had interchanges that were

*The low pollen abortion is the result of a high proportion of alternate segregations from the ring of four, i.e. zigzag configurations at meta phase I.

different. Arbitarily the chromosomes involved in one parental interchange were assigned a-b, those in the other interchange, c-d. If each of these, when crossed with another interchange stock had 2 ⊙4, the third one would be e-f. If an intercross between parent 1 and another interchange had a ⊙6, chromosome a would be assigned as the chromosome involved in both interchanges.

Eventually interchanges were found that involved each chromosome and a tester set was selected that could identify the chromosomes involved in any other interchange.

The general principle in selecting a tester set of interchanges is: divide the total number of chromosomes into groups of three, a species with nine pairs would have three groups. If the three chromosomes in a group are designated a, b, c, two interchanges, properly chosen, will identify any interchange involving either or both. There are three possible sets:

1. a-b, b-c   2. a-c, b-c   3. a-b, a-c

One chromosome is common to the members of each set. Hence for a 9-paired species, a set of six will be sufficient

The set for barley included:

a - b and b - d which will identify a, b, d

c - e and c - f which will identify c, e, f

b - g was added to complete the set.

Although two barley chromosomes have satellites, one satellite longer than the other, their association with the nucleolus is not reliable for identification of rings involving them. This contrasts with corn in which a ring involving chromosome 6 is almost always found associated witht the nucleolus at diakinesis. Also diakinesis, in my experience, is found much less frequently in barley, more often metaphase I is found. Meanwhile data were being accumulated for tests for linkage between partial sterility and gene markers. If several different interchanges showed linkage with the same markers, the chromosome common to those interchanges must carry that linkage group (Burnham, 1957; Kramer et al. 1954). Eventually the linkage group carried by each of the chromosomes, a, b -- g was identified. Linkage tests were made as follows: F_2 progenies were classified for pollen abortion by collecting a flowering head from each numbered plant, storing them in 70% alcohol and classifying them later as having pollen sterility or fertile pollen. The characters were recorded for each individual numbered plant. This furnished F_2 data for linkage tests. Each F_2 plant was harvested and F_3 progenies grown. This added more data for linkage tests, data from each F_2 phenotype. When a linkage was found with the sterility produced by an interchange with chromosomes identified by crossing with the tester set, e.g., a - b, that linkage group must be carried by a or b. Whichever one it was, other interchanges involving that chromosome showed linkage with markers in that group. Two linkage groups, previously thought to be independent, were found to be in the same chromosome: group III n-ac2 and VII wx fc (Kramer et al. 1954, Burnham, 1957). All interchanges involving the b chromosome showed linkage with markers in both groups.

With no more information than this, identification of linkage groups with arbitrarily assigned designations, a - - - g, various applications of cytogenetics to breeding would be possible.

Although barley has only seven chromosome pairs, thus far no one has devised a satisfactory technique for using pachytene chromosomes to locate interchange breakpoints. Sarvella et al. (1958) published a paper on pachytene cytology of barley, but pachytene cytology has not been used in subsequent studies. Possibly the short chromosome mutant might be useful, but this recessive would have to be introduced into each interchange stock.

Tjio and Hagberg (1951) established an idiogram for the barley chromosomes based on root-tip smears. Treatment with colchicine or other chemicals shortened the chromosomes so they could be measured: total lengths and arm lengths. The centromere positions showed as gaps. Examination of interchange homozygotes allowed them to correlate the letter designations with the chromosomes in the idiogram. For example an interchange with an exchange of end segments differing greatly in length made it possible to identify the chromosomes involved, especially if the new chromosomes also differed from the other five pairs (Hagberg and Tjio, 1952). If interchanges that had one chromosome in common had an identifiable change in the same chromosome, that served to correlate the letter designation with that in the idiogram.

The three longest chromosomes were so difficult to distinguish morphologically that the proper order of length was in doubt for several years, although each had been associated with its proper linkage group. New information that distinguishes these chromosomes and changes their ranking as to length has been reported (Noda and Kasha, 1978).

Later, trisomic tests by Tsuchiya (1960) served to confirm the correlations. Crosses between them and the interchange stocks were used. The use of telocentric trisomics placed genes to arm. Orientations had been achieved from the genetic and the cytological information also.

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15 DETAILS OF THE SMEAR TECHNIQUE FOR STUDYING CHROMOSOMES IN MAIZE

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1. Collection of sporocyte material: corn

In corn the microsporocyte material for meiotic stages is taken usually about 5 to 10 days before the tassel tip first begins to make its appearance. The young developing tassel may be located inside the tightly rolled leaf sheaths by squeezing the upper part of the plant between the thumb and forefinger. When this region feels soft and spongy the oldest portions are usually at or near meiosis. Another method of checking the stage of growth of the tassel is to cut or twist off the upper portions of the central whorl of leaves. When this exposes the tip of the tassel, the portions again are at or near meiosis. By checking cytologically, experience can be gained as to the appearance of the tassel, spikelets and anthers at the desired stages of meiosis.

If well-developed tillers are available, or if pollen from the same plant is not required, the entire tassel may be taken. If the ear is not needed, pull up on the central portion of the whorl of leaves at the top of the plant. The stalk usually breaks at a point several internodes below the tassel. Remove the leaves and place the entire tassel in a vial of freshly-mixed killer. Portions obviously too old can be discarded. A small cork attached to the end of a dissecting needle or pencil can be used to push the material into the killer, thus avoiding contact between one's skin and killer. Repeated contact often causes the skin to peel.

When mature pollen from the same plant is desired, a different procedure is used in which only a portion of the tassel is collected. Make a longitudinal slit with a razor blade on one side of the culm at the spongy region to expose the young tassel. Bending the plant slightly at that point opens the slit so that the main branch or several of the side branches may be removed with tweezers or fingers. Place the material directly into freshly-mixed killer. Removal of the branches without breaking them facilitates locating the desired meiotic stages later. The remaining portion of the tassel is pressed back, the stalk straightened up to bring the edges of the slit together and the stalk then is wrapped with masking tape to prevent drying out and to keep the top of the plant upright as it grows.

It may be necessary to tie the top to a stake. Dusting with Orthocide may be helpful if corn smut is a problem. If properly done, the tassel will continue to develop so that it can be used for pollinations and the ear also will develop.

Progression of stages in the tassel

The first anthers to shed pollen and hence the oldest ones, are in the spikelets located a little above the middle of the central spike of the tassel. The tassel branches below begin to flower a little later, and again the first anthers to shed are a little above the middle. The spikelets are progressively younger from that point toward the tip and toward the base of each branch. If the central spike is collected when the oldest spikelets are undergoing meiosis, the side branches usually will all be too young. Collections made when anthers in the middle spikelets on the side branches are at meiosis will furnish the greatest amount of material at that stage. If the tassel is old, it is important to include the lower branches and all florets to the base of each. Often in very old tassels, a few of the basal florets will be young enough for stages of meiosis.

2. Length of time in killer (fixative)

The anthers from freshly collected material may be smeared in acetocarmine stain without previous killing. With fresh material or material that has not been in killer long enough heating has a tendency to remove the protoplasm, leaving only the chromosomes. Only gentle heating can be used. Better results are obtained after the sporocytes have been in killer for at least 24 hours at room temperature.

For pachytene study in corn, there is an optimum length of time to leave the material in the killer before preparing the slides in order to get the sharpest staining of the chromomeres; but this can be determined only by preparing slides at intervals after the material is collected. At room temperature the best slides with corn are usually made at from 12 to 24 hours after the material has been collected. When placed in the refrigerator immediately after collection, the material gives satisfactory pachytene slides for a much longer period of time.

Storage at temperatures below freezing (e.g. in a deep-freeze box) prolongs their usefulness still further (several years). To avoid poor fixation, place at that temperature only after they have been at room temperature for at least 24 to 36 hours after collection. Material that has been stored in the refrigerator or below freezing should be allowed to stand at room temperature for a time (the optimum time to be determined as above by making slides at intervals) before making the smear preparations. For later use: for pachytene study, the killer should be replaced by 70% alcohol after 24 to 36 hours at room temperature, after 7 to 10 days in the refrigerator, after 1 to 2 months if below freezing. One change is sufficient if an excess of alcohol is added. Material in 70% alcohol possibly need not be stored in the refrigerator. Material in 70% alcohol stored in the refrigerator has given good pachytene preparations in maize after as long as 6 years, but the results are not as consistent, and some changes in technique are necessary to get the proper intensity of staining. For gross diakinesis, metaphase, or microspore studies the material may be left in the original killer. As a routine procedure, however, it is best to change all material to 70% alcohol even if stored below freezing.

3. Preparation of the slide

Place the sporocyte material in a petri dish and add killer solution or 70% alcohol to prevent drying out. The black background of the laboratory table permits the anthers to be seen more easily than if white filter paper is placed in the petri dish. Spread out the material so that one or two branches can be followed from end to end.

a. When seeking diakinesis stages tease out three long anthers of a single floret using rusty needles and place the anthers in a drop of aceto-carmine. Anthers placed on a dry slide may shrivel before a drop of stain can be added and become very difficult to smear. Nickel-plated needles are not satisfactory. Cut the anthers transversely with a razor blade or between the tips of the needles. By gently tapping the anther pieces with the needle, the sporocytes are forced out of the open or cut ends of the anther without breaking the anthers into small pieces. This makes it easier to remove the anther pieces before adding the cover slip. Stir the drop vigorously to separate the cells and also to incorporate iron into the stain. Add the cover slip (7/8" square #1 in thickness). Cover slips now are more uniform in thickness, but a thickness gauge can be used to avoid those that are too thick. Examine the slide under low power to determine the stage (note that cells near the edge of the drop may be stained more densely, and the stage more easily recognized). Cell size and shape can be used to distinguish between separated microspores and pollen mother cells. Cells that have a very lightly stained central area are usually at pachytene or earlier. Ability to recognize the stage of meiosis without the cover slip will save time in slide preparation.

b. When preparing slides for pachytene stages, tease out one anther and place it in a small drop of aceto-carmine stain. The drop should be small enough so that, when the cover slip is applied, the solution just barely fills out to the edges. It should not be necessary to apply pressure to squeeze out an excess of carmine on a blotter. If the drop is small, surface tension pulls the cover slip to the slide. An excess of stain also results in a slide in which the cells become too darkly stained after a few hours or overnight.

c. If the pollen mother cells are at the desired stage, and are stained intensely enough (see "General Notes" section) remove the pieces of anther by picking them up between the points of the two needles. This seems to leave more cells in the drop than when the anther pieces are dragged off to one side. There are 3 to 4,000 pollen grains in an anther and hence 750 to 1,000 P.M.C.'s. A common problem is the loss of all but a few cells. Remove all dust particles in the area outside the drop, in the area to be covered, by brushing them off with the finger tip before applying the cover slip. Dust, lint or anther material in the drop will prevent the cover slip from being pulled down properly to the slide. If the drop is too large, remove a little of it with the finger tip. Good slides cannot be made if the working space is dusty or if dirty slides or cover slips are used. When the cover slip is added, if it

fails to spread out at one or more spots, local light pressure with the point of the dissecting needle will aid in getting the carmine to spread and fill out to the edges of the slip. Care must be taken not to move the cover slip after it is applied. After applying the cover slip, do not try to remove the small trapped air pockets. They do no harm, and do serve as an indicator of when to stop heating when the slide is being heated.

In preparing slides for diakinesis stages, a larger drop of stain may be used. After removing the anther pieces and adding the cover slip, excess stain may be removed by inverting the slide on a blotter and applying gentle pressure while taking care not to move the cover slip.

d. After adding the cover slip, heat over an alcohol flame. Pass the slide (middle portion) back and forth over an alcohol flame, allowing it to heat to a point just short of boiling. Examine under low power of the microscope. Note that the cytoplasm becomes lighter and the chromosomes more densely stained. Repeat the heating until there is no further change in differentiation. Slides may need to be heated as many as 10-15 times. If they boil, the cells and chromosomes are ruined, but some very good figures have been found at the edges of slides that had been boiled. Some workers have used a heating plate, or a steam bath, but it is important to check the progress under the microscope.

   A test often seen is that of touching the back of one hand with the heated slide. This is not satisfactory, since the proper temperature is one that will burn the hand.

e. Apply the temporary seal, covering the top edges of the cover slip as well as the sides to make certain it is completely sealed.

f. With a wax pencil, record the culture and plant number on the slide, as well as the stage. If made properly, the slides will keep for some time, but will gradually become dark or the stain may crystallize out. An excess of stain in the original preparation results in a more rapid deterioration. The slides may be kept longer in a refrigerator, although they are more likely to dry out unless kept in the hydrator pan or a petri dish with moisture.

   A variation of the above technique which results in slides which will keep longer before being made permanent was used by Dr. A.E. Longley: Just before adding the cover slip, i.e. after the chromosomes are well stained, a small drop of glacial acetic acid is added at the edge of the drop of carmine and stirred into it gradually. Then the cover slip is added, and heat applied. The larger drop size presents some difficulty, and may have to be squeezed out on a blotter. This method appears to result in a better destaining of the cytoplasm and in slower crystallizing of the carmine as the slide ages. He used a larger, rectangular cover slip.

General notes

Tassel morphology in relation to locating desired stages

In corn, the spikelets on the side branches of the tassel are paired. The sessile one is usually at a slightly earlier stage than the adjacent long stalked one. Within each spikelet, there are two flowers, each with three anthers, those in the first or upper flower being much longer and hence older than those in the lower or second flower. Anthers from the first flowers in the spikelets show the greatest regularity in succession of stages along the branch. Also the three anthers in the first flowers are usually at about the same stage of meiosis. This is very useful in studying pachytene stages. If half an anther is used to make a slide, as many as 6 slides at a given stage may be made. This increases the chance of getting some well-made preparations from that flower.

Under some conditions, not all the stages are on a single branch, the jumps being too great between successive spikelets. The missing stages may be found on a different branch. When this occurs, the three anthers of one flower may not be at the same stage. If all the first flowers are too old, the desired stages may be found in the second flowers with the shorter anthers. In these, the succession is usually not as regular, i.e. not in the expected order from one spikelet to the next. In some cases where diakinesis is desired, a few cells in this stage may be present in an anther in which most of the cells are at metaphase I. (For a description of the staminate inflorescence, see Hayward, pp. 130-132).

Miscellaneous notes

In maize the spore-mother cell wall in most cases has slipped off the cell either in the killing or in the smearing process. The empty shells are frequently seen in the smear. The nuclear membrane is removed also by the killer. Both enhance the spreading of the cell contents.

The sporocytes are easily broken if the anther is crushed or if pressure is applied to the cover slip. For this reason spear-point needles are not recommended. It is best to prepare your own needles, replacing the commercial dissecting ones by a size 7 needle or smaller. The pointed end of the needle should be curved by bending it in a hot flame. The curved ends make them easier to manipulate in removing the anther pieces.

In some cases slides made immediately after removing the tassel material from the refrigerator will be free from "bubbles" in the cytoplasm, whereas slides made later may have them.

Since the configurations in permanent slides may not be as good as in freshly stained ones, all observations, camera lucida (C.L.) drawings, and photographs should be made before making the slide permanent.

Intensity and color of stain is influenced by the following and may therefore be controlled by varying these factors:

1. Intensity of the aceto-carmine solution.
2. The amount of iron added by the iron needles. This is determined by the extent the needles are used in stirring and teasing the material (also the degree of rustiness of the needles).

Another method is to add to the killer a few drops of a solution of ferric hydrate dissolved in 45% acetic acid. For tomatoes, Barton (1950) used a series with increasing amounts of iron. As mentioned earlier, some investigators add ferric chloride to the killer. For pachytene study, too much iron stains the nucleolus so heavily that chromosomes above or below it cannot be studied.

3. Oxidation: Longer exposure to the air will intensify the staining. If it is still too light in color after applying the cover slip, its intensity may be increased by allowing the slide to stand for a time before heating the slide.

Method for making centromeres clearly distinguishable
(McClintock, 1957, personal communication)

"Preparations are made in the usual way with carmine. The slides should stand for at least four days or a week. Then, the seal on two opposite sides should be removed and a stain made from acetic acid, lactic acid and orecein should be drawn under the cover slip, replacing the carmine stain. (The stain: 1 part water, 1 part acetic acid, one part lactic acid and 2% orcein). After a day, the orcein stain will have replaced the carmine stain. The DNA-containing parts of the cell are then deeply stained and the other parts are very lightly stained. In maize, this makes the positions of the centromeres very conspicuous as they are not stained." She reports this gives excellent results in maize.

Making aceto-carmine or propiono-carmine smears permanent (McClintock, 1929)

Remove the temporary seal with a razor blade, off the top of the cover slip first, then along the sides; removing the remainder with a xylol-moistened camel's hair brush. Examine the slides; if the cytoplasm is too dark, run 45% acetic acid under the cover slip by adding a drop of acid along one side, pulling it through with a blotter held against the opposite side. Then heat to destain, repeat until destained satisfactorily.

Place right side up in a petri dish of 10% acetic acid. The solution should soak under the cover slip and loosen it. If not, then while pressing the spread ends of the forceps gently against one side or edge of the cover slip to keep it from sliding, run the edge of the razor blade under the opposite side to raise the cover slip from the slide. Keeping the cover slip and slide in the same relative position they originally had, run both through the series of solutions listed below. The original position of the cover slip is usually indicated by a line of aceto-carmine crystals on the slide, or its position may be marked on the bottom side with a diamond pencil. If carried out properly the figures will be found very near their original vernier readings.

Pass cover slip and slide through the following freshly prepared solutions in coplin jars, about 2 minutes each:

1.	10 cc 95% alcohol + 30 cc of 45% acetic acid
2.	20 cc " " + 20 cc of " " "
*3.	20 cc absolute alcohol + 20 cc of glacial acetic
4.	30 cc " " + 10 cc of " "
*5.	36 cc " " + 4 cc of " "
*6.	40 cc absolute alcohol
*7.	40 cc " "

*These steps alone as a short method have been satisfactory for corn and for barley.

Then add balsam of proper consistency to the slide and quickly place the cover slip in its original position, using a needle to shift it about. Turn the slide bottom side up. Check cover slip position again. Then place on a piece of paper toweling or a blotter and press out the excess balsam, being careful not to move the cover slip out of position. Transfer the slide to a fresh piece of paper towel, still bottom side up, flood with xylol, being certain the towel under the slide is saturated. When dry, the slide will be relatively free of excess balsam. Place in a dry, warm place to harden. Euparol may be used in place of the balsam. My experience with clarite has been that one application is not sufficient to seal the cover slips; unsealed areas appear as the slide dries.

General notes on the method: Slides that are a few days old seem to lose fewer cells than ones made into permanents immediately. Most of the sporocytes adhere to the cover slip, very few are on the slide.

When step #7 with absolute alcohol was omitted, the edges of the cells frequently were darker or folded over, possibly due to moisture since this additional step (#7) with absolute alcohol has corrected this.

In a moist atmosphere a milky-white precipitate soon forms on the surface of the drop of balsam, hence the need for adding the cover slip quickly.

The alcohol-acetic acid mixtures in the series probably remain usable longer if stored in a refrigerator.

A step using pure xylol preceding balsam might logically be expected, but McClintock found that it results in inferior slides. It was only when the xylol became mixed with alcohol from the preceding steps that the slides were satisfactory. No xylol is used in the method now.

Quick-freeze method of making slides permanent, as described by Conger and Fairchild (1953).

Place the temporary slide on the flat side of block of dry ice. When thoroughly frozen and while still on the ice, pry off the cover slip by inserting a razor blade under one corner of the cover slip. The cells adhere to the slide. Place the frozen slide and cover slip immediately in 95% or absolute alcohol. After about 5 minutes, place in the second and last, absolute alcohol. Remove the slide, without draining, add a very large drop of balsam or a synthetic resin at an edge of the cell-bearing area. With the edge of the cover slip, draw the balsam or synthetic resin up over the cells and gently lower the cover slip. The advantages are: ease and speed of

removing the cover slip with a minimum loss of cells, superiority of the permanent slides, and vernier readings of cell positions for the temporary mount remain the same for the permanent preparation.

For the study of the nucleoli in microspore quartets, more iron must be worked into the drop than is used for sporocytes. After the nucleoli are densely stained, the cytoplasm may be destained by applying heat or a combination of the Longley method (addition of a small drop of glacial acetic acid) plus heat.

Cooper's staining method: The use of aceto-carmine to which ¼ part of Ehrlich's haematoxylin has been added will also give darker staining, especially of the nucleolus (weak carmine solutions may be made fairly usable for sporocytes in this manner). Delay in heating after applying the cover slip will also result in darker-stained nucleoli. If the first division plane is to be determined, a larger-sized drop should be used, and heat applied cautiously.

For first division of microspores in corn

An anther is teased into aceto-carmine in a hollow-ground hanging drop slide or in a watch glass, and allowed to stain for 20-30 minutes, adding more stain as it evaporates. A small drop of the suspension may be transferred with a wire loop to a slide, cover slip added, heat applied, and sealed. In corn, the pollen grains show a characteristic furrowed or wrinkled appearance when at the proper stage. This is usually at about 7 to 10 days after meiosis, or when the tip of the tassel first begins to show. With corn, the percentage of microspores in division stages is much higher in greenhouse than in field grown plants.

Determination of pollen sterility

The pollen of plants heterozygous for various chromosomal aberrations is partially serile. The visible abnormal grains may be completely empty (devoid of starch) and smaller than the normal ones, partially filled with starch, or well-filled but smaller in size, the proportions varying with the aberration.

For checking in the field, a "Leitz Taschen Mikroskop" with a magnification of 40X is the most convenient. It is about 1" in diameter, 1½" long and uses a small glass slide which is inserted in a slot below the lens which can be focused by screwing the top in or out. It is no longer manufactured by Leitz, but an instrument similar mechanically to this, and with only slightly less magnification, called the Midgard, is manufactured by:

Nippon Microscope Works Company
35-2 Minami Cho
Aoyama, Akasaka
Tokyo, Japan

The opening in the bottom of the instrument can be enlarged with a metal reamer to let in more light (Burnham, 1961). The magnification is not sufficient for classification of species with small pollen, such as barley and tomato.

Field classification: An anther freshly extruded or about to extrude is removed, the tip pinched off with a fingernail and the anther rolled between the fingers to dust the pollen on the small glass slide. Young anthers with mature pollen, but not ready to shed can be classified by crushing and smearing the anther on the slide with a finger; but this is too slow for general use. Fertile plants are marked by tearing off all but about 2" of the top 2 or 3 leaves, partially sterile plants are marked with a strung tag on which the degree of sterility can be recorded. Where there is only one partially sterile class, with rare exceptions, a 2' long string is looped around the plant.

For accurate counts in the laboratory a 5- or 6-inch section of tassel which includes a portion that has not shed but also a portion that has shed (thus assuring the inclusion of spikelets with mature anthers) can be collected and placed in 70% alcohol. A small tag with culture and plant number can be attached to each piece.

To determine the degree of visibly aborted pollen, the pollen from an anther about ready to shed is teased out into a drop of I_2-KI solution and examined under the microscope. Experience gained by estimating the degree of abortion and then checking the same slide by counting enables one with practice, to distinguish between 25, 50, or 75 percent sterility and intermediate values.

By screwing off the front lens of the low power objective sufficient magnification is obtained (32 or 40X) with a larger field which aids in making estimates. A special 4X, 32 mm low power objective will give a flatter field.

For making accurate counts, 1/3 of an anther is teased out into a very small drop of I_2-KI solution, and a small cover slip (narrow strip, about 1/3 of the 7/8" square) added. This may be ringed with glycerine to prevent drying out during the counting. A narrow piece of a glass slide may be used to cover the drop.

A circular disc of note card cut to fit on the shelf in the ocular and with a rectangular opening cut out gives a field with parallel sides. Strips across the slide are counted by recording the total of a single class in each trip across the strip. Since the aborted grains tend to float to the outside the entire slide is counted.

If glycerin is added to the solution, the grains will stay in place better (Pittenger and Frolik, 1950).

Classification for waxy pollen or kernels

For classification of pollen for non-waxy (Wx) vs. waxy (wx), the stock solution (see below) should be diluted with three parts of water. Tease out an anther in a drop on a slide, examine with the light cut off from the mirror but with light shining on it from above (a method discovered by McClintock). The starch of Wx is blue, while that of wx is brick red.

For classifying waxy in sugary seeds, use the undiluted solution.

Aceto-carmine stain: preparation

Method 1: Corn pachytenes require a very strong aceto-carmine stain. Carmine in large excess (2g./100cc) of what will dissolve (about 14 mg per 100 cc) is added to boiling 45% acetic acid, and boiled for 1 to 2 minutes or until there is a sudden change to a darker color. Cool, then filter. If not sufficiently dark, boil up again with carmine.

Method 2: Using the same proportions as above, simmer the acetic acid and carmine for one to four hours in a flask with a reflux condenser. Refluxing 6 to 8 hours gives the best results for corn (Stout, 1966). Cool and filter. This method gives more consistent results and is recommended. Store the stock supply in a brown bottle in the dark. It will keep indefinitely.

Propiono-carmine stain: preparation

Prepare as for acetocarmine, substituting 45% propionic acid for the 45% acetic acid. Propionic acid dissolves more carmine stain than does acetic acid and may give a clearer cytoplasm (McCallum, as given by Johannsen, 1940; see also Stout, 1966).

Aceto-orcein stain: preparation

Orcein source: G.T. Gurr, London. The U.S. source behaves differently - more sensitive to acid, and also requires a greater dye concentration.

Dissolve 4.4 gm orcein in 100 cc glacial acetic acid, reflux about 30 minutes by gentle boiling, cool, filter. Dilute when needed for use (it deteriorates in dilute acid). Dilute about ½ with distilled water to a strength of 45% acetic acid--this gives about a 2% orcein solution.

It is further diluted ½ (with 45% acetic acid) to make up the 1% aceto-orcein which is used for staining.

Fixing or killing solutions

Farmer's fluid		Carnoy's fluids A	Carnoy's fluids B
3 parts	95% alcohol	6 parts	6
1 part	glacial acetic acid	3 parts	1
--	chloroform	1 part	3

These killers should be mixed fresh immediately before use. For corn Farmer's fluid is satisfactory and 95% alcohol does as well as absolute alcohol. A large excess of killer over the quantity of tissue may give better fixation, especially when 95% alcohol is used. For some material absolute alcohol may be better. Other proportions of alcohol to acetic acid may be better under certain conditions or for different species.

Temporary seals

A satisfactory temporary seal must prevent the slide from drying out, must not smear the front lens of the microscope objective, and yet must be capable of being removed easily in case the slide is to be made permanent.

1. Dahl's varnish-beeswax paraffin
   ½ part Turtox Ringing Varnish
   1 part beeswax
   2 parts paraffin (parowax)
   The ingredients should be heated to melt them together.
   This seems to make a very good airtight seal, satisfactory in all respects.

2. Gum-mastic and paraffin, in equal parts, are melted and stirred together. It may be necessary to strain the melted mixture through cheesecloth or wire screen to remove the hard material which is usually found in the gum mastic. Presence of this in the mixture might scratch the lens of the objective when changing from low to high power for figures near the edges of the cover slip. This mixture has a tendency not to stick to the slide if there is skin grease on it.

3. Lanolin-paraffin. Equal parts of each are melted together. This makes an airtight seal, but tends to be a little too soft, resulting in a tendency for the cover slip to slide when removing the seal in preparation for making it permanent, and also to leave a smear on the lens if it touches the seal. Possibly a mixture including beeswax would correct these faults.

4. Beeswax plus vaseline might be worth a trial.

Strong I_2-KI solution to stain starch in pollen or endosperm

0.3 gm I_2
1.0 gm KI
100 cc H_2O

This may be diluted several times for the pollen sterility classification.

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16 PREFIXATION AND STAINING OF THE SOMATIC CHROMOSOMES OF CORN

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Producing good somatic metaphase spreads of Zea mays L. is hindered by the combined difficulties of obtaining large numbers of dividing cells and of spreading and staining the chromosomes well. The present contribution describes a chemical prefixation technique using mono-bromonaphthalene and dimethyl sulfoxide (DMSO).

Over the years many methods have been developed for preparing somatic chromosomes, and they are generally made up of four stages. First, the seed is germinated and the actively-growing root tip is collected. Second, a treatment prior to fixation (i.e., chemical or temperature) is used to inhibit spindle formation preventing the congression of chromosomes to the metaphase plate. Third, fixation in any of a large range of solutions. Fourth, preparation of the root tips by hydrolysis followed by staining.

Assuming that the material to be examined contains dividing cells, it would be desirable to have a prefixation method that resulted in a quick and uniform penetration of the prefixative. Consequently, experiments were conducted utilizing several species in which the prefixative was combined with DMSO (Sallee and Kimber, 1981). During these experiments it was determined that the use of DMSO, along with mono-bromonaphthalene, reduces the amount of prefixative needed, the time of treatment required, and increases the proportion of cells that are suitable for analysis.

Procedure

Seeds are placed in Petri dishes lined with moist filter paper and germinated for two to three days at 30°C. Three to four cm long roots are collected and placed in the prefixation solution consisting of five drops of mono-bromonaphthalene, four drops of DMSO and 100cc tap water for two to three hours at room temperature and then transferred into glacial acetic acid for fixation. It is important that the DMSO be added to the water after the mono-bromonaphthalene. The minimum time of fixation is two hours; ideally, they should be left overnight. If the roots are kept longer than two days, the acetic acid should be replaced by 70 percent ethyl alcohol. When ethyl alcohol is used for storage, the root tips should be rinsed in tap water for 10 minutes prior to hydrolysis. Roots are hydrolysed for 14 minutes in 1N HCl at 60°C and then placed into Feulgen stain for 10 to 15 minutes. Slides are made from squashes of small portions of the root tips in propriono-orcein (see preceding article by Burnham for recipe). Plastic cover slips are preferable over glass due to the tendency of glass to break under vigorous tapping needed to ensure proper cell separations.

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17 IN SITU HYBRIDIZATION WITH MAIZE MEIOTIC CELLS

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In situ hybridization of RNA to maize chromosomes allows the localization of specific nucleotide sequences. The procedure given here will detect the location of genes repeated 50 times or more at a particular site. We have employed this method to further localize the 17/26 S ribosomal RNA genes within the nucleolus organizer region of chromosome 6 (Phillips et al., 1979) and to confirm the location of the 5S rRNA genes to the long arm of chromosome 2 (Mascia et al., 1981).

Slide Preparation:

1. Fix freshly collected microsporocytes in 3 parts 95% ethanol and 1 part glacial acetic acid for 1-3 days at room temperature.

2. Use one of three anthers in a floret to identify appropriate meiotic stage and keep remaining anthers in a vial containing fixative.

3. Squash 3-5 anthers on an acid-cleaned slide in a drop of 45% acetic acid and add an acid-cleaned cover slip.

4. Place slides on dry ice for 5-10 minutes. Use a razor blade to flip off the cover slip.

5. Keep slides in 2 x SSC (1 x SSC: 0.15 M NaCl and 0.015 M Na citrate, pH 7.4).

Denaturation of DNA:

1. Dissolve RNase in 2 x SSC (0.2 mg/ml) and heat at 80°C for 10 minutes.

2. Digest the meiotic cells with the heat treated RNase for 2 hrs. at room temperature (or 1 hr. at 37°C) to remove endogenous RNA which may compete with the hybridizing RNA.

3. Remove the RNase by washing 3 times with 2 x SSC.

4. Treat the slides with 0.2 N HCl for 20 min., at room temperature.

5. Wash the denatured slides 3 times with 2 x SSC.

RNA-DNA Hybridization:

1. Place 20 μl of ^{125}I-RNA or ^{3}HcRNA on each slide (conc.: 0.25 μg/ml with 10 μg E. coli RNA as carrier). RNA is dissolved in 4 x SSC and mixed with an equal volume of formamide resulting in a final solution that is 2 x SSC and 50% formamide.

2. Cover the slides with an acid-cleaned cover glass and place in a pan containing 2 x SSC or deionized water and support the slides with glass rods.

3. Seal the pan with aluminum foil and keep in 45°C oven overnight (16 hrs.).

4. Float the cover glasses off the slides with 2 x SSC and wash three times with 2 x SSC.

5. Digest with 80°C heat treated RNase (0.2 mg/ml) for 2 hours at room temperature or at 37°C for 1 hour.

6. Wash the slides 3 times with 2 x SSC and keep in 2 x SSC.

Autoradiography:

1. Melt Kodak NTB-2 emulsion (112 ml) at 45°C and dilute with two volumes of distilled water in absolute darkness.

2. Pour 30 ml of the diluted emulsion in a small container and save the remainder for future use.

3. Dip the slides into the emulsion and withdraw slowly. Place slide vertically in a rack for 10 minutes and wipe the wet end.

4. Place the emulsion-coated slides in a light-tight plastic slide box with Dririte inside. Seal the box with black electrical tape, wrap with aluminum foil, and store at 4°C to expose.

5. After proper exposure time (2-4 days), develop the slides in Kodak D-19 developer for approximately 15 seconds and rinse 3 times with distilled water.

6. Fix slides in Kodak fixer for 1 minute and wash with distilled water for 20 minutes through several changes of water.

7. Stain the slides with 5% Giemsa stain (Harleco) before drying (dilute the Giemsa solution with 0.01 M phosphate buffer at pH 6.8).

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18 GENE-ENZYME RELATIONSHIPS IN ANTHOCYANIN BIOSYNTHESIS IN MAIZE

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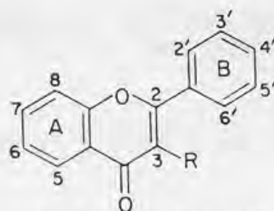
The genetic control of anthocyanin pigmentation in the aleurone layer of the maize kernel has long been regarded as a textbook example of genic interaction. In the 70 years that have elapsed since the first pair of complementary genes, the aleurone color factors c and r, were described in maize (East and Hayes, 1911), the genetics of anthocyanin production has been studied extensively. During this period geneticists have accumulated a wealth of information concerning the structural organization and interaction of the many factors that are involved in the formation of anthocyanin pigments in the aleurone layer of the endosperm (Coe and Neuffer, 1977), The conspicuous and nonvital nature of anthocyanin pigments and the suitability of the endosperm as an object for genetic research are the main factors that have caused the genetics of anthocyanin formation in the aleurone to be more fully studied than that of any other constituent in any other tissue of the maize plant.

At least nine loci, mapping to seven different chromosome arms, are required for anthocyanin synthesis in the aleurone, the outermost layer of the seed endosperm. These loci and their respective locations are: a (3L); a2 (5S); bz (9S); bz2 (1L); c (9S); c2 (4L); dek1 (1S); r (10L) and vp (3L). The dek1 and vp are pleiotropic since they affect, respectively, embryo development (Neuffer and Sheridan, 1981) and dormancy (Robertson, 1955), in addition to aleurone pigmentation. The remaining anthocyaninless mutants can be ordered in a tentative gene action sequence on the basis of several criteria. Current evidence places c and r early in the anthocyanin biosynthetic pathway, followed by c2, a, a2, bz, and bz2, in the indicated order. This evidence stems principally from an intertissue complementation test between all possible pair of mutants (Reddy and Coe, 1962), and from analyses of accumulated intermediates in single and double mutants (Coe, 1955: Kirby and Styles, 1970; Reddy and Reddy, 1971; Styles and Ceska, 1972, 1977), an examination of the effect of the different mutations on the anthocyanin biosynthetic enzyme UFGT (Dooner and Nelson, 1977a, 1979a) and a study of the response of mutant genotypes to exogenously supplied precursors (McCormick, 1978). However, because the rather complex anthocyanin biosynthetic pathway is still being worked out, because some of the enzymes in the pathway have proved to be unstable, and because the enzymes in the endosperm are low-abundance proteins, (restricted to the aleurone, the conspicuously pigmented, outermost mono-cell layer), few studies have concerned themselves with the primary biochemical lesion in the maize anthocyaninless mutants.

The purpose of this communication is to acquaint the reader with current information that reveals a complex system of genetic control of the anthocyanin biosynthetic enzymes in corn.

Anthocyanins belong to the more general class of phenolic compounds known as flavonoids. These compounds possess a common basic structural unit - the C-15 skeleton of a flavone.

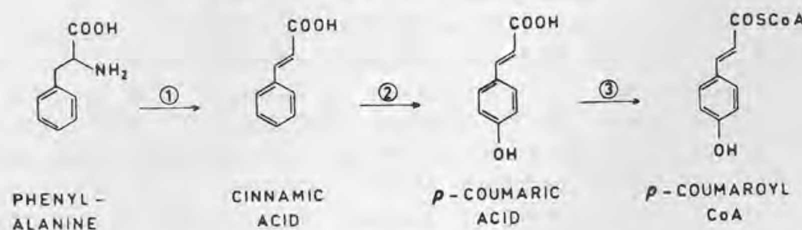


Hydroxyl groups may be found at almost any position but more commonly at the 3, 5, 7, 3' and 4' carbons (Harborne, 1967).

The primary metabolites from which flavonoids are derived are the phenylpropanoid aromatic amino acids, phenylalanine and tryosine. Higher plants, in addition to incorporating these amino acids into proteins, have developed an alternate metabolic pathway by which these amino acids are converted first into cinnamic acids, and then incorporated into compounds such as flavonoids and lignins, known as secondary plant metabolites.

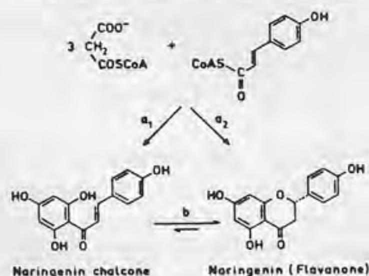
The enzymology of flavonoid and lignin biosynthesis has been considerably advanced in the past decade or so by the work of Grisebach, Hahlbrock and colleagues at Freiburg (see reviews by Hahlbrock and Grisebach, 1975, 1979). These investigators have successfully exploited a parsley cell suspension system to confirm the general scheme for the biosynthesis of lignins and flavonoids that had been originally proposed on the basis of results from tracer studies with radioactive substrates.

The first three steps in the conversion of phenylalanine to cinnamic acid derivatives are common to all phenylpropanoid pathways. Therefore, this sequence of reactions has been termed "general phenylpropanoid metabolism."



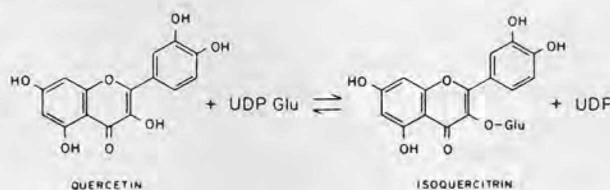
The first enzyme in this pathway, phenylalanine ammonia lyase (PAL), catalyzes the deamination of phenylalanine to cinnamic acid and is considered to be a key enzyme since it channels aromatic amino acid metabolism in the direction of lignin and flavonoid biosynthesis. The next two enzymes in the pathway are cinnamic acid hydroxylase, which is involved in the hydroxylation of cinnamic acid at the 4-OH position to give coumaric acid, and p-coumaroyl CoA ligase, which catalyzes the synthesis of the CoA ester of p-coumaric acid. Hahlbrock et al. (1971) refer to these enzymes as Group I enzymes, Group II enzymes being the seven or so responsible for the biosynthesis of flavonoid glycosides from CoA esters of p-coumaric acid and malonic acids. The anthocyanin pigments, found ubiquitously in vascular plants, are flavonoid glycosides.

The other two enzymes that concern us here are flavanone synthase (FS) and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT). FS is the first enzyme unique to flavonoid biosynthesis: it catalyzes the synthesis of the C-15 flavonoid skeleton from the activated precursors p-coumaroyl CoA and malonyl CoA.



The nature of the immediate product of the reaction is still unclear. Recent evidence suggests that the product may be a chalcone rather than a flavanone (Heller and Hahlbrock, 1980).

UFGT, on the other hand, is concerned with one of the last steps in flavonoid biosynthesis, the glucosylation of flavonoids at the 3-OH position to give the respective glucosides.



The genetic control of only one anthocyanin biosynthetic enzyme, UFGT, has been studied in corn. Larson and Coe reported in 1968 that bz pollen was deficient in UFGT activity. Subsequently, it was shown (Larson and Coe, 1977; Dooner and Nelson, 1977a) that bz mature endosperms also lacked UFGT activity and that the Bz allele had a clear linear dosage effect on activity, an observation that would suggest that Bz is the structural gene for UFGT. In addition, Dooner and Nelson (1977a, 1979a) found that three other mutations, namely, c, r, and vp, drastically affected UFGT levels in mature endosperms. In c, r, and vp mature seeds, the amount of UFGT is only three percent of that present in normal. Enzyme activity, however, is directly proportional to Bz dosage only and is not affected by dosage variations at either C, R, or Vp. Actually, endosperms receiving one or two doses of a paternally transmitted R allele do have similar, reduced UFGT levels. But, this is a transmission rather than a dosage effect (Kermicle 1970).

There is other support for the contention that Bz is the structual gene for UFGT. (1) Stable bz mutants are null, totally lacking UFGT at all stages of development, whereas the other mutants (c, r, and vp) that affect UFGT activity in mature endosperms are not null. Furthermore, early in endosperm development, the enzyme is present in an uninduced low level that is independent of the allelic state of C, R, and Vp, and dependent solely on the presence of Bz (Dooner and Nelson, 1979a). (2) Several fully or partially functioning alleles of Bz, all derived from a common progenitor allele, have been shown to possess a modified UFGT, as judged from the

altered thermal and electrophoretic stability of the enzyme (Dooner and Nelson, 1977b; 1979b). (3) Finally, immunological characterization of ten ethyl methanesulfonate (EMS)-induced bz mutants has revealed that, while all are UFGT-null, two of these (bz-E2 and bz-E5) are CRM+ (CRM=cross-reacting material), whereas the remaining ones are CRM-(Dooner, 1981). The demonstration of both CRM+ and CRM- types among allelic null mutants argues strongly against the possibility that Bz might be involved in the post-translational modification or activation of UFGT.

Attempts to measure flavanone synthase (FS) levels in maize endosperms had, until recently, been unsuccessful (Dooner, unpub.). FS is quite unstable and its levels appear to fluctuate from season to season, but attempts are currently underway to find conditions that stabilize the enzyme. Some interesting preliminary data (Dooner, 1980) suggest that the genetic control of FS parallels that of UFGT. Mutations at C, R, and Vp affect both enzymes. However, mutations at C2 (spontaneous and induced) affect only FS. Dosage comparisons reveal that variation in the number of normal R and Vp alleles in the endosperm does not affect FS levels, but varying dosage of C and C2 does. Neither of the latter two gene dosage-enzyme relationships is strictly linear. In general, the C dosage series shows more FS activity than expected on a linear model whereas the C2 dosage series shows less activity than expected. The allelic dominant inhibitors at both of these loci (c-I and c2-Idf) completely eliminate activity in either two or three doses. The meaning of the C and C2 dosage relationships is unclear at this point. However, the differential effect of c and c2 on UFGT (Dooner and Nelson, 1977a) and the differential response of these two mutants to exogenous precursors (McCormick, 1978) would suggest a structural role for C2 and a regulatory role for C in the control of FS in maize.

The studies with UFGT and with FS have revealed an unexpectedly complex pattern of control of the flavonoid biosynthetic enzymes in the maize aleurone. The genes Vp, C, and R regulate production of UFGT, and at least two of them, and possible all three, also regulate production of FS.

Several mechanisms can be considered to explain the interaction between Vp, R, and (possibly) C and the genes they regulate (Bz, C2, and very likely A, A2, and Bz2). First, one or more of them could act as regulatory genes coding for trans-acting molecules directly involved in turning on the structural genes. Such a macromolecule could even be an early enzyme in the biosynthetic pathway itself. Alternatively, they could specify early enzymes in the anthocyanin pathway that are involved in the synthesis of a flavonoid precursor responsible for UFGT and FS induction. This precursor-induction would most likely be mediated via an activated regulatory protein. If the interaction of a flavonoid precursor and a regulatory macromolecule is required for the induction of the flavonoid biosynthetic enzymes during aleurone development, the effects of the vp, r, and c mutations could be due to failure to synthesize either the macromolecular activator or the flavonoid precursor.

The primary function of the putative regulatory loci, Vp, C, and R, is not known. The evidence now at hand indicates that if these genes specify any enzymes at all, such enzymes would be concerned with reactions of general phenylpropanoid metabolism. The first of these enzymes, PAL, has been examined in the respective mutants and found to be reduced drastically

in vp endosperms (together with several unrelated enzymes) and to be somewhat affected by mutations at C and R (Dooner, unpub.). However, PAL is known to be sensitive to product inhibition and conceivably could be inhibited by compounds accumulating in c and r endosperms.

R and C are loci that have been widely studied by maize geneticists. The elucidation of their mode of action remains an interesting challenge awaiting further study.

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19 STARCH MUTANTS AND THEIR PROTEIN PRODUCTS

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Maize endosperm is the major site of starch deposition in the plant during kernel development. Many mutants leading to altered levels of starch or its quality are available (Creech, 1968). Although a large number of these mutants are mapped, many remain unmapped. Because their phenotype readily identifies the area of metabolic lesion, these mutants are considered ideal systems for analysis of the gene-enzyme relationship. However, to date, only four mutants, viz., shrunken (sh), shrunken-2 (sh-2), brittle-2 (bt-2) and waxy (wx) have been identified with their enzyme products. The following discussion will attempt to elaborate on these genetic systems.

The shrunken (sh) locus

The shrunken (sh) mutation on chromosome nine in homozygous form conditions a shrunken or collapsed phenotype of the kernel due to a reduced level of starch content in the endosperm. The mutation is highly tissue specific and no other part of the plant is affected. Schwartz (1960) reported that the sh mutations is associated with the loss of a major protein designated as Sh protein. Chourey and Schwartz (1971) obtained several new sh mutants from Sh kernels by using ethyl methanesulfonate. Some of these mutants showed the presence of Sh protein by electrophoretic as well as immunochemical criteria (i.e., they were CRM+). Co-occurrence of electrophoretic mobility alterations of this protein in two of the six CRM+ mutants suggests that Sh locus is the structural gene for this protein. These observations, however, did not provide any information on the function of this major protein in the endosperm. Chourey and Nelson (1976) examined starch biosynthesis enzymes in developing endosperms of wild (Sh) type and sh genotype. They concluded on the basis of several observations (Chourey and Nelson, 1976, 1979) that the Sh locus codes for the enzyme sucrose synthetase and its deficiency leads to a rate limiting step in starch biosynthesis and hence the sh phenotype.

A puzzling observation, however, remained; although all spontaneously induced sh mutants and Ds suppressed Sh alleles showed a complete loss of Sh protein (Schwartz, 1960; Chourey and Nelson, 1976), they still retained a certain residual level (approximately 5 percent, as compared to the wild type) of sucrose synthetase activity (Chourey and Nelson, 1978). Recently, three isolates of Ds suppressed Sh alleles, designated as sh bzm-4, sh-m5933, and sh-m6233 (originally obtained by Barbara McClintock) have been examined for the enzyme activity. They also have a similar level of residual activity (Chourey, unpubl.) and are CRM- for the Sh protein. Biochemical characterization studies revealed that the two enzyme activities were very similar to each other (Chourey and Nelson, 1976, 1978) except for the following characteristics: (1) A slight difference in electrophoretic mobility in native gels, presumably due to a difference in the net charge of the molecules is seen. (2) The relative abundance of the enzyme molecules in wild type and sh endosperms is very different. Because of these two simultaneous alterations in the protein, it was proposed that the minor enzyme is specified by a locus elsewhere in the genome and not by the sh

locus (i.e., the sh mutational event is not leaky). This genetic inference was based on biochemical observations. However, this hypothesis is now considerably strengthened by recent data. First, a sh mutant, presumably due to a deletion of the sh locus and designated as sh bzm-4 is similar to sh-R (a reference allele) in terms of enzyme activity as well as sucrose synthetase protein in native and SDS gels (Chourey, 1981). Secondly, according to the two gene hypotheses, the wild type endosperm should show both sucrose synthetase proteins on the native gels. Indeed, this has been recently demonstrated (Chourey, unpubl.). Su and Preiss (1978) have purified and characterized sucrose synthetase from wild type kernels and have found the enzyme to be a polymer; the predominant form is a homomeric tetramer (M. W. of 360,000). The subunit MW is 88,000. Amino acid analysis shows the subunit protein to be rich in hydrophobic amino acids with methionine as the least frequent amino acid residue.

Interallelic or Intragenic Complementation

The sh locus also exhibits an important genetic phenomenon: some of the EMS-induced sh mutants when inter-crossed show interallelic (intracistronic) complementation (Chourey, 1971). It is probably the only locus in higher plants, where positive complementation as evident by the occurrence of wild type phenotype (plump kernels) in the F1 hybrid of two homozygous mutants, has been reported. It was observed to occur only in specific combinations of certain CRM+ mutants. Inclusion of the sh-S allele in the heterozygote was obligatory for the restoration of the wild type phenotype. Enzymatic analyses of the homozygotes and heterozygotes has been done, the latter showed a two fold elevation in sucrose cleavage activity compared to the former (Chourey and Nelson, 1979).

Attempts to visualize this enzyme on a gel after electrophoresis using a histochemical stain specific for sucrose synthetase reaction, have been unsuccessful (Chourey and Nelson, 1976). However, this enzyme can be readily seen as a major protein band(s) among the slower migrating proteins on the gel. Electrophoretic analyses of complementing heterozygotes has shown two protein bands and no hybrid band has been detectable (Chourey 1971; Chourey, 1981a). The latter study has shown that the sucrose synthetase protein band pattern in a complementing hybrid was different from that of the corresponding in vitro mixture of extracts from the homozygotes. A random polymerization of subunits of the tetramer coded by each allele in the heterozygote should have generated a multiple of five sucrose synthetase proteins. The observed band pattern, however, deviated from both the above expectations and is under further investigation. Sucrose synthetase analyses strongly suggest the existence of a heteromer(s) in complemeting heterozygotes (Chourey and Nelson, 1979). Association of the hybrid enzyme with complementation has been demonstrated in various microbial systems. Further analyses of this phenomenon would enable not only a better understanding of the physiological function of this enzyme but also, more importantly, show how a single locus heterozygosity leads to the formation of a "better" enzyme. Preliminary observations on some of the complementing reciprocally-crossed heterozygotes also show evidence of differential expression of alleles at the protein level (Chourey, 1981a). Homozygous alleles apparently show no difference in the level of expression at the protein level; critical and conclusive assays are currently in progress.

The shrunken-2 (sh-2) and brittle-2 (bt-2) locus

The shrunken-2 (sh-2) allele on chromosome 3 and brittle-2 (bt-2) allele on chromosome 4, each in homozygous form, leads to a severe reduction in endosperm starch content and a shrunken endosperm phenotype. Tsai and Nelson (1966) noted that both of these mutants were associated with a complete loss of adenosine diphosphate glucose (ADPG) pyrophosphorylase activity. Later, Dickinson and Preiss (1969) using a more sensitive assay found that sh-2 and bt-2 endosperms have a residual level of 8 to 10 percent of normal activity. The complexity of genetic control of ADPG pyrophosphorylase in maize endosperm, however, was first suggested in detailed studies by Hannah and Nelson (1975 and 1976). They found that both sh-2 and bt-2 affected enzyme activity in a fashion analogous to each being a structural gene for the enzyme. They hypothesized that enzyme subunits were coded by sh-2 as well as bt-2 loci (as in the case of the hemoglobin molecule). It is an attractive hypothesis and can be tested experimentally.

Hannah and Nelson (1976) were the first to show that the association of a controlling element with a locus can lead to a qualitative alteration in an enzyme. The Km of ADPG pyrophosphorylase in the sh-2m stock (Ds suppressed Sh-2 allele) was two to three times higher than that of the enzyme synthesized in the absence of Ds. Tuschall and Hannah (1980) examined the wild type revertants derived from removal of the Ds and found ADPG pyrophosphorylase to be altered as compared to that of sh-2m as well as the wild type progenitor allele. These studies confirm the role of sh-2 locus as being a structural gene for ADPG pyrophosphorylase. On the basis of the structural role of the sh-2 and bt-2 genes for this enzyme, it was expected that the activity level in a sh-2 bt-2 double mutant should be much lower than found in either single mutant. However, analysis of one double mutant (Hannah and Nelson, 1975) showed that the level of enzyme activity was similar to a single mutant, sh2-R (a reference allele). The genetic basis of the residual enzyme in a double mutant is not known. So far, this enzyme has neither been purified to a homogeneity nor has it been possible to associate it with certain protein species in partially purified preparations. Hence, the nature of the lesion at the protein level in sh-2, bt-2, and various sh-2m alleles is not known.

The waxy (wx) locus

The wx mutation on chromosome nine is unique as it affects the type of starch and not the quantity, in the affected tissue. Also, the tissue specificity of this mutation is not as stringent as that of several other starch mutants. The wx allele is expressed in endosperm, pollen, and embryo sac. The starch produced in normal (Wx) genotype is 25 percent amylose and 75 percent amylopectin; the homozygous wx condition is associated with starch, which has 100 percent amylopectin (Sprague et al., 1943). Nelson and Rines (1962) observed that starch granules from developing endosperms of homozygous wx maize did not possess the starch granule bound enzyme transferring glucose from UDP-glucose to the non-reducing end of the starch molecule. Later, Nelson and Tsai (1964) found about one-tenth as much activity in preparations from 17 different waxy mutants as in the homozygous wild (Wx) type genotype. Recent analyses (Nelson et al., 1978) relating to Km determinations for ADP-glucose on wx starch granules have been found to be 7.1 x 10-5 M. The Km on non-waxy preparations was estimated as 3 X 10-3 M. The large Km differences clearly indicates that there are two starch

granule-bound glucosyl transferases in developing maize endosperm. It was also suggested that the two forms of the enzyme were coded by two separate genes. The large Km enzyme is believed to be encoded by the Wx allele (Akatsuk and Nelson, 1969); the genetic basis of the second enzyme is still unknown.

Due to the entrapment of the enzyme in starch granules, it was not possible to analyze wx alleles at the protein level. However, this problem is now apparently solved. An ingenious method for the extraction of starch bound proteins by Echt (1981) has enabled identification of a protein which is presumably encoded by the Wx locus. This protein, designated as Wx protein, contributes 85 percent of the total heat extractable starch granule bound proteins and has a subunit M.W. of 60,000 daltons. Although it is not possible to demonstrate directly that the Wx protein is the enzyme NDP-sugar-starch glucosyl transferase (as the extraction methods inactivate enzyme activity), Echt and Swartz (1981) have provided sufficient evidence that the Wx protein is the monomeric form of the enzyme coded by the Wx locus (see Table 1). One of their most important observations is that some of the wx alleles show coincident qualitative alterations in the Wx protein, while no natural polymorphism for this protein in several inbred lines (Wx genotype) is seen. The staining intensity of this protein is also gene-dose dependent.

Table 1

Alterations in the Wx protein in various wx alleles (compiled from: Echt and Schwartz, In Press).

Nature of Mutation	Total No. of alleles analysed	No. of alleles with protein		Alterations in the protein		
		Absent	Present	Quantitative (as compared to wild type)		Qualitative (net charge)
				None	Reduced	
spontaneous	26	22	4	2:C31,R	2:a*,90	3:R,C3,90**
controlling element	5	3 ml, m6 and B4 (all due to Ds)	2 m8	1:B3 (due to Mp)	1:m8* (due to Spm)	1:B3

* Not analysed for alteration in the net charge.

**The protein in this mutant shows both qualitative and quantitative alterations.

Conclusion

A mutation in each of the four gene systems discussed here allows identification of a rate limiting step in starch biosynthetic reactions in endosperm cells. It also enables in each case, the uncovering of a second isozyme coded by a gene elsewhere in the genome. A set of at least two genes is thus involved in the expression of isozymes of each of the enzymes. Of these two genes, the gene encoding the major isozyme (responsible for 90-95 percent of the total activity) is highly specific to the endosperm tissue. This is not surprising in view of the knowledge that starch biosynthesis is one of the major biosynthetic functions of the developing endosperm. At present very little is known about tissue specificity of genes encoding the minor enzymes.

Acknowledgements

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20 CATALASE: A SYSTEM FOR STUDYING THE MOLECULAR BASIS OF DEVELOPMENTAL GENE REGULATION

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A number of model systems exist which have proven useful in studies on gene structure and regulation, each of which has its own set of strengths and weaknesses. An ideal system for studying developmental gene regulation would have a number of important attributes including: 1) suitability for a genetic analysis of the gene(s), 2) the availability of mutants affecting the developmental expression of the gene product, 3) variation in the temporal and/or tissue expression of the gene during development, 4) suitability for studying the expression of the gene in cultured cells where certain types of experiments (e.g. pulse-chase labelling) are more efficiently conducted, 5) suitability for the purification and biochemical characterization of the gene product and 6) suitability for isolation of the gene sequences themselves for structural analysis and for use as hybridization probes.

My laboratory has been involved in the development of a model system in maize which has many (though not all) of these characteristics. In this brief review I shall attempt to summarize the current status of work on this system, and of our understanding of the regulation of these genes.

Maize catalase is a tetrameric enzyme with a subunit molecular weight of 56,000 daltons. There are three catalase structural genes in maize (Cat1, Cat2, and Cat3) (Roupakias et al. 1980), which differ in their temporal and spatial expression. Cat1 is the only one of these genes expressed in the immature endosperm and embryo. The single catalase isozyme observed in these tissues is a homotetramer composed of Cat1 subunits. Cat1 and Cat2 are both expressed in the aleurone layer of the immature endosperm, and five isozymes can be observed in this tissue (the two homotetramers and three heterotetramers).

In the scutellum of the germinating seedling there is a developmental shift in the expression of catalase loci. Scutella from newly imbibed seeds contain a single catalase isozyme (the Cat1 homotetramer). By the second day of germination expression of the Cat2 locus can be observed. This locus continues to be expressed until the scutellum degenerates. There is a transition period of several days during which both genes are expressed simultaneously. Cat1 expression can no longer be detected in the scutellum after the fourth or fifth day of germination, although de novo synthesis of Cat1 polypeptides can be detected until that time (Quail and Scandalios 1971, Sorenson et al. 1977). Cat3 expression can be detected in the coleoptile sheath of the germinating seedling and in mature leaves (Sorenson and Scandalios 1975). In most inbred lines, it is the only isozyme observed in these tissues, although Cat1 expression can also be observed in some lines. In most of these cases it appears that Cat1 and Cat3 are being expressed in different cells since no heterotetramers are observed. There is some evidence that Cat3 may be light inducible.

The Cat1 locus maps on the short arm of chromosome 5 (Roupakias et al. 1980). Six electrophoretic alleles have been found at this locus. Cat2

maps on the short arm of chromosome 1 and has 3 electrophoretic variant alleles. *Cat3* is located on the distal half of the long arm of chromosome 1 and also has 3 known alleles. In addition, a genetically distinct locus has been reported which may regulate some aspects of *Cat2* expression (Scandalios *et al*. 1980).

Most of our attention to date has been focused on the developmental shift from *Cat1* to *Cat2* expression which occurs in the scutellum of the germinating seedling. The CAT-2 isozyme is synthesized at about twice the rate of the CAT-1 isozyme as measured by either a pulse-chase experiment (Quail and Scandalios 1971) or a synthesis inhibition experiment (Sorenson *et al*. 1977). It is not yet clear whether this differential synthetic rate is due to differences in transcription (and/or processing), translation, or messenger RNA stability.

We have also studied the post-translational aspects of CAT-1 and CAT-2 expression in the scutellum of the developing seedling. Immature endosperm catalase (CAT-1) does not appear to be associated with any subcellular organelle. Both isozymes are associated with scutellar glyoxysomes for approximately three days during seedling germination (from days 3-6). Prior to day 3 and after day 6 catalase is soluble. This compartmentation process is somewhat atypical in that: (a) it is transient; (b) catalase appears to be synthesized on free ribosomes (Sorenson and Kelly, submitted); (c) catalase synthesized *in vitro* in response to purified catalase mRNA is identical in size to mature catalase, and thus does not appear to be synthesized as a large precursor protein; and (d) catalase contains no detectable carbohydrate (Tsaftaris *et al*. 1980). Substantial evidence has been obtained which suggests that the compartmentation of catalase in glyoxysomes may involve an "anchor protein" which binds catalase to the glyoxysomal membrane post-translationally. An analogous mechanism has been thoroughly documented for mouse glucuronidase (Lusis and Paigen 1977).

The putative maize "anchor protein" is a small glycoprotein (Tsaftaris *et al*. 1980) which had been identified initially by its ability to inhibit catalase *in vitro* (Sorenson and Scandalios 1975, 1976, 1980; Tsaftaris and Sorenson 1980a). This protein is associated with the glyoxysomal membrane. ^{125}I-labeled catalase will bind specifically to glyoxysomes *in vitro*, and this binding can be at least partially prevented by preincubating the glyoxysomes with antibodies prepared against the putative anchor protein (Tsaftaris and Sorenson, submitted). We have screened a number of inbred lines using a radio-immunoassay for this protein (Tsaftaris and Sorenson 1980b), and have found a mutant which has less than 1% of normal levels. We are currently increasing this line so that we can evaluate the effects of this mutation on the catalase compartmentation process.

Our primary efforts over the past few years have been at developing the tools to examine the molecular basis of differential expression of the catalase genes. The first phase of this study was to isolate the two catalase mRNAs. The approach used involved the immunological precipitation of polysomes synthesizing catalase with antibodies prepared in rabbits against each of the catalase isozymes. CAT-1 antibodies labeled with ^{125}I will bind to polysomes isolated from immature endosperm but not to polysomes isolated from a tissue (coleoptile) which is not synthesizing the enzyme. This binding is prevented by predigesting the polysomes with ribonuclease.

Preimmune antibodies do not bind to the polysomes. Similar patterns were observed with CAT-2 antibodies and polysomes isolated from 2-day scutella.

These antibody-polysome complexes can be precipitated by the addition of either goat antirabbit antibodies or Staph aureus cell walls containing protein A. The polysome-antibody-Staph cell wall aggregates can be observed in the electron microscope, and the polysomes show a characteristically narrow size distribution (8.9 $\pm$ .64 ribosomes/message). After considerable manipulation of experimental parameters, conditions were found which allowed the Cat1 mRNA to be obtained at 85% translational purity. This mRNA migrates on methyl mercury denaturing gels as a discrete band with a length of 1850 nucleotides and two presumptive degradation products with lengths of 975 and 850 nucleotides. All three of these bands hybridize with a cloned Cat1 cDNA probe.

Cat1 mRNA directs the synthesis of a single major polypeptide in the wheat germ cell-free translation system with a mobility which is indistinguishable from that of ^{125}I-labeled native catalase. This polypeptide has a molecular weight of 56,000 and is precipitable with antibodies raised against purified CAT-1 protein.

Cat2 mRNA has been isolated from the scutella of germinating seedlings using a similar approach. Due to high ribonuclease activity in this tissue, it is necessary to perform the polysome immunoprecipitation under non-optimal conditions (relatively large amounts of antibody for short incubation times) with the result that the mRNA obtained is not as pure as the Cat1 mRNA. We have recently, however, obtained evidence that Cat1 and Cat2 have sufficient sequence homology that we may be able to use the Cat1 mRNA to detect Cat2 sequences in cloning experiments.

We have prepared both genomic (native) libraries and cDNA libraries from various inbred lines and developmental stages, in order to isolate the catalase gene sequences.

We have generated three cDNA libraries from various developmental stages in the inbred W64A. The first library was constructed from immature endosperm cDNA. The cDNA was synthesized against polysomal polyA(+) RNA isolated from 18 day post pollination endosperm, and cloned into the tetracycline resistance gene of pBR322. We have screened this library and recovered several catalase clones. These in turn have been used to screen genomic libraries prepared in bacteriophage Charon 4A. A number of potential catalase genomic clones have been identified and we are currently characterizing them. Experiments which estimate cross-hybridization suggest that these clones may represent the Cat1 and Cat2 genes (and possibly even Cat3).

Studies currently in progress are directed at elucidating the structural features of the catalase genes as well as the details of their expression at the transcription level.

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21 MOLECULAR GENETIC ANALYSIS OF THE MAIZE ANAEROBIC RESPONSE

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Introduction

In studying the structure and function of genes in higher plants, maize offers advantages in terms of physiology, biochemistry, tissue culture and especially genetics. Studies on the maize anaerobic response stemmed from the extensive analysis of the maize alcohol dehydrogenase (ADH) system by Schwartz and coworkers (reviewed by Freeling and Birchler, 1981). When maize seedlings are subjected to anaerobiosis, ADH activity increases (Hageman and Flesher, 1960) at a zero-order rate in roots, epicotyls and mesocotyls (Freeling, 1973). This increase in ADH activity reflects an increase in cross-reacting material to anti-ADH-antibodies and is due to the simultaneous expression of two unlinked genes, Adh1 and Adh2 (Freeling, 1973). In addition, it has been shown that ADH activity is required to allow maize seeds and seedlings to survive anaerobic treatment (Schwartz, 1969). In light of this, the analysis of protein synthesis in maize seedlings during anaerobic treatment was of great interest. The finding that ADH1 and ADH2 are two of a small group of proteins selectively synthesized during anaerobic treatment has led to further molecular genetic analysis and development as a model system for studying the control of gene expression in plants.

The Maize Anaerobic Response

Maize seedling roots respond to anaerobic treatment by redirecting protein synthesis with an immediate repression of most pre-existing protein synthesis and a rapid increase in the synthesis of the transition polypeptides (TPs), a class of polypeptides with an approximate molecular weight of 33,000 daltons. After approximately 90 minutes, the synthesis of an additional group of 20 polypeptides is induced (Sachs, et al., 1980). The synthesis of these 20 anaerobic polypeptides (ANPs) represents 70 percent of the total label incorporation after five hours of anaerobic treatment. By this time, synthesis of the TPs is minimal (Sachs, et al., 1980). The synthesis of the ANPs continues in a quantitatively stable ratio for up to 72 hours of anaerobic treatment, at which time the roots begin to die. ADH1 and ADH2 have been identified as ANPs by using available genetic variants (Sachs and Freeling, 1978; Ferl, et al., 1979); however, the 18 remaining ANPs and the TPs serve unknown functions. Pyruvate decarboxylase activity has been shown to increase during anaerobic treatment (A. Laszlo, personal communication) and, therefore, may also be an ANP.

In the presence of air, each maize organ examined such as root, endosperm, scutellum, anther, and leaves synthesizes a tissue-specific spectrum of polypeptides. Under anaerobic conditions no detectable protein synthesis

is observed in the leaves, and they die after a short treatment. On the other hand, all other organs examined synthesize the ANPs during anaerobic treatment. Moreover, except for a few characteristic qualitative and quantitative differences, the patterns of anaerobic protein synthesis in these diverse organs is remarkably similar (Okimoto, et al., 1980).

It has been reported that maize seedlings subjected to heat shock synthesize a unique set of polypeptides, perhaps similar to those observed in heat treated Drosophila. However, heat treatment does not induce the ANPs (Laszlo and Sung, 1980). It was observed that wounding of maize seedlings also induces the synthesis of a few new polypeptides, but once again not the ANPs (Okimoto, et al., 1980). These results indicate that unlike the heat shock proteins in Drosophila (Ashburner and Bonner, 1979), the ANPs are not a response to general stress, but a specific response to anaerobic conditions as would occur in flooding. Similar ANP profiles are found in other plant species including rice, sorghum, and carrots (M. L. Roose, C. H. Chen, and R. Okimoto; Freeling Lab., unpublished observations).

The rapid repression of pre-existing protein synthesis, as in soybeans (Lin and Key, 1967), is correlated with a near complete dissociation of polysomes in anaerobically treated maize roots (Dennis, unpublished) and has been shown to occur at the level of translation, since the mRNAs encoding them remain translatable in an in vitro system at least five hours after initiation of anaerobic treatment (Sachs, et al., 1980). This is in agreement with the observations in soybean seedlings returned to air that anaerobically dissociated polysomes rapidly reform up to 80-90 percent their pretreatment levels even in the absence of new RNA synthesis (Lin and Key, 1967).

Pre-existing protein synthesis is not repressed in roots which are immersed in water while the rest of the seedling is left exposed to air. However, this treatment does induce synthesis of the ANPs including the two ADHs (Sachs, unpublished observations). It seems that even trace amounts of oxygen, which might mix with a nitrogen atmosphere during experimental manipulations, allow aerobic protein synthesis (Sachs and Okimoto, unpublished observations).

When total RNA from aerobic roots is analyzed with an in vitro translation system, little or no ADH synthesis occurs. However, RNA from anaerobically treated roots clearly does stimulate ADH1 and ADH2 synthesis in an in vitro system (Ferl, et al., 1980). This suggests that the induction of ADH and presumably the other ANPs by anaerobic stress is accomplished by increasing ANP-mRNA levels; perhaps by increasing transcription rates at the ANP genes.

Molecular Analysis

One of the anaerobic genes, Alcohol Dehydrogenase 1, is among the best characterized gene systems in higher organisms (reviewed by Freeling and Birchler, 1981). Molecular genetic analysis of this gene and the other genes of the anaerobic response could be valuable in telling us what genes are and how they work.

The molecular analysis of the maize anaerobic response was initiated by synthesizing cDNA clones from high molecular weight poly(A)-RNA from

maize seedling roots subjected to anaerobic treatment for 24 hours. Anaerobic specific clones were identified by colony hybridization analysis with labelled cDNAs of RNA from anaerobic roots and aerobic roots. Colonies were selected that hybridized specifically with anaerobic RNA. The anaerobic specific clones were further analyzed by hybrid release translation (HRT). HRT polypeptide products were run on SDS gels and each clone was correlated with a particular polypeptide molecular weight class. Several different families of clones were obtained. One clone coded for a polypeptide with an approximate molecular weight of 40,000 daltons (pZML84), the molecular weight of ADH polypeptide. This clone, and the family of clones which strongly hybridize to it, were further analyzed using a line of maize with the mutant Adhl-U725 allele whose product has altered migration in both IEF and SDS gels (Ferl, et al., 1979, 1980). The expected U725 HRT product was detected in SDS gels and 2-D IEF-SDS gels. In addition, the clone was identified by antibody precipitation of the HRT polypeptide product, using anti-ADH1-IgG. The results of these experiments strongly suggest that the pZML84 clone family encodes ADH1. A class of clones which only weakly hybridize to pZML84 (the pZML841 class) was also isolated.

The nucleotide sequences of pZML84 and pZML841 are about 80 percent homologous in the presumptive coding region, but there is no homology in the 3'-noncoding region. Two-thirds of the differences in the presumptive coding region did not change the amino acids coded, giving a 80 percent identity in the amino acid sequence derived from the nucleotide sequences. The amino acid sequence of ADH1 and ADH2 proteins are also being determined in order to correlate them with their respective nucleotide sequences. A short region of the ADH1 protein sequence corresponds to that expected from the nucleotide sequence of pZML84 (A. S. Inglis, personal communication).

Maize RNA from anaerobically treated roots or shoots was analyzed by "Northern" blotting (Alwine, et al., 1977), using nick translated 84 class and 841 class clones as labeled probes. Aerobically grown maize lines show very low amounts of RNA hybridizing to either probe. After anaerobic treatment, there is up to a 50-fold induction of RNA. The 84 class probes hybridize to two size classes of mRNA in lines with an Adhl-F allele, of approximately 1,650 and 1,750 bases. However, lines homozygous for an Adhl-S allele show the 1,650 mRNA class but very little 1,750 class. When 841 is used as the probe, it hybridizes to a 1,750 class mRNA in both Adhl-F and Adhl-S lines. This band comigrates with the larger class seen in Adhl-F lines. 841 hybridizes only weakly to the 1,650 mRNA class. A line which is Adhl-S and null for Adh2 (Ferl, et al., 1979) shows only very faint hybridization to the 1,650 mRNA class and no hybridization to 1,750 class when probed with 841. These results suggest that clones of the 84 class encode ADH1, while clones of the 841 class encode ADH2. A number of the other anaerobic specific clones have been identified on the basis of their mRNA sizes and molecular weights of proteins they code.

The anaerobic specific clones are being used as probes for in situ hybridization. It is expected that Adhl will be cytologically located to chromosome 1 (Schwartz, 1971) and Adh2 to chromosome 4 (Dlouhy, 1980). It would be interesting to determine if any of the other anaerobic genes are linked to either Adhl or Adh2 or to each other.

Isolation of the Adh genes from genomic libraries of various maize lines will permit an analysis of the nucleotide sequences of several interesting Adh1 alleles which show quantitative regulatory differences. The Adh1 gene sequences will also be compared with sequences of Adh2 and other anaerobic specific genes to determine if there are common sequences which might account for their coordinate expression and selective translation during anaerobic treatment. In addition, we hope to analyze the functions of these genes by transforming genomic clones into tissue culture cells.

Protoplast, Cell and Tissue Culture

The overall situation of plant cell culture of grasses and particularly the cereals has been reviewed in a recent issue of PMBN (Vasil, 1981). Although significant progress has been made over the last few years, cereals generally still cause serious problems in developing satisfactory tissue and cell culture methods. Maize is no exception, but because of our interest in the maize ADH system, it is valuable to attempt to develop these systems.

Immature embryo cultures have been used to establish tissue cultures as described originally by Green and Phillips (1975). The 22 lines we have tested are Black Mexican Sweet (BMS), several Adh variants including Adh-null mutants, and F1 hybrids of BMS and the Adh variants. BMS is known to have a good tissue culture ability. The initiation of all cultures was highly efficient, i.e., more than 80 percent of the plated embryos showed proliferation of the scutellum and formed a callus. During the first two months after culture initiation, plants were regenerated without difficulty. Cultures transferred to medium with reduced auxin and finally to auxin-free medium readily formed shoots and plants which could be transferred to soil. Immature embryos of the regenerants are being used for a second tissue culture cycle. Clear-cut differences and limitations among the lines became obvious after prolonged subculture of the callus material. Whereas BMS and heterozygous lines showed a slow but steady growth, homozygous Adh-null mutants failed to survive for longer times as tissue cultures. Preliminary experiments aimed at establishing embryogenic suspension cultures showed similar results with respect to genotypic differences. Embryogenic suspensions are of special interest (1) as a totipotent cell culture system and (2) as a possible source for the isolation of totipotent single cells and protoplasts.

Another approach for establishing a single cell/protoplast system is based on an established maize cell culture line. This line, Z4, originally derived from stem protoplasts of *Zea mays* (Potrykus, *et al.*, 1977) grows well as callus and as suspension. Protoplast isolation and protoplast culture conditions have been studied extensively and are well defined (Potrykus, *et al.*, 1979). However, the line Z4 is non-morphogenic, aneuploid and shows additional features typical of established cell culture lines (King, 1980). Although haploid cells are advantageous over diploid or aneuploid material with respect to mutant or variant selection (Gebhardt, *et al.*, 1981) experiments are in progress aimed at selecting variants of Z4 with reduced ADH activity. This selection is based on allyl alcohol treatment (Schwartz and Osterman, 1976). Adh+ cells convert the allyl alcohol to the toxic acrylaldehyde, whereas Adh- cells do not and are thus more resistant to allyl alcohol exposure.

Cultured cells of Z4 have been used also to study the influence of culture conditions (physical parameters, media composition) on ADH induction and activity. ADH activity under standard culture conditions, callus or agar medium or liquid cultures as shaking suspensions, is rather high. Only limited success in defining and maintaining aerobic cultures with low levels of ADH activity has been achieved. However, strictly anaerobic culture conditions (under argon) increase the activity of ADH about ten times.

Tissue culture work will continue in two directions. Existing genetically defined mutant stocks will be used as the source of material for the establishment of new cell cultures. Where such cultures are not available, attempts will be made to find similar mutations in an existing cell culture line.

Cell and tissue culture will be extremely valuable for developing plant transformation methods using ADH as a model system. Some of the prerequisites for defined, controlled plant transformation have been previously discussed (Langridge, 1979). The requirement for a gene carried on a transforming vector that is selectable in plant cells may be achieved with the ADH system of maize. It is already obvious from preliminary studies that cultures established from *Adh-* genetic stocks have low viability under most conditions of culture and particularly under anaerobiosis. This is in contrast to cultures having an active *Adh+* gene. Thus, it seems reasonable that this observation may provide the basis of a system for selecting cells having an active *Adh* gene derived from transforming DNA.

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22 AN ANNOTATED BIBLIOGRAPHY OF THE Adh GENES OF MAIZE, FROM 1966 THROUGH 1981, AND PREDICTION ON THE FUTURE OF CLASSICAL GENETICS

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PREFACE

What we know of the alcohol dehydrogenase genes (Adh1 and Adh2) in maize derives largely from the efforts of Dr. Drew Schwartz at Indiana University, USA, and those who studied with him. Beginning with the discovery in 1966 of naturally occurring allozyme variation of Adh1, there has been a steady accumulation of genetic and molecular information that, like pieces of a jigsaw puzzle, fit together into a particularly useful system for obtaining answers to big problems of gene regulation and programming during development. A detailed review has been published in a relatively obscure volume (Freeling and Birchler, 1981). In the annotated bibliography that follows, particular papers are denoted with an asterisk; the study of these might provide efficient access to the history of the Adhs and the other anaerobic genes, and their products.

Under anaerobic conditions, the ADHs and several other proteins are induced at the level of protein synthesis and mRNA levels while nonanaerobic protein synthesis halts. Naturally occurring variants have been found for a few of the anaerobic genes. Over a hundred mutants have been selected for altered Adh1 expression; some are clearly in coding sequence, some are insertions, and some affect the regulation of Adh1 without altering the ADH1 polypeptide. All have a future at the level of nucleotide sequence.

One cannot help but speculate that the huge quantitative and qualitative diversity of the anaerobic genes and their expression among cultivars of maize and among higher plants in general reflects differences in adaptation to flooding. This has not been adequately tested. That this gene system might permit extrapolations from nucleotide sequence to ecological adaptedness makes it unique among other groups of coordinately regulated genes.

To study each anaerobic gene as if it were an autonomous unit of function is an important first step, but just a first step. There is compelling evidence that the anaerobic genes respond to signals from more fundamental levels of information that might be called levels of "developmental programming." Owing to the wealth of mutants of Adh1 and variants of the other anaerobic genes, one can safely predict that we will soon know exact molecular details of the DNA sequences that receive the trans-acting molecules encoded in these levels of developmental programming. To move backwards from regulatory sites of genes (that merely serve developmental programs) to developmental programs themselves, is a quest worth some thought.

There is a rumor that classical genetics is on its way to being replaced by in vitro alterations of DNA assayed in a transformation system. There is some truth in this. The question of whether classical geneticists should continue inducing, selecting and recovering mutants in whole, complicated organisms deserves examination. Of course, the answer will depend on exactly what sort of mutant is desired, and the ability of the biological assay system to permit recognition of this mutant. Assuming that a mutant or two altering the anaerobic gene program would be useful, there is little question that classical genetics has the greater chance of success. Since we know nothing about the rules or mechanisms governing how development controls groups of genes, there is wisdom in allowing the organism maximum freedom to give us clues.

The judicious use of in vitro genetics presupposes some minimal knowledge about the molecules involved in the mechanism to be altered. For example, to alter the sequence arrangement of a "programming gene" requires a recombinant DNA containing the gene. However, to identify the "programming gene" recombinant requires an expression system that one could imagine to be as informationally complex as the organism itself. When the phenomenon to benefit from mutational analysis involves sequential action of molecules compartmentalized in space or time, or all but the most simple molecular interactions, classical genetic technology is the cutting edge.

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23 ZEIN: GENETICS AND BIOCHEMISTRY

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The endospermic proteins of maize were first classified by Osborne (1924) according to their solubility. The main classes he recognized were albumins (soluble in water), globulins (soluble in saline), zeins (soluble in alcohols) and glutelins (soluble in alkali).

Zeins represent the most abundant class accounting at maturity for more than 60 percent of the total endosperm proteins. These polypeptides are synthesized by membrane-bound polysomes as precursor proteins containing a 10-20 amino acids long signal peptide and after processing, they are deposited in enlargements of the endosplasmic reticulum (protein bodies) (Burr and Burr, 1976; Burr _et al_., 1978; Larkins and Hurkman, 1978; Burr and Burr, 1981; Khoo and Wolf, 1970). They accumulate in the endosperm for nitrogen storage to be used later by the embryo during germination. From the chemical point of view, zeins exhibit distinctive features: they are soluble in alcoholic solutions (70% v/v ethanol or isopropanol), especially if disulphide bridges are reduced with 2-mercaptoethanol; their amino acid composition is rich in glutamine, proline, leucine and alanine and poor in lysine and tryptophan (Mosse _et al_., 1966).

By SDS - polyacrylamide gel electrophoresis zein polypeptides with a M. W. of 23, 22, 20, 19, 14, and 10K can be identified (Misra _et al_., 1975; Soave _et al_., 1976); in genetics studies they have been grouped into four classes of 22, 20, 14, and 10K. It should be, however, underlined that these molecular weights are likely not to represent absolute values, since their estimate depend on the markers used in SDS electrophoresis (E. Vitale, personal communication); moreover, a zein cDNA clone sequenced by Geraghty _et al_., (1981) encodes for a zein peptide with a M. W. of 23K while in hybrid selected translation, it selects a peptide with an apparent SDS M. W. of 19K.

When fractionated by charge, zeins appear heterogeneous: up to 15-20 components can be easily detected in isoelectric-focusing (IEF) gels depending on the inbred analyzed (Righetti _et al_., 1977). This extensive heterogeneity is not an artifact since 1) zein polypeptides differ slightly in their amino acid sequence (Bietz _et al_., 1979; Vitale _et al_., 1980); 2) an _in vitro_ protein-synthesizing system supplemented with zein mRNA produces zeins with the same charge heterogeneity as observed for zeins synthesized _in vivo_ (Viotti _et al_., 1978); 3) zein cDNA clones that bind mRNAs which _in vitro_ synthesize zeins of the same size class, yield different fragments when treated with the same restriction enzyme (Park _et al_., 1980); 4) zein genes are reiterated in the maize genome (Wienand and Feix, 1980): about 100 copies of zein genes per haploid genome have been estimated on the basis of kinetic studies (Viotti _et al_., 1979); 5) in crosses between inbreds with

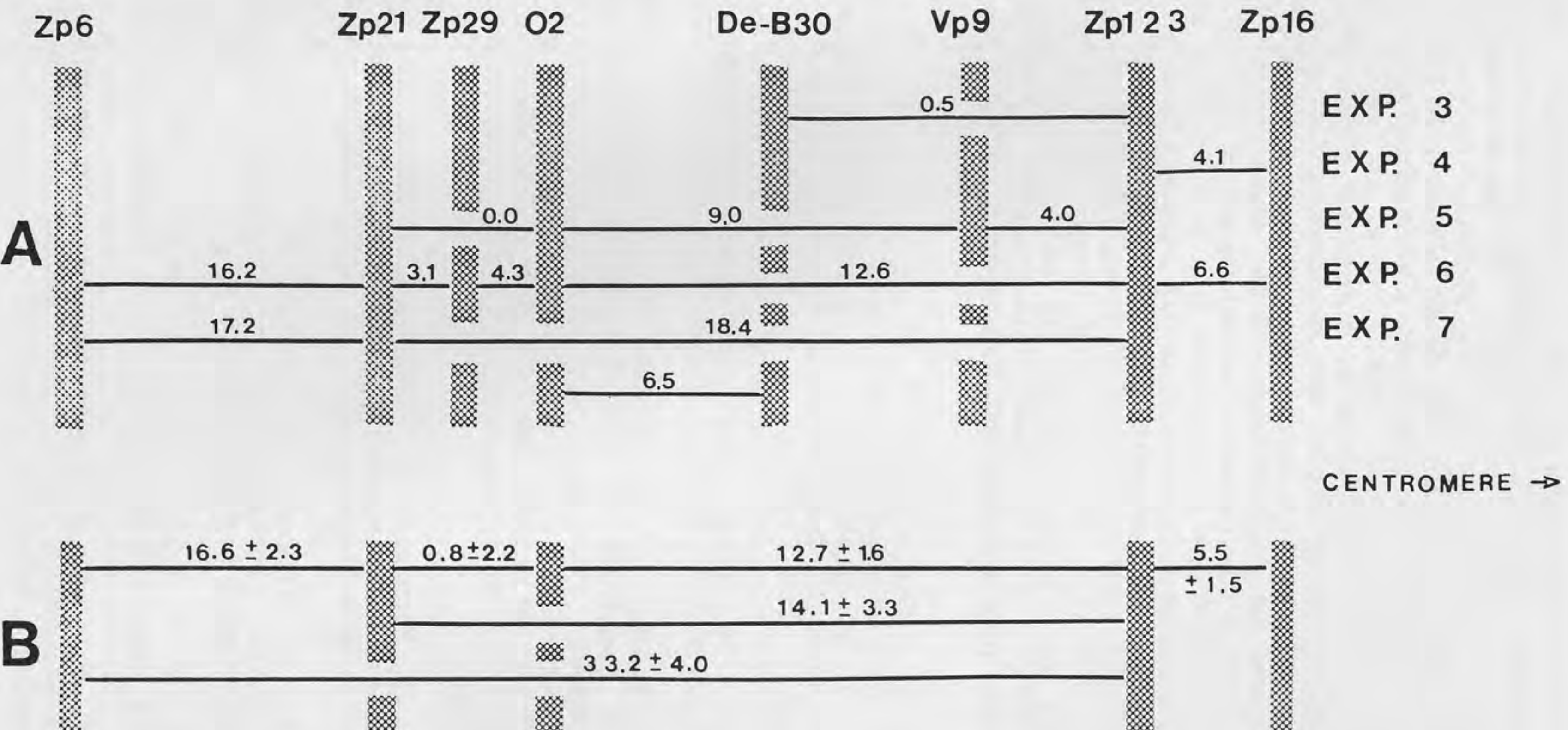
different zein IEF pattern, genetic factors coding for individual zein components are inherited as simple Mendelian units (Soave et al., 1978a). These data support the conclusion that the zein heterogeneity is due to the existence of many structural genes coding for zein polypeptides.

Considering the location of zein genes in the maize genome, we took advantage of the genotype specifity of the zein IEF pattern together with the large variability of this pattern among maize inbreds (Gentinetta et al., 1975). Thus, studying the inheritance of single IEF zein components in connection with that of other genes of known location, we have associated some zein structural genes to maize chromosome 4 and 7 (Soave et al., 1978a; Valentini et al., 1979). Up to now seven genes encoding different zein polypeptides belonging to the 20K family have been mapped in a region of about 30 crossover units in the short arm of chromosome 7 (Fig. 1) (Soave et al., 1981). According to preliminary data, the location of a gene encoding a 14K zein peptide is also in the same region of chromosome 7. Recently six elements belonging to the 22K family have been associated to a region of chromosome 4 near Fl2 and Su1 loci (Soave et al., unpublished observations).

The picture emerging from our genetic studies is that zein structural genes are clustered in two main sites of the maize genome: the 20K genes plus a 14K gene on chromosome 7 and the 22K genes on chromosome 4. Inside each chromosomal location, the individual zein elements are not contiguous to each other but rather dispersed. This situation shares some features of the multigene families as defined by Hood et al. (1975): multiplicity, homology, overlapping phenotypic function and linkage. A similar situation was observed for maize histone genes (Stout and Kermicle, 1979), for chorion proteins of Bombyx mori (Goldsmith and Basehoar, 1979) and for certain Drosophila gene families (Spradling et al., 1979). The genetic organization found for zein genes implies the idea that duplications of short chromosomal regions occurred during maize evolution giving rise to clustered gene families as those observed. Furthermore, since the 20 and 22K zein genes appear related at the DNA level (Park et al., 1980; Wienand and Feix, 1980) and because they are controlled by a common regulatory locus (Floury-2) (Soave et al., 1978b), it is also possible that they diverged from a common ancestor; however, they seem to occupy two distinct and specific chromosomal regions.

The expression of the zein genes is controlled by several loci acting on the onset and on the rate of zein accumulation (Mertz et al., 1964; Nelson et al., 1965; McWhirter, 1971; Ma and Nelson, 1975; Salamini et al., 1979; Manzocchi et al., 1980). In wild type endosperms zein polypeptides appear simultaneously around 15 days after pollination and then they are synchronously accumulated. Mutants altering the timing and the rate of zein synthesis have been described and they can be grouped into two classes: those delaying or reducing the accumulation of all the zein polypeptides and those acting more specifically on some of the components. For instance, the accumulation of the two major zein M. W. classes (the 20 and 22K classes) appears to be specifically affected by two mutations, opaque-2 (o2) and opaque-7 (o7), the first reducing mainly the 22K class, the second the 20K class. Since in the double mutants o2o2o7o7 both the alleles act additively in reducing zein accumulation, we suggest that in zein regulation at least two pathways are operating: one related to the accumulation of the 22K zeins, the other to the 20K family: o2 and o7 are involved respectively in the first and second pathway (Di Fonzo et al., 1980).

Figure 1. - A. Results of 5 mapping experiments among 20K zein genes and chromosome 7 markers. In B are reported linkage intensities of loci for which estimates are available from more than one experiment.



Among the loci controlling the level of zeins, O2 has been particularly studied because the recessive alleles at this locus confer a superior nutritive value to maize meal (Mertz et al., 1964). At the molecular level, however, the mechanism of action of O2 on zein synthesis has not been elucidated. The recessive alleles at the locus preferentially reduce the 22K zein family; in o2 extracts, moreover, the cytoplasmic mRNAs encoding for this family are almost absent (Pedersen et al., 1980). It is not clear, however, if the primary effect of the mutant allele is on the zein mRNAs concentration or if the lower level of zein mRNAs is the consequence of a reduced efficiency of their translation followed by degradation. Whatever the mechanism may be, the O2 locus must act by a diffusible factor which interfers with zein production, since the locus controls the expression of several zein genes not linked to it. Recently we demonstrated that a salt-soluble protein of M. W. 32,000 (b-32) is under the control of O2 (Soave, Tardani, et al., 1981). This protein, apparently located in the soluble cytoplasm of endosperm cells, is present in wild type but absent in seven recessive alleles of the locus. The O2 gene, however, does not itself encode the protein b-32, but it is apparently the gene product of the O6 locus; o6 endosperm is not only devoid of b-32 protein but almost without zeins. This indicates that the b-32 protein may play a role in zein accumulation.

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24 ISOLATION OF DNA AND DNA RECOMBINANTS FROM MAIZE

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We have found the following method useful for the preparation of maize nuclear DNA that is suitable for restriction and cloning. The general scheme of the isolation is from Bendich et al. (1979), with a number of buffers and procedures derived from Bedbrook et al. (1979). The method of tissue maceration was suggested to us by Peter Mascia. The advantages of this procedure are that it is fast, the yield is good and the DNA is clean and of high molecular weight.

Procedure	Comments
1. Surface sterilize fresh leaf tissue by soaking it in a solution of 5% bleach for 15 minutes. Rinse well with clean water and air dry.	1. Surface sterilization is very important for leaf tissue. We also use immature anthers and ears for which surface sterilization is probably unnecessary.
2. Freeze the tissue in liquid N_2 until it is very brittle.	2. Mature leaf tissue needs to be left in liquid N_2 at least 5 minutes. Etiolated seedling leaf takes less time and thicker tissues may take longer.
3. Grind the frozen tissue to a powder using a blender at high speed for 30 to 60 seconds. Keep the tissue frozen by adding a small amount of liquid N_2 to the blender jar.	3. Small amounts of tissue (2-15g) are more effectively powdered by grinding in liquid N_2 using a mortar and pestle. The tissue can be stored frozen at -20° at this stage.
4. Add a large excess (8-20 volumes) of ice cold Grinding Buffer (0.3 M sucrose -- 50mM Tris-HCl pH 8.0--5mM $MgCl_2$) to the powder. Allow it to sit on ice for 5 min.	4. We find powder from 5-100g fresh weight starting material suitable to use for a single DNA isolation.
5. Grind the swollen tissue with a polytron at the highest setting for 30 seconds. Repeat once. Keep the solution in an ice bath during this step.	5. From this step until step 9 (addition of CsCl), it is important to work quickly and keep everything cold. When processing a large amount of tissue or many different samples, we find it works best to take one or two samples from step 4 to step 9, before starting to process the next ones.

Procedure	Comments
6. Using a vacuum flask and a Buchner funnel, filter the ground tissue through 3 layers of miracloth or a 60 micron mesh. Wash through with a little Grinding Buffer.	6. If there is a large amount of residue left on the filter, resuspend it in more Grinding Buffer and repeat steps 5 and 6.
7. Pellet nuclei by centrifuging the filtrate at 350Xg for 10 minutes. Quickly pour off the supernatant and briefly drain the tube. Discard the supernatant. Keep the pellet on ice.	7. For dealing with large amounts of tissue, it is convenient to use the large swinging bucket HS-4 rotor at 1500 rpm.
8. Keeping the sample on ice, gently but rapidly resuspend the pellet in cold Lysis Buffer (20mM EDTA--50mM Tris-HCl pH 8.0 - 1% sarkosyl).	8. The pellet is rather sticky. Gently scraping the bottom of the tube with a glass rod facilitates resuspension.
9. Add 1.5 volumes of saturated CsCl (in 20mM EDTA-50mM Tris-HCl pH 8.0) or .95 g solid CsCl/ml lysate.	9. Don't try to adjust the CsCl concentration at this point.
10. To remove insoluble proteins and polysaccharides, centrifuge at 17,000XG for 15-30 minutes at 4°. Pour the supernatant away from the pellicle and pellet.	10. If the pellicle and/or pellet are large, it is useful to repeat this step. If the pellicle is loose and won't cling to the wall, it can be removed with a cotton swab or by pouring the supernatant through cheesecloth or miracloth. We use an SS34 rotor at 12,000 rpm for this step. Plastic centrifuge tubes are preferred, as glass corex tubes frequently break at this speed.
11. Add ethidium bromide to 300 μg/ml. Adjust the CsCl concentration and bring up to volume for ultracentrifugation with 50% CsCl. The refractive index should be about 1.3895. Centrifuge 15-20 hours at 40,000 rpm (approx. 100,000XG, check a nomogram).	11. In a vertical rotor, 15 hours of centrifugation is sufficient to band the DNA-EtBr. In other angle rotors (we use Ti 75 and Ti 70) the band will still be quite diffuse.
12. Remove the fluorescent orange band carefully with a wide bore pipette.	12. In preparations using mature leaves there will be a dark pigment layer at the top of the tube. This can be removed with absorbent cotton without disturbing the gradient before taking off the DNA band.
13. Optional: Reband the DNA-EtBr band in more CsCl without additional EtBr.	13. This step gives cleaner DNA (see below), but adequate results are obtained with a single CsCl gradient, especially when the vertical rotor is used.

Procedure	Comments
14. Remove the EtBr by mixing with several changes of n-butanol equilibrated with buffer. Do an additional extraction after no more pink color can be seen in the n-butanol layer.	14.-16. An alternative method for steps 14-16 is to:
15. Dialyze thoroughly against TE buffer (10 mM Tris-HCl pH 7.5 - 1mM EDTA).	Collect the DNA-EtBr band into a screw cap tube and add 4 volumes of CsCl saturated isopropanol. Mix gently but thoroughly to extract EtBr out of the aqueous layer. Remove the top (isopropanol) layer and repeat until no more pink color is seen.
16. Add 0.1 volume 3M NaAcetate and 2 volumes of ice cold ethanol to the dialysate in order to precipitate the DNA. Chill at -20°, 2 hr.	Transfer the DNA in CsCl to a 30 ml Corex tube and measure the volume. Add 2 volumes water and 3 volumes of isopropanol. Mix and chill at -20°C for at least 2 hours. Pellet in SS34 rotor, 9,000 rpm, 4°C for 10 minutes. Air dry the pellet.
17. Collect the DNA by spooling on a glass rod or by pelleting in the centrifuge. Resuspend in 1-2 mls TE.	17. It is easier to resuspend the spooled DNA than DNA that has been pelleted. Partially resuspended DNA in TE can be heated to 65° for 10-20 minutes to aid complete resuspension.
18. The DNA samples can be stored in TE in the refrigerator. Excess sample can be stored at -20°C in 1.5 ml Eppendorf tubes, wrapped with Parafilm to prevent desiccation.	18. For long term storage in the refrigerator, it is helpful to keep a drop of chloroform in the sample. Frozen samples should be stored in the refrigerator after they are thawed. Do not repeatedly freeze and thaw the DNA.

Yield and purity: Depending on the starting tissue, 5-20 μg DNA/g fresh weight tissue is isolated by this method. We generally judge the purity of our preparations by comparing the OD at 260 and 280 nm. DNA isolated through one CsCl gradient has a 260:280 ratio of 1.6-1.8. A second CsCl gradient improves this to 1.9-2.0. Nonetheless, we find a single gradient is sufficient to make DNA that is fully restrictable and clonable.

Restriction and cloning: The DNA isolated is mostly greater than 60 kb in length, as judged by agarose gel electrophoresis. It shows no inhibition of restriction endonuclease activity and no nuclease activity. Using standard techniques we have used this DNA to clone with good efficiency into pBR322, pBR325, Charon 4A and Charon 30. To make a representative DNA library from corn, 300-500 ug of DNA is needed (Slightom et al., 1980). A mature plant of moderate size has enough leaf tissue to make this much DNA, although we prefer to use younger material if more plants are available.

Contamination with mitochondrial and chloroplast DNA: Mitochondrial DNA sequences represent 0.2-0.5% of the DNA isolated by this method, irrespective of the tissue or of the particular maize line. Chloroplast DNA sequences represent from 2% to 10% of the DNA isolated by this method. Contamination with chloroplast DNA varies both with the tissue used and with genetic background.

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25 A SIMPLE METHOD FOR THE ISOLATION OF HIGH MOLECULAR WEIGHT DNA FROM INDIVIDUAL MAIZE SEEDLINGS AND TISSUES

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In order to facilitate genetic analyses of DNA sequence variation, detected as differences in restriction enzyme banding patterns, we have been using the method outlined below for isolating DNA from individual mature kernel and seedling tissues in maize. Comparisons of restriction enzyme patterns produced from the DNA of single individuals after hybridization to specific labeled probes have proven powerful diagnostic tools in clinical and population genetic studies in a variety of organisms (Kan and Dozy 1980, Ferris et al. 1981, Endow and Glover 1979, Arnheim et al. 1980, Krystal et al. 1981). While we are using this method to examine the F2 progeny of crosses between parents carrying different restriction enzyme polymorphisms of the highly-repeated ribosomal RNA genes, the yields of DNA from single total 3 day-old etiolated seedlings appear adequate for detection of differences among single copy gene sequences.

The advantages of this technique are:

(1) Rapidity--only 3-4 hours are required to prepare DNA from 20 seedlings
(2) Seedling screens save space, which is especially valuable when greenhouse facilities are limited
(3) If the tissues are chosen carefully (e.g., portions of the scutellum of a 24 hour-soaked kernel, the primary root of a 3-5 day-old etiolated seedling, or 1-2 leaves of a 10 day-old plant), the individuals need not be sacrificed, yet enough DNA can be isolated to examine repeated gene sequences.

Method for single seedling nucleic acid minipreps:

(1) Kernels are surface-sterilized in 10% chlorox, rinsed extensively in distilled water, and soaked for 24 hours in dilute fungicide solution. Kernels are then rinsed and set out to germinate on 5 layers of moistened paper towels or 3 MM filter paper, in the dark, at 26°C.

(2) Excise away endosperms from 3 day-old seedlings. After weighing, rinse root, shoot and scutellum tissues in cold sterile water. (Alternatively, portions of 24 hour-soaked scutella, roots and/or shoots from 5 day-old seedlings or leaves from 10-14 day-old plants may be used.)

(3) Grind the tissue, using a cold mortar and pestle, for 30-60 seconds, with at least 10 volumes of ice cold extraction buffer--solution (A). Filter the extract through 1 layer of miracloth in a small funnel, directly into 1.5 ml Eppendorf tubes (usually 2 tubes/seedling).

(4) Rapidly spin out the nuclear pellet at low speeds (1-2 minutes at about 350 X g) in a table top centrifuge or a speed-vac centrifuge, keeping the tubes as cold as possible during the spin.

(5) Using a cut-off 200 μl Pipetman tip, resuspend each nuclear pellet in 100 μl of nuclei lysis buffer--solution (B). The pellet should turn viscous as it lyses. Combine the lysates into 1 tube (200 μl total).

(6) Add 5 μl of a Proteinase K solution (10 mg/ml stock). Mix well, by gently inverting the tubes. Incubate the lysates in a water bath at 50°C for 40-60 minutes.

(7) Remove the tubes from the water bath and extract with an equal volume (about 200 μl) of phenol saturated with solution (C). Mix well by inverting the tubes, taking care not to shear the DNA. Separate the aqueous and phenol layers by spinning at 4-12°C in an Eppendorf microfuge for 2 minutes.

(8) Remove the upper aqueous layer with a cut-off Pipetman tip and transfer it to a fresh tube. Extract this solution with an equal volume (150-200 μl) of chloroform. Separate the aqueous and organic layers in the Eppendorf microfuge as in step 7.

(9) Again remove the aqueous layer to a fresh tube and repeat the chloroform extraction and centrifugation steps.

(10) Transfer the aqueous layer to a fresh Eppendorf tube; add 1 volume 4 M NH4OAc (100-200 μl) and mix well. Add 2-3 volumes of ice cold EtOH (600-1000 μl). Gently invert the tubes and observe whether there is a nucleic acid precipitate.

(11) Store the EtOH-precipitated samples at -20°C for at least 2 hours or immerse them in a dry-ice EtOH bath for 10-20 minutes. Spin out the pellets in the microfuge for 2-5 minutes. Wash the pellets 1-2 times with ice cold 70% EtOH. Remove the supernatant and air dry the pellets until the EtOH is gone, but the pellet is still moist (15-30 minutes).

(12) Resuspend the DNA in 200-400 μl of solution (C). Be careful to get the DNA fully resuspended in solution (C), using a 5-10 minute incubation at 65°C, if necessary. Resuspension can be further speeded up by drawing the liquid up and down, using a Pipetman with a cut-off, 200 μl capacity tip.

(13) At this point, the DNA can be run on an agarose gel and the concentration determined by comparison to phage DNA concentration standards. Restriction digests of the DNA can then be made, using standard assay conditions.

(14) After restriction, RNA can be removed from the samples by adding 1-2 μl of a boiled RNase solution (1 mg/ml RNase A in 10 mM TrisHCl, pH 7.5, 1 mM EDTA) to the restriction enzyme digest and incubating the samples for 15-60 minutes at 37°C, prior to loading the gel. Alternatively, the RNase treatment of the sample can be carried out prior to the Proteinase K digestion (step 6).

Solutions

Solution (A)--Extraction Buffer (Riven *et al*. 1982)
0.3 M sucrose; 50 mM Tris-HCl, pH 8.8; 5 mM $MgCl_2$ (optional: add DEPC to a final concentration of 0.1%)

Solution (B)--Nuclei Lysis Buffer (Riven *et al*. 1982)
50 mM Tris-HCl, pH 8.0; 20 mM EDTA; 1% Sarkosyl

Solution (C)--DNA Storage Buffer = 1 X TEN
10 mM Tris-HCl, pH 7.5; 1 mM EDTA; 10 mM NaCl

Yields

The yields of DNA are variable and highly dependent on the genetic background of the seedlings. The range of yields that we observed are listed below:

Material	Yields of DNA
3 day-old seedlings (fresh weight of 0.1-0.3 grams)	4-12 micrograms
shoot (3-5 cm)	2-4 micrograms
primary root (8-10 cm)	1-2 micrograms
24 hr-soaked scutellum--whole (approx. 40 mg fresh weight)	2-3 micrograms
24 hr-soaked scutellum--excised around embryonic axis so that kernel can be subsequently germinated (20 mg fresh weight)	1-2 micrograms

Optical density measurements of the DNA obtained with this procedure give A260:A280 ratios ranging from 1.7 - 1.92.

Additional Comments

We have been able to digest DNAs prepared using this method with a variety of restriction endonucleases: *EcoRI*, *BamHI*, *HindIII*, *BglII*, *SstI* and *XbaI*. DNA digested with *EcoRI*, for example, was separated by electrophoresis in agarose gels and transferred to nitrocellulose filters according to the method of Southern. Hybridization of the DNA on these filters, with ^{32}P-labeled ribosomal gene-specific probe from soybean, gives patterns comparable to those seen when a large scale plant DNA isolation procedure (Riven *et al*. 1982) is used.

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26 MAIZE RNA POLYMERASES AND *IN VITRO* TRANSCRIPTION

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Each genome-containing compartment in a maize cell contains one or more unique DNA-dependent RNA polymerases (nucleosidetriphosphate: RNA nucleotidyl-transferase, EC 2.7.7.6). Since multimeric enzymes are responsible for the synthesis of RNA from the DNA template, study of RNA polymerases may reveal mechanisms central to the regulation and coordination of eukaryotic gene expression.

For several reasons, maize is an excellent experimental plant system for examining RNA polymerases' role in the control and integration of transcription during development and for studying the intracellular interaction of nuclear and plastid genomes. First, a large variety of inbred lines and hybrids that have been extensively studied, are readily available from industrial and foundation maize breeding programs. Second, kilograms of maize tissues at various stages of seedling development can be obtained from greenhouse-grown material. Also, a number of cell types from several differentiated states and with different parental genetic information (e.g., diploid pericarp, diploid embryo, triploid endosperm) can be obtained by gross dissection or from cereal processors. Third, the protein and RNA synthetic machinery from rapidly-growing or greening maize tissues is highly responsive to environmental stimuli, such as light and nutrients.

Here we briefly review the literature pertinent to maize RNA polymerases and their role in transcription.

CHLOROPLAST RNA POLYMERASE

Purification and Characterization of the Maize Chloroplast RNA Polymerase

Kirk (1964) showed that purified broad-bean chloroplasts could form polyribonucleotides. In 1967, an RNA polymerase activity tightly associated with maize chloroplasts was described (Bogorad, 1967), and this activity was shown to increase very soon after leaves of etiolated plants were illuminated. Early attempts to solubilize the enzyme from the membrane were unsuccessful because polymerase activity (and almost all of the chloroplast DNA) remained associated with the chloroplast membrane fraction.

Solubilization of maize chloroplast RNA polymerase was first achieved in 1971 (Bottomley *et al.*, 1971). Critical to solubilization were low magnesium concentration (decreased by addition of EDTA) and higher temperature. Purification of the chloroplast enzyme was accomplished by using standard chromatographic methods. The enzymological properties of this maize RNA polymerase are listed in Table I.

Smith and Bogorad (1974) reported that the chloroplast enzyme contains at least two polypeptides of molecular weight 180,000 and 140,000. Polypeptides of 100,000, 95,000, 85,000, and 40,000 daltons were associated with

enzymatic activity in relatively constant ratios, but each polypeptide was removed by at least one specific purification step. No polypeptides below 40,000 daltons were detected. When maize chloroplast and type II nuclear RNA polymerases were mixed and electrophoresed on a SDS polyacrylamide gel, the 180,000 dalton subunits of the two enzymes were not resolved (Hardin et al., 1975). Despite their similarity in molecular mass, both one- and two-dimensional peptide maps revealed that these 180,000 dalton subunits were not the same (Kidd and Bogorad, 1979). Comparisons of tryptic peptide maps, generated from other maize nuclear and chloroplast subunits of similar size, indicate that each polypeptide is unique.

TABLE 1. PROPERTIES OF NUCLEAR, CHLOROPLAST, AND MITOCHONDRIAL RNA POLYMERASES OF MAIZE

Property	Nuclear I	Nuclear II	Nuclear III[a]	Chloroplast	Mitochondria[b]
Intracellular Location	Nucleolus	Nucleoplasm	Nucleoplasm	Chloroplasts, Cytoplasm	Mitochondria
Product	rRNA	HnRNA, mRNA	RNA, 5S RNA	Chloroplast RNAs	
Elution from DEAE-cellulose ammonium sulfate (M)	0.1	0.2		0.2	0.2
Relative inhibition with alpha-amanitin	None	Highly sensitive		None	None
Salt Optima					
Magnesium (mM)	25	10		15-40	
Manganese (mM)	8	2		8	
Magnesium/Manganese ratio at optimum	2.5	1		5	
Ammonium Sulfate (mM)		100		0	
Temperature Optima (°C)	44	37-44		48	

a) Too little research has yet been done on maize nuclear RNA polymerase III to warrant discussion of its properties.

b) Only preliminary evidence is available currently.

Recently, Kidd and Bogorad (1980) described a facile procedure for purifying maize chloroplast RNA polymerase from whole cell homogenates. The yield of this soluble RNA polymerase (average 0.5 mg/kg leaves) was 50 percent better than that of RNA polymerase prepared from isolated chloroplasts. Also, this soluble chloroplast enzyme was stable for long periods and lacked detectable nuclease activity.

Effects of Light on Maize Chloroplast RNA Polymerase and Transcription

When etiolated seedlings are transferred into light, a temporal sequence of complex changes ensues. In this greening process, etioplasts develop into mature, functional chloroplasts (Bogorad, 1967). Following illumination, the rate of RNA synthesis and the activity of RNA polymerase in dark grown maize seedlings increase dramatically. Most of the radio-labeled ribonucleotides fed to etiolated maize seedlings in the dark were incorporated into etioplast RNA. This incorporation was greatly and preferentially enhanced during the first two hours of illumination, and the synthesis persisted after the leaves were returned to the dark (Harel and Bogorad, 1973).

RNA polymerase activity in isolated plastids increases about four-fold after etiolated plants have been illuminated for 16 hours. However, this enhanced activity was not paralleled by a comparable increase in the amount of enzyme protein. Also, no differences in polypeptide composition were observed when denatured electrophoretic patterns of the purified chloroplast enzymes from etiolated and 16-hour illuminated plants were compared (Apel and Bogorad, 1976). So, the plastid RNA polymerase may be subject to greater modulation in activity than in actual enzyme concentration during greening. A vast amount of research on correlation between polymerase activity in greening plastids and altered synthesis of specific RNAs remains to be done.

In Vitro Transcription of Maize Chloroplast DNA by Maize Chloroplast RNA Polymerase

Along with the physical characterization and fragmentation of the maize chloroplast genome (Kolodner and Tewari, 1975; Bedbrook and Bogorad, 1976), many have imagined homologous *in vitro* transcription systems. Jolly and Bogorad (1980) have recently provided a key ingredient by using chimeric bacterial plasmids, containing the chloroplast ribosomal cistrons (pZmc134, Bedbrook *et al.*, 1977), to construct an *in vitro* transcription system with homologous RNA polymerase. During development of the transcription system, Jolly and Bogorad (1980) discovered a 27,500 dalton polypeptide from maize plastids (called S Factor) that accelerates transcription of circular plasmid DNA by maize chloroplast RNA polymerase and that promotes this enzyme's preferential transcription *in vitro* of maize chloroplast DNA (both ribosomal and RuBP carboxylase sequences) located in the bacterial plasmid. The selectivity for chloroplast genes was strongly dependent upon the template being supercoiled. Even though several questions remain to be determined, such as the optimal stoichiometry between the S factor and RNA polymerase and the exact initiation sites on the chimeric plasmids, this homologous transcription system is a fascinating first step. Nucleotide sequences (e.g., Koch *et al.*, 1981) of cloned maize chloroplast DNA fragments will reveal subtleties of this genome as a template.

One can envision that similar homologous *in vitro* transcription systems from maize plastids could be used to analyze developmental programs for which transcriptionally regulated genes have been cloned. Several examples of transcriptional regulation *in vivo* are known. For example, the large subunit of the chloroplast enzyme, RuBP carboxylase, is found almost

entirely in the bundle sheath cells, but the structural gene coding for this polypeptide is contained in the chloroplast DNA of both mesophyll and bundle sheath cells. RNA complementary to this coding sequence and translatable mRNA for this polypeptide have been detected in RNA from bundle sheath cells but not from mesophyll cells (Coen et al., 1977; Link et al., 1978; Lind and Bogorad, 1980). In addition, expression of the chloroplast chromosome's photogene 32 is transcriptionally controlled during photoregulated development (Bedbrook et al., 1978; Grebanier et al., 1979).

NUCLEAR RNA POLYMERASES

Purification and Characterization of the Maize Enzymes

The first report of the isolation of maize RNA polymerases appeared in the mid 1960s (Mans and Novelli, 1964; Stout and Mans, 1967). This was followed by the partial purification of nuclear RNA polymerase II (responsible for mRNA synthesis) (Strain et al., 1971) and documentation of that enzyme's catalytic properties (Table I). Nuclear enzyme II can be resolved into two forms by DEAE-cellulose chromatography or by glycerol gradient centrifugation. One enzyme form prefers a less denatured template than the other enzyme form. The difference in template preferences between the two enzyme forms may be due to a factor that enables transcription of native DNA, to a nuclease that co-chromatographs with one of the enzyme forms, or to artifacts produced when polymerases are assayed with native DNA (Dynan et al., 1977).

Purification of the alpha-amanitin sensitive, nuclear RNA polymerase II has been achieved by combinations of salt precipitation, ion exchange and affinity chromatography, and molecular sizing chromatography or gradients. Polypeptides associated with highly purified maize RNA polymerase II preparations have molecular weights and molar ratios of 180,000 (1); 160,000 (1); 40,000-43,000 (1); 26,000-28,000 (2); 22,000 (1); 20,000 (1); 17,800 (1); 17,500 (1); 16,300 (1); 16,100 (1); and 14,000 (variable stochiometry) (Mullinix et al., 1973; Jendrisak and Guilfoyle, 1978; Kidd and Bogorad, 1980).

Clear similarities exist among maize RNA polymerase II and the same enzyme from dicotyledonous and other monocotyledonous plants. The subunit structures of the various plant type II nuclear enzymes are virtually identical in the number, molecular weights, charge densities, and molar ratios of the polypeptides associated with each of the enzymes. Antibodies against hexaploid wheat RNA polymerase II react with the same enzymes purified from several dicotyledonous plants, as well as with other monocotyledonous species, but do not crossreact with yeast nuclear RNA polymerase II or *Escherichia coli* RNA polymerase (Jendrisak and Guilfoyle, 1978). However, despite antigenic similarities among type II RNA polymerases, two-dimensional tryptic maps comparing the large subunits of the same size from maize, parsley, and wheat type II enzymes revealed that each large polypeptide is unique to each individual plant species (Kidd et al., 1979).

The bulk of the research on maize nuclear RNA polymerases has been focused on the type II enzyme. However, maize type I RNA polymerase, which synthesizes rRNA (Table I), has been partially purified and characterized (Strain et al., 1971; Lee et al., 1977). The extreme lability and low activity of the maize type I RNA polymerase has hindered elucidation of its

structure and transcriptional specificity. Type III nuclear RNA polymerase has not yet been isolated from maize tissues.

Over the past several years, reports on the abundance, excellent activity, and ease of purification of nuclear RNA polymerases from wheat germ (Jendrisak and Burgess, 1975, 1977; Jendrisak and Guilfoyle, 1978) have stimulated interest in cereal polymerases. In a similar vein, corn germ may be a rich source of RNA polymerases and the germination of this embryonic tissue could provide for the study of transcription during plant development. Preliminary experiments with wet-milled corn germ point to the presence of active homopolymer polymerases (Kidd, unpublished results).

In Vitro Transcription of Maize Nuclear DNA by Maize Nuclear RNA Polymerase

Even though the template requirements for maize nuclear RNA polymerase II have been described using heterologous DNAs (Gardner *et al.*, 1976), the development of homologous templates and specificity assays has just commenced. For instance, cloned complementary DNA copies of mRNA representing several of the zein mRNA families are now available, and these DNAs are being used as probes to locate and quantify zein sequences in the maize genome (Pedersen, *et al.*, 1980; Hagen and Rubenstein, 1981). Additional work will be required to define assay conditions for transcriptional specificity.

Study of transcription in maize may be further enhanced by the discovery of a low molecular weight inhibitor of maize nuclear RNA polymerase II (Arens and Stout, 1974). The inhibitor affects the polymerization reaction and acts at the level of chain elongation to prevent nucleotide incorporation in a manner similar to *alpha*-amanitin.

MITOCHONDRIAL RNA POLYMERASE

An RNA polymerase has been isolated from maize mitochondria (Maheshwari *et al.*, 1975). This RNA polymerase was solubilized from isolated mitochondria which had been purified by treatment with deoxyribonuclease and by density gradient centrifugation. Solubilized mitochondrial RNA polymerase was further purified by DEAE-cellulose chromatography and by glycerol-gradient centrifugation. This purified RNA polymerase was completely inhibited by rifampicin, was resistant to *alpha*-amanitin, and was composed of polypeptides of about 60,000-70,000 daltons. This preliminary evidence should lead the way to further study.

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27 CLONING OF MAIZE ZEIN GENES

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For the past several years we have been studying the genetic and biochemical mechanisms regulating the synthesis of the maize storage proteins collectively known as zein proteins. Several comprehensive reviews have been published that describe the properties of these proteins as well as their biosynthesis (Wall and Paulis, 1978; Larkins 1981). Although our primary purpose here is to describe the cloning of the zein genes, it is useful to briefly review some of the significant properties of the proteins and their mechanism of synthesis.

The zein proteins were originally classified as prolamines because, like the storage proteins of many cereals, they contained a high content of proline and glutamine, and were soluble in aqueous alcohol solutions such as 70% ethanol or 55% isopropanol (Larkins, 1981). Amino acid analyses of the zein fraction revealed an especially high content of glutamine (30%), leucine (20%), alanine (10%), and proline (10%), with only trace amounts of lysine (Mosse, 1966). It is their low lysine content that causes maize kernels to be an inferior source of protein for nutrition of monogastric animals (Nelson, 1969).

Separation of zein proteins on SDS polyacrylamide gels reveals several groups of proteins differing in molecular weights (MW) (Larkins, et al., 1980). There are slight differences in the MW which have been assigned to the proteins by various investigators (Lee et al., 1976; Gianazza et al., 1977), but we classified them as MW = 22,000, 19,000, 15,000, and 10,000 (Larkins et al., 1980). The different MW groups contain several polypeptides with different charges as shown by 2-dimensional analysis on O'Farrel gels (1975). The heterogeneity within the MW groups has also been demonstrated by NH2-terminal sequence analysis (Larkins et al., 1979), and it has been shown to have a genetic basis (Righetti, et al., 1977; see also the article by Salamini and Soave in this publication). Altogether the studies on the structural characteristics of these proteins have demonstrated that the zein fraction consists of a family of related polypeptides having some sequence homology.

In normal maize genotypes the zein proteins are synthesized between 10 and 50 days after pollination depending on the temperature and growing conditions, and they account for 50-60 percent of the total endosperm protein. The proteins are synthesized by polyribosomes bound to the endoplasmic reticulum (ER) (Larkins, 1981). The mRNAs directing the synthesis of these proteins code for a "signal peptide" which is presumably responsible for directing their transport into the lumen of the ER. Inside the ER, the "signal peptide" is removed and the proteins associate with one another to form dense masses called protein bodies (Larkins, 1981; Hurkman et al., 1981).

Construction of Zein DNA Clones

Since the membrane-bound polyribosomes are particularly enriched with zein mRNAs, we and others (Burr and Burr, 1980) have used them as a source of mRNA for synthesizing zein cDNA clones. However, these mRNAs are so abundant in endosperm tissue that clones corresponding to these sequences can be readily screened from a cDNA library of total endosperm mRNA (Weinand et al., 1979).

In order to obtain large cDNA inserts, it was necessary to use a strain of E. coli carrying the rec A-mutation (e.g., HB101). Transformations using strains such as C600 or SF8 resulted in clones containing very small cDNA inserts. The small size of these inserts appears to result from recombination events which remove homologous or repeated sequences within the zein cDNAs. Whether or not this is the correct explanation, we did find repeated nucleotide sequences in several of the clones we examined by DNA sequencing.

The zein cDNA clones have been initially identified by mRNA hybrid-selected translation (Wienand et al., 1979; Park et al., 1980). By this method the recombinant plasmid is hybridized to excess mRNA, and after removing unbound mRNA, that which is specifically retained is eluted at an increasingly stringent criterion. SDS gel analysis of the in vitro translation products of this mRNA allows classification of the cDNA clone with respect to the appropriate protein MW group. Park et al., (1980) were also able to show charge heterogeneity among the clone selected translation products. However, since the isoelectric points of the in vitro translation products differ from the native proteins, specific correlation of the isoelectric focusing (IEF) forms of the native polypeptides cannot be made.

We have sequenced several of these cDNA clones using the method of Maxam and Gilbert (1977), and several clones have also been sequenced in Dr. Irwin Rubinstein's laboratory (personal communication). These analyses have revealed some expected and some unexpected structural features of the proteins and mRNAs (Pedersen et al., in preparation). Of the clones we have sequenced, the first methionine in phase for translation was 21 amino acids, 5' of the NH2-terminus of the protein. Signal peptides of 21 amino acids are relatively common (Habener et al., 1978), and a peptide of this length would correspond with the difference in MW of the native proteins and the in vitro translation products (Larkins and Hurkman, 1978).

A clone corresponding to the 19,000 MW group of zein proteins was found to contain 214 amino acids, while a clone corresponding to the 22,000 MW class contained 244 amino acids (these values do not include the signal peptide). These proteins would have MW equivalent to 23,540 and 26,840, respectively, which is larger than any of the estimations predicted by SDS polyacrylamide gel electrophoresis. The underestimation of polypeptide sizes probably results from incomplete denaturation of the proteins by SDS.

Each of these clones contained short amino acid sequence repeats, although the length of the repeats varied. There was not extensive sequence homology between the repeated sequences, but more extensive analyses of their sequence relationships remains to be done. It is quite possible that the instability of the clones in rec A+ bacteria is due to these sequence repeats.

Isolation of Zein Genes from Nuclear DNA

The isolation and partial structural characterization of zein genomic clones has been reported by several laboratories (Lewis *et al.*, 1981; Wienand *et al.*, 1981; Pedersen *et al.*, 1981). These genes were identified among clones of maize nuclear DNA constructed in lambda phage. Methods for the construction of the recombinant phage have been simplified (Hohn, 1979), and improved methods for isolating DNA from plants (Bendich *et al.*, 1980) have contributed to the efficiency of constructing genomic clones.

Zein genes have been identified by screening lambda libraries with zein cDNa clones as probes. Alternatively, we screened an Eco RI library with a mixture of zein cDNAs to obtain a mixed population of zein genomic clones (Pedersen *et al.*, 1981). After the initial Benton-Davis plaque hybridization (Benton and Davis, 1977), individual phage were purified by repeated cDNA hybridizations. Our clones were finally screened by hybridizing at a low stringency with specific cDNA clones.

The purified genomic clones have been characterized by one of several techniques including: (1) mRNA hybrid selection to identify the corresponding protein, (2) restriction enzyme digestion and Southern hybridization (Southern, 1975) as well as R-loop analysis to localize the coding region, (3) DNA sequence analysis to define the coding and potential regulatory sequences of the gene.

Based upon current data zein genes do not contain intervening sequences. Heteroduplex analysis of several of our clones revealed only a single R-loop, and Wienand *et al.* (1981) also found a single R-loop in one of their genomic clones. This result suggests the absence of intervening sequences in the gene, and we have confirmed this through DNA sequence analysis of one of the genomic clones (Pedersen *et al.*, 1981). While it may be premature to generalize the structure of all zein genes, thus far they do not appear to have interrupted coding sequences.

We were able to deduce the structure of the 5' non-coding portion of the gene by comparing it with a cDNA clone having a long 5' sequence (Pederson *et al.*, 1981). Although the first base in this cDNA clone may not correspond to the first base transcribed into mRNA, we predict that transcription begins very near this base. Based on this assumption, we identified a TATAAATAT (the "Goldberg-Hogness box") sequence 33 bases, 5' of the first nucleotide in the cDNA clone, which is very close to the position of this promotor sequence in other eukaryotic genes. We identified an AATAAA polyadenylation sequence at a site 24 bases from the end of the amber sequence terminating translation. Whether these are indeed the correct sequences regulating gene expression remains to be determined, but additional experiments should help answer these questions. Based simply upon the DNA sequence analysis, zein genes appear to be similar to a number of other eukaryotic genes except perhaps that they lack intervening sequences.

Reiteration of Zein Genes in the Maize Genome

While there is good agreement among the results of different groups studying zein gene structure, there is a substantial discrepancy in the estimates of the number of copies or reiteration frequency of the genes in

the genome. Using a mixed population of zein cDNAs as a probe and hybridizing to sheared nuclear DNA from the inbred W64A, we found that cDNAs hybridized at a rate two times that of the single copy DNA (Pedersen et al., 1980). When the hybridization was done at a lower stringency (58° vs. 68°), or with zein cDNAs synthesized using short fragments of calf thymus DNA as primers (random primed cDNAs), we calculated a hybridization rate that was between two and four times the single copy rate. By doing a similar experiment with mixed zein cDNAs as a probe and also using nuclear DNA from the inbred W64A, Viotti et al., (1979) determined a hybridization rate that was eight to ten times the single copy rate.

The twofold difference in these rate determinations is probably not significant; however, since Viotti and coworkers concluded that there was no cross-hybridization between the mRNAs and cDNAs, they accounted for 15 different mRNA species. As a result, they calculated a much larger number of total genes in the genome than we did (i.e., 15 mRNAs x 10 gene copies = 150 genes cf. 4 mRNAs x 2-5 gene copies = 8-20 genes). Although the numbers of genes calculated from these two measurements are substantially different, it results primarily from differences in interpretation.

Both of these estimates of the reiteration frequency are significantly less than those obtained by Southern blot hybridization analysis. Wienand and Feix (1980) and Hagen and Rubenstein (1981) found multiple restriction fragments hybridizing to zein cDNA clones. Although Wienand and Feix did not present a gene reconstruction analysis, they concluded that the hybridization intensity of certain bands represented single gene copies while other bands represented multiple gene copies. The reconstruction analyses of Hagen and Rubenstein using DNA from Illinois High Protein indicated the presence of between 50-70 genes homologous to a single cDNA clone, with different clones hybridizing to different groups of restriction fragments. These results suggest that the genes are much more highly reiterated in the genome than indicated by the solution hybridization results.

It is not yet clear what is responsible for the variation in these data. They may result from the different genetic backgrounds of the DNAs, the construction and nature of the cDNA clones used as probes, or the inherent different stringencies in the methods of hybridization. Additional experiments will be necessary to resolve the discrepencies. It is clear, nevertheless, that the zein proteins are coded by a family of related genes.

Much remains to be learned regarding the regulation of expression of maize storage protein genes. It will be particularly interesting to analyze the mechanisms by which mutants such as opaque-2 alter the expression of the zein genes. The availability of specific cloned zein gene sequences should further these studies.

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28 ZEIN GENOMIC CLONES FROM MAIZE

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It has tended to be rather typical of the plant sciences that developments that have occurred in other fields have been slow to be applied to plants. In this respect the application of molecular cloning techniques is no exception. Only within the last year or two have reports appeared, firstly of the preparation of cDNA clones (Hall et al., 1980; Brandt, 1979; Wienand et al., 1979) and subsequently, of genomic clones for a variety of genes from a range of plants. The earliest genomic clones to be described were for leghaemoglobin from soybean (Sullivan et al., 1981) and for the phaseolin storage protein of french bean (Sun et al., 1981). To date only two other types of genomic clones have been prepared from plants and these are both for maize genes; sucrose synthetase (Burr, personal communication) and the zein proteins of maize seed (Wienand et al., 1981; Lewis et al., 1981; Pedersen et al., 1981). The cloning of sucrose synthetase was motivated by the possibility made available with such clones to isolate and study the curious maize controlling elements (Fincham and Sastry, 1974). We and several other laboratories have prepared clones of the zein genes in order to investigate the mechanism of storage protein synthesis and accumulation.

The study of the storage protein genes is particularly interesting since these genes are expressed at very high levels at a specific developmental stage and only in one tissue, the endosperm (Spencer and Higgins, 1979). The level and specifity of expression has always implied the existence of a specialized control mechanism. These points of interest apply to most plant seed storage proteins. However, for several reasons the maize storage proteins are particularly suitable for study. The main storage proteins of maize seed are the alcohol soluble zeins which are comprised of only two major polypeptide size classes (19,000 and 21,000 daltons) (Burr and Burr, 1976) as compared to the complex composition of the storage proteins of peas, beans, barley, wheat, etc. Within the two zein size classes are a number of isoelectric variants but these appear to differ from each other by only one or two amino acids (Gianazza et al., 1976 and 1977).

There is also an extensive background of maize genetics and the existence of several mutants known to alter the levels of expression of the zein genes (Lee et al., 1976; Tsai et al., 1978). Particularly important are the opaque-2 mutation, which reduces the zein content of the seed from 60 percent in normal seeds to 30 percent of the total seed protein (the effect is strongest on the 21,000 dalton polypeptides), and the floury-2 mutation which reduces the zein content to about 40 percent (Lee et al., 1976). The mutants are important in the search for maize endosperm that contains an amino acid composition more suited to human nutritional needs than the normal endosperm (Alexander and Creech, 1977).

In order to investigate the organization and expression of the zein genes, it is important to isolate genomic clones that contain these genes and their flanking sequences where control sites may be located. The first stage in such a project is the preparation of cDNA clones from messenger RNA of

endosperm where the genes are strongly expressed (Larkins et al., 1978; Wienand et al., 1978). Using techniques that are now standard, two non-hybridizing classes of cDNA clones were prepared that correspond to the two major protein size classes of zein (Wienand et al., 1979). Such clones provided the first direct evidence that the two classes of zein protein are coded by distinct non-homologous messenger RNAs. With the availability of cDNA clones, it has also become feasible to analyze the general organization of the zein genes by hybridization to restriction endonuclease digested and electrophoretically fractionated maize DNA (the Southern technique; Southern, 1975). These analyses have demonstrated that the zein genes comprise a multigene family since a large number of different sized maize DNA fragments will hybridize to the cDNA probes. It was also found that the two types of cDNA probes gave patterns with many differences, implying further complexities in the organization of the genes (Wienand and Feix, 1980).

The comparison of the Southern patterns of DNA prepared from maize seedlings with those patterns with DNA prepared from endosperm has suggested that major structural differences exist that may be correlated with the transition from silent to highly active genes. Furthermore, we have found that specific base modifications, present on both sides of the zein genes in seedling DNA, are no longer detectable in the endosperm DNA (Langridge et al., in preparation). Both observations may be related to the regulation of zein gene expression.

Genomic clones were prepared by cloning total EcoRI restricted maize seedling DNA into λgt WES and screening the resultant plaques with radioactively labelled cDNA clones (Wienand et al., 1981). The first screen of 40,000 plaques yielded a single clone that hybridized specifically to the cDNA for the 19,000 dalton polypeptide. The gene was further identified and localized by the electronmicroscopic analysis of R-loops formed between the clone and polyA RNA from maize endosperm known to contain high concentrations of the zein mRNAs. Subsequently, four clones were isolated that contain the gene for the 21,000 dalton polypeptide, this time after screening 400,000 plaques. All the clones contain the zein gene in the center with flanking sequences of varying sizes.

Only the clone for the 19,000 dalton zein protein has so far been analyzed by electron microscopy. The gene is 800 ± 50 bases long to which is added a poly-A tail of 150 bases to produce a messenger RNA approximately 950 bases long. No intervening sequences were seen in the electron microscope when the R-loops were examined. Although the microscopic technique would not detect introns of smaller than 50 nucleotides, it has been possible to eliminate even small introns since the hybrids between the genomic clones and the zein mRNA were found to be resistant to digestion by mung bean nuclease. This single strand specific nuclease will cleave at mismatches of a single nucleotide (Kowalski et al., 1976). Therefore, the resistance of the hybrids to digestion implies not only the absence of any intervening sequences but also that the clones represent actively transcribed genes.

The genomic clones have not yet been sequenced. However, from restriction endonuclease mapping, it appears that extensive differences exist between clones, in the sequences flanking the zein genes. This is not a surprising result since the zein genes are present in many copies. It also

seems that the zein genes are very rich in A + T since R-loops form preferentially at low temperatures (42°C for zein as compared to 57°C for the immunoglobulin and interferon genes under identical buffer conditions, Brack, 1981). In addition, there are remarkably few restriction sites within the cloned sequences, particularly within the zein gene, and this also implies an unusual base composition.

The genomic clones have been used for Southern analysis of restricted maize DNA both as intact probes and separated into the zein gene and flanking regions. The patterns obtained were all similar to those seen using the corresponding cDNA clone as probe. This indicates that the sequences around the zein genes do not contain highly repeated DNA.

A major drawback with the clones we have isolated is their small size. We are not able yet to determine whether or not the zein genes are arranged in the chromosome proximal to each other or in widely scattered positions. It is also of obvious interest to know if the sequences flanking the genes exert an influence on zein expression or carry some completely unrelated function. The situation is complicated by the multigenic nature of zein system and the absence of any clear data on the exact number of zein genes present since estimates from reassociation experiments vary from 5 to 120 copies per haploid genome (Pedersen et al., 1980; Viotti et al., 1979). Genetic and in situ hybridization experiments have suggested at least three principle chromosomal locations of the zein genes (Viotti et al., 1980). This number is, however, only tentative and it does not appear that these techniques will be able to provide a more refined figure (however, see the report of Salamini and Soave in this publication). Progress is more likely to occur with the study of more genomic clones, particularly larger clones, and also with the application of "chromosome walking" techniques. With these methods it may be possible to move further into the regions surrounding the zein genes in search of adjacent genes and functions.

Initial problems encountered with the preparation of plant DNAs for cloning experiments no longer appear to exist as quite crude preparations of maize DNA have yielded clones. There is, however, some indication that the structure of large DNA fragments confers instability on many clones. We and other groups have encountered problems, with the cloning and isolation of clones larger than 5 kilobase pairs, that would seem to be associated with reduced stability of these clones.

Indications at present suggest that within a short period several new cDNA and genomic clones for maize seed proteins will become available. It may, therefore, be possible in the future to compare the organization and regulation of several different maize seed protein genes.

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29 THE ZEIN MULTIGENE FAMILY

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I. Introduction

Multigene families were defined by Hood et al. (1975) to consist of "a group of nucleotide sequences or genes that exhibits four properties--multiplicity, close linkage, sequence homology, and related or overlapping phenotypic functions. In addition to these features, multigene families share a novel evolutionary characteristic: natural selection appears to operate on these gene families as a whole, and not upon the individual gene members." The zein genes fulfill the characteristics of multiplicity, sequence homology, and overlapping functions (Park et al., 1980; Hagen and Rubenstein, 1981). The genes appear to be clustered and have been mapped to maize chromosomes 4 and 7 (Soave et al., 1978; Valentini et al., 1979; Soave et al., 1981).

Our study of this multigene family is centered on understanding the interrelationships among its members at the protein, mRNA, and the genomic levels of organization and on the detailed nucleotide sequences of zein cDNAs and genes. From these sequences we can deduce the amino acid sequences of zein proteins.

We have found that the zein multigene family can be subdivided into sub-families (in our previous publications we have used the word "family" to describe a subgrouping of the total zein mRNA fraction. This causes confusion; therefore, I will use the word family to refer to the entire zein mRNA population and the word sub-family to refer to the various subdivisions that can be experimentally obtained) of mRNAs and their corresponding genes on the basis of the relatedness of their nucleotide sequences. The nucleotide sequences of the zein cDNAs and genes have allowed us to suggest some general principles about the structure of zein proteins.

II. The Zein Proteins

Large amounts of the zein proteins are synthesized in the endosperm of the developing corn kernel (Larkins and Dalby, 1975; Burr and Burr, 1976; Burr and Burr, 1979) and stored in the protein bodies (Duvick, 1961). The complexity of the zeins was obscured for a long time by the apparent homogeneity of their molecular weights; two prominent relative molecular weight classes of about 19 and 22 kilodaltons (kd) (Misra, et al., 1975) and two minor molecular classes of about 10 and 15 kd are seen in SDS-polyacrylamide gel electrophoresis (Wall and Paulis, 1978). The work of Righetti, et al. (1977), however, indicated that a much more complex pattern could be discerned if the mixture was analyzed by isoelectric focusing (IEF) gel electrophoresis. This complexity is also apparent in 2D gels (Hagen and Rubenstein, 1980). The work of a number of laboratories now indicates that the zein proteins represent about 100 products of a complex multigene family. (Viotti et al., 1979; Wienand and Feix, 1980; Hagen and Rubenstein, 1981). The data of Pederson et al., 1980 presents a lower estimate of 8-20 genes.

III. The Zein mRNAs

We investigated the interrelatedness of the zein mRNA population by hybridization-release translation using cDNA clones prepared from zein mRNA isolated from the developing endosperm of Illinois High Protein corn (Burr, et al., 1978; Park, et al., 1980). Three cDNA clones, A20, A30, and B49, were chosen from a collection of cDNAs generated by Ben and Frances Burr, Tom St. John, and Ron Davis at Stanford University (Burr et al. 1982). The selection was based on the uniqueness of their restriction enzyme maps and the high percentage of zein mRNA to which each of these clones could hybridize. Since zein mRNAs are approximately the same size (1100 nucleotides; Burr et al., 1978) it is not possible to cleanly subfraction the mRNA population by ordinary physical means (Wienand and Feix, 1978). Instead, we used the cDNA clones for hybridization-selection (Noyes and Stark, 1975). We observe that each of these cDNA clones hybridizes to a sub-group of mRNAs that when translated in vitro result in a group of polypeptides that produce a unique pattern in an isoelectric focusing gel (Park et al., 1980). The A20 clone hybridize-selects mRNAs that correspond to the 19 kd zeins; the A30 clone selects RNAs that correspond to the 19 kd zeins but also selects some mRNAs that correspond to the 22 kd zein; and the B49 clone selects mostly mRNAs that correspond to the 22 kd zeins but also some mRNAs that correspond to the 19 kd zeins. As judged by the protein bands obtained by their translation, these three clones are collectively able to bind most of the major classes of zein mRNAs. In addition, we find that the mRNAs that bind to the A30 cDNA clone are closely related in nucleotide sequence. Under our experimental conditions they "melt off" in a 3-5°C range indicating that they are some 95-97% homologous in their nucleotide sequences. Additional experiments indicated that the A20 and A30 sub-families were more closely related to each other than to the B49 sub-family (Park, et al., 1980).

Our general model of the zein multigene family is shown in Fig. 1. Each of the cDNA clones identifies a sub-family of zein mRNAs of related nucleotide sequence. As a group these three cDNA clones are able to hybridize to the major sub-classes of zein mRNA. This model somewhat over-simplifies the situation since there is evidence (Park, et al., 1980; Lewis, et al., 1981) that limited cross-hybridization can occur between one or more members of these sub-families. Hence, the designation into sub-families is arbitrary since it depends on the conditions of hybridization. Nevertheless, the concept of sub-families is useful since we know that most of the nucleic acid components of one sub-family can be easily distinguished from another sub-family if the conditions of hybridization are closely controlled.

IV. The Genomic Organization of Zein Genes

The model given in Fig. 1 makes a strong prediction: if a given cDNA clone is related to a number of mRNAs by close sequence homology then that cDNA clone should also be homologous to each of the genes that coded for these mRNAs. We tested this idea with the use of genomic blots (Southern, 1975). High molecular weight DNA was obtained from Illinois High Protein leaves making use of ethidium bromide in the extraction buffer to inhibit double and single strand breaks (Kislev and Rubenstein, 1980). The DNA was then digested with various restriction enzymes and Southern blots were produced. The blots were probed with each of the cDNA clones (A20, A30, B49); the resulting hybridization patterns are complex and unique for each

of the cDNA clones (Hagen and Rubenstein, 1981). The patterns consist of numerous bands of differing intensities and cover a wide range of molecular weights. For example, the Bam HI digest gel when probed with the cDNA B49 reveals 20-22 bands of different molecular weight ranging from 1.7 to over 20 kb; the relative intensity of hybridization of B49 to the various bands also differs. A similar experiment and result had been reported by Wienand and Feix, 1980. In our experiments the use of simulation lanes involving known amounts of probe DNA demonstrated that we could detect a single copy of a zein gene. This result, as well as controls for the uniformity of gel transfer and specificity of hybridization, allowed us to quantify the number of genomic segments that were homologous to any one of these cDNA clones. We estimated that some 30-50 equivalent copies were present for each of these sub-families. This implies that there were some 100 zein genes present in the maize genome and is in agreement with the work of Viotti, et al. (1979). We can not, however, prove by these experiments that these genes are functional; they may be pseudogenes (Vanin et al., 1980).

THE ZEIN MULTIGENE FAMILY

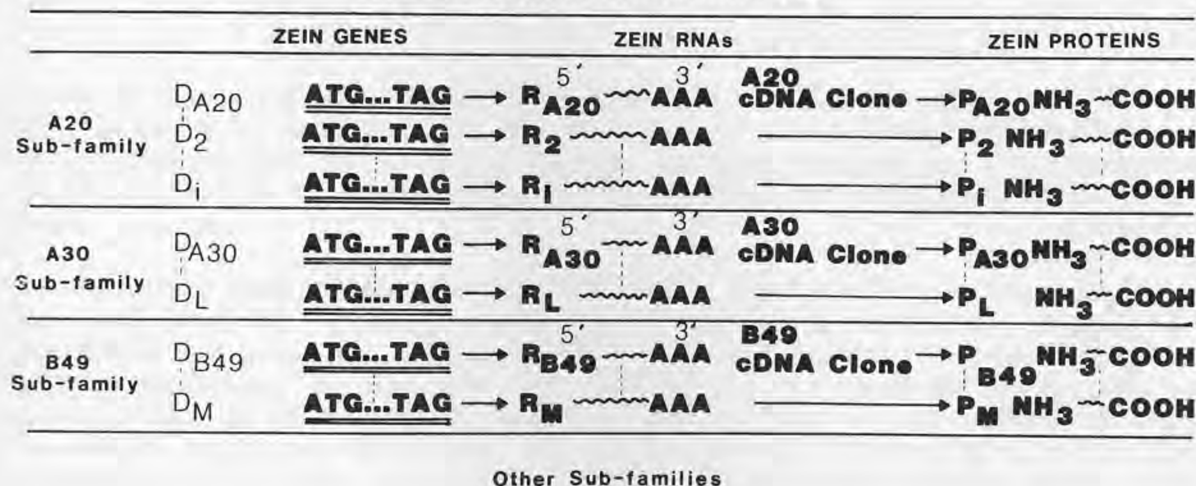

Figure 1. The Zein Multigene Family Model. The figure is divided into three columns: the first column represents the zein genes, the second column the zein mRNAs, and the third column the zein proteins. The arrows show the relationship between a given gene and its mRNA and protein product. Each of the zein cDNA clones A20, A30, and B49 when hybridized to the entire zein mRNA population is able to select a sub-group of related mRNA sequences. Each of these sub-groups (named after the cDNA used in the hybridization) is called a zein sub-family. The number of members in a given family is unknown; this situation is acknowledged by the use of the letter subscripts. When the mRNAs of a given sub-family are translated in vitro the resulting protein products represent a sub-group of all the zein proteins. Each of the zein cDNA clones A20, A30, and B49 when hybridized to the maize genome is able to bind to the sub-group of genes that code for the mRNAs in its sub-family. The situation is probably more complex than indicated since some members of a given sub-family are able to cross-hybridize to members of another sub-family.

V. Isolation of Zein Genes

These data and the model shown in Fig. 1 allowed us to make additional predictions. Zein genes should be relatively easy to isolate since there are so many of them in any one sub-family; and when isolated the gene should hybridize to a sub-group of zein mRNAs similar to the cDNA used as a probe to find the clone containing the gene. We have constructed a lambda bacteriophage Charon 4 library of Eco RI fragments of W22 leaf DNA and isolated a number of zein genes from it (Lewis et al. 1981). We have chosen for futher study two lambda bacteriophage which contain sequences homologous to zein cDNAs and which we term Z4 and Z7. The lambda Z4 DNA is homologous to the A30 cDNA clone and the lambda Z7 DNA is homologous to the B49 cDNA clone. The Z4 DNA also shows some homology to B49 cDNA. When used in a hybridization-release translation experiment, each of these genomic clones hybridizes to a sub-group of zein mRNAs similar to those that hybridize to their related cDNA (Lewis, et al., 1981). For each of these genomic clones the region of zein cDNA homology is on an Eco RI fragment of about 7.5 kb and this fragment apparently contains the entire structural region of the zein protein as well as considerable flanking bases.

VI. The Primary Amino Acid Sequence of a Zein Protein

The determination of the primary amino acid sequence of a zein protein by standard chemical means would require the isolation of a single pure component from a complex mixture of closely similar proteins (Bietz et al., 1979). The availability of cDNA clones of zein allowed us to take a different approach.

Both of the cDNA clones A20 and A30 contain inserts that are about 1000 bases long and therefore we expected them to contain the entire nucleotide sequence coding for the zein structural protein. By determining the nucleotide sequence of these clones, we are able to deduce the complete amino acid sequence of zein storage proteins (Geraghty et al., 1981). The sequence of amino acids predicted by the nucleotide sequence of the A30 cDNA is in agreement with the known sequence of amino acids at the N-terminus of zein proteins (Bietz et al., 1979). The predicted amino acid composition also agrees with the published values (Lee et al., 1976). The mature protein consists of 213 amino acid residues and the calculated molecular weight is 23,329. This is some 4 kd larger than the relative molecular weight measured by SDS gel electrophoresis. We also discovered that the C-terminal region of this zein contains about 7 or 8 tandem repetitions of a highly conserved 20 amino acid repeating unit.

We have now sequenced the B49 and A20 cDNA clones (Geraghty et al., 1982); the amino acid sequence of these zeins also contain a C-terminal repeated region similar to that found in A30. In addition, we found that A20 and A30 are closer in nucleotide sequences homology to one another (80-90%) then they are to B49 (70-80%). This finding agrees with the results obtained by hybridization-release translation (Park, et al., 1980).

VII. The Nucleotide Sequence of a Zein Gene

The nucleotide sequence of the genomic clone Z4 that is in the A30 cDNA sub-family has been determined (Hu et al., 1982). The Z4 nucleotide's sequence is about 97% homologous to that of A30. In contrast to the

storage protein phaseolin in French bean (Sun, et al., 1981) the Z4 genomic sequence does not contain introns. The Z4 sequence does contain, however, a interesting feature that distinguishes it from the A30 cDNA sequence; it has an internal repeat of 96 nucleotides that is found but once in A30. This sequence differs by only a few nucleotides from the sequence of 96 nucleotides that preceeds it in the Z4 sequence. The 32 amino acids specified by each repeat are identical and the region of the repeat is near the center of the amino acid sequence.

Thus, the lambda Z4 clone that was isolated from a genomic library constructed from W22 DNA codes for a protein which is very similar to the cDNA A30 that was obtained from mRNA isolated from Illinois High Protein. The Z4 protein, however, would be 32 amino acids larger than that coded for by A30. We note with interest that this difference is approximately the difference in molecular weight between the large "22 kd" and small "19 kd" zeins. Thus, although A30 apparently codes for one of the "19 kd" zein proteins and hybridizes mainly to mRNAs that are translated into zeins of this molecular weight class, the sequence of A30 is almost completely homologous to a genomic clone that codes for a zein of the "22 kd" class. This seems to imply that a zein sub-family can consist of members of both the "19 kd" and the "22 kd" molecular weight size classes. These findings are in agreement with our earlier hybridization-release translation experiments; these indicated that A30 cDNA was able to hybridize to mRNAs that code for the "22 kd" zeins and that the genomic and mRNA members of a zein sub-family have very similar nucleotide sequences.

VIII. Conclusions

A clearer picture of the zein multigene family is now emerging. The number of genes in the family is apparently large (about 100). The individual members can be clustered into sub-families with closely related nucleotide sequences. Among these sub-families there exists varying degrees of relatedness. The zein gene that has been sequenced does not contain introns. The "19 and 22 kd" zein proteins have an absolute molecular weight that is some 4 kd larger. About two-thirds of a zein protein consists of tandem repetitions of a highly conserved 20 amino acid unit.

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30 THE CONSTRUCTION OF MAIZE DNA LIBRARIES

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A major advance in DNA cloning technology was the development of what are now called DNA libraries. DNA libraries made possible the isolation of specific DNA sequences from unfractionated DNA of high complexity (Blattner *et al.*, 1978, Maniatis *et al.*, 1978). Previously, specific genomic DNA sequences had been clonally isolated only if the specific sequences were present in many copies (see review by Long and Dawid, 1980), or if the specific sequences were from low complexity genomes, such as yeast (Clarke and Carbon, 1976), or if the specific sequences could be partially purified by physical means (Tilghman *et al.*, 1977, Tonegawa, *et al.*, 1977). Although single copy genes have been isolated by physical means from high complexity genomes (Tilghman *et al.*, 1977, Tonegawa *et al.*, 1977) the DNA library method allowed the isolation of single copy genes with far less starting material. In addition, unlike methods involving partial physical purification, many single copy genes could be isolated from the same DNA library. Possession of a DNA library also enables the making of a quick estimate of the minimum frequency of occurrence of a specific DNA segment in a genome. Finally, DNA libraries facilitate the study of genes in their chromosomal environment, since the DNA surrounding a previously cloned gene can often be obtained by rescreening the already existing library with a probe derived from the cloned gene.

The application of the DNA library procedure to plants is a critical advance for plant molecular genetics. Until recently, only repeated sequences from the nucleus, such as the tRNA genes and the ribosomal genes, (Gerlach and Bedbrook, 1979) and sequences from the low complexity organellar genomes had been cloned from plants (Bedbrook, Kolodner, and Bogorad, 1977; Thompson, Kemble, and Flavell, 1980).

For the DNA library procedure to succeed, large numbers of recombinant molecules must be replicated in *E. coli* and large numbers of recombinant molecules must be screened. These prerequisites for the use of DNA libraries were successfully fulfilled by groups cloning mammalian DNA (Blattner *et al.*, 1978, Maniatis *et al.*, 1978). The low efficiency of transformation of *E. coli* was the barrier to replicating large numbers of recombinant molecules in *E coli*. This barrier was overcome by using lambdoid bacteriophage vectors which can be encapsidated *in vitro* (Becker and Gold, 1975) in good yields, thereby allowing the efficient process of infection to be substituted for the inefficient process of transformation. The problem of screening large numbers of recombinants was solved by scaling up the Benton-Davis plaque assay (1977). For screening, recombinant lambdoid phages were plated onto *E. coli* lawns on cafeteria trays and large sheets of nitrocellulose where used to take prints of the resultant plaques. Hybridization to the nitrocellulose prints allowed the desired recombinant phages to be located after autoradiography (Blattner *et al.*, 1978).

The principal barrier to the construction of a corn DNA library was the lack of a good preparation of high molecular weight corn DNA. Using a

simple method of rapid lysis of corn tissue with SDS and buffered phenol, Dr. John Stout and I were able to prepare corn DNA averaging about 50 kbp. From this DNA, with the assistance of Dr. Jerry Slightom, I constructed a library from Black Mexican maize DNA using the method described by Slightom, Blechl and Smithies (1980). The Black Mexican maize DNA library contained enough maize DNA so that it should fully represent the maize genome except for the small fraction in which Eco R1 sites are farther apart than 22 kbp. This library should allow the isolation of virtually any single copy sequence for which a hybridization probe is available.

Materials and Methods

1. Materials

RNAse was purchased from New England Biolabs (Beverly, Massachusetts, USA). T4 DNA ligase was from Miles Laboratories (Elkhart, Indiana, USA) and from New England Biolabs. Eco R1 was isolated from RY-13 cells by T. Szeto and Dr. Jerry Slightom, using a modification of the method of Sumegi *et al.* (1977).

2. Preparation of Maize DNA

DNA was prepared from Black Mexican suspension culture cells generously provided by Ko Shimamoto. A 19 gram cell pellet was ground in a pestle with a mortar at room temperature in 10 ml 1% SDS, 0.15 M NaCl, 0.1 M EDTA pH 8.0, and 5 ml phenol pH 8.0 containing 0.1% 8-hydroxyquinoline and saturated with DNA dialysis buffer (10 mM NaCl, 1 mM EDTA, 10 mM Tris pH 8.0). The phenol had been previously equilibrated to pH 8.0 by successive washes with 1 M Tris pH 8.0 followed by DNA dialysis buffer. After grinding, the extract was incubated for 10 minutes at 60°C. The phases were then separated by centrifugation for 5 minutes at 10,000 x g. The aqueous phase was extracted once more with equilibrated phenol and centrifuged again. The DNA was ethanol-precipitated from the aqueous phase, wound out on a glass rod, and redissolved in DNA dialysis buffer. The DNA was then treated with 50 μg/ml RNAse, phenol extracted, ethanol-precipitated, and dialyzed against DNA dialysis buffer. DNA from glossy maize shoots, W22 leaves and W23 leaves, was prepared in a similar way. The yield of Black Mexican DNA was about 500 mg from a 19 gram cell pellet. The yield from shoots and leaves was about 1 mg per 10 grams of leaves or shoots.

3. Construction of Recombinant DNA Bacteriophage

A Black Mexican maize Charon 4A library representing almost the complete maize genome, and a glossy maize Charon 3A library representing part of the maize genome was constructed. The Black Mexican, W22 and W23 maize libraries were constructed with the vector Charon 4A (Blattner *et al.*, 1977) using maize DNA partially and totally digested with Eco R1 (Slightom, Blechl, and Smithies, 1980). The glossy maize library was made with the vector Charon 3A using maize DNA completely digested with Eco R1 as described by Blattner *et al.* (1978).

A check on the size distribution of the fragments was made by randomly picking eight Black Mexican maize Charon 4A phages; all had inserts of 10-20 kbp. We also established that none of the eight randomly picked phages had the bacterial inserts lac 5 or bio 256 used to grow the vector.

To further demonstrate the absence of the bacterial inserts lac 5 or bio 256 in the Black Mexican maize library, lac indicator plates were used to detect beta galactosidase-producing phages (Blattner et al., 1977). The lac indicator plates showed no beta galactosidase-producing phages in 550 plaques tested. This test is expected to detect about 25% of the phages that had randomly picked up either one or both of the two bacterial inserts, since only the lac 5 inserts in one of two possible orientations with respect to the promoter site will allow production of beta galactosidase. Since Charon 4A is inviable without inserts, this estimate indicates that more than 99% of the plaque forming units should have maize DNA inserts.

Results and Discussion

Table 1 shows that the Black Mexican maize DNA library, prepared from relatively little maize DNA, potentially contains several maize genomes. It is also clear from Table 1 that the highest yield of recombinants is from the maize DNA prepared from a suspension culture of the Black Mexican strain. However, the data show that, with some scaling up, DNA libraries as complete as the Black Mexican suspension culture library could be constructed with DNA prepared from the leaves of the W22 and W23 maize strains. By increasing the amount of ligated W22 or W23 DNA packaged in vitro tenfold to 60 μg, enough recombinant phages could be obtained to represent almost all of the maize genome.

Table 1. This table shows to what extent each library represents the maize genome. The estimate of the average size for W22 and W23 is based on the measurement of the insert size of the Black Mexican library which used fragments of the same size for ligation.

According to Bennett and Smith the maize genome is 4×10^9 bp (1976). Only ten percent of the Charon 3 phages had inserts as shown by electrophoresis of DNA from randomly picked clones.

Source of DNA	Maize DNA Ligated	Phages In Vitro Packaged	Total Recombinants	Average Insert Size	Total bp of Maize Inserts	Percentage of Maize Genome
glossy shoots	1 μg	10^6	10^5	4×10^3	4×10^8	10%
W22 leaves	6 μg	7.5×10^4	7.5×10^4	1.5×10^4	1×10^9	23%
W23 leaves	6 μg	6×10^4	6×10^4	1.5×10^4	9×10^8	18%
Black Mexican Suspension Culture	8 μg	10^6	10^6	1.5×10^4	1.5×10^{10}	375%

Although the Black Mexican maize DNA library contains 1.5×10^{10} bp of maize DNA, some maize DNA sequences may be underrepresented in this library. The fraction of maize DNA in which adjacent Eco R1 sites are more than 22 kbp apart is not represented in this library because the Charon 4A vector cannot take an insert larger than 22 kbp (Blattner et al., 1977).

However gel electrophoresis, after complete Eco R1 digestion, reveals that only about 5% of the maize genome is contained in fragments larger than 22 kbp. Selection against replication in E. coli of certain classes of inserts might also cause them to be underrepresented. This potential problem is minimized by growing the bacteriophages as isolated plaques in an E. coli lawn, since independent growth prevents competition between bacteriophages for bacteria. To show that inserts remain unchanged after several cycles of growth, eight clones were picked at random from the Black Mexican maize library and have been shown to have inserts stable in size through several cycles of growth in liquid medium.

Assuming equal representation of all classes of maize DNA (except the fraction in which adjacent Eco R1 sites are separated by more than 22 kbp) 10^6 Black Mexican maize DNA clones with inserts averaging 15 kbp should contain almost every sequence in the maize genome. According to Clarke and Carbon (1976) the probability of finding a specific fragment, P, is equal to 1-(1-F) N, where F is equal to the fraction of the genome represented by one fragment and where N is equal to the number of clones in the library. F equals $1/2.7 \times 10^5$ because the 4×10^9 bp maize genome (Bennett and Smith, 1976) can be divided into 2.7×10^5 fragments averaging 15 kbp. Since N equals 10^6, the probability of the library containing a specific fragment is 0.975.

Several different DNA fragments have been isolated from the Black Mexican maize library and from the glossy maize DNA library. In collaboration with John Devereux, a clone with a polydeoxypyrimidine·polydeoxypurine tract associated with a segment with homology to origins of DNA replication was isolated from the glossy maize DNA library (Sheldon, 1980). In collaboration with Dr. John Stout and John Devereux, clones with short stretches of homology to histone DNA sequences were isolated from the Black Mexican maize DNA library (Sheldon, 1980). Finally, clones putatively coding for zein, the storage protein of maize, were isolated from the Black Mexican maize library, in collaboration with Dr. Brian Larkins and Karl Pedersen of Purdue (Pedersen et al., 1981). The Black Mexican maize DNA library has also been used to measure the frequency of occurrence in the maize genome of specific sequences (Sheldon, 1980).

In conclusion, the DNA library procedure, employing a lambdoid bacteriophage vector, has been successfully applied to maize so that the isolation of single copy maize nuclear sequences is now feasible. Obtaining suitable hybridization probes is now the limiting factor in the isolation of additional nuclear genes from maize.

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31 INTRODUCTION TO TRANSPOSABLE CONTROLLING ELEMENTS IN MAIZE

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The basic properties of maize controlling elements are similar to those of transposable elements in prokaryotes and other eukaryotes. Insertion of a controlling element into a locus can disrupt gene expression and result in a recessive mutant phenotype*. Controlling element insertion mutations can be quite unstable, showing a high frequency of both somatic and germinal reversion. Alleles of a locus that display somatic instability are designated "mutable" alleles, indicated by an "m" subsequent to the allele designation (e.g., bz-m).

Controlling element insertion mutations are of two basic types. One type is inherently unstable and is associated with insertion of an element capable of autonomous excision and transposition. Such mutations have been termed "autonomous" and I will refer to the elements causing such mutations as "autonomous" controlling elements. The second type of controlling element mutation is not inherently unstable, but becomes unstable when an autonomous element is present elsewhere in the genome. The elements causing such mutations do not appear to be capable of autonomous transposition and I will refer to them as "non-autonomous" controlling elements. The relationship between elements is simply represented in Figure 1. There are several lines of evidence suggesting that non-autonomous elements can be

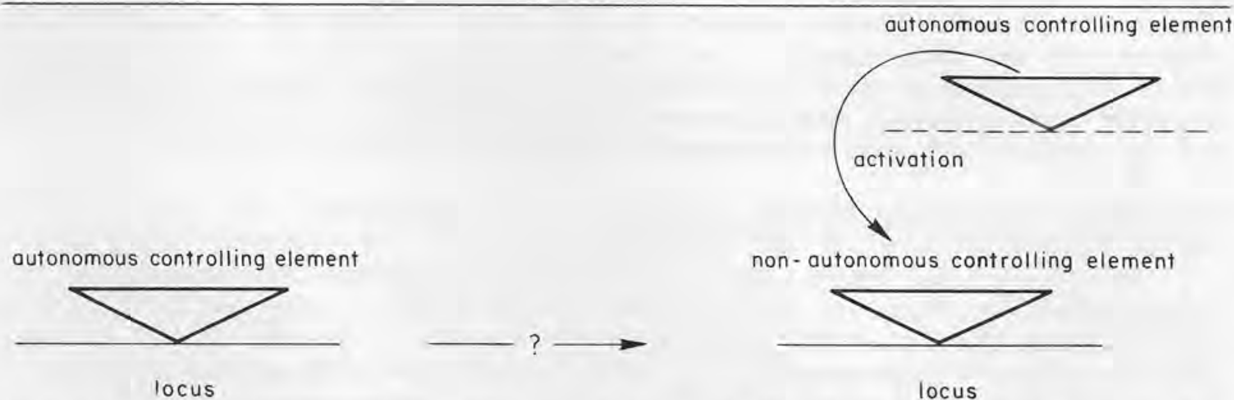

Figure 1. A diagrammatic representation of the interaction of autonomous and non-autonomous controlling elements. The uninterrupted arrow designates the activation of genetic changes at a non-autonomous element by an autonomous element. The arrow interrupted by a question mark designates the possibility that a non-autonomous element can be derived from an autonomous element.

*For reviews of controlling element phenomenology and original references, the reader is referred to McClintock (1951, 1956, 1965) and Fincham and Sastry (1974). I have inserted original references only for relatively recent work and when referring to specific authors by name.

derived from autonomous elements, leading to the suspicion that a non-autonomous element represents a transposition-defective autonomous element. That possibility is represented by an arrow interrupted by a question mark in Figure 1 to emphasize the absence of direct evidence for the implied sequence relationship.

An autonomous element can activate a non-autonomous element to transpose or initiate other types of genetic changes. A given autonomous element can activate some, but not all, non-autonomous elements. The selective interaction of controlling elements with each other has provided a basis for their classification into groups or families. Six or more controlling families have been distinguished by this criterion and three have been studied extensively. These include the Ac-Ds (Mp), Spm (En-I) and Dt families.

The Ac-Ds family was the first one studied by McClintock (1947). Activator (Ac) designates the autonomous element and Dissociation (Ds) designates the non-autonomous element belonging to this family. Early in the century Emerson (1917) studied variegation caused by an autonomous element later identified, named Modulator (Mp) and shown to belong to the AC-Ds family by Brink and his colleagues (Brink and Nilan, 1952; Barclay and Brink, 1954).

McClintock (1954) and Peterson (1953) independently discovered the Spm family at about the same time. McClintock designated the bi-functional autonomous element Suppressor-mutator (Spm), while Peterson designated it Enhancer (En). McClintock left the non-autonomous element unnamed, while Peterson designated it Inhibitor (I).

The Dotted (Dt) controlling element family was studied primarily by Rhoades (1941) and Nuffer (1961), and Doerschug (1973). Dotted (Dt) designates the autonomous element in this family, while the non-autonomous element has not been named. Each family of elements differs from the others in its detailed mode of action, although all share the capacity of producing unstable mutations. I will illustrate these differences by briefly describing the Ac-Ds and Spm controlling element families.

The Ac-Ds Controlling Element Family

In the Ac-Ds family, the elements are named for the property of the system that was observed first by McClintock (1947). Dissociation refers to the fact that the non-autonomous element provides a specific site of chromosome breakage. Site-specific chromosome breakage requires the presence of the autonomous controlling element, which was designated Activator for its ability to activate the process of chromosome breakage. Chromosome breakage at Ds activated by Ac is diagrammed in Figure 2. The broken ends of the sister chromatids fuse, giving an acentric fragment and a dicentric chromosome. The dicentric chromosome connects the spindles at mitosis and is subsequently broken at random. The broken ends fuse once again during or after the next round of chromosome replication, perpetuating the cycle of breakage. This was termed the chromatid type of breakage-fusion-bridge cycle by McClintock (1942).

Ds is stable in the absence of Ac. In the presence of Ac, Ds not only acts as a specific site of chromosome breakage and acentric-dicentric formation as outlined in Figure 2, but also transposes to new locations. Ds can

integrate into or near loci affecting morphological gene expression. When Ds inserts into a locus, it can cause a recessive mutation. Such a mutation is stable in the absence of Ac and unstable in its presence. In the presence of Ac, such an insertion mutant allele gives the mosaic or variegated phenotype characteristic of transposable element mutants. The variegation is

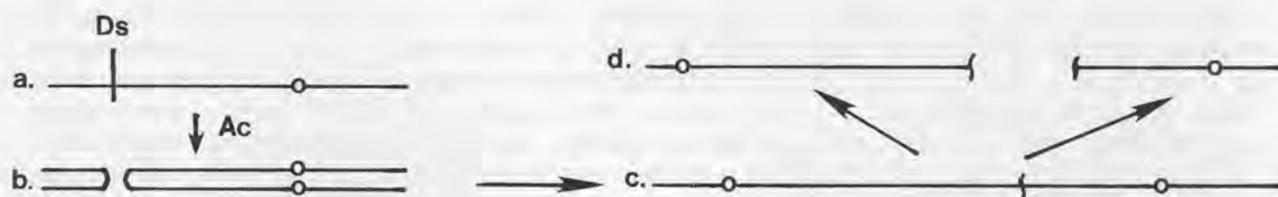

Figure 2. The chromatid types of breakage-fusion bridge cycle. Breakage at the site of Ds (a) is followed by fusion of the sister chromatids at the site of Ds during or after replication (b). The acentric fragment is lost at mitosis, while the dicentric breaks (c) and the broken chromatids segregate to the daughter cells (d). The process continues in subsequent cell divisions as a consequence of fusion of the ends of the broken sister chromotids during subsequent rounds of replication.

due to element-associated genetic changes. For some mutable alleles, the most frequent genetic change is excision of the Ds element from the locus, with (and possibly without) its concomitant transposition to a new site. Phenotypically, the pattern of variegation is one that gives revertant sectors on a recessive background. Premeiotic mutation gives stable revertants in which Ds is no longer at the previously mutant locus. The frequency of acentric-dicentric formation at Ds is inversely related to the frequency of Ds excision/transposition, suggesting that they are concomitant to or consequent of the same molecular events.

Strains have also been isolated in which Ds transposes from its initial site of insertion at a much lower frequency or does not transpose at all, but is responsible for changes in the expression of the locus where it is integrated, or of nearby loci. The three mutable alleles of the Sh locus that have been analyzed in greatest detail at the molecular level were derived from two strains with a non-transposing Ds inserted just distal to the Sh locus. Both stable and unstable mutations affecting the Sh, C and Bz loci in the immediate vicinity of Ds occurred at a relatively high frequency in these strains. Ds remained at or near its original location in both the stable and unstable strains, as well as in revertants of the unstable mutants. The Sh locus encodes sucrose synthetase, a tetrameric protein having a monomer molecular weight of about 89 kD (Chourey and Nelson, 1976; Su and Preiss, 1978). Molecular analysis of this locus and its gene product is somewhat complicated by the existence of a related but not identical gene encoding a protein which is indistinguishable from the Sh-encoded sucrose synthetase in size and enzymatic properties and also appears to be antigenically related (Chourey, 1981). The proteins differ in sequence and the coding sequences are only distantly homologous, permitting their discrimination at the nucleotide sequence level (Chaleff et al., 1981). Of the three known Ds mutations of the Sh locus, the sh-m6233 and sh-m5933 alleles permit synthesis of 0.5-1

percent of the normal level of sucrose synthetase mRNA (Chaleff et al., 1981). Although analyses of the sucrose synthetase gene have not yet been altogether reconciled between laboratories, it is generally agreed that both mutants have insertions or rearrangements in the immediate vicinity of the gene (Burr and Burr, 1981; Chaleff et al., 1981; Doring et al., 1981). Figure 3 shows a partial restriction site map of the locus, indicating the region detected by the cDNA probe used in our laboratory (Fedoroff et al., 1980; Chaleff et al., 1981). The arrows indicate the approximate point at which the Sh and sh-m alleles differ and do not imply that the alteration is an insertion, since the data are insufficient to discriminate between insertions and rearrangements. The mutant designated sh-m6258 and a derivative (sh-m6795) isolated as an sh mutant from an Sh revertant of sh-m6258, contain an altered transcription unit in which the 3' terminal portion is separated from the 5' terminal portion by an insertion or rearrangement. The site of insertion or the rearrangement break point is indicated in Figure 3 and may lie within an intervening sequence in the gene, indicated by a gap in the cDNA sequence (Chaleef et al. 1981). Immature endosperm tissue from both strains contains an abundant gene transcript slightly shorter than sucrose synthetase mRNA and lacking the normal mRNA sequence beyond the insertion or inversion breakpoint (Federoff, unpublished observations). The aberrant mRNA can be translated in vitro and has been found to encode two polypeptides antigenically related to, but shorter than the 89-kD sucrose synthetase monomer (Fedoroff and Mauvais, unpublished observations). Additional hybridizable restriction enzyme fragments are present in both sh-m6258 and sh-m6795 DNA, suggesting a complex origin of the mutations. These studies indicate that Ds mutations can interfere with gene expression in rather different ways.

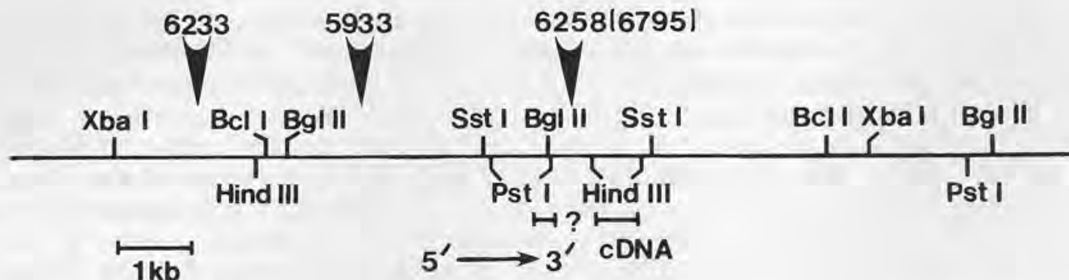

Figure 3. A restriction map of the Sh locus. Data were obtained from blot hybridization analyses of the progenitor strain from which the sh-m alleles were derived, using a cloned cDNA representing approximately 25 percent of the mRNA length. The interruption in the cDNA clone may correspond to an intervening sequence in the DNA. The approximate position at which the sh-m maps differ from the Sh map are indicated. Specifically, the differences are between the Pst I and Hind III sites at the 3' end of the transcription unit in sh-m5933 and between the Bcl I and Xba I sites in sh-m6233.

Studies on the Ds mutations of the Wx and Bz loci indicate that Ds can alter gene expression in yet other ways, as well. There is suggestive

evidence that the primary structure of the protein and the timing of gene expression in development can be altered in wx-m and bz-m strains (Chaleff et al., 1981; Echt and Schwartz, 1981; Dooner, 1980). Some studies have also been done on stable mutants derived from unstable controlling element mutants (Dooner and Nelson, 1979; Dooner, 1980; Echt and Schwartz, 1981). The results of these studies indicate that although some revertants appear to synthesize normal levels of the normal enzyme, others produce low levels of enzyme or produce high levels of an enzyme with altered activity or physical properties. These observations suggest that Ds may excise both precisely and imprecisely, leaving the gene permanently altered.

The Ds-associated genetic changes described above occur only in the presence of Ac and their frequency and developmental timing are determined by the number of Ac elements present, as well as the nature of the Ac element. The time in development at which Ds-associated changes occur is delayed with increasing numbers of Ac elements in the genome. Thus somatic reversion and acentric-dicentric formation at Ds are delayed in the presence of more than one element, with the result that the somatic sectors showing the results of the genetic changes are smaller. Ac elements also undergo heritable changes that either stably or reversibly affect their ability to transpose and trans-activate the Ds element. Ac can itself insert at a locus to give an unstable mutation. In some cases, Ac insertion is associated with the origin of an unstable recessive allele. Subsequent mutations can give stable mutants (nulls, intermediates and revertants) that no longer have Ac at the locus, as well as strains having a non-autonomous element at the locus. The Mp element, which is similar or identical to Ac, transposes during chromosome replication in such a way that the element is removed from the donor site on one of the two sister chromatids and inserted either into a replicated or an unreplicated recipient site elsewhere in the genome. The recipient site is most commonly on the same chromosome, within a few recombination units of the donor site.

The Spm Controlling Element Family

The Spm controlling element family differs in several ways from the Ac-Ds family. The systematic acentric-dicentric formation commonly observed at Ds is not a regular feature of mutations caused by elements belonging to the Spm family. In addition, there are mutations associated with insertion of a non-autonomous element belonging to the Spm family which have relatively little effect on expression of the locus in the absence of the Spm element. In its presence, however, such mutations show a completely recessive phenotype. Thus the element suppresses, or inhibits expression of the affected locus in such mutants. This trans-acting function of the element is designated the suppressor (sp) function (Figure 4). In addition, the element trans-activates somatic mutation, as does the autonomous element Ac in the Ac-Ds family. The element function necessary for somatic reversion was designated the "mutator" or m function hence the name Suppressor-mutator (Spm).

Mutations caused by non-autonomous elements belonging to the Spm family are stable in the absence of Spm and unstable in its presence, giving rise to secondary mutations of various kinds. These are either stable or unstable. Many stable mutants, ranging in phenotype from null to the full dominant, have been analyzed and are generally found to be insensitive to the presence of Spm, indicating that the non-autonomous element has either been excised or has transposed away from the locus. Unstable mutations

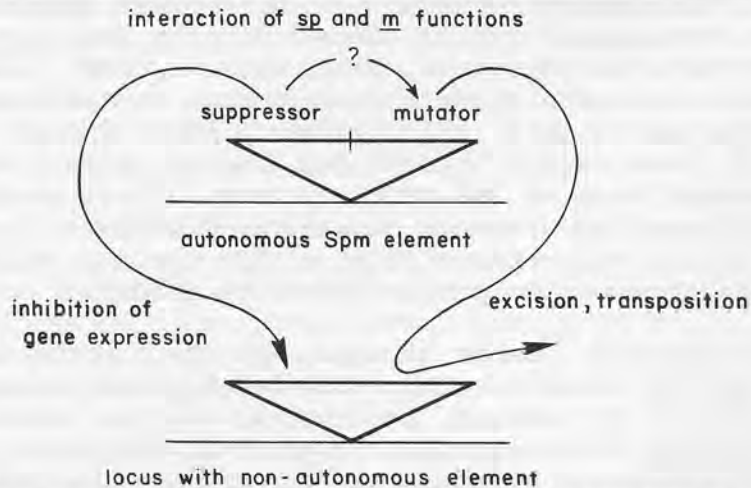

Figure 4. A diagrammatic representation of the interactions between autonomous and non-autonomous elements belonging to the Spm family. The arrows indicate that the suppressor function of Spm acts in trans to inhibit expression of a locus with an integrated non-autonomous element and the mutator acts in trans to promote excision/transposition of the non-autonomous element. Expression of the suppressor and mutator functions are likewise interrelated.

comprise the other basic category and these are mutations that affect the frequency and timing of somatic mutation, as well as the level of gene expression in revertant sectors. Each mutant strain has its own characteristic heritable pattern, defined by the number and size of revertant sectors, as well as their phenotype. The determinant of this pattern resides at the locus and is cis-acting. Heterozygotes between mutants having different patterns display both patterns. How the controlling element sequence is involved in pattern determination is not known. It may be that the mutations that affect the somatic reversion pattern result from changes in the location of the element within the locus or from changes in the sequence of the element itself.

Many types of Spm mutations are also known, some of which mimic the mutations discussed above. They can be distinguished from mutations affecting the non-autonomous element at the locus by having two different unstable genes responsive to the same controlling element present in the test strain. Mutations in the autonomous controlling element simultaneously affect the phenotype associated with both unstable genes, while the effect of a mutation in a non-autonomous element is restricted to one of the two unstable genes. Mutations are known which affect the developmental timing and frequency of somatic reversion (m function) with little or no effect on the ability of the Spm to suppress gene expression (sp function). The converse is not true. The mutations analyzed so far which affect sp function also affect m function. This suggests that the sp function is either involved in regulating expression of the m function or that both are needed for excision/transposition.

Spm elements that undergo cyclic changes in activity have been studied. An Spm element in an inactive phase shows neither sp- nor m-function. Elements have been identified that undergo frequent changes in activity phase during development of a single plant and which remain in an inactive phase for a prolonged period extending over several plant generations. Although no dosage effect comparable to that seen in the Ac-Ds system has been observed with Spm, cyclic Spm elements showing frequent transitions from the active to the inactive phase give an interesting dosage effect. The observation is that the total tissue area showing the phenotype characteristic of an active Spm increases with increasing numbers of cycling Spm elements. This is intelligible if each Spm element cycles independently of the others and produces sp gene product when in the active phase. Because the sp gene product is trans-acting, only one Spm element need be active to give the corresponding phenotype, while all resident Spm's must be inactive to give the phenotype characteristic of the inactive Spm. Since the probability of the coincidence of inactive phases decreases with an increase in the number of Spm elements, it follows that the area displaying the corresponding phenotype will also decrease. Curiously enough, when a frequently cycling Spm is combined with one in an inactive phase of very long duration, the pattern of gene expression obtained is that expected for two short-period Spm elements. The cycling Spm element therefore appears to impose its pattern of expression on the inactive one, although the two elements appear to undergo phase changes independently in the heterozygote. This is a transient effect, and the two Spm elements segregate from each other unchanged. The implication is that the cycling Spm differs from the non-cycling one by the production of a trans-acting substance required for the active ⟷ inactive transition. So simple an explanation is unlikely to suffice however, since a stably active Spm is not affected in trans, nor is the phase of a stably inactive one heritably altered in a heterozygote.

Spm and En elements have also been isolated whose activity phase is determined by the position of the element within a plant or a given tissue of the plant. As an example, Peterson (1966) isolated En elements which are active only in the crown of a kernel or only in the base of a kernel. These observations suggest that the elements can respond to signals differentially distributed within a tissue and within a plant.

Transposable Elements in Maize and Other Organisms

Controlling elements in maize transpose, cause chromosomal aberrations and unstable mutations, as do transposable elements in bacteria and other eukaryotes. Although the molecular study of controlling element mutations is just beginning, it is already evident that the elements can alter gene structure and expression in a variety of ways. What is unique to maize controlling elements is their ability to sense developmental time, as reflected in the observation that mutations and transpositions tend to occur within a relatively narrow developmental window.

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32 CONTROLLING ELEMENT Ds AT THE SHRUNKEN LOCUS IN ZEA MAYS

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Transposable genetic elements were first described by B. McClintock (1951, 1965). As they were detected from the beginning by the effects exerted on genes in their vicinity, more physiological detail is known about them than about some of the transposable elements studied in other eucaryotes that have been studied from the beginning as DNA sequences by biochemical methods. It would be desirable to extend the study of the maize transposable elements also to the level of DNA. It can be hoped that biochemical studies will help to answer some of the questions that can be asked by applying the present knowledge of molecular biology to the genetic data gathered nearly 30 years ago. This has been discussed in several review articles (Fincham and Sastry, 1974; Nevers and Saedler, 1977; Starlinger, 1980).

A possible approach to the isolation of maize controlling elements has been outlined by Doring et al. (1980). In brief, it consists of isolating DNA fragments carrying a certain gene both from wild type genomic DNA and from the genomic DNA of a mutant caused by the insertion of one of these elements. In the most simple situation, the fragment obtained from the mutant would be larger than that from the wild type, and the additional DNA would not have a sequence related to that of the wild type fragment. It could then be inferred that the additional DNA was the transposable DNA element, and tests could be designed to prove this assumption.

The attempts to isolate the DNA fragments mentioned above would have to include the characterization of the protein and its purification, the production of an antiserum, the identification of the mRNA for this protein in a cell-free system for protein synthesis, and the synthesis of cDNA to this mRNA and its cloning in an appropriate plasmid vector. If the cDNA were produced from a mixture of mRNAs, the correct clone could be found by its ability to hybridize to the mRNA, and this could be seen by hybrid-arrested or hybrid-promoted translation. The cDNA clones could then be used to prepare radioactively labelled DNA, and to identify the appropriate genomic DNA fragments by the Southern blotting technique. The fragments of the right size could then be cloned in the appropriate lambda vectors, and identified by plaque hybridization.

In order to choose an appropriate gene for these studies, two conditions should be fulfilled: (i) the gene should produce an abundant protein, and (ii) mutants of that gene caused by the insertion of one of these elements should be available. We have chosen the gene Sh that encodes a major protein (Chourey and Schwartz, 1971). The protein is endosperm sucrose synthetase (Chourey and Nelson, 1976). Recessive sh mutants lacking most of this endosperm enzyme have been obtained by McClintock (1952, 1953). These mutants, however, have arisen in two steps. The first step was the insertion of Ds in the vicinity of Sh without altering the phenotype of the latter. In a second step, a series of mutants was obtained which affected either Sh alone, or Sh and the closely linked

gene Bz. Some of the mutants were stably recessive, others were mutable and frequently reverted to the wild-type phenotype. These reversions, however, only occurred in the presence of a second transposable element, Ac. This is an indication that the reversion also is mediated by an event at Ds, which is under the influence of Ac (McClintock, 1951). The appearance of the double mutants and the lack of single bz mutants in this series of experiments led McClintock to the assumption that the secondary mutations are not transpositions of Ds to a new location within the mutated genes, but rather deletions extending from Ds either to gene Sh or beyond that gene to gene Bz. It is, therefore, possible that DNA rearrangements in these mutants are more complicated than the simple insertion of Ds within the mutated gene.

Starting from these considerations, we have done the following experiments: Sucrose synthetase mRNA was identified in a cell-free system (Wostemeyer et al., 1981). A clone with a 620 bp insert of sucrose synthetase cDNA was obtained (Geiser et al., 1980). This clone was used for Southern blotting experiments with genomic DNA from wild type, from two mutable sh mutants (sh-m5933 and sh-m6233), and from a double mutant (sh bz-m4). The wild type and the three mutants were separated from each other by only a few plant generations and thus it could be hoped (but not be proven) that any DNA differences at the Sh locus were not due to a restriction polymorphism unrelated to the mutations.

The hybridization experiments have yielded the following results: After single or double digestion with several restriction enzymes, one major band and, in some of the experiments, a few minor bands hybridizing to the cDNA clone were seen. When the minor bands were visible, their pattern did not differ for wild type and mutant DNAs. Differences were seen for the major band with some of the restriction enzymes used. It was concluded that the major band corresponds to a DNA fragment containing (part of) the sucrose synthetase gene.

No major band was seen with the DNA of the double mutant sh bz-m4. This indicated that (part of) the sucrose synthetase gene is deleted in this mutant, in accord with the genetic evidence. It also lends support to the notion that some of the single mutants are deletions adjacent to Ds rather than transpositions.

A restriction map could be constructed for wild type and mutant DNAs. These maps show the same pattern of restriction sites on one side of the region hybridizing to the probe. Deviations are seen on the other side. The deviation point may be the insertion point of Ds, or it may be the endpoint of a deletion removing the DNA segment between the original site of Ds insertion and one terminus of the gene. This deletion would have to remove DNA necessary for gene expression, but no part of the coding sequence (because of reversion) (Doring et al., submitted).

Our results support the findings by Burr and Burr (1981a, b), but deviate from them in some details. Similar results to ours have been obtained by Nina Fedoroff (personal communication).

We have now cloned DNA from the wild type and from sh-m5933 in the lambda vector 1059 (Karn et al., 1980) using BamHI and BclI fragments, respectively. The DNA of these clones is presently being characterized, and

we hope to use it to answer some of the questions raised and to investigate transposable element Ds at the DNA level.

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33 GENOMIC DNA CLONES OF ZEA MAYS

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Transposable elements are widely spread in nature. In bacteria, the structure of these genetic entities is known, and the mechanism of transposition is reasonably well understood (for reviews, see Starlinger and Saedler, 1976; Cohen and Shapiro, 1980). They not only transpose from one position in the genome to another, but they also can induce various DNA rearrangements such as deletions and inversions (Saedler *et al.*, 1981).

The presence of such elements in *Zea mays* becomes apparent upon transposition from an unknown site into an indicator gene where it induces a mutant phenotype. Such mutations are somatically unstable, i.e., the element frequently is excised thus restoring the wild-type locus. This results in a variegated phenotype. For example, if the element had integrated into one of the several genes involved in anthocyanin synthesis, the somatic instability of the element would generate colored spots on colorless kernels.

Occasionally, however, the excision of the element is inaccurate and leaves part of the element behind at the locus. This segment of the element now can respond to trans-acting signals issued by a complete element located somewhere else in the genome. This constitutes a two-element system consisting of a receptive part located at a given locus and a trans-acting regulatory element. Due to such a visitation of the element at a locus, this locus has come under the control of the element, hence the name "controlling element."

Most of our current knowledge about controlling elements is based on genetic experiments done by B. McClintock and P. A. Peterson (McClintock, 1951, 1965; Peterson, 1970). Controlling elements respond to tissue signals in a programmed way (McClintock, 1967; Peterson, 1966) and hence they have the capacity to be involved in differentiation.

A model which summarizes the properties of the element *En* (Peterson) or *Spm* (McClintock) has been presented (Nevers and Saedler, 1977). To understand the structure and the function of *En* at the molecular level, it seems necessary to clone the element *in vitro* and subsequently to probe various tissues in a developing plant for expression of its functions. A prerequisite for *in vitro* isolation of *En* or any other such element is the *in vitro* isolation of a gene into which the element is known to integrate.

En transposes into many genes including the "anthocyanin genes" A1, A2, C1, C2, and Pr (Fincham and Sastry, 1974). There is evidence based on precursor feeding studies (McCormick, 1978) and precursor accumulation (Balaravi and Reddy, 1981) that C2 encodes flavanone synthetase (see article by Dooner in this publication).

Recently, Hahlbrock and collaborators purified this enzyme from parsley tissue cultures. With the aid of antibodies produced against purified enzyme, they were able to isolate the corresponding mRNA. Using reverse transcriptase, this was converted into complementary DNA (cDNA), which then was cloned into pBR322 in E. coli. The specificity of this clone was tested by the hybrid release translation technique (Woolford and Rosbash, 1979).

One 1400 bp cDNA clone from parsley was used in our approach to isolate the C2 gene of Zea mays. However, in experiments where radioactively labelled parsley flavanone synthetase cDNA was hybridized to Eco R1 generated genomic maize DNA fragments, only weak hybridization was occasionally detected indicating only weak homology in this heterologous system. Nonetheless, when DNA fragments from maize wildtype W22 DNA (containing all dominant anthocyanin genes) were cloned into gtWES, a clone which hybridized to the parsley cDNA probe was obtained. The clone contains a 5.2 kb insert of maize DNA, which gives only one signal in autoradiograms when used to probe EcoR1 generated fragments from wildtype W22 DNA. This suggests that a unique DNA sequence from Zea mays was indeed cloned.

To relate this fragment to the C2 locus, we probed DNA fragments from various C2 mutants and other maize lines. Some lines showed an increase of about 800bp in the only Eco R1 fragment hybridizing. This fragment was cloned and compared to the genomic wildtype fragment using various restriction endonucleases. It became clear that the two fragments differ by an insert of about 800 bp located roughly in the middle of the fragment. This insertion seems to be characteristic for some maize lines, while it is missing in others. Whether we have cloned the C2 gene and a standard derivative thereof, or rather another unique DNA segment of maize which apparently differs in various lines by an insertion of 800 bp, remains to be determined.

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34 TYPES AND AVAILABILITY OF MALE STERILE CYTOPLASMS

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Cytoplasmic control of male sterility has important economic and logistical advantages in production of hybrid corn seed. The occurrence of the 1970 southern corn leaf blight epidemic on cmsT or Texas male-sterile cytoplasm corn demonstrated the dangers of using one cytoplasm exclusively for hybrid corn production and forced the seed corn industry into hybrid production using non-male sterile cytoplasms, which necessitate either manual or mechanical detasseling. Alternate types of male sterile cytoplasms which are resistant to Helminthosporium (Bipolaris) maydis, race T (HmT), the causal agent of southern corn leaf blight, are available, and the use of these cytoplasms in hybrid seed production is increasing. Studies conducted over the past few years have produced a lot of information about the nature of cytoplasmic control of disease resistance and male sterility that has broadened our knowledge of the various types of male sterile cytoplasms.

The 40 or more sources of cytoplasmic male sterility in maize can be classified into three major groups by differential fertility restoration reactions (Beckett, 1971; Duvick, 1965; Gracen and Grogan, 1974).

The T group includes T, P, Q, HA, RS, and SC (or South Carolina) cytoplasms. All are relatively stable sources of male sterility but all are susceptible to race T of Helminthosporium maydis and, therefore, not recommended for hybrid production in the U. S. today. Fertility restoration of the T group of male sterile cytoplasms has been extensively studied (see Duvick, 1965, for a review). The major genes for pollen fertility restoration in the Texas cytoplasms have been designated as Rf1 and Rf2. Both the Rf1 and Rf2 genes must be present for complete pollen fertility restoration of the T cytoplasm. In addition, one or more modifying genes must also be present to insure complete fertility.

The S group contains the majority of cytoplasmic sources of male sterility. It also appears to be the most diverse group in terms of stability of male sterility over environments and response to nuclear fertility restorer genes. Fertility restoration of the S group of cytoplasms is substantially different from that in T cytoplasm. In T cytoplasm restoration is determined by the restorer genotype of the female plant. Plants heterozygous for Rf genes in T cytoplasm produce all normal pollen because the genotype of the sporophytic tissue (Rf/rf), rather than that of the pollen, determines restoration. Individual pollen grains carrying either the Rf or rf alleles are fertile. This type of pollen restoration has been called sporophytic. In contrast, plants heterozygous for the Rf factors in the S cytoplasm produce 50 percent fertile pollen grains and 50 percent shrunken, inviable pollen. In this case, the genotype of the pollen determines restoration. Pollen with the dominant Rf allele is viable, whereas pollen with the recessive rf allele is not. This has been called gametophytic restoration.

The S cytoplasms exhibit partial fertility restoration more often than T cytoplasm, and the degree of fertility restoration of S cytoplasms appears to

be more sensitive to environmental variation than the T cytoplasms. Fertility restoration of the S cytoplasms is reported to be controlled by a single locus, Rf3, (Duvick, 1965) which is different from the Rf factors responsible for restoring the T cytoplasm. Recent evidence, however, suggests that fertility restoration of the S type of cytoplasms may be much more complex. Singh and Laughnan (1972) have reported a number of cases of instability of the S male-sterile cytoplasms. These could have arisen through changes at either the cytoplasmic or nuclear gene level. Genetic analyses indicate that cytoplasmic mutants in S cytoplasm were involved in some of these variants (Singh and Laughnan, 1972), although other reversions to male fertility arose through apparent nuclear mutations giving rise to S cytoplasm restorer genes (Laughnan and Gabay, 1973, 1978). These are the first reported mutations in nuclear fertility restoration factors, and interestingly, these apparent S Rf mutants can be mapped to several different chromosomes (Laughnan and Gabay, 1973). All of these new Rf genes, except one, are associated with deleterious phenotypic side effects which suggest that most restorer "mutations" originating in nature are deleterious and at a disadvantage for survival when compared to the Rf3 locus.

In addition to the instability of male sterility identified by Laughnan and coworkers, additional variability appears to exist in the fertility restoration patterns of other sources of S cytoplasms. Studies of 10 maize lines that are natural restorers of the S cytoplasm indicate that all carry the Rf3 allele for restoration of the VG or S sources tested (Laughnan and Gabay, 1978). However, some inbred lines have been identified that apparently restore certain sources of S cytoplasm but do not restore others (Gracen and Grogan, 1974). Cytoplasms B, D, ME, and MY often give fertility restoration responses different from the typical S restoration pattern. Also, backcrosses involving the W, J, R, M, VG, and CA cytoplasms with certain inbred lines were reported to segregate in 3:1 ratios suggesting the involvement of more than one S Rf gene (Josephson et al., 1978). These data suggest that for some sources of S cytoplasm, either genes other than Rf3 are necessary for restoration or multiple alleles that have slightly different fertility restoration effects occur at the Rf3 locus.

The C group of cytoplasms originally contained only the C and RB sources. This group has been expanded in recent years to include the El Salvador (ES) source and the Bb and Parana (PR) Sources from Brazil. New sources: Cuarentenos, Italy IB, IR-1, IR-2, IR-3, and Pl are probably C types, although they have not been fully classified as yet. All are relatively stable in some inbred backgrounds but some combinations do exhibit a "late" break of sterility about 5-10 days post-silking. Fertility restoration of the C group cytoplasms more closely resembles that of the T group. Restoration is sporophytic in the C as in the T. Although a recent report (Josephson et al., 1978) suggested that three or more Rf factors (designated Rf4, Rf5, and Rf6) were involved in C restoration, data from our laboratory, involving a systematic series of crosses, backcrosses, and selfs, indicate that fertility restoration of the C, RB, PR, ES, and Bb cytoplasms is controlled by a single dominant gene (designated as Rf4) in the lines we have studied (Kheyr-Pour et al., 1981).

A major problem encountered with the C cytoplasm in our studies is the tendency of C steriles to exhibit a delayed breakdown of sterility in some inbred backgrounds. This late breaking characteristic appears to be

influenced by nuclear genes different from the Rf4 allele and selection of C sterile versions of these inbreds with less tendency to break is possible.

Most of the cytoplasms in the T, C, and S groups have been converted at Cornell to a series of inbreds adapted to the United States and they were released in 1974. Seed of most of these combinations is still available, although supplies are limited. The exact combinations available with fertility ratings using a 1-5 scale (1 = full sterility, and 5 = full pollen fertility) are presented in Table 1 (see reverse side of this page).

Although the mechanism(s) of cytoplasmic male sterility is not known, it is reasonable to speculate that either mitochondria or chloroplasts could be involved. Several studies have indicated that mitochondria of T, C, and S cytoplasms differ in structure and/or function while differences in chloroplasts are less apparent. Mitochondria of the T cytoplasms are certainly involved in sensitivity to H. maydis race T toxin (see Gregory et al., 1977, for a review). The following articles in this section will describe some of the differences between mitochondria and chloroplasts and the relationship of such differences to cytoplasmic control of male sterility.

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Table 1. Relative degree of pollen sterility and maturity of different sources of cytoplasmic male sterility in different maize inbred backgrounds.

Inbreds	A239	A495	A619	A632	A636	AyX65	AyX138	AyX145	AyX157	AyX187y-1	AyX187y-2	Ay499	Ay191-71	Ay303E	Ay490-2A	B8	C153	CO113	CO150	CO192	CO220	CrS4HLA	MS64-7	MS89A	MS1334	NYD410	NY63-71-1	NY821	NY821LERf	Oh43	Oh51A	Pa33	Pa884P	SD10	Va20	W64A	W182BN
Days to Flowering	84	78	81	82	82	84	73	74	78	80	80	79	73	73	81	75	75	69	74	76	70	81	77	76	76	80	75	79	79	79	80	75	88	78	82	79	75
Cytoplasms																																					
C Group																																					
Bb			5	1							5										1	5							5	5							1
C	1*	5	5			5	5	5	1	1	5	5		3	1	1	5	5	5	5	1	4	1		3	1		2	5	5	1		5		1	5	1
ES			5	3					1		5											5							5				5			4	1
PR			5	3							5											5							5	4							1
RB	3	5	5	1	1			3	1	1	5	5		3	1		5		5	4		4	1	4	3	1	1	2	5	4	1		5	1	1	5	1
T Group																																					
HA		1	1	1	1				1	5	5	3		1	1	1	5			2		1	1	1	1		1	1	5	1	1	1	1	1	1	1	1
P	1		3								4					1	3			1		1		1				3	4		1				1		1
Q	1		1	1	1			1	1	5	5	3		1	1	1	5			1		1		1	1	5		1	5	1	1				1	1	1
RS		1	1		1			1		5	5	3		1	1		5	1	1	1	1	1	1	1	1	5		1	5	1	1	1		1	1	1	1
SC							1		1																												1
T	1	1						1			5	1			1				1	1	1			1	1	5	1	1							1		1
S Group																																					
B			5	5							4		1	3		5	5		2	4		4						4	5		5				5		3
CA	3		3	3	3	3		3	3	3	3	3	1	5	1	3	3			3		1	5	1	1	1		1	5	4	3	3	1	1	5	1	1
D	3	5	5	5		5		3	5	5	5				3	5	5			5		4		1	4	1	3	3	5	5	4	5		5		1	3
RK	1	4	3	3	5		1	3		3	3	3	1	2	3	3	3	3	2	3	5	1	5	1	1		2	1	5	4	3	3	1	1	4	1	1
F		3	3		3						3					3	3			3		1						3	5		1				5		1
G	1	3	3	3	3	3			3	3	3	3	1	5	3	3	3		1	2		1		2	3	1	2	1	5	5	1		1		5	1	1
H		3	3				1	3	3	3	3	3	1	5	1	3		3	1	2	4	1		2	1	1	2	1	5		3		1		5	1	1
I		3	3	3				3	3	3	3	3	1	3	3	3	3	4	1	2		1	5	2	1	1	1	2	5	4	3	5		1	5	1	1
IA		3	3	3	3				3	3	3	3			3	3	3		1	2		1			2	1	2	1	5	4	3		1	2	4	3	1
J	1	3	3	3	3			3		3	3	3	1	5	1	3	5			3		1		2	1			2	5		3				5	1	1
K	1		3	3	3	3			3	3	3	3		5	3		3		1	2		1			3	1	2	2	5	5	2	3	3	1	4	1	1
L								3						5				3	1	3	4		5									3			5		1
M			3	3	3	3	1				3				1	3	3	4		3	5	1	5	1				2	5	4	3		1		5	1	1
ME	3		3	3	3	3		3	3	3	3	3				3	3			5		1	5	1	3	1		2	5	4	5	5		2	5	1	3
ML	1	3	3	3	3			3	3	3	3	3		5	1	3	3		2					1	1	1	1	2	5	5	2	3		1	5		1
MY	1	3	3	3	3	3		1		3	3	3	1		3	3	3		1	2		1		2	1	1	1	2	5	5	3	3		1	5	1	
PS	3	3		3	3			3	3	3	3	3	1		1	3	3	3	1	2		1	5	1	1	1	1	2	5	4	2	1		1	5	1	1
R		3	3					3		3	3		1			3	3	4		2		1						1	5		3		1		4		1
S	1		3	3						3	3					3	3			3		1		1				1	5		3				4	1	1
SD	1		3	1	3		1		3	3	3	3		5	1	3	3	4		2			5	1		1		1	5	5	1	3		1	5	2	1
TA	1	3	3	3	3			3		3	3		1			3	3		1	2		1	5	2				1	5		2				5	1	1
TC	1	3	3	3	3			3	3	3	3	3				3	3		1	2		1						1	5		3	5		1	5	1	1
VG		3	3								3			3		3	3		1	2		1						1	5		1				5		1
W		3	3					1			3		3			3	3		1	2		1	5					1	5		1				5		1

*Fertility ratings: 1. male sterile; 2. sterile anthers exserted; 3. partially fertile; 4. slightly subnormal; 5. fully fertile.

35 CYTOPLASMIC MALE-STERILE SYSTEMS IN MAIZE AND RECENT APPROACHES TO THEIR MOLECULAR INTERPRETATION

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In higher plants, the normal production and function of pollen grains is necessary in sexual reproduction. Pollen production represents the successful culmination of a complicated series of developmental events that are not well understood at the molecular level. Microsporogenesis commences with diploid pollen mother cells that undergo meiosis and yield haploid microspores which, in microgametogenesis, develop into pollen grains. These pollen grains carry the sperm cells that, after growth of the pollen tube and its entry into the embryo sac, are involved in the fertilization events that establish the basis for growth of embryo and endosperm, and ultimately, the development of the mature kernel.

Pollen grains that fail to develop normally are said to abort, and plants that fail altogether to produce functional pollen grains are said to be male-sterile. Certain structural alterations, such as chromosome inversions and chromosome translocations, produce meiotic complications so that plants carrying these chromosome aberrations in a heterozygous condition produce both normal and aborted pollen grains; such plants are said to be semisterile. Certain nuclear gene mutations, for example polymitotic (po), asynaptic (as) and ameiotic (am) in maize, also produce aberrant cell or chromosome behavior that leads to pollen abortion in the recessive homozygote. In the cases of many other strains arising from nuclear mutation events there are currently no clues concerning the underlying causes for the male-sterile condition; these strains are commonly designated ms ms, signifying the generally recessive nature of these mutations. In addition to the above categories of male-sterile plants, all of which trace their origins to underlying nuclear events, certain strains exhibit a type of male sterility that is not inherited according to Mendelian rules, but is instead transmitted maternally. The inheritance is basically extranuclear (see below) and the trait is commonly referred to as cytoplasmic male sterility (cms).

Although crossing experiments indicate that the primary determination of the male-sterile trait in cms strains is extranuclear, it is established, in many cases, that certain nuclear genes, called restorers of fertility (Rf) can overrule the male-sterile effect of the cytoplasm. Thus, cms plants are phenotypically male sterile unless they carry an Rf nuclear gene that can lead to the production of functional pollen in the presence of male-sterile cytoplasm. As indicated below, Rf genes are specific with regard to the cms strains that they restore.

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The first reported case of cytoplasmic male sterility (Bateson and Gairdner, 1921) involved two strains of flax that yielded male-sterile F_2 offspring when crossed in one direction, but not in the other. Since then, cms has been found in many higher plant groups. Edwardson (1970) indicates that cms has been identified in 153 species, 51 genera and 22 families of plants. It is apparent from this that both nuclear and extranuclear genetic determinants play an important role in the production of pollen.

The first case of cms in maize was described by Rhoades (1931, 1933). This male-sterile strain, of Peruvian origin, was lost but many other discoveries of cms in existing strains were later encountered by maize geneticists and breeders. Studies based on fertility restoration indicate, however, that these many cms strains can be placed in one of just several major categories. Thus, two types of cms were early recognized in maize (Duvick, 1965; Edwardson, 1970); one of these is cms-T (Texas) and the other is cms-S (USDA). A third major category, cms-C (Charrua), was identified by Beckett (1971), and it is possible that still others exist that have not yet been identified.

The three recognized types of cms in maize were originally identified on the basis of differing restoration responses in field studies. Thus, male-sterile strains T, P, Q, HA and RS represent independent discoveries of cms strains but all have been assigned to the cms-T group because they give similar male-fertile or male-sterile responses in the presence of a series of inbred line nuclear genotypes (Beckett, 1971), indicating that some inbred lines do, and others do not, carry the nuclear restorer genes for the cms-T group. On the same criteria, male-sterile strains C, RB and EL, as examples, were placed in the cms-C group, and male-sterile strains S, VG, RD, ML, I and J, as examples, were assigned to the cms-S group.

Two restorer gene loci, Rf and Rf2 are known to be involved in restoration of cms-T; Rf is located in chromosome 3 (Duvick, Snyder and Anderson, 1961), and Rf2 is assigned to chromosome 9 (Snyder and Duvick, 1969). Rf and Rf2 are dominant but both are required for phenotypic restoration. The restorer of cms-S, designated Rf3, carried by a number of established inbred lines, is located in chromosome 2 (Laughnan and Gabay, 1975). As indicated below, there is now evidence that a transposable element may also be involved in cms-S restoration, and that the restorer element may occupy other sites in addition to the standard one in chromosome 2. Many established inbred lines of maize are restorers of cms-C but there is, at this time, no published evidence of chromosome location for such a restorer. On the basis of ratios of male-fertile and male-sterile individuals in certain backcross and F_2 progenies, it was concluded (Kheyr-Pour, Gracen and Everett, 1981) that a single gene locus, designated Rf4, is involved in cms-C restoration, but a similar study (Josephson, Morgan and Arnold, 1978) suggests that several restorer loci may be involved. Preliminary evidence (Laughnan, Gabay-Laughnan and Patterson, unpublished) from experiments involving cms-C restorer strains and a standard series of translocation testers indicates restorer linkage with both T1-9 and T2-9; the data suggest there are at least two cms-C restorers, one in chromosome 1, and another in chromosome 2, and that they exhibit duplicate, rather than complementary, gene action.

While T, C and S clearly represent distinct cms groups, it is not necessarily true that the several independent discoveries represented by cms strains that have been placed within an individual cms group are identical. In fact there is some evidence, based on specific restoration responses, that minor heterogeneity exists within the S group (Gracen and Grogan, 1974), and that cms-T and cms-P, while being very similar, are not identical in their responses (Laughnan and Gabay-Laughnan, unpublished).

Aside from the differential restorer responses and the molecular evidence to be discussed later, cms-T, cms-S and cms-C have other distinguishing features. The T and C male-sterile cytoplasms are restored sporophytically, whereas S-type cytoplasm is restored gametophytically. For example, a cms-T plant that is heterozygous at both restorer gene loci, that is Rf rf Rf2 rf2, will produce all normal pollen even though being postmeiotic products, only one-fourth of the pollen grains from such a plant carry both restoring alleles Rf and Rf2. In the case of cms-S, on the other hand, it is the genotype of the pollen grain that is important for restoration (Buchert, 1961); thus the cms-S Rf3 rf3 plant produces one-half normal and one-half aborted pollen grains, the former having the restorer genotype Rf3, the latter the genotype rf3. It appears that in cms-T and cms-C plants that are heterozygous for the restorer, the "product" of the Rf allel in Rf-containing microspores is shared with rf microspores, but not so in the case of cms-S Rf rf plants. This suggests that the Rf "product" may be different in the two systems, or that, whether it is or not, it is produced at different developmental stages. In any case, these differences in mode of restoration must be considered in interpreting the molecular evidence to be presented later.

There has been a long-standing interest in the use of male-sterile plants in hybrid seed production. In maize, where the male and female reproductive elements are borne on the same plant, but separately in tassel and ear, respectively, the first commercial hybrid seed was produced by growing male-fertile inbred strains A and B, for example, in adjacent rows in a production field. Tassels of inbred line A, previously identified as a high quality seed bearing strain, were physically removed to insure that the vast majority of pollen in the field was from the strain B pollen plant and therefore that seeds borne on ears of the strain A plants carried hybrid embryos and were suitable for sale as superior hybrid seed. It was appreciated that if the female parents in production fields carried some form of genetic male sterility, the chore of hand detasseling could be avoided. Ordinarily, male sterility based on nuclear gene mutations (ms ms) is not suitable for this purpose since the male-sterile ms ms plants are produced from crosses that are either Ms ms x Ms ms or ms ms x Ms ms, and therefore, in either case, would occur along with male-fertile siblings in the progeny that would have to be detasseled. Patterson (1973) has shown that through the use of certain special genetic engineering approaches involving highly selected ms nuclear gene mutants and translocation hemizygotes it is possible to produce progenies that have close to 100% male sterile (ms ms) plants, and that are therefore suitable for use as female parents in commercial seed production. This method is now being reduced to practice.

Another obvious solution to this engineering problem lies in the use of cms. As a result of certain crosses, cms plants not carrying a nuclear restorer can be produced in pure stands, and when these are crossed in a

production field with restorer strains, the resulting seed will produce exclusively male-fertile hybrid offspring. One of the consequences of this method, and it has turned out to be a highly significant one from the commercial standpoint, is that all the hybrid plants grown in the farmers' fields carry the cms of the original female parent in the cross. Cms has been used in hybrid seed production in numbers of plant species, including onions, sorghum, sugar beet, sunflower and maize. In maize, all three types of cms have been involved in commercial production of hybrid seed, most notably cms-T. The use of cms-T in hybrid seed corn production was so successful that by 1970 more than 90% of corn grown in the U.S. carried cms-T. Its use was abruptly discontinued after the 1970 southern corn leaf blight epidemic caused by race T of the ascomycete Helminthosporium maydis. This race of H. maydis, it turned out, is specifically virulent on the plants with Texas cytoplasm.

The cause and consequences of the 1970 corn leaf blight epidemic have been reviewed by Ullstrup (1972). Numbers of studies indicated that the epidemic was caused by a specific interaction between a strain, race T, of the pathogen and maize plants carrying cms-T. Using a root assay method originally developed by Luke and Wheeler (1955), it was shown (Hooker et al., 1970; Wheeler, Williams and Young, 1971) that pathotoxin isolated from race T inhibits the elongation of primary roots of cms-T seedlings, but not those of seedlings with normal (nonsterile) cytoplasm, or with cms-C or cms-S. Similar inhibition of root growth of cms-T seedlings was observed in studies (Lim and Hooker, 1972) using extracts of leaf tissue from cms-T corn plants infected with race T of the fungus. This highly specific interaction was confirmed by studies (Laughnan and Gabay, 1973) showing that the pathotoxin extracted from lesions on cms-T plants infected with race T of H. maydis inhibited in vitro germination and growth of pollen from cms-T plants at concentrations that had no inhibitory effect on pollen from cms-S, cms-C and normal cytoplasm plants.

Some understanding of the molecular basis for the highly specific pathogenic response of cms-T plants to the H. maydis, race T pathogen has since been achieved. It was shown (Miller and Koeppe, 1971) that when mitochondria isolated from etiolated shoots of cms-T plants were placed under different physiological regimes and exposed to the pathotoxin from race T, they gave responses quite different from those of mitochondria from normal strains of maize. Specifically, the addition of pathotoxin caused organelle swelling and changes in oxidative phosphorylation and in respiratory rates. Other studies (Gengenbach, Koeppe and Miller, 1973; Gengenbach et al., 1973) have confirmed that cms-T mitochondria exhibit unique physiological responses to race T pathotoxin. In addition, the insecticide Lannate 90 (methomyl), is specifically toxic to cms-T plants (Humaydan and Scott, 1977), and has been shown (Koeppe, Cox and Malone, 1978) to produce physiological effects similar to those of race T pathotoxin on isolated cms-T mitochondria. The underlying cause of the altered physiological state of cms-T mitochondria, however, remains obscure. Since the mtDNA of cms-T plants is shown, on the basis of restriction enzyme analyses, to differ from that of normal cytoplasm maize (see below), it is tempting to consider that the basic genetic alteration resides in the mtDNA, but there is still no convincing evidence for this from mutational analysis.

Maize geneticists and breeders who have worked with cms-T maize in many different nuclear backgrounds, have had an unusual opportunity to

identify changes from male sterility to male fertility in such strains, but there have been no reports of such spontaneous reversions. As indicated earlier, however, there have been repeated discoveries of naturally occurring strains of maize that carry the Texas type of male-sterile cytoplasm. Interestingly, in each such case, the male-sterile trait was associated with susceptibility to infection by race T of H. maydis. One interpretation of these results is that the numbers of independent discoveries of cms-T originated from a single mutational event involving a cytoplasmic change from normal to Texas-type male sterility in a strain that already carried race-T susceptibility, or from resistance to susceptibility in a strain that already carried the Texas-type male sterility; according to this view, the male-sterility and susceptibility traits represent different and independently occuring lesions. On the other hand, T-type male sterility and race-T sensitivity may represent the phenotypic expression of a single genetic defect and, according to this interpretation, the independent discoveries of cms-T strains may represent independent mutational events in different strains of maize.

Recent studies in a number of laboratories dealing with induced or selected changes in cms-T strains support the notion that the male-sterile and susceptibility traits exhibited by such strains result from a common mutational defect. It was shown (Gengenbach and Green, 1975; Gengenbach, Green and Donovan, 1977) that certain cms-T strains of maize, when grown in tissue culture media containing H. maydis race T pathotoxin, produce callus growth that is toxin resistant, and that plants regenerated from such cms-T callus cultures are not only resistant to H. maydis and its pathotoxin but are male-fertile as well. Brettell, Thomas and Ingram (1980), employing similar strains of maize and corresponding culture conditions, confirmed these results but added the important finding that numbers of male-fertile, T-toxin resistant plants are also obtained from unselected (control) cultures grown in the absence of race T pathotoxin. In addition, there was one case of an unselected regenerant strain that was male-fertile but T-toxin sensitive. In all cases, the revertant traits, male fertility and T-toxin resistance, were shown to be maternally transmitted. These results support the idea that male sterility and T-toxin sensitivity are the result of a single cytoplasmic lesion. Further support for this notion comes from studies (Cassini et al., 1977; Cornu, Cassini and Berville, 1980) in which strains of cms-T maize were treated with ethyl methanesulfonate in some instances, and with gamma rays in others; seven mutant derivatives from these treatments proved to be both male-fertile and resistant to H. maydis race T.

Restriction endonuclease analyses of mitochondrial DNA isolated from the male-fertile, T-toxin resistant revertants described above give inconclusive results. In one case (Gengenbach et al., 1981), three of the four resistant lines analyzed showed slight differences in band patterns, but all were similar to cms-T rather than normal cytoplasm maize. Two other revertant strains showed a banding pattern indistinguishable from cms-T (Brettell, Thomas and Ingram, 1980). It was proposed earlier (Gengenbach, Green and Donovan, 1977) that cms-T strains of maize may have heterogeneous mitochondrial genomes, that is, cms-T and normal mitochondria, and that selection of cells with normal mitochondria occurs in toxin-containing tissue cultures. Since the male-fertile, resistant revertant strains described above appear to have mtDNA characteristics similar to those of cms-T strains, this interpretation appears not to be supported.

A basic mechanism for the occurrence of the male-fertile, T-resistant revertants described above is not apparent at the present time (Cornu and Gabay-Laughnan, see this volume). Some of the revertants occurred in tissue culture media that did not contain the T-toxin, and the mtDNA characteristics of revertant strains do not suggest a straightforward mutational basis for the changes. Indeed, while an altered physiological state of mitochondria in cms-T strains is clearly established, the question of whether or not the basic genetic alteration in cms-T strains resides in the mitochondrial genome remains unanswered at the present time (However, see Gengenbach and Pring, this publication).

While no spontaneous reversions from male-sterile to male-fertile phenotype have been reported in cms-C and cms-T strains, cms-S strains not infrequently revert spontaneously to the male-fertile state (Jones, 1956; Singh and Laughnan, 1972). It has since been established (Laughnan and Gabay, 1973) that such reversions to male fertility, initially recognized as male-fertile tassels or as tassel sectors on plants in male-sterile strains, are based on genetic changes at either the cytoplasmic or the nuclear level. We postulated (Laughnan and Gabay, 1973) that the male-fertility element in the cytoplasm may in fact be identical with the nuclear restorer gene. When the analysis of a sample of ten independently occurring nuclear revertants revealed that the restoring element in these strains was located in several different sites in different chromosomes, we proposed (Laughnan and Gabay, 1975, 1978) that S male-sterile plants carry an episomal fertility element (F) that is capable of being fixed either in the cytoplasm (cytoplasmic revertants), or in the nucleus (nuclear revertants), and thus leads to the male-fertile manifestation. Cytoplasmic and nuclear revertants are easily distinguished by straightforward crossing procedures and, in both cases, the newly-arisen male-fertile phenotype is inherited in ensuing generations and remains stable.

The advent of restriction enzyme technology provided an opportunity to characterize the DNA of cytoplasmic organelles at the molecular level. It was first shown (Levings and Pring, 1976) that samples of mtDNA from normal and cms-T strains of maize exhibit different banding patterns following endonuclease digestion and agarose gel electrophoresis. Later (Pring and Levings, 1978) it was shown by the same procedures, that each of the four maize cytoplasms, that is, normal, cms-C, cms-T and cms-S, has distinctive mtDNA characteristics, and that chloroplast DNA from the same sources exhibits only minor differences. These findings suggested that mtDNA may be the site of the genetic defects in cms strains of maize, and that mtDNA is the carrier of the genetic determiners of male fertility at the cytoplasmic level. This viewpoint was further supported by the finding that two low molecular weight plasmid-like double-stranded DNA molecules are associated with mtDNA preparations from cms-S maize, but are not found in mtDNA preparations from cms-C, cms-T or normal (male-fertile) maize (Pring *et al.*, 1977).

These plasmids, designated S1 and S2 (formerly S-S and S-F), have molecular weights estimated to be 4.1 and 3.5 x 10^6, respectively, are linear molecules, and in most cms-S strains occur in equimolar amounts. The S1 and S2 plasmids have similar reverse repeat sequences of approximately 200 base pairs at the termini of the duplex molecules (Levings and Pring, 1979), and restriction enzyme mapping studies reveal that S1 and S2 have approximately 1300 additional base pair sequences in common. Since the terminal reverse repeat sequences carried by the S1 and S2 mtDNA plasmids of cms-S

maize are reminiscent of the insertion sequences (IS) that have been described in numbers of transposable elements in prokaryotes (Bukhari, Shapiro and Adhya, 1977) it was inviting to consider that they equip the S1 and S2 plasmids for a similar role in integration and that the S1 and S2 plasmids may correspond to the episomal F factor proposed earlier (Laughnan and Gabay, 1975, 1978) on the basis of genetic studies.

As it turns out, the S1 and S2 plasmids are indeed involved in cytoplasmic reversion of cms-S strains to male fertility. Analyses of unrestricted mtDNA from seven cytoplasmic revertants reveals that in all such strains the S1 and S2 plasmids are no longer present in electrophoretograms (Levings et al., 1980). Moreover, restriction enzyme studies of mtDNA from such revertants, coupled with hybridization analyses using the 32p labelled S1 and S2 plasmids as probes (Kim et al., 1980; Levings et al., 1980) reveal that the reversion step from male-sterile to male-fertile in cms-S strains involves integration of plasmid sequences into the main mtDNA genome. It also appears, from these studies, that independently occurring cytoplasmic reversions of cms-S to male fertility are associated with the integration of plasmid sequences at different sites in the high molecular weight mitochondrial genome. This conclusion is based on the interesting finding that restricted mtDNAs from cytoplasmically reverted strains display unique bands that hybridize with labelled S1 and S2 plasmid probes.

In addition to the seven cytoplasmic revertant strains discussed above, 16 other cytoplasmically reverted male-fertile strains have recently been analyzed in our laboratory; in every case, the S1 and S2 plasmids present in the cms-S male-sterile source strains were found to be absent in the cytoplasmically reverted male-fertile strains. This association of cytoplasmic reversion of cms-S strains to male fertility with disappearance of S1 and S2 as unique plasmid species from the mtDNA represents the first unequivocal evidence that the genetic basis for extra-nuclear inheritance of cms-S male sterility is encoded in mtDNA.

The relationship between behavior of the S1 and S2 plasmids carried in the mtDNA of cms-S maize and reversions of some of these strains to male fertility has stimulated other investigations. Molecular hybridization studies involving the use of cloned partial S1 and S2 derivatives as probes (Thompson, Kemble and Flavell, 1980) indicate that the cloned labelled sequences exhibit strong homology with endonuclease restriction fragments from the high molecular weight mtDNA of normal, male-fertile maize, but show much weaker hybridizations with similar mtDNA preparations from cms-T, cms-C and cms-S strains. These results are consistent with the idea that the various cms strains originated by excision of integrated plasmid sequences from the higher molecular weight genome of a male-fertile ancestor (Levings et al., 1980). In another study, the mtDNA of normal-cytoplasm maize was cloned (Lonsdale, Thompson and Hodge, 1981) and use of S1 and S2 labelled probes indicated that the S1 and S2 sequences occur in the normal genome as single copies at unique sites, and that each is flanked by a 26 kilobase repeated sequence. Additional evidence is also now available concerning the occurrence of lower molecular weight plasmid-like elements in the mtDNA of normal and male-sterile maize. As indicated above, the S1 and S2 plasmids are found only in cms-S (Pring et al., 1977) strains and are implicated in cytoplasmic reversion to fertility (Levings et al., 1980). Mitochondria of all four cytoplasms, that is normal, cms-T, cms-C and cms-S, appear to contain a 1.94 kb. pair supercoiled circular DNA species (Levings

et al., 1980; Kemble and Bedbrook, 1980). In addition, normal, C and S mitochondria contain a DNA species of about 2.35 kb. pairs that is not present in T cytoplasm; and C mitochondria contain two circular DNA species, having about 1.57 and 1.42 kb. pairs, that are not found in the other cytoplasmic types (Kemble and Bedbrook, 1980). In summary, at least six plasmid-like mtDNA species have been identified among the four described maize cytoplasms. Except for S1 and S2, none of these is implicated in cytoplasmic reversion from male-sterile to male-fertile phenotype. In fact, it remains to be seen whether the characteristic distribution of the four smaller plasmid species among the normal and male-sterile mitochondrial genomes is functionally related to the male-fertile and male-sterile phenotypes. Alternatively, these differences in plasmid species may represent independent mutations that have occurred during the isolated evolutionary histories of the various male-sterile strains, and that may therefore have no role in determination of the male-sterile phenotype.

In most cms-S strains the S1 and S2 plasmids occur in approximately equimolar amounts (Pring et al., 1977) and restriction endonuclease analyses indicate that the replication of these plasmids is amplified about five-fold in comparison with the main high molecular-weight mitochondrial genome. There are, however, certain interesting exceptions; in the M825 inbred line background, cms-VG plants characteristically exhibit a striking reduction in the molar equivalents of S2 as compared with the S1 plasmid (Levings et al., 1980). While others have interpreted this difference to be a function of the cms-VG version of S-type cytoplasm, studies in our laboratory clearly indicate that it is, in fact, determined by the M825 nuclear genotype. As it turns out, M825 is the nuclear background in which cytoplasmic reversion occurs at an extraordinarily high rate (Laughnan and Gabay, 1975; 1978). Although there is not direct evidence to support the argument at this time, it is tempting to consider that the differential replication of S1 and S2 plasmids in the M825 nuclear background is related to the high reversion rate in M825 strains. That the nuclear genotype is in essential control of replication characteristics of the S1 and S2 plasmids in cms-S type strains is further indicated by the finding (Carlson, Gabay-Laughnan and Laughnan, this volume) that molar equivalents of the S1 plasmid are strikingly reduced in comparison with S2 in cms-S versions of the inbred line 38-11, and again, it is the nuclear genotype rather than the cytoplasmic genome that determines this difference.

Among 44 South American maize accessions whose mtDNA was examined by agarose gel electrophoresis, endonuclease fragment analysis and electron microscopy, twelve strains with unusual plasmid characteristics were identified (Weissinger et al., 1981). In each of these strains the mtDNA carried a plasmid that appears to be identical to the S2 plasmid carried by cms-S strains; each also exhibited another plasmid whose molecular weight exceeds that of S1 but which has a high degree of homology with S1. Since male sterility has so far not been identified in these strains, the significance of the presence of the S2-like plasmid and of the modified S1 plasmid in these sources remains obscure. S1 and S2 have heretofore been identified only in cms-S male-sterile strains; if the twelve South American strains that carry the modified version of the S1 plasmid are indeed cytoplasmically normal, that is, male-fertile, further studies of this plasmid, similar to those that led to restriction maps of S1 and S2 (Kim et al., submitted), may provide an important clue to the molecular basis for male sterility in S-type cytoplasms.

Are the differences in mtDNA exhibited by the several cms strains of maize reflected in unique patterns of messenger RNA and protein synthesis? In particular, do plasmids such as S1 and S2, and perhaps others, that appear to be uniquely associated with mtDNA of male-sterile strains such as cms-S, have coding function, and are they associated with identifiable functional products? Very little is known about altered transcriptional patterns in these strains but such studies are under way (Mans, this volume). Meanwhile, studies involving biosynthesis in isolated mitochondria (Forde, Oliver and Leaver, 1978; Forde and Leaver, 1980; Forde *et al*., 1980; Leaver and Gray, 1982) have identified discrete differences in mitochondrially synthesized polypeptides in normal maize and in the several cytoplasmic male-sterile strains, in both their restored and nonrestored versions. Mitochondria from each of the male-sterile cytoplasms C, T and S synthesize unique polypeptides, and in the case of cms-S, mitochondria synthesize eight characteristic high-molecular weight polypeptides that are not synthesized by mitochondria from cms-T, cms-C or normal male-fertile strains. Recent analysis of six cytoplasmic male-fertile revertant strains originating from cms-S male-sterile sources in our laboratory indicate (Leaver, personal communication) that mitochondria isolated from these revertant strains no longer synthesize the eight high molecular weight polypeptides that are characteristically synthesized by cms-S mitochondria. It is exciting to speculate that these high molecular weight polypeptides are encoded by the S1 and/or S2 plasmids of cms-S strains and that, if they are, they will provide an important clue to the functional basis for the S-type male sterility, but there is presently no evidence to support this view.

If the S1 and S2 plasmids represent a fertility episome whose presence in a certain form in the high molecular weight portion of the mtDNA genome of maize is necessary for the expression of male fertility, it is reasonable to expect that reversion of cms-S from male-sterile to male-fertile phenotype would be accompanied by transpositional events involving S1 and S2, and such appears to be the case (Levings *et al*., 1980). Whether the reversions to fertility that occur at the nuclear level, and these occur frequently in some strains (Laughnan and Gabay-Laughnan, this volume), involve corresponding transpositions in which plasmid sequences are integrated into nuclear chromosomes is uncertain at this time. The hypothesis is currently under investigation in a number of laboratories.

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36 NUCLEAR CONTROL OVER REVERSIONS TO MALE FERTILITY IN S MALE-STERILE MAIZE*

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In maize three different kinds of cytoplasmic male sterility (cms) have been identified, cms-C, cms-T and cms-S (see review, Laughnan, Gabay-Laughnan and Carlson, this volume). We have found cms-C and cms-T, measured in a variety of nuclear backgrounds, to be highly stable to reversion, but cms-S strains not infrequently revert spontaneously to the male-fertile state (Laughnan and Gabay, 1975). This event occurs more frequently in some inbred lines (nuclear backgrounds) than in others, and involves either a change in the cytoplasm, corresponding to a change from cms-S to normal (fertile), or a nuclear event, following which fertility is inherited in Mendelian fashion. We have shown (Laughnan and Gabay, 1978) that nuclear reversion is associated with a fertility element that maps at different chromosomal sites, a property that led us to describe the element as an episome.

Some genetic backgrounds are highly favorable to these reversions and others are relatively stable. Moreover, the genetic background also influences the proportion of such mutations that are cytoplasmic versus nuclear in origin. Among several strains of maize whose cms-S versions produce frequent mutations to male fertility one, designated M825, has a high frequency of cytoplasmic and low frequency of nuclear events. In another strain, WB4, the frequency of cytoplasmic events is low while the frequency of nuclear events is high. A third strain, M825/Oh07, has about equal frequencies of both.

To determine the relative influence of cytoplasm and nucleus on both the frequency and nature of the reversion event, we undertook a back-crossing program involving a number of inbred lines carrying different subgroups of S cytoplasm as the nonrecurrent female parent, and the inbred line M825 as the recurrent male (maintainer) parent. Seven inbred lines that do not restore cms-S, that is WF9, 38-11, N6, K55, M14, I153 and IllA, and five subgroups of S cytoplasm, S, VG, I, ML and RD, were represented among the nonrecurrent female parents. In all, 14 different line-cytoplasm combinations were employed. Each of these, as an original female parent, is relatively stable, with reversion frequencies ranging from 0.0 to 1.9%. By contrast, 10.0% of M825 cms-VG plants exhibited reversion.

The backcrossing program referred to above, undertaken without selecting for high mutation rate, is now in the tenth backcross. The second (3X) and sixth (7X) backcross generations of this experiment were grown in numbers and plants were scored for reversion events. Plants from the 14 inbred line-cytoplasm combinations representing the original nonrecurrent

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parents (OX) in the experiment were also searched for reversions. On the basis of subsequent analysis of the revertant strains it was determined whether the change occurred at the cytoplasmic or nuclear level. For some revertants it was not possible to make this determination.

Continued backcrossing by the M825 male parent maintainer line in this experiment is expected to produce plants that have the nuclear genes of M825 line (high frequency reversion), while retaining the cytoplasmic genotypes of the original nonrecurrent S male-sterile inbred lines (low frequency reversion). This provides an opportunity to determine the relative influence of cytoplasm and nucleus on reversion rates. The results indicate a preponderant influence of the nuclear genome of M825 in this regard. Table 1 summarizes data on reversion frequencies in the original line-cytoplasm sources and in progenies after 3 and 7 crosses with M825.

Table 1

Summarized frequencies of male-fertile revertants in inbred line and two backcross generations involving inbred line M825 as recurrent male parent*

Generation	Number of plants	Number of revertants	Reversion frequency (%)
Inbred line (no cross x M825) (= OX)	5355	24	0.45
2nd backcross x M825 (= 3X)	2286	48	2.1
6th backcross x M825 (= 7X)	2044	169	8.3

* 14 line-cytoplasm combinations were involved as nonrecurrent female parents and as the sources of cytoplasms in ensuing backcross generations.

The data indicate that after 3 generations the reversion frequency is increased over four-fold, from 0.45% in the inbred line male-sterile backgrounds (OX) to 2.1% in the 3X generation. After four more crosses with M825, in the 7X generation, the reversion frequency is 8.3%. While statistical analysis indicates that this value is still significantly lower than the rate of 10.9% for the M825 strains, it closely approaches it. Moreover, two of the 14 pedigrees, WF9-RD and N6-VG, after 7 crosses with M825, had reversion rates that were significantly higher than the M825 strain, and three others had rates that were not significantly different from M825. To summarize, the near replacement of the inbred line nuclear genomes of 14

line-cytoplasm sources with the M825 nuclear genome results in an increase in spontaneous reversion rate from below 1% to an average rate of 8.3%, which is close to the frequency characteristic of the recurrent M825 strain itself.

The M825-VG strain, whose maintainer counterpart was used as the recurrent parent in this experiment, produces overwhelmingly cytoplasmic reversions, these being estimated at greater than 95% of the total. As indicated in Table 2, about 32% of the reversions occurring spontaneously in the inbred line male-sterile sources (OX generation) are cytoplasmic.

Table 2

Origin of male-fertile revertants summarized in Table 1 *

Generation	No. of revertants analyzed	No. of cytoplasmic revertants	No. of nuclear revertants	Frequency of cytoplasmic revertants (%)
Inbred line** (no cross x M825) (= OX)	19	6	13	31.6
2nd backcross*** x M825 (=3X)	33	25	8	75.8
6th backcross*** x M825 (= 7X)	129	125	4	96.9

* Not all revertants could be analyzed.
** Cytoplasmic vs. nuclear origin of revertants determined by pollen analysis.
*** Cytoplasmic vs. nuclear origin of revertants determined by progeny test.

After three generations of M825 crosses (3X), the proportion of cytoplasmic cases rises to 76%, and after four more M825 crosses (7X), the proportion of revertants that are cytoplasmic, 96.9%, has reached or exceeded the frequency in M825 itself. It should be noted that this shift in proportion of cytoplasmic:nuclear cases is based on both an absolute increase in frequency of cytoplasmic reversions as well as an absolute decrease in frequency of nuclear revertants. It should be mentioned also that the 14 male-sterile sources used as the nonrecurrent parents in this experiment probably differ in reversion characteristics. For example, in six of these sources no reversions have yet been identified, and in the remaining 8 sources, reversion frequencies range from 0.2% to 1.9%, and the total numbers of cases in individual sources are too few to allow us to characterize them in regard to the basis for reversion. Nevertheless, the shift in favor of cytoplasmic reversion that occurs from 3X to 7X (Table 2) was noted in all 14 pedigrees. We conclude that the nuclear genome has predominant influence

not only over the frequency of the reversion event, but over the site of reversion as well.

Cytoplasmic reversion to male fertility is associated with behavior of the S1 and S2 plasmids found in the mitochondrial DNA of cms-S strains (Pring et al., 1977). There is evidence (Levings et al., 1980) that the reversion event involves transposition of these plasmid molecules. The information presented here indicates that the molecular events connected with cytoplasmic and nuclear reversions are controlled predominantly by nuclear genes which, in the former case at least, govern S1 and S2 plasmid behavior. A companion paper in this volume (Carlson, Gabay-Laughnan and Laughnan) provides direct evidence that nuclear genes influence the replication of S1 and S2 mtDNA plasmids.

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37 NUCLEO-CYTOPLASMIC INTERACTIONS IN cms-S OF MAIZE*

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The S type of cytoplasmic male sterility (cms-S) in maize can be distinguished from cms-T and cms-C not only on the basis of its restoration pattern (Duvick, 1965), but by the presence of unique plasmid-like DNAs in cms-S mitochondria (Pring et al., 1977; Kemble et al., 1980) and by the greater relative instability of cms-S strains (Laughnan and Gabay, 1973).

Spontaneous reversions to fertility in cms-S occur by heritable mutations at either the cytoplasmic or nuclear level (Singh and Laughnan, 1972). As indicated elsewhere in this volume (Laughnan and Gabay-Laughnan), the nuclear genotype strongly influences both the frequency of cms-S reversions and the origin of the event. For example, reversions of cms-S plants to fertility in inbred M825 occur at a high frequency and are predominantly cytoplasmic, while reversions are observed at a much lower frequency and typically segregate in a Mendelian fashion in the inbred WB4.

We have found as well that the nucleus exhibits some control over the plasmid-like S1 and S2 DNAs in the mitochondria of cms-S plants. When Levings et al. (1980) reported that a "VG" cytoplasmic strain of cms-S has a greatly reduced amount of S2 relative to Sl DNA, while an "S" isolate of cms-S yields equimolar quantities of the two plasmids, it was suspected that the cytoplasm conditions this difference. An alternative hypothesis holds that the difference could be traced to the nuclear genotype since the VG cytoplasm was carried by hybrid M825/Oh07 while the S cytoplasm was carried by inbred B37. In fact, the latter explanation is supported.

Regardless of which version of cms-S cytoplasm (be it S, VG, RD, ML, I or J) the mitochondrial DNA is isolated from, in most nuclear backgrounds (e.g. inbreds W23, WF9, N6, K55, M14, 0h51A) the S1 and S2 plasmid-like DNAs are represented in nearly equimolar amounts, as determined with densitometry. Thus, VG cytoplasm has a reduced amount of S2 DNA in the M825/Oh07 background but equimolar levels of the plasmid-like DNAs in many other inbreds. Moreover, each S-type cytoplasm examined in inbred line M825 nuclear background exhibits the reduction in S2 level, with the ratios of S1:S2 DNA falling around 5:1. It appears, therefore, that the nuclear contributions of M825 alone causes the reduction in S2.

Another interesting finding is that cms-S cytoplasms, when carried by the inbred 38-11, are characterized by the opposite situation in which the S1 DNA level is greatly reduced while the S2 level is maintained. The amount of S2 DNA from 38-11 cms-S plants exceeds the amount of S1 DNA by a factor of 3 or more. Figure 1 is a diagrammatic representation of the altered levels of S1 and S2 DNA in the two strains discussed above compared with the equimolar levels characteristic of inbred line W23. Thus the influence of

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putative nuclear genes over components of maize mitochondrial DNA appears to be quite specific, not just an overall regulation of mitochondrial DNA replication.

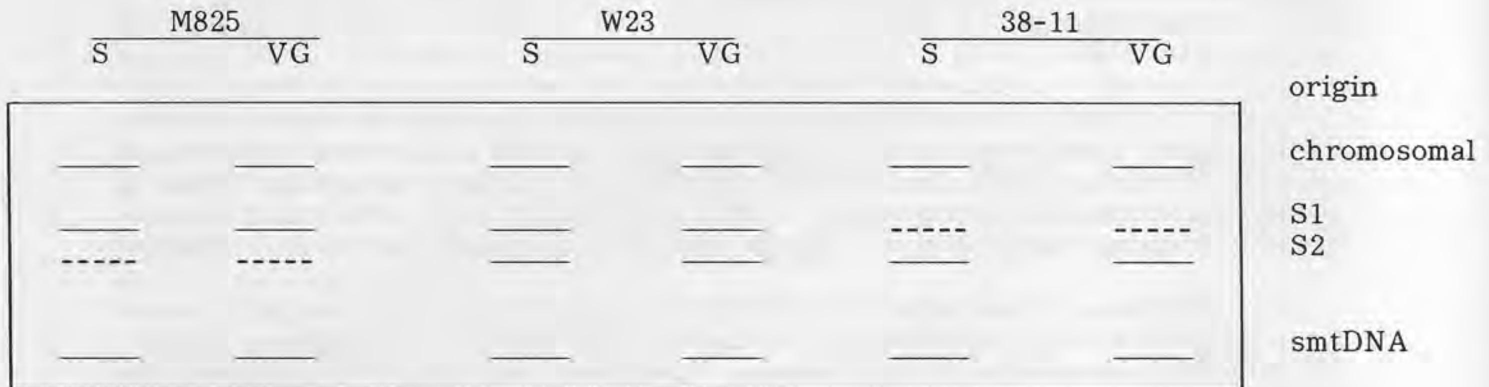

Figure 1. Diagrammatic representation of agarose gel electrophoresis of mitochondrial lysates prepared from S and VG strains of cms-S cytoplasms in three inbred nuclear backgrounds, M825, W23, and 38-11. Bands correspond to DNA stained with ethidium bromide and labelled to the right of the figure. "smt" refers to small (1.2 x 10^6 Daltons) mitochondrial DNA present in all lines.

Progeny from the backcrossing program detailed by Laughnan and Gabay-Laughnan (this volume) were available for analysis of mitochondrial DNA. As frequencies and types of reversion shift from those characteristic of the initial female parent to those characteristic of the recurrent male parent over succeeding generations of backcrossing there is a concurrent conversion of S1 and S2 DNA levels to those of the recurrent male parent. For example, the inbred 38-11 containing cms-S cytoplasm was used as the initial female parent in a series of backcrosses with M825 maintainer as the recurrent male parent. By the third cross to M825 the relative plasmid levels had switched from the S2 greater than S1 pattern typical of the 38-11 background to the equimolar pattern. Two generations later the pattern had shifted entirely to that of an M825 inbred, that is S1 greater than S2 pattern. In each of the backcross schemes examined no changes in DNA levels could be detected after only one cross. This lag could be the result of dominance involving nuclear controlling genes, or of a delay in attainment of an equilibrium with regard to mitochrondrial elements.

In summary, the phenomena observed in the cms-S system clearly exemplify nuclear genetic control over cms-S reversion to fertility, as well as over the mitochondrial DNA itself. More in-depth studies are currently underway in the hope of gaining a better understanding of these nucleo-cytoplasmic relationships.

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38 REVERSIONS OF T MALE-STERILE CYTOPLASM TO MALE FERTILITY

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Many types of cytoplasmic male sterility (cms), characterized by abortion of pollen after meiosis and maternal inheritance, are known in maize. They have been classified into three main groups, C, T and S, according to the results of their testcrosses with maintainer and restorer lines. Within each group, all male sterile types are restored by the same specific lines (Beckett, 1971; Gracen and Grogan, 1974). This classification was recently confirmed by work related to mitochondrial DNA (mt-DNA). Each group can be recognized by its mt-DNA species detected by agarose gel electrophoresis (Kemble et al. 1980), by restriction endonuclease banding patterns of its mt-DNA (Pring and Levings 1978) and by its mitochondrial translation products (Forde et al. 1980).

Widely used for hybrid seed production until 1970, cms-T was abandoned because of its specific susceptibility to the fungus Helminthosporium maydis, Nisikado and Miyake race T. The studies performed on the mode of action of H. maydis race T (HmT) and its toxin led to the discovery that this toxin acts at the cellular level. Therefore, it is possible to set up methods for measuring the susceptibility of a given plant to this fungus or its toxin at any developmental stage. This apparent facility of screening prompted some research teams to undertake experiments in order to obtain plants with T male-sterile cytoplasm resistant to HmT.

Two main techniques were employed to reach this goal: (1) tissue culture selection and (2) mutagenesis.

1) Using an immature embryo culture method, Gengenbach et al. (1977) obtained tissue cultures of T male-sterile cytoplasm maize on medium containing pathotoxin of HmT (Experiment 1A). After several selection cycles, regenerated plants appeared toxin-resistant, most of them being male-fertile. Using the same culture method and the same material, Brettell et. al. (1980) initiated tissue cultures in medium with and without toxin. "Fertile, T-toxin resistant plants were obtained from the unselected control cultures as well as from the selected material. In addition, one regenerant from an unselected culture was fertile and T-toxin sensitive" (Experiment 1B).

2) Mutagen treatments (E.M.S. and x-rays) were applied to dry seeds of an inbred line carrying T male-sterile cytoplasm. Plants originating from treated seeds were crossed by a restorer line and produced male fertile progeny (M2). These yielded, by self-pollination, numerous M3 progenies. Among these M3 plants, several families proved to be toxin-resistant (Experiment 2). After several screenings in subsequent generations, seven families appeared totally HmT-resistant and male fertile (Cassini et al. 1977; Cornu et al. 1980).

Analyses of resistant families obtained in the three series of experiments (1A, 1B and 2) gave similar results that can be summarized as follows:

1) The new traits obtained, i.e., male fertility and resistance to HmT, are transmitted as extranuclear traits. Mitochondria isolated from resistant plants (Experiments 1A and 2) were shown to be resistant to the HmT toxin (Berville 1978; Gengenbach et al. 1977).

2) Typical T male sterility disappeared in such families except in B - type families of Experiment 2 which are characterized by an unstable resistance (Cornu et al. 1980). Abnormal male-sterile plants sometimes appeared in the progenies of resistant families (Experiments 1A, 1B and 2). However, these male-sterile types cannot be maintained by the usual maintainer lines and cannot be attributed to a stable cytoplasmic factor (Gengenbach et al. 1981; Cornu et al. 1981; Brettell et al. 1980).

3) The newly HmT-resistant plants are also resistant to the toxin of Phyllosticta maydis, Arny and Nelson, (Experiments 1A and 2) and to the insecticide methomyl (Experiment 2) (Gengenbach et al. 1981; Cornu et al. 1981). According to the host-pathogen relations, these new cytoplasmic types seem to be identical to the N (normal) cytoplasm types.

Analyses of mt-DNA isolated from revertant plants gave divergent results:

1) Restriction endonuclease digestion patterns: resistant lines from Experiment 1A exhibited patterns distinct from each other and from T and N cytoplasms (Gengenbach et al. 1981). However, preliminary analyses of the resistant lines from Experiment 2 suggest a mt-DNA organization similar to that of N plants (Leaver, personal communication; Paillard and Berville, in preparation).

2) Electrophoretic analyses of (nondigested) mt-DNA: Kemble and Bedbrook (1980), using material from Experiment 1B, found the same mitochondrial DNA banding as cms-T.

3) Analyses of mitochondrial translation products: mt-DNA isolated from plants of Experiment 2 yielded the same products as those of N mitochondria (Forde and Leaver 1979).

A general explanation of the genetic events leading to phenotypic reversions of the T-plasmon is not evident. It is clear that these genetic events are not necessarily dependent on a selection pressure. While revertants were obtained from mutagen treated material (Experiment 2), some revertants originated from in vitro culture (Experiment 1B) and were induced without toxin selection. In the three series of experiments, the phenotypic characteristics of the revertants were very similar. On the contrary, the mt-DNA changes observed in revertants from the different sources were divergent. Nevertheless, these differences between the revertants and their source material seem to suggest that discrete alterations of mt-DNA could be related to the phenotypic traits observed.

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39 EFFECTS OF *HELMINTHOSPORIUM MAYDIS* RACE T TOXIN ON MITOCHONDRIA AND PROTOPLASTS FROM T CYTOPLASM MAIZE

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The 1970 epidemic of Southern Corn Leaf Blight, which caused serious crop losses in the U.S., drew attention to an attractive experimental system for study of the involvement of mitochondria in disease-resistance and male-sterility. Crop damage resulted from a combination of two factors: 1) much of the maize grown in 1970 contained Texas (T) cytoplasm, a male-sterile cytoplasm widely used for hybrid production because it reliably sterilized many inbreds in many environments and 2) the fungal pathogen *Helminthosporium maydis* Race T (HmT) caused much more severe damage on T cytoplasm maize than on maize with normal male-fertile (N) or other male-sterile (e.g. C or S) cytoplasms.

The crop losses in 1970 led to prompt abandonment of T cytoplasm for hybrid maize production and heightened concern about genetic uniformity of crop plants. The biological basis of the two cytoplasmically inherited traits (susceptibility to disease and T male-sterility) remains an intriguing problem which has been studied at many different levels (for review, see Gregory *et al.*, 1977).

Studies of the interaction of HmT with T cytoplasm are facilitated by the fact that the fungus produces a toxin (HmT toxin) with the same specificity for T material as the pathogen itself (Smedegard-Petersen and Nelson, 1969; Lim and Hooker, 1972). The toxin can be obtained either from infected plant tissue or, more commonly, from culture medium in which the fungus has grown. Diluted culture filtrate has been successfully used in some experiments but extraction and purification of the filtrate raises activity and specificity. Kono and Daly (1979) have isolated and analysed a highly active and T cytoplasm-specific toxin preparation for which they propose a polyketide structure with some 41 carbon atoms.

Treatment with HmT toxin causes many different effects on T cytoplasm material, including formation of leaf lesions, inhibition of root growth and dark CO_2 fixation, stimulation of electrolyte leakage, etc. (summarized in Gregory *et al.*, 1977). Most of these are clearly secondary effects of earlier damage. Evidence currently available suggests that mitochondria are the primary site of toxin action. The cytoplasmic inheritance of toxin sensitivity and the marked effects of HmT toxin on non-green tissue like seedling roots direct attention to mitochondria as a plausible site for relevant genes and gene products. Differences seen between mitochondrial (but not chloroplast) DNA of the various maize cytoplasms also tend to implicate the mitochondria (Levings and Pring, 1979; Kemble and Bedbrook, 1980). Moreover, low levels of toxin cause rapid uncoupling of oxidative phosphorylation and inhibition or stimulation of respiration (depending on substrate supplied) in mitochondria isolated from T material (Miller and Koeppe, 1971). Spectrophotometric and ultrastructural studies of such isolated mitochondria indicate that toxin induces swelling and disruption of cristae (Gengenbach *et al.*, 1973; York *et al.*, 1980). Mitochondria isolated from N, C or S cytoplasms show no such changes.

Involvement of mitochondria in toxin action is further supported by studies of toxin-treated leaf mesophyll protoplasts (Earle, 1982). Good yields of such protoplasts (5×10^6/g.F.W.) can readily be obtained from young maize leaves abraded with Carborundum and incubated for 4 hours in 2% cellulysin in 0.5M sorbitol + 10mM $CaCl_2 \cdot 2H_2O$ (Earle et al., 1978). The protoplasts remain intact for more than a week even when cultured in a simple osmoticum (e.g. 0.5M sorbitol + 10mM $CaCl_2 \cdot 2H_2O$); they survive longer and form walls but do not divide when cultured in a more complete nutrient medium. Addition of HmT toxin to the culture medium has no obvious rapid effect (e.g. shrinkage or bursting) on T protoplasts but after 12-24 hours treated T protoplasts look markedly different from controls (Pelcher et al., 1975; Earle et al., 1978). They look distorted or bulging, fail to expand, and no longer have distinct disc-like chloroplasts. Within 1-3 days (depending on toxin concentration used) all toxin-treated T protoplasts collapse, leaving clusters of damaged dark-green chloroplasts. Thirty minutes of exposure to toxin followed by thorough washing causes the same effects on T protoplasts as continuous culture in toxin. Apparently the protoplasts are irreversibly damaged within 30 minutes (Earle et al., 1978).

The results described above are obtained using freshly isolated green leaf mesophyll protoplasts from inbred W64AT, cultured in the dark. Change of nuclear genotype, use of cultured rather than freshly isolated protoplasts, incubation in the light, or use of non-green roots or albino leaves can increase the time or toxin concentration required for collapse of all treated T protoplasts (Earle et al., 1978; Earle, 1982). When these variables are carefully controlled, T protoplasts are destroyed by only a few ng/ml of the Kono and Daly (1979) toxin preparation, a concentration comparable to that active against isolated T mitochondria (Gregory et al., 1980; Payne et al., 1980). Normal protoplasts are unaffected by long-term culture in very high levels of purified toxin and by levels of crude culture filtrate 1000x higher than those that cause severe damage to T protoplasts (Gregory et al., 1980; Earle et al., 1978). Thus light microscope examination of protoplast populations provides a very sensitive and cytoplasm specific assay for HmT toxin.

Light microscopy, of course, cannot pinpoint the subcellular basis of HmT toxin action. Suspensions of protoplasts do provide large numbers (10^5-10^6/ml) of individual 'cells', uniformly exposed to surrounding solutions; absence of walls or multicellular clumps minimizes diffusion gradients. These features, coupled with high sensitivity, make protoplasts excellent material for ultrastructural and biochemical studies of early effects of HmT toxin on T cytoplasm cells.

The first ultrastructural changes seen in toxin-treated T protoplasts are alterations of the mitochondria similar to those seen in isolated T mitochondria (York et al., 1980). Some swelling, disruption of cristae, and leaching of the matrix can be detected within 5 minutes; by 60 minutes (at high toxin concentrations) all mitochondria are severely damaged. Such rapid mitochondrial damage occurs both in fresh and cultured, green and non-green T protoplasts, cultured in light or dark.

It is striking that comparable mitochondrial changes have been reported as the earliest ultrastructural feature of pollen abortion in T anthers (Warmke and Lee, 1977). In contrast, C and S male-sterile anthers show no

mitochondrial changes early in pollen abortion (Lee et al., 1979, 1980). Flavell (1974) has hypothesized that during pollen development a substance analagous to HmT toxin is produced; such an 'anther toxin' might be responsible for mitochondrial damage and subsequent pollen abortion in T anthers.

The ultrastructural evidence for a primary (or at least very early) effect of HmT toxin on T mitochondria is supported by biochemical studies of ATP levels in T protoplasts. Luciferin-luciferase assays show that within 1-2 minutes after toxin treatment, steady state ATP levels of dark-grown T (but not N) protoplasts drop sharply (Walton et al., 1979). Apparently oxidative phosphorylation of T mitochondria is inhibited in situ as well as in isolated mitochondria. The rapid decrease in ATP supply may be responsible for the many later degenerative changes seen in toxin-treated T material.

When green toxin-treated T protoplasts are cultured in light, their ATP content decreases much more slowly than in the dark. This probably accounts for the observation that such light-grown protoplasts look healthier in the light microscope and also at the EM level (except for mitochondrial damage) than dark-grown ones. In the light the chloroplasts produce sufficient ATP to maintain cellular functions for a significant period after the mitochondria have been destroyed.

It is not yet clear how HmT toxin affects oxidative phosphorylation of T mitochondria, how differences between the mtDNA and mtDNA translation products of T and other maize cytoplasms affect toxin sensitivity, and how toxin-sensitivity of T somatic cells relates to pollen abortion in T anthers. Even now, however, the ability of HmT toxin to distinguish T protoplasts and mitochondria from those of other maize cytoplasms makes the toxin a promising selective agent for genetic manipulations. 'Hybrid' protoplasts created by fusion of T mesophyll protoplasts with toxin-resistant protoplasts from maize or other species survive prolonged exposure to HmT toxin (Earle and Gracen, 1979; Earle, 1982). Attempts to transfer mitochondria isolated from toxin-resistant tissues into T protoplasts are now underway at Cornell. Success in conferring stable toxin resistance by a mitochondrial transfer would be strong evidence that mitochondrial genes control response to the toxin. Because HmT toxin destroys all sensitive protoplasts within a few days, successful transfer of sufficient functional mitochondria to give toxin-resistance could be detected rapidly. Transfer techniques developed in this simple system might facilitate experiments testing the role of mitochondria in other cytoplasmically inherited traits detectable only in tissue masses or in whole plants.

Analysis of mitochondrial DNA and proteins in callus or plants obtained via protoplast fusion or organelle transfer would be of great interest. Unfortunately, maize protoplasts capable of division and callus formation have been obtained only from a few N cytoplasm cell cultures (Potrykus et al., 1979; Chourey and Zurawski, 1981). No comparable T cytoplasm cultures are available, and no success in inducing division of maize leaf protoplasts has yet been reported. Some hybrid cell clumps have been obtained after fusion of T leaf protoplasts with protoplasts from soybean or sorghum cell cultures (Kao et al., 1974; Brar et al., 1980), but callus from fusion of maize protoplasts with different cytoplasms and the same nuclear genotype would be more valuable material. Plant regeneration from such callus is at present an even more remote goal.

Still, the combination of cell culture techniques with selection for a mitochondrial trait via HmT toxin provides a very attractive experimental system. Selection for toxin-resistance in regenerating maize scutellar callus has already yielded disease-resistant plants and has confirmed the linkage between T cytoplasm and sensitivity to H. maydis race T (Gengenbach et al., 1977). As maize protoplast culture techniques improve, genetic manipulations with protoplasts, enucleated protoplasts or cytoplasts, isolated mitochondria or isolated mtDNA may also provide cells and/or plants of great value both to molecular biologists and to plant breeders.

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40 ISOLATION OF REVERTANTS FROM CMS-T BY TISSUE CULTURE TECHNIQUES

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Texas male-sterile cytoplasm (CMS-T) conditions an extranuclear state which, in the presence of homozygous recessive alleles for either of two nuclear genes (Rf_1/rf_1 and Rf_2/rf_2), causes pollen abortion and male sterility. CMS-T was discovered in 1944 (Rogers and Edwardson, 1952) and has since been studied extensively and used widely in the production of hybrid corn seed (see Duvick, 1965, for review). The expression and inheritance of CMS-T in sexually propagated lines is a very stable trait. In the proper nuclear backgrounds CMS-T is uniformly expressed across a wide range of field environments. Also there have been no documented cases of CMS-T reverting to an extranuclear state that conditions male fertility (i.e., "normal" or N cytoplasm) among the numerous CMS-T plants that have been grown in hybrid seed production fields. This article describes and summarizes work which shows that changes from the CMS-T state to a male-fertile cytoplasm do occur in plants regenerated from CMS-T maize tissue cultures.

The tissue culture work summarized here was done at Cambridge University (Brettell et al. 1979, 1980) and the University of Minnesota (Gengenbach and Green, 1975; Gengenbach et al., 1977, 1981); details and much of the data may be found in the above references. Three factors led to the initiation of this work: (1) the development of an effective procedure for regenerating plants from maize tissue cultures (Green and Phillips, 1975), (2) the availability of a cytoplasm-specific pathotoxin produced by the southern corn leaf blight fungus, Helminthosporium maydis (Nisikado and Miyake) race T (Hooker et al. 1970) and (3) ready access to toxin-resistant (N) and toxin-susceptible (CMS-T) maize lines.

In the Minnesota study, maize tissue cultures were initiated from immature embryos of the inbred A188N (normal or non-sterile cytoplasm) and a backcross-derived A188 line with CMS-T. Suitable toxin preparations were obtained from shake cultures of H. maydis race T after modest purification steps. When incorporated into media the toxin had no deleterious effects on growth of A188N cultures, but caused a concentration-dependent inhibition of A188CMS-T tissue culture growth. Thus, the cytoplasm-specific effects of the toxin were manifested at the cellular level.

Selection of toxin resistance in A188CMS-T cultures was accomplished by a series of transfers (at about 4-5 week intervals) to media containing successively higher concentrations of toxin. Growth of cultures was only slightly inhibited (ca. 0-25%) during the initial selection cycle. The fastest growing tissue that also appeared to retain plant regeneration capability was

chosen at each transfer. During the fifth selection cycle, two rapidly growing cultures were obtained on media containing toxin sufficient to kill nearly all other cultures. From these two cell lines and from 15 other resistant lines subsequently obtained 167 plants were regenerated over a period of 18 months.

These plants were grown to maturity and characterized for toxin reactions by making a paper-punch hole in a leaf and inserting a toxin-soaked piece of filter paper or pipe cleaner into the hole. All 167 plants were resistant based on the absence of chlorotic lesions emanating from the point of treatment (such lesions are characteristic of CMS-T control plants, but absent from A188N controls). In addition to being resistant, 97 plants were male fertile as evidenced by their ability to shed fertile pollen from exserted anthers. The remaining 70 resistant plants did not produce fertile pollen which in many cases could be attributed to abnormal plant morphology. Although no progeny were obtained from pollinations of ears of such sterile resistant plants, several "tassel seeds" were obtained from one sterile plant that had a pistilloid tassel. These progeny were resistant and male fertile. Our results indicated that many plants regenerated from the selected toxin-resistant tissue cultures also were fully male fertile even though the cultures were initiated from a toxin-susceptible, male-sterile CMS-T line. In addition to the 167 resistant regenerated plants, nine plants were regenerated from susceptible cultures after the fourth selection cycle. These plants were toxin susceptible and male sterile and indistinguishable from CMS-T controls.

Regenerated plants were self-pollinated or crossed by A188N. Many male-fertile plants also were crossed onto CMS-T female plants. Genetic tests have now been completed through five sexual generations for lines derived from six resistant male-fertile plants and four susceptible, male-sterile plants. Results of all genetic tests conducted to date at Minnesota are consistent with the following conclusion: toxin resistance and male fertility are stable, cytoplasmically-inherited traits that are not separable either by selection for resistance in CMS-T tissue cultures or by subsequent sexual crosses.

The inheritance data reported by Brettell _et_ _al_. (1980) support the above conclusion. Their study was very important because it also revealed that analogous changes could be obtained in plants from CMS-T cultures that had not been subjected to selection for toxin resistance. Thirty-five of 60 plants regenerated from their unselected CMS-T cultures were toxin resistant. The remaining plants were toxin susceptible and, except for one male-fertile plant, also were male sterile.

Umbeck and Gengenbach (unpublished) also regenerated plants from unselected A188CMS-T cultures and obtained six fully male-fertile plants, two plants with fertile tassel sectors, and 154 male-sterile plants. Two of the fertile plants were toxin susceptible, but progeny from ears of those plants were either resistant and fertile (224 plants) or susceptible and sterile (3 plants). Somatic sorting out of different cytoplasmic conditions could account for this apparent disassociation of male sterility and toxin susceptibility within individual regenerated plants. The fact remains, however, that no new stable combination of these traits has been obtained in progeny from regenerated plants to date. These results and those described by Brettell _et_ _al_. (1980) indicate that CMS-T cultures undergo changes that

result in regenerated plants with an apparent "revertant" phenotype corresponding to that of N cytoplasm. From the data to date it is clear that the frequency of recovering "revertant" plants varies; 35/60 in Brettell's study, 8/162 in Umbeck's study and 0/67 from a third independent set of A188CMS-T cultures (Gengenbach, unpublished). The factors that mediate or influence such changes in CMS-T cultures are not known but could involve culture age and genotype, or unintentional stresses.

Restriction endonuclease analyses of organellar DNAs, especially mitochondrial DNA (mtDNA), have been very useful in delineating differences among different sources of maize cytoplasms. Similar analyses are beginning to reveal several interesting and important mtDNA features of both the selected and nonselected toxin-resistant, male-fertile lines obtained from CMS-T tissue cultures. Mitochondrial DNA was isolated from young green plants according to the procedure of Pring and Levings (1978) or from etiolated seedlings according to the procedure of Kemble et al. (1980) for many lines derived from plants regenerated in the Minnesota studies. Some of the initial results have been published (Gengenbach et al., 1981) and Table 1 is a current summary of our mtDNA analyses.

The restriction endonuclease XhoI has been the most useful in comparing mtDNA fragment patterns of different lines. Compared to XhoI mtDNA patterns for sexually propagated CMS-T controls, lines derived from regenerated plants have exhibited zero, one, two or three variations. One prevalent variation in banding pattern (column 1) could be accounted for by an asymmetric chromosomal inversion about an XhoI cleavage site that generates two new sizes of fragments (Gengenbach and Pring, unpublished). A second variation (column 2) appears on gels as the absence of a fragment of about 10.5 Kb. It should be noted from Table 1 that the first and second type of variation have been found both in male-fertile, toxin-resistant and in male-sterile, toxin-susceptible lines from CMS-T cultures (i.e. these two changes in mtDNA are not associated with the "reversion" in phenotype).

The most interesting variation is visualized on gels as the apparent loss of a 6.5-7.0 Kb fragment (column 3). This variation has appeared only in mtDNA from resistant, male-fertile lines from CMS-T cultures. Of 19 such lines examined to date (nine selected and six unselected lines from Minnesota and three selected and one unselected line from Brettell), 18 lines showed this variation while one unselected Minnesota line retained the parental CMS-T pattern. None of the 34 male-sterile, toxin-susceptible lines from CMS-T cultures has shown any detectable change in this fragment. Therefore, it is possible that the 6.5-7.0 Kb fragment is involved in expression of male sterility and toxin susceptibility in CMS-T.

The 6.5-7.0 Kb fragment was isolated from a preparative gel of control CMS-T mtDNA and was nick-translated. Hybridizations to Southern blots of XhoI restriction fragments from male-fertile, resistant variants revealed strong homology with a fragment about 500 bp smaller for the six Minnesota lines examined to date (Pring and Gengenbach, unpublished) and three of four Brettell lines (Pring and Brettell, unpublished). In the fourth Brettel line, there was no homology apparent for fragments of the original or slightly smaller sizes. The fate of the sequence representing this 500 bp difference in the nine other lines is not known at this time because the sequence might not have been present on the gels used for Southern blots. A fragment of 500 bp could be generated from a new XhoI site near the end

Table 1. *Xho1* restriction endonuclease patterns of mitochondrial DNA from lines obtained from CMS-T tissue cultures.

			Variation in restriction pattern		
				Absence of	
Source culture	Regenerated plant	No. plants	"Inversion"	10.5Kb	6.5Kb
A188CMS-T(BC)$_2$[a/]	Sterile, suscept.	1	No	No	No
		2	Yes	Yes	No
		1	No	Yes	No
	Fertile, resistant (selected)	4	No	No	Yes
		1	No	Yes	Yes
		3	Yes	No	Yes
		1	Yes	Yes	Yes
A188CMS-T(BC)$_2$[b/]	Fertile, resistant (selected)	3	?	?	Yes
	Fertile, resistant (unselected)	1	?	?	Yes
A188CMS-T[c/]	Sterile, suscept. (unselected)	7	No	No	No
		1	Yes	No	No
	Fertile, resistant (unselected)	4	No	No	Yes
		1	No	No	No
		1	Yes	No	Yes
A188CMS-T[d/]	Sterile, suscept. (unselected)	19	No	No	No
		3	Yes	No	No

a/ Gengenbach *et al.*, 1977

b/ Brettell *et al.*, 1980

c/ Umbeck & Gengenbach (unpublished)

d/ Gengenbach (unpublished)

of the original fragment and as such could have migrated off the end of the gel under the electrophoresis conditions used. It also could be deleted entirely from the genome or be associated with other fragments as a result of rearrangements. Studies currently are underway to determine its fate in the male-fertile, toxin-resistant lines from CMS-T cultures. "Reversion" to male fertility and toxin resistance might be conditioned by nucleotide base changes or by rearrangements within a portion of the 6.5-7.0 Kb fragment.

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of the original fragment and its small size would have migrated off the end of the gel under the electrophoretic conditions used. It also could be deleted entirely from the genome or be associated with other fragments as a result of rearrangements. Studies currently are underway to determine its fate in the male-fertile, toxin-resistant lines from CMS-T cultures. 'Reversion' to male fertility and toxin resistance might be conditioned by nucleotide base changes or by rearrangements within a portion of the 6.6-kb Xho fragment.

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41 EPISOMAL DNA AS A MOLECULAR PROBE OF CYTOPLASMIC MALE STERILITY IN S ZEA MAYS

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The discrete DNA molecules isolated from the mitochondria of genetically defined male sterile maize offer a facile entree into the molecular genetics of an agronomically important plant. Maternal inheritance of the failure of mature corn plants to exsert pollen grains, referred to as cytoplasmic male sterility (cms), has been known for fifty years (Rhoades, 1931). The recognition of nuclear genes that suppress the maternally inherited trait and that themselves obey the Mendelian Laws of Inheritance offer still another valuable probe into the molecular basis of inheritance and the regulation of gene expression in maize (Edwardson, 1970). Appropriate genetic crosses, utilizing inbred lines of maize exhibiting specific nuclear, restorer to fertility (Rf) genes, facilitated the classification of sundry cms from all over the world into one of three groups: S, T or C (Beckett, 1971). The molecular organization of the cytoplasmic genome of the S group of cms maize is particularly amenable to experimental exploitation. S cms plants have a propensity to spontaneously revert to fertility at high frequency as a consequence of an alteration in the cytoplasmic component (Singh and Laughnan, 1972) on the one hand or the nuclear component (Laughnan and Gabay, 1973) of their genome on the other. The frequency of these exceptions to expected phenotypic ratios led Laughnan and Gabay (1975) to postulate the presence of a male "fertility element" that is alternately fixed in the cytoplasm or in the nucleus; i.e., a mobile episome.

Molecular evidence for the existence of a "fertility episome" in maize has come from direct examination of the cytoplasmic components of S cms plants and through serendipity. Levings and Pring (1976) confirmed an earlier inference from physiologic studies (e.g., Miller and Koeppe, 1971) that, of the two known maternally heritable organelles in the cytoplasm of a corn cell, the mitrochondrion bore the cms character and not the chloroplast. They definitely implicated the mitochondrion by demonstrating significant and reproducible differences in the restriction endonuclease cleavage products of mitochondrial DNA (mtDNA) for T cms plants as compared with those from the mtDNA of normal fertile plants. Serendipity played its role when these authors (Levings and Pring, 1977a) utilized endonuclease restriction fragment analysis to distinguish among the mtDNAs from the three types of cms, i.e., S, T and C. Digests of mtDNA from S cms consistently showed a few significantly overrepresented fragments, i.e., multiple copies of a few DNA fragments with identically spaced cleavage sites. When undigested S mtDNA was compared electrophoretically with that from fertile or from cms plants other than S, two linear DNA molecules were detected (Pring et al., 1977). These linear molecules peculiar to S cms plants are now referred to as S-1 and S-2. The authors speculated that this pair of linear DNAs (6.2 and 5.2 Kb) in S mitochondria represented the episomes postulated by Laughnan and Gabay (1975). Kemble et al., (1980) have subsequently distinguished the three types of male steriles and the normal cytoplasms of maize by the presence of these and additional low molecular weight bands detected among

undigested mtDNAs resolved by agarose gel electrophoresis. A significant contribution to the molecular characterization of extrachromosomal inheritance is the discovery of these discrete linear as well as super coiled circular DNAs as regularly occurring components of an organellar genome of the corn plant.

Directly relevant to S cms is the fate of S-1 and S-2 DNAs in the fertile revertants that were genetically characterized by Laughnan and Gabay (1978). The linear plasmid-like DNAs that characterized the mitochondria of the sterile S plants were not detected as discrete entities in the mitochondria isolated from the progeny of several different plants that had cytoplasmically reverted to fertility (Levings and Pring, 1979). On the other hand, dominant alleles of nuclear restorer genes, although enabling the plants to exsert viable pollen, did not alter the electrophoretic pattern of the mtDNA in the progeny, i.e., S-1 and S-2 were present. The transient behavior of the S-1 and S-2 DNAs in the cytoplasmic revertants and their apparent suppression in the presence of nuclear restorer genes was consistent with the well-characterized role of episomes in altering prokaryotic phenotypes.

It was at this juncture that our laboratory became experimentally involved with cms in maize. We had pursued, over the years, the isolation and reconstitution of in vitro protein synthesizing (Mans and Novelli, 1964), DNA transcribing (Stout and Mans, 1967) and RNA polyadenylating (Walter and Mans, 1970) systems from maize seedlings. The S-1 and S-2 DNAs from the mitochondria of S maize seedlings seemed to be the small defined templates with associated genetic function that we were casting about for (Mans et al., 1979) as templates for the purified RNA polymerase II, that exhibited a preference for homologous maize DNA (Brooks and Mans, 1973). We experimentally approached the fate of S-1 and S-2 sequences in the cytoplasmic revertants in two stages. First we asked, are the deoxynucleotide sequences in S-1 and S-2 "stabilized" in another portion of the genome as postulated by Laughnan and Gabay (1975). By Southern blotting mtDNA restriction fragments and hybridizing them with isolated S-1 and S-2 DNAs (radioactively labeled by nick translation), we observed the apparent integration of portions of each into fragments unique to the high molecular weight mtDNA of the cytoplasmic revertants (Levings et al., 1980). The data showed a correlation between the apparent integration of the S-1 and S-2 sequences into the high molecular weight mtDNA and the plants' ability to exsert pollen. This inference is further supported by the detection of S-1 and S-2 sequences among the endonuclease restriction fragments derived from the mtDNA of normal fertile maize (Thompson et al., 1980). In the latter experiments, cloned segments of S-1 and S-2 DNAs were used as hybridization probes. Recently, we (Kemble and Mans, unpublished) have shown that these same cloned sequences hybridize with the unique fragments derived from the fertile revertant DNAs. Further definition of the apparent transposition event is being pursued. To facilitate investigation of the fate of the S-1 and S-2 sequences in fertile revertants, we prepared endonuclease restriction maps of each and terminally oriented regions of sequence homology between S-1 and S-2 (Kim et al., 1981). The physical organization as well as the behavior of S-1 and S-2 is strikingly similar to the transposable elements characterized in both prokaryotic and eukaryotic systems.

The S cms system of maize provides an excellent experimental milieu to approach the regulation of gene expression in an agriculturally important

crop. Differences among the proteins accumulated in the mitochondria of fertile and cms maize have been reported (Forde et al., 1978). We are utilizing the S-1 and S-2 sequences to probe mitochondrial RNAs for altered transcription of free versus integrated sequences on the one hand and as templates for in vitro transcription on the other. We look forward to having a well-defined maize mitochondrial genome on which to hang the fertility episomes of S cms. Detection of altered transcription will then initiate the second stage of our program, i.e., the molecular definition of the nuclear restorer gene product.

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42 MITOCHONDRIA AND CHLOROPLASTS: SPECULATIONS AND REFLECTIONS ON THE MOLECULAR MECHANISM OF HETEROSIS

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We are seeking an explanation for the mechanism of hybrid vigor which is responsible for an increase of activity above that expected for an average of the two parents of a hybrid. From the breeder's point of view hybrid vigor is the increase of yield (or other criterion) in comparison with the best parent.

1 - Hybrid vigor: a widespread phenomenon

Although hybrid vigor causes an increased yield in several crops the plant physiologists are embarassed because of failure to develop an explanation of the mechanism. We are not going to tackle the role of heterosis in populations or in evolution but at the single plant level. We shall analyze the hypotheses which would account for a faster growth and the larger amount of plant material produced by hybrids.

Heterosis is a well known way to increase the yield, and is widely utilized by plant breeders. We have to consider the involvement of the breeding methods both in the way that the hybrids are made and on the assistance which should be brought to the breeder in order to increase the efficiency of well fitted genotype combinations.

The effects of inbreeding described both for plants and for animals have been reported since antiquity. Since in animals the sexes are separated as a general rule, fertilization must occur between gametes from different partners, leading to a mixture of genetic traits. In contrast, with plants, as a general rule, ovules and pollen grains are produced on the same individual. In nature several devices prevent self-fertilization; moreover foreign pollen is favored as soon as it falls on the stigma so that heterozygosity occurs at fertilization. If the heterozygous state is associated with vigor and high yield, while on the other hand, inbreeding leads to the homozygous state associated with poor vigor, this may not be due to inbreeding itself but because of the uncovering of unfavorable hidden alleles.

2 - Improving combining ability by recurrent selection

Crop plants may be divided up between those which are grown under the homozygous state as lines (wheat, barley, beans, peas ...) and those which are grown under the heterozygous state as populations, cultivar, synthetic varieties, double crosses, three way or single cross hybrids (maize, sunflower, sorghum ...). Theoretically the F_1 hybrid remains the breeder's aim, since it is the more productive and the less variable. Double crosses or three way hybrid seeds become less expensive when the female line used in the F_1 seeds is too weak. But in order to produce the seeds for most of the crops certain questions have to be solved.

The first requirement for producing hybrid seed is to obtain or develop the parent lines. The breeder then has to determine what are the best crosses between the lines to raise the yield. The most efficient way to improve the lines is the reciprocal recurrent selection method which combines the selection for both general and specific combining abilities (Allard, 1960). This allows the improvement of one population of lines with respect to the other.

3 - Field checking for combining ability

To determine the best combinations between the lines a great many trials have to be made in the fields. The hybrid plant behavior in the field depends on both the potential yield of the specific cross and the limiting environmental factors (i.e., fertilizer, disease, drought). In practice the limiting steps of a breeding program are at this level. Among 100 thousand lines only 60 can be used in hybrid seed production because of their high level of heterosis.

Such trials have to be repeated to estimate climatic effects since the rank of hybrids may depend on year or location. It is obvious that the best combination according to the potential yield can hardly be estimated.

4 - The explanatory theories

The genetic theories account for the required heterozygosity. Several interactions at the gene locus: dominance, overdominance or interactions between genes (epistasis) could support the basis of the mechanism of hybrid vigour. Nevertheless these genetic approaches are unable to "pin down which tracks" the physiologist has to follow to explain how and why the hybrid plant grows faster and exhibits more efficient metabolic pathways.

Furthermore a cumulative effect of increase for a large number of enzymes might lead to a well "balanced metabolism" according to Hageman et al. (1967). On the other hand the abundance of isozymes and their sequential appearance might be advantageous for the hybrid during changes in the environment and during development. This ability for the hybrid is called homeostasy (Lerner, 1954), it leads to a phenotype less sensitive to environmental conditions.

5 - The physiological approach is a necessity

Several mechanisms might converge to make hybrids more efficient: basic functions of the cell - respiration processes (Hanson et al., 1960) carbon dioxide fixation (Moss, 1960) and nitrogen metabolism (see Hageman et al., 1967 for review) appear to be involved. The energy production required for the hybrid growth must be recovered at the single plant level, at the cell level as well as at organelle levels. On the other hand the molecular interactions between the enzymatic proteins and the regulatory process called homeostasy might be another aspect of efficient metabolism.

Heterosis (activity for the hybrid greater than expected according to the average activity of the parents) is more an exception than a rule since all the enzymes involved cannot account for the excess of energy production inasmuch as the heterotic enzymes which are known: - alcohol dehydrogenase

(Schwartz and Laughner, 1969) - amylase (Sarkissian et al., 1964) - pH 7.5 esterase (Schwartz et al., 1965) - catalase (Scandalios et al., 1972), have a limited role in relation to metabolic processes.

There are several reasons to focus interest on the physiological approach of hybrid vigor. Firstly, the breeder has no way to determine the hybrid combinations which possesses the highest potential yield and consequently to set apart those which should be improved by correcting their specific failure. So that whatever way to approach the potential yield that could help the breeder, save field area and time, will increase the efficiency of the screening process. Secondly most of the hybrids are produced by using cytoplasmic male sterility. The mitochondria, at least, might be the source for both male sterility and hybrid vigor. Such a hybrid will inherit its set of mitochondria and chloroplasts from the female line. We wonder if the interactions at the mitochondrial (or the chloroplast) level leading both to male sterility and to vigor are compatible.

6 - Is there an in vitro test that could account for all the aspects of hybrid vigour?

The question raised here is the relationship between heterosis observed *in vitro* and the yield in the field. Since 1966 Sarkissian and his co-workers have compared the tissue respiration and the mitochondrial respiration with the heterosis yield and found a correlation between them. Most of their results showed a positive correlation between the rate of respiration for the tissue and for isolated mitochondria with the yield of the hybrids.

A tentative approach was made by Hanson et al, (1975) to find a correlation between mitochondrial activity (i.e. ADP/O) and the yield in grains. This important observation on genotype specificity has to be extended to commercial hybrids. Several authors compared hybrids with their parents. We refer to those who have studied methodically for several stages and/or several substrates: i.e. Sarkissian and Srivastava (1967) 60 hours; McDaniel and Sarkissian (1968) 2.5 days with different substrates; Berville et al. (1976) 21 hours to 165 hours for oxoglutarate; Charbonnier (1981) 2 to 8 days for NADH malate oxoglutarate. All these authors concluded that mitochondria from hybrids exhibit (at least for one stage and one substrate) an advantage called mitochondrial heterosis.

Reports from the literature on single checks are less illustrative. According to the stage, mitochondrial heterosis was not observed by Sen (1981) 60 hours; Van Gelder and Miedema (1975) at 3 cm shoot length: i.e. 2.5 to 4 day-old-seedlings, while it was observed by Yakovlev et al (1971) 96 hours, and Hanson et al (1975) at 4 day-old-seedlings.

7 - Mitochondrial complementation: a tool or a fantasia conception?

During the last fifteen years the investigations about respiration in relation to hybrid vigor have led Sarkissian to propose a theory based on the polymorphism of mitochondria (Sarkissian and McDaniel 1967), revealed by shape and density, which could account for a part of the explanation of the hybrid vigour. Furthermore the complementation for the mitochondrial functions postulated in the hybrids (Sarkissian and McDaniel, 1967, Sarkissian, 1972) and experimentally controlled leads the authors to develop

an experimental technique which would predict the yield before making the cross. The questions raised in the publications have now died down but the interest of such a method for breeders should be so fruitful that dreams are permitted. We do not want to speculate longer on this technique having not worked with it.

The coexistence of mitochondria from the female and the male gametophyte in the hybrid is not supported by the recent findings on inheritance nor by the origins of the mitochondrial components; Schatz and Masson (1974), Levings and Pring (1976).

8 - Questions and remarks

Heterozygosity and hybrid vigor are not synonymous since several cases of single point mutations have exhibited a change at the heterotic level. On the other hand the cytoplasm is involved in the heterosis process as revealed by differences in reciprocal hybrids. So, in our mind, the molecular basis of heterosis should be in the interactions between gene products specified by nuclear DNA, mitochondrial DNA and chloroplast DNA. Of course the regulatory genes might play a large role in these processes, nevertheless, the genetic situation in which they should be revealed are not easily recognized or are too difficult to unravel.

9 - Mitochondria as a target for hybrid vigor

The mitochondrion holds several DNA molecules coding for their own translation system (ribosomal RNA, transfer RNA and ribosomal proteins) and for at least 10 to 12 polypeptides which are involved in complexes belonging to the inner mitochondrial membrane. Some of these peptides are a part of the cytochrome b-c complex, or the cytochrome oxidase (the 3 largest units) and of the adenosine triphosphatase (oligomycine resistant unit). Besides the mitochondrial DNA encoded peptides, several hundred others are the translation products of nuclear genes (on the cytoplasmic ribosomes) which moved towards the matrix through the mitochondrial membranes (Schatz and Masson, 1974).

It is well established in maize that the inheritance of the mt DNA is strictly maternal (Pring and Levings, 1978) therefore we should keep in mind that the hybrid will possess the same mitochondrial information as the female parent.

Let us try to investigate what happens after the fertilization of the ovule by the male gamete. First, the metabolism in the egg is dependent upon the mitochondria coming from the female line. As soon as the nuclear genes brought in by the male are derepressed, their translation products enter the mitochondria, which enlarge and divide, to participate in the structure of the mitochondria. Thus a change in the gene structure might lead to unite the nuclear information of the male to the nuclear and/or the cytoplasmic information of the female. Consequently this change might carry along disturbances, positive or negative, into the mitochondrial function, as the rate of oxidation or the efficiency of ADP phosphorylation. Moreover the alleles coming from the male express themselves in sequence depending upon: the stage of the seedlings (it is well known that the germinating process causes a deep change in the mitochondria); the pre-existing structures in the mitochondria; and the tissue in which the mitochondria are located.

10 - Chloroplast as a target for hybrid vigor

A literature survey focusing on the relationship between the yield in the field and the rate of photosynthesis per unit area of the leaves does not lead to a correlation (for review see Sinha and Khanna 1975). Depending upon both the stage of the plants and the genotype, the clearest conclusion is that the rate of photosynthesis measured from the leaves varies according to the genotype, the hybrid either follows the best parent or is intermediary (Hageman et al. 1967; Monma and Tsunoda, 1979). As it has been described for the mitochondria, the chloroplast contains several multisubunit complexes, i.e., the apoprotein of the chlorophyll a/b complex, the ATP synthase complex (CF1) and the RuBP carboxylase (large subunit) and the elongation factors T and G. (for review see Ellis, 1981).

The hybrid did not exhibit heterosis in photosynthetic rate even if the rate is divided into elementary pathways. Protoplast fusions (Belliard et al., 1978) have shown the independence of the nuclear genome and the chloroplast genome when Nicotiana plants are screened for cytoplasmic male sterility. In other experiments, Iwai et al. (1980) observed for the RuBP carboxylase the coexistence in the same plant of different nuclear encoded subunit types with always only one of the chloroplast encoded large subunit types due to rapid segregation of the chloroplast.

11 - Mitochondrial and/or chloroplast structures as a basis for heterosis

Mitochondrial and chloroplast heterosis could result from the polypeptide cooperative - interactions and should be checked at the level of these complexes but could also result from the whole structure of the inner membrane according to the set of polypeptide arrangement. It could be expected that the part played by a unique component (wherever it is encoded) might change several activities and thus the yield of the energy production by the respiratory chain. A change in the mitochondrial DNA (Levings and Pring, 1976) which corresponds to a new set of polypeptides (Forde et al., 1978) might require a new set of nuclear origin polypeptides of the hybrid genotype to maintain the same efficiency of the respiratory chain.

Let us sum up the genetic causes leading to a likely change in the mitochondrial or chloroplast function, keeping in mind that for a given cytoplasm the genotype is a source of variation. The degree of heterozygosity as well as the loci involved are different and for a given genotype the cytoplasmic constitution seem to have an influence.

The reassociation of peptides encoded by nucleus and chloroplast should be generalized to mitochondria as might be shown in protoplast fusion experiments or in either interspecific or intergeneric crosses. A large variability has been recorded by RuBP carboxylase according to subunit associations, however the literature is totally devoid of studies on correlated activity.

Our aim is to define a general combining ability between mitochondria, chloroplasts, and nucleus and also a specific combining ability. The products of protoplast fusion experiments will be useful for moving in this direction.

In the future if the screening for combining ability determination is to be made at the mitochondria and/or at the chloroplast level (not exclusive of any other way) much basic work has to be carried out. Molecular biologists must provide evidence to explain the biogenesis of mitochondria and chloroplast, especially for those polypeptides which cooperate and will help identify those functions the physiologist have to integrate into new knowledge to help the breeder.

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43 RESTRICTION ENDONUCLEASE CLEAVAGE MAP OF THE MAIZE CHLOROPLAST GENOME

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The maize chloroplast genome exists as a circular double-stranded DNA molecule approximately 126 kb in size (Bedbrook and Bogorad, 1976). A restriction endonuclease cleavage map compiled from various reports cited below is shown in Figure 1. Approximately 15% of the chloroplast genome exists as an inverted repeat (Bedbrook et al., 1977). This repeated area is shown by the arrows (Fig. 1).

Certain areas of the genome have been analyzed in greater detail than others. The structure of these areas are shown in the expansions. In addition portions of these areas have been further defined by transcript mapping and DNA sequence analysis as shown in the figure. Of particular interest is the area which includes the 5' end of the transcript containing the 23S rRNA and the complete 16S rRNA. The spacer between the 23S and 16S rRNAs contains two tRNA genes each of which have large intervening sequences (Schwarz and Kossel, 1980; Koch et al., 1981). An additional area of interest defines the gene for the large subunit of ribulose bisphosphate carboxylase. The complete sequence of this gene has been obtained (McIntosh et al., 1980). The chloroplast genome also contains at least one example of overlapping genes. A chloroplast tRNAhis gene overlaps by at least a few nucleotides another as yet unknown gene and these genes are transcribed divergently from complementary DNA strands (Schwarz et al. 1981).

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Figure 1. Restriction endonuclease cleavage map of the maize chloroplast genome.

44 MAIZE POLLEN AS A UNIFORM TESTING MATERIAL FOR BIOCHEMICAL STUDIES

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Maize pollen, the wind borne product of an imperfect flower, is ideal material for biochemical studies. It is easily collected because the anthers are in the tassel away from other parts. Being wind borne, each pollen grain (speaking teleologically) must minimize weight and maximize biological and biochemical activities. Each pollen grain is a separate package of active material in a semipermeable membrane within a tough sporopollenin envelope making handling easy. Furthermore, because each grain is so small and yet representative of an individual genotype, small sized pollen samples represent essentially all possible recombinants within a test cultivar or variety.

The male tassel, separate from the female earshoot, can be cut from the plant with a portion of culm or stem attached approximately 1 to 2 days before anthesis and kept in the laboratory or a draft-free room. Insects, dust, and other foreign material can be washed off the unopened anthers, and the anther surfaces sterilized with 3 percent aqueous calcium or sodium hypochlorite. If the culm is immersed in tap water and the tassels left hanging over smooth, preferably white shelf paper, clean shed pollen may be collected for several successive mornings before anthesis terminates. Empty anthers among the pollen may be hand picked or screened from the pollen before it is studied or stored.

A less clean but easier bulk pollen collection method is tassel bagging. A brown paper tassel bag is paper clipped or stapled tightly around the culm below the tassel, and shed pollen is dumped from the bag each morning in the field. These samples require air drying in the laboratory to prevent pollen clumping and incipient or peg germination. Once dried for one or more hours, the pollen may be sieved from insects, anthers, and larger extraneous matter, although dust will pass through the sieve with the pollen.

Pollen storage is determined by the subsequent studies. Maize pollen viability is short, and these comments on biochemical studies concern fresh, enzymatically-active but not necessarily viable pollen.

Although biochemical studies of pollen began early in the 19th centruy and much research has been published ranging from apian nutrition to human allergens, relatively few studies have pertained to maize pollen. Goss (1968) reviewed maize pollen constituents and found a modicum of research dealing with carbohydrates, proteins, and vitamins but a lack of mineral and lipid research. Togasawa and coworkers published a series of maize pollen papers about the same time, one of which dealt with various inorganic constituents (Togasawa et al., 1967).

Ortega and Bates (1980) found few maize pollen protein fractionation studies and no applications of recent isoelectrofocusing techniques. We developed a continuous maize pollen extraction and fractionation system via a tandem ultrafiltration cascade and characterized the water-soluble proteins by

molecular weight, isoelectric point, and enzymatic activity. The system was somewhat more efficacious than electrophoretic analyses (Ortega and Bates, 1980). An earlier ultrafiltration system designed to minimize phenolic interferences during pollen protein extraction and fractionation (Bates, 1979) remains to be tested for efficaciousness via subsequent isoelectrofocusing and enzymatic assays.

Increasingly sensitive protein and other analytical techniques are opening opportunities to study pollen enzymes and metabolism. Few new techniques have been applied to maize pollen studies. Therefore, the importance of maize as an economic crop, the relative paucity of maize pollen studies and the ease of sample handling and collecting makes maize pollen an ideal candidate for future research.

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45 MAIZE POLLEN: COLLECTION AND ENZYMOLOGY

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Maize is monoecious, having male and female flowers on the same plant. The male or staminate flowers are located in the tassels at the top of the plant. The female flowers are located in the ears growing from along the stalk at the base of the central leaves. Each of the numerous florets within a tassel contains three anthers, the male-spore-producing organs, and within each of these anthers is produced about 2,000 (Wallace et al., 1949) to 7,500 (Goss, 1968) pollen grains, depending on the plant, the variety, and the conditions of growth. Estimates of the number of pollen grains per plant have been from 14,000,000 (Wallace et al., 1949) to 50,000,000 for a medium-sized plant (Paton, 1921). Goss (1968) also estimates 21,000 pollen grains per ear (about 21 pollen grains/kernel) and Kiesselbach (1949) about 42,500 pollen grains per square inch of corn field. This high ratio of pollen grains per female flower available for fertilization is typical of plants fertilized by wind pollination or anemophilous plants such as corn and pine.

The maize pollen grain is larger than most pollen yet falls in about the middle of the potential size range for the plants reported. It is basically spheroidal with a diameter of 90 to 116 microns, a volume of about 700 x 10^{-9} cm^3 and a weight of about 247 x 10^{-9} grams (Stanley and Linskens, 1974; Goss, 1968).

When the pollen is mature and the anthers are exposed (anthesis), the tip of the anthers breaks open leaving a pore through which the pollen is shed (dehiscence). A corn plant sheds its pollen on several successive days with dehiscence occurring in the mornings between 630 and 1100 hours, although it may be delayed by two hours in cold, cloudy weather (Khudoyan, 1961).

The pollen grain represents the male gametophyte of flowering plants and hence the cells of a pollen grain contain the reduced number of chromosomes (10 in corn) typical of that produced by meiosis in the formation of microspores and megaspores. Meiosis can be observed in maize microspore mother cells about two weeks before dehiscence. At this stage, "the tassel, as felt through the layers of sheaths and leaves, should not feel hard (too young) or soft (too old), but like an inflated football--resilient" (Smith, 1947). The first mitotic division in the pollen grain is about 5-7 days following meiosis--at the stage when the tassel is emerging. The second mitotic division occurs 5-7 days later, the pollen being shed in the tri-nucleate stage. Material collected a little before midday for mitosis and slightly earlier for meiosis shows more nuclei undergoing division than material collected at other times.

There are several excellent reviews on the development of maize tassels and pollen (Weatherwax, 1917, 1919; Kiesselbach, 1949; Pfahler, 1978; Goss, 1968), pictures of which may be seen in the text of Bold et al., (1980).

For the past few years our laboratory has been interested in the control of organelle heredity. One of the proposed mechanisms of this control is through the activity of nuclease enzymes. With this in mind we decided to explore the nature of these enzymes in pollen. One of the advantages of working with maize pollen is the ease with which large amounts of the pollen can be collected. In order to obtain the relatively large amounts of pollen necessary for enzyme purification, tassels can be collected from local farm fields. From any one spot between corn rows 5 or 6 tassels, just starting to dehisce, are taken by severing the stalk 10 to 15 cm below the tassel. Then moving up the row about ten paces, the tassel collection is repeated until the row is finished. Then moving 3 or 4 rows further into the field, this procedure is continued until the field has been covered. The tassels are taken to the lab where they are suspended from a string in a dry draft-free room. They are suspended several centimeters above sterile paper, which catches the fallen pollen. If the tassels are allowed to rest on the paper, we have found that fungal contamination may occur due to the moisture content in the fresh tassel. That afternoon and the following afternoon, the fallen pollen is collected, passed through successively finer sieving screens until the pollen is relatively pure, and then immediately stored by freezing at -20°C.

Paton (1921) used a similar method for collecting the tassels, but the cut ends were placed in a pan of water with the paper lying under the pan. Efron (1971) and Ray Sarkar _et al_., (1949), during the evening hours, placed paper bags over tassels, which were just beginning to dehisce. The next morning, the tassels were shaken and the bags containing the pollen collected. The pollen was then cleaned by sieving. Stanley and Linskens (1974, pgs. 87-115) describe how bees may be used for the collecting procedure. Once the bees have collected the pollen in their normal manner, the pollen is then collected from the bees and used. However, it has been noticed that some biochemical alterations can occur in pollen collected by bees (Stanley and Linskens, 1974).

To test the amount of bacterial and fungal contamination on collected pollen, Paton (1921) and I have plated pollen out onto nutrient media and monitored the amount of contamination which appeared. In both studies, the contamination was found to be low.

There have been several studies into the optimal storage conditions for the maintaining of pollen viability (see Pfahler, 1978). On occasions, viability has been measured by the ability of the pollen grain to germinate, forming a pollen tube, _in vitro_ (Goss, 1968). Several media for growing pollen _in vitro_ have been developed (Pfahler, 1967; Cook and Walden, 1965). Pfahler (1968) used 15% sucrose, 0.6% bacto-agar, 0.03% $Ca(NO_3)_2$ 0.01% boric acid; he found optimum levels for _in vitro_ germination of calcium and boron concentrations varied with the genetic source of the pollen. Some required both Ca and B for germination, but others did best with one or the other alone. However, _in vitro_ germination is not an accurate measurement of the capacity to bring about fertilization. At the optimum conditions of storage, 2-4°C and 90-100 percent relative humidity (Knowlton, 1921; Jones and Newell, 1948; Sartoris, 1942), pollen is capable of germination _in vitro_ for about seven days after storage. However, Pfahler and Linskens (1972) found that pollen which was no longer capable of _in vitro_ germination was still capable of fertilization. Hence, the best test for viability in the sense of fertilization ability would be a method such as the "K assay" of Walden (1960) in which the actual fertilizing capacity of the pollen was measured.

Other methods used to test viability with partial success are the vital stain tests, e.g., 2,3,5-triphenyltetrazolium chloride (TTC) (Daikonu, 1961; Pylinev and Diakonu, 1961) or benzidine-hydrogen-peroxide (Vereshchazin, 1963). TTC (1%) in phosphate buffer, pH 7.2, tests the activity of certain enzymes, especially dehydrogenases and presumably stains live pollen grains red while dead pollen does not stain at all, remaining yellow.

With the possible exception of pollen storage at -180°C there are biochemical changes which continue to occur during storage (Frova and Feder, 1979). Amino acid content is altered both quantitatively and qualitatively (Linskens and Pfahler, 1973). Isozyme patterns may change quite rapidly after dehiscence (Hamill and Brewbaker, 1969). Certain enzymes, e.g., acid phosphatases and certain peroxidase isozymes, are known to be lost quickly (Anderson et al., 1978; Hamill and Brewbaker, 1969), while other enzyme activities, e.g., glucosyl-transferase (Goss, 1968), amylase, invertase (Knowlton, 1921), and DNase activity (Miller et al., 1981), will be maintained long (months) after viability appears to have been lost. The cause of loss of viability is not understood or known. However, since at least some degradative enzymes in pollen remain active for relatively long periods of time, perhaps maintenance of the pollen grain is overcome by degradative activities and hence degeneration occurs.

A great deal of work has been done on the physiology and biochemistry of pollen (two excellent books on this subject are by Stanley and Linskens, 1974, and Heslop-Harrison, 1971; reviews of the topic are given by Rosen, 1968, and Pfahler, 1978). Studies on maize pollen represent a good percentage of these data. The variation in the values reported for various chemical constituents in corn pollen (and any pollen) probably reflects varietal, fertilizer, and other environmental variations among the experiments (Tseluiko, 1968; Pfahler and Linskens, 1974; Khoo and Stinson, 1957; Stanley and Linskens, 1974). Maize pollen is typical and very roughly average for many of the values of its chemical components. Due to relatively high levels of protein, sugar and nutrients, maize pollen also has been used as a nutrient additive in the feed of certain animals: piglets (Salajan, 1970), calves (Popa et al., 1970), chickens (Salajan, 1972; Costantini and Dalborne, 1971), and athletes (Stanley and Linskens, 1974). Table 1 contains a listing of many of the elements reported for maize pollen, and Table 2 is a list of biochemical components and their contribution to the overall composition of maize pollen.

A considerable amount of enzymology on maize pollen has been reported, but a great deal remains to be done. The enzyme activities detected tend to vary with the developmental stage so that during microsporogenesis, the most active enzymes appear to be those associated with nucleic acid replication and synthesis and cell wall (intine and exine) synthesis. Enzyme activities associated with pollen tube growth are also present in the mature microspore (Stanley and Linskens, 1974). Many enzymes of maize, e.g., acid phosphatase, nitrate reductase, isocitrate lyase, isoctric dehydrogenase, and alcohol dehydrogenase, exist as isozymes with each one's appearance depending on the tissue under consideration (Efron, 1970) and the genetic complement of the plant (Efron, 1970; Paton, 1921; Makinen and MacDonald, 1968; Dickinson, 1965; Dickinson and Davies, 1971; Poddubnaya-Arnoldi et al., 1960). Many enzymes in the pollen are common to all parts of the corn plant, while several of the enzyme patterns indicate enzymes or enzyme

Table 1. Elementary Composition of Maize Pollen

Mineral Elements[1]	Percent Fresh Pollen[2]	Percent of Ash[2]	Percent of Ash[3]	μg/g dry Weight[1]	mequiv./100 g Dry Weight[4]
N	4.30	---	---	---	303
P	0.63	18.92	10.2	69.57	21
K	1.24	35.58	26.3	105.09	32
S	0.34	0.69	---	---	18
Cl	0.19	0.80	---	---	---
Mg	---	4.60	8.2	11.58	13
SiO_2	---	3.76	---	---	---
Ca	---	1.02	3.9	9.20	3
Fe	---	0.25	0.00059	0.48	---
Al	---	0.22	---	0.46	---
Na	---	0.69	---	5.93	1
Cu	---	---	---	0.20	---
Mn	---	---	---	0.24	---
Zn	---	---	---	1.90	---
ash, % of total	---	---	2.55	2.8-3.77	3.5
water (H_2O)[1]	------------------------ _ 50% by weight at anthesis------------------Heslop-Harrison, 1971				

1. Pfahler and Linskens, 1974; they have shown that many of these values vary significantly with the genotype.
2. Anderson and Kulp, 1922.
3. Todd and Bretherick, 1942.
4. Knight et al., 1972.

Table 2. Biochemical Composition of Maize Pollen

Total carbohydrates	76.1%	Pfahler and Linskens, 1971
	36.59%	Todd and Bretherick, 1942
Sugars	40.1%	Pfahler and Linskens, 1971
	14.1%	Todd and Bretherick, 1942
major ones	fructose and glucose	Istatkov *et al.*, 1964
others	sucrose, rhamnose	Istatkov *et al.*, 1964
pentosans	10.6%	Anderson and Kulp, 1922
inositol	30 mg/g (Nielson et al., 1955)	Miyake, 1922; Vinson, 1927
Reducing sugars	6.88%	Todd and Bretherick, 1942
Non-reducing sugars	7.31%	Todd and Bretherick, 1942
Starch	28.6%	Pfahler and Linskens, 1971
	22.4%	Todd and Bretherick, 1942
Amylopectin	86.0%	Goss, 1968
Amylose	14.0%	Zuber *et al.*, 1960
Paraffins	n-pentacosane (C_{25})	Nilsson *et al.*, 1957
	n-heptacosane (C_{27})	Nilsson *et al.*, 1957
	n-nanocosane (C_{29})	Anderson, 1923
Waxes		Goss, 1968
Cellulose		Goss, 1968
Pectic materials		Goss, 1968
Uric acid	17 mequiv./100 g dry wt.	Knight *et al.*, 1972
Methylated uric acid	3.2 mequiv./100 g dry wt.	Knight *et al.*, 1972
Lipids	3.67%	Todd and Bretherick, 1942
	1.8%	Pfahler and Linskens, 1971
Fatty acids		
palmitic	54.1%	Pfahler and Linskens, 1970
linolenic	34.4%	Pfahler and Linskens, 1970
Choline		Vinson, 1927
Phytosterols		Miyake, 1922; Anderson, 1923
total sterols	0.1%	Miyake, 1922
24-methylene cholesterol	59%	Devys and Barbier, 1966
-sitosterol	17%	Standifer *et al.*, 1968
campesterols	15%	Standifer *et al.*, 1968
stigmasterol	12%	Standifer *et al.*, 1968
Triterpenoid	squalene -- 0.02%	Davys and Barbier, 1966
Flavonoids	quercetin	Redemann *et al.*, 1950
source of yellow color	kaempferol	Stanley and Linskens, 1974
	isorhamnetin (an aglycone)	Stanley and Linskens, 1974
	3-glycoside isoquercitrin	Larson, 1971

Table 2. Biochemical Composition of Maize Pollen (Continued)

Protein	20.32%	Todd and Bretherick, 1942
	albumin	Vinson, 1927
	glutelin	Vinson, 1927
	2 mucoproteins	Gladushev, 1962
Amino acids	a partial list of 21 amino acids	Goss, 1968
DNAs		Goss, 1968
RNAs		Das, 1965; Moss and Heslop-Harrison, 1967
Vitamins		
B_2-riboflavin	5.7-6.2 μg/g dry wt.	Nielson et al., 1955
B_3-nicotinic acid	40.7 μg/g dry wt.	Nielson et al., 1955; Togasawa et al., 1967
B_5-pantothenic acid	14.2 μg/g dry wt.	Nielson et al., 1955
B_6-pyridoxine	5.5-5.9 μg.g dry wt.	Nielson et al., 1955
C-ascorbic acid	58.5 μg/g dry wt.	Togasawa et al., 1967
H-biotin	0.52-0.55 μg/g dry wt.	Nielson et al., 1955
folic acid	2.2 μg/g dry wt.	Nielson and Holmstrom, 1957
Folic acid conjugates		Nielson and Holmstrom, 1957
Hormones	IAA (indole-3-acetic acid)	Fukui et al., 1958
	2 growth promoters from acid fraction	Fukui et al., 1958
	1 growth promoter from neutral fraction	Fukui et al., 1958
	hydroxy pyridin -- growth stimulator	Redemann, 1949
Coenzyme A		Seifter, 1954
-carotene		Togasawa et al., 1967

Table 3. Enzyme Activities Reported for Maize Pollen

Enzyme	Reference
Carbohydrases	Vinson, 1927; Mac Donald, 1969
Amylase (diastase)	Knowlton, 1921; Katsumata and Togasawa, 1968; Paton, 1921
Polygalacturonase (pectinase)	Paton, 1921
-fructofuranosidase (invertase)	Paton, 1921; Katsumata and Togasawa, 1968; Knowlton, 1921
Acid invertase (soluble and insoluble forms)	Bryce and Nelson, 1979
ADP-glucose pyrophosphorylase	Bryce and Nelson, 1979
UDP-glucose pyrophosphorylase	Bryce and Nelson, 1979
Glucosyltransferase (substrates = quercetin, glucose)	Larson, 1971
Starch-glucosyltransferases	Bryce and Nelson, 1979
ADP-glucose starch glucosyltransferase	Bryce and Nelson, 1979
Nucleoside diphosphate kinase	Bryce and Nelson, 1979
Hexokinase	Bryce and Nelson, 1979; Katsumata and Togasawa, 1968
Phosphoglucomutase (insoluble form)	Bryce and Nelson, 1979
Glucose phosphate isomerase	Bryce and Nelson, 1979
Xylose isomerase	Pubols and Axelrod, 1959
Xylulose kinase	Pubols and Axelrod, 1959
Phosphorylase	Katsumata and Togasawa, 1968
Hydrolases	Knox and Heslop-Harrison, 1969, 1970
Amino acylase	Umebayashi, 1968
Reductase	Paton, 1921
Conjugases (substrate = folic acid)	Neilson and Holmstrom, 1957
Catalase	Paton, 1921; Scandalios, 1964; Istatkov et al., 1964
Peroxidase	Hamill and Brewbaker, 1969; Scandalios, 1964; Istatkov et al., 1964
Polyphenol oxidase (catechol oxidase)	Istatkov et al., 1964
Cytochrome oxidase	Walden, 1960; Tsinger and Petrovskaya, 1961
Proteases (proteinases)	Paton, 192
Leucineaminopeptidase	Scandalios, 1964; Makinen and MacDonald, 1968
Trypsin	Paton, 1921
Pepsin	Paton, 1921
Aspartate-amino transferase (glutamate-oxaloacetate transaminase)	MacDonald, 1969
Glutamate-aspartate transaminase	Sawada, 1960
Glutamate-alanine transaminase	Sawada, 1960
Esterases	Scandalios, 1964; Knox and Heslop-Harrison, 1969, 1970
Carboxylic ester hydrolase (-esterase)	MacDonald, 1969
Aryl ester hydrolase (-esterase)	MacDonald, 1969
Acetic ester acetyl esterase	MacDonald, 1969
Acid phosphatase	Knox and Heslop-Harrison, 1969
DNase activities	Miller et al., 1981
Ribonuclease	Knox and Heslop-Harrison, 1969, 1970
Lipase	MacDonald, 1969
Alcohol dehydrogenase	Freeling, 1969
Transcription and translation machinery	Goss, 1968; Das, 1965; Heslop-Harrison, 1971

forms which are unique to the pollen grain (MacDonald, 1969). The enzyme activities have been looked at in several ways as per enzyme source: whole pollen (Larson, 1971), grinding of whole pollen (Miller et al., 1981), cell fractionation studies (Weeden and Gotlieb, 1980), isozyme studies (Hamill and Brewbaker, 1969), histochemical demonstration (Vaughn, 1981), and autoradiographic studies (Das, 1965). Table 3 contains a list of enzymes or enzyme activities reported for maize pollen.

In searching for nuclease activity which might play a role in the inheritance of organelles, one activity to be sought was one with a specificity toward covalently closed circular (supercoiled) DNA. We have found and isolated an enzyme which appears to be a DNA-nicking or topoisomerase-type enzyme from maize pollen. Although a DNA-topoisomerase activity was recently reported to be found in wheat germ (Hager and Burgess, 1980), this is the first report of this type of nuclease activity to be found in pollen.

Procedure for isolation of DNA-topoisomerase from maize pollen

All steps of the enzyme purification are carried out at 4°C, with different stages of the enzyme purification being stored at -20°C. All equipment is sterilized before use to protect against extraneous nucleases and proteases. 40 grams of dry pollen are homogenized in Tembrock homogenizers in 400 ml of 75 mM Tris buffer, pH 7.5, 0.5 mM EDTA, 7 mM 2-mercaptoethanol and 15 percent glycerol. The homogenate is passed through two layers of cheese cloth on top of two layers of Miracloth (Chicopee Mills, Inc.) and the filtrate is centrifuged at 27,000 g for 20 minutes. The supernatant is decanted and recentrifuged until relatively clear (though still yellow in color). The supernatant is decanted and centrifuged for one hour at 105,000 g. This high speed supernatant is collected and made 0.2 M NaCl. This solution is then made 1 percent Polyethyleneimine (PEI) (Sigma Chemical Co.) by the slow addition of a 10 percent (^{v}/v) solution of PEI in water, pH 7.5 with HCl, and stirring is continued for at least two hours. This procedure precipitates the nucleic acids for a cleaner protein preparation (Bickle et al., 1977). After precipitation is complete, the solution is centrifuged for 20 minutes at 27,000 g. The proteins are precipitated from this supernatant at 65 percent $(NH4)_2SO_4$ by the slow addition of a saturated $(NH4)_2SO4$ solution. Stirring is continued for at least one hour. The protein solution is centrifuged 20 minutes at 27,000 g and the pellets are drained. The pellets are resuspended in about 5 ml of dialysis buffer, same as the isolation buffer, and this solution is dialyzed in the same buffer for 12-20 hours. The dialyzed sample is centrifuged for 20 minutes at 27,000 g. The supernatant is collected and approximately 7 ml are loaded onto a heparin-agarose (Bethesda Research Labs, Inc.) affinity column and the column is allowed to sit for 20 minutes. The column width is 1.5 cm and the bed volume approximately 10 ml. The column is previously equilibrated with at least 10 bed volumes of dialysis buffer. Ten bed volumes are then passed through the column to rinse out those compounds with low affinity to the nucleic acid analogue (Pirrotta and Bickle, 1980). A 100 ml linear NaCl gradient from 0.0 to 1.0 M is then passed through the column. Two milliliter fractions are collected and activity in the fractions is monitored on agarose gels. A typical sample which is loaded onto an agarose gel (usually 0.7 percent agarose in a 40 mM Tris-20 mM Na acetate buffer, pH 7.8, 2 mM Na_2EDTA, same as the electrophoresis running buffer) consists of 10 μl of enzyme fraction, 0.1 μg DNA, 40-50 μl of reaction buffer (75 mM Tris,

pH 7.5, 2 mM 2-mercaptoethanol), with the addition of $MgCl_2$ to 10 mM. This mixture in incubated for one hour at 37°C before electrophoresing. The enzyme activity may be further purified by molecular sieving chromatography or preparative acrylamide gel electrophoresis.

An enzyme activity such as this one, relatively easy to obtain, has several valuable uses. I have used it to identify the various bands on an agrose gel from a mixture of linear, relaxed circles and supercoiled DNAs. It could also be used in studies where the relaxed-circle conformation is needed, e.g., electron microscopy sizing studies, DNA conformation studies, and DNA binding studies (Shishido et al., 1980). As far as speculation on the in vivo function of such an enzyme, several possibilities come to mind: a role in DNA replication; a role in the maintenance of DNA molecules; a role in the alteration or degradation of DNA molecules.

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46 GENETIC FACTORS AFFECTING PLANT DEVELOPMENT

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Genetic analysis has been used to address three major problems in plant development: the regulation of gene expression, the cell lineage of the plant body, and the organization of morphogenetic pathways. The first two of these approaches are discussed in more detail elsewhere in this publication and will be given attention here only enough to emphasize the background upon which we can describe morphological mutations that may provide information about the factors involved in maize morphogenetic pathways.

Gene expression is regulated quantitatively and in space and time. Early evidence for spatial regulation is given by the work of East and Hayes (1911) and Emerson (1921) on the expressions of genes affecting anthocyanin pigmentation in the kernel and in parts of the plant body. The genetic basis of allelic variation in tissue specificity has been probed most extensively at the R locus, starting with Stadler and Emmerling's (1956) identification of recombinationally distinct elements responsible for the seed color effects (S) and the plant color effects (P). More recently, Kermicle (1980) has presented evidence for an element that provides quantitative control of R expression distinct from the tissue-specificity elements, while Dooner (1979) has concluded, from fine-structure analysis, that tissue specificity is controlled by at least two interacting sites at the locus. Evidence for genetic units regulating tissue-specific variation has also been sought by profiling the variations at the B locus (Coe, 1979). The existence of temporal regulation among alleles is evident in the stage specificity of different alleles at the R and B loci (Styles, Ceska and Seah, 1973).

That genetic markers can be used to analyze cell lineages in corn was first made evident by the studies of Emerson (1917), whose superb photographs (his figure 3) show well-defined somatic sectors in the ear, occurring under the influence of what is now known to be a controlling element (Mp) at the P locus. Brink and Nilan (1952) and Greenblatt (1968) studied similar controlling element-induced changes during ear development, some of which produced twin spots, useful for determining the orientation of cell divisions. To study the apparent sorting out of mutant and normal plastids during the formation of the ear, Anderson (1923), Demerec (1927), Rhoades (1943) and Stroup (1970) employed the technique of ear mapping, in which seeds are planted according to their physical position on the ear in order to visualize the clonal distribution of the affected lineages. All of these investigations were concerned with genetic problems rather than developmental ones, and hence did not consider questions concerning cell lineage. For the purpose of cell lineage analysis, it is important to know when in development a particular sector arose. This can be accomplished by inducing the loss of genetic markers with ionizing radiation at selected times. Extensive definitions of the lineages giving rise to the ear, as well as to the tassel and the plant body, are described elsewhere (Johri and Coe, this publication). For endosperm development, the extensive information derived from controlling-element events by McClintock (1978) shows a theme of spherical expansion of clones from a central focus. Losses of genetic markers, induced at specific

times following fertilization, have shown (Coe, 1978) that the first division of the endosperm is in a vertical plane, dividing the kernel into symmetrical left and right halves. A second division is approximately at right angles to the first. The last several divisions, forming the aleurone layer at the surface, show a highly patterned alternation of division planes at right angles to one another, leading to striking clonal groups of 2 x 2 cells, 2 x 4, and 4 x 4 (Coe, 1978).

Detailed descriptions of many of the genetic variations affecting the vegetative and reproductive morphology of the corn plant are found in Coe and Neuffer (1977), to which the reader is referred for references that are not repeated here. The variations can be classified into two broad groups: those that prevent the formation of an organ or have deleterious effects on its structure, and those that modify the morphology of an organ in a specific way. Both types can yield important information about the nature of morphogenetic processes. However, because relatively little attention has been paid to this area, most of the information about morphological factors is descriptive in nature and of limited definition in a morphogenetic sense.

Tassel and Ear

Because of their complexity, the inflorescences of maize are subject to a number of different kinds of variation. Perhaps the most fascinating are those that lead to homoeotic transformations; that is, the transformation of one kind of differentiated structure into another, presumably related, type. This type of transformation is brought about not only by genetic factors but by a variety of other factors as well. The most familiar variation is the formation of female (pistillate) flowers in the tassel, a feature common on tillers or on plants grown under physiologically unusual conditions (e.g., under short day in the greenhouse). Similar effects are produced by the tassel seed factors (ts, ts2, Ts3, ts4, Ts5, Ts6). Each of these factors causes the "suppressed" pistil in tassel florets to develop silks and a functional ovary, without greatly affecting the branching pattern of the tassel. Some types produce functional anthers, others do not. In the most extreme cases, the tassel is so profusely filled with seeds that it is reminiscent of a sorghum head.

Transformation of tassel structures into vegetative organs can occur when short-day plants (e.g., homozygous for the indeterminate, id, factor) are exposed to a short day regime early in development followed by a period of long days. In such plants, a variable number of floral appendages become transformed into leaflets, and in some cases, tassel spikelets form plantlets. This phenomenon is also characteristic of the crazy top disease, caused by the mildew fungus Sclerophthora macrospora. In the latter, all floral appendages are transformed into plantlets on the tassel. Among the genetic variants producing related changes are the three dominant factors Tp, Tp2, Cg (Teopod, Teopod-2, Corngrass), which cause plants to become teosintoid (i.e., highly tillered, with narrow leaves and leaf-like floral appendages). Cg and Tp can be normalized by gibberellic acid, and Cg by autumn conditions. Plants affected by the recessive tb (teosinte branched) are highly tillered and have staminate inflorescences in place of ears, but have normal tassels. Related transformations are brought about by the andromonoecious dwarfs and anther-ear factors d, d2, d3, d5, D8, and an. Each of these factors reduces plant height and leaf length and causes proliferation of the "suppressed" stamens in the ear; all, except for the

dominant, D8, are normalized by gibberellin treatments. The andromonoecious dwarfs are so greatly altered in plant proportions that they have been called "cabbage-like."

Another major class of factors acts by changing the size or degree of proliferation of specific parts of the tassel and ear. Tunicate (Tu), for example, elicits an enlargement of all the glumes in the tassel and ear (where each kernel is surrounded by miniature husk-like structures), while vestigial glumes (Vg) has the opposite effect, inhibiting glume growth and thereby leaving the anthers and cob virtually naked. Papyrescent (Pn) produces long papery glumes, in contrast to the thicker structures characteristic of Tu. The number of lateral branches and the length of spikelet internodes is increased by the ramosa factors (ra, ra2). Ramosa tassels have about twice the number of major lateral branches, and have a "Christmas-tree" aspect because the branches increase in length from the tip to the base of the tassel; this is particularly pronounced in ramosa-1 tassels, and similar branching is found in the ear as well. In ra2 individuals, lateral tassel branches are held upright rather than being perpendicular to the tassel axis, and there is an extra internode between the base of each spikelet pair and the rachis to which they are attached, both in the tassel and in the ear. Unbranched (ub) plants have the opposite effect of suppressing lateral branch formation in the tassels, so that only a single main spike is produced. Branched silkless (bd) produces additional spikelets in the tassel and suppresses the formation of silks in the ear. Polytypic (Pt) causes an unregulated proliferation of ear and tassel spikelets, while silky (si) causes silks to form profusely in both inflorescences. Silkless (sk), on the other hand, suppresses silk formation, making the plant female sterile.

Leaves

Most, if not all, of the factors that affect leaf shape (i.e., its outline) also affect other organs, particularly the stem (see section on pleiotropic factors). The only factor thought to specifically affect leaf shape is narrow leaf (nl), a factor that reduces leaf width. In contrast, factors that cause defects in leaf structure are usually specific for this organ. The collar-like ligule and auriculate tissue at the transition between the leaf sheath and the blade is reduced or eliminated in liguleless plants (lg, lg2, and Lg3). Auriculate outgrowths occur in the leaf blade with Kn, causing knot-like protrusions and cups. In cr, as in the andromonoecious dwarfs and other reduced-stature types, the blade is wrinkled (presumably due to hyperexpansion of the lamina) to a greater extent than is typically found in normal materials. Superficially, at least, the opposite effect is found in ragged, Rg, and slashed, sl, where tearing occurs over linear stretches of the blade. Ragged seedlings, rgd, have greatly reduced, often thread-like leaves. Adherent (ad) plants express a clinging together of young leaves and of the tassel, presumably reflecting cell-cell adhesions at surfaces that normally are free from one another. The surface of hs sheaths is hairy; Rs and rs2 result in rough sheaths. The effects of green-striped (gs) include reversible wilting between the veins, suggesting a possible vascular alteration.

Roots

Relatively few factors affecting root development have been identified. The rootless (rt) factor reduces the number of secondary roots with the

result that the plants have difficulty remaining upright. Ageotropic growth of the radicle is conditioned by the recently isolated agt mutation (Doyle, 1978), and by photosynthetic mutations that prevent the accumulation of starch statoliths in the root apex (Miles, 1981).

Stem

In contrast to the homoeotic modifications that accompany the dwarf factors listed previously, the numerous brachytic, nana, and reduced types (br, br2, br3, bv, cr, mi, na, na2, rd, td) have more nearly normal form. The shortening in some is a consequence of more or less uniform reduction of internode length, while in others, it is due to a specifically localized reduction. Most of these types have normal inflorescences and none are normalized by gibberellins.

Lazy (la) plants are normal in height but grow prostrate to the ground. This behavior is due to a defect in either their geotropic or phototropic sensing mechanism and is correlated with an abnormal distribution of auxin across the stem. Extensive tillering at the base of the stem is characteristic of grassy tillers (gt). The wilting caused by wi has been traced to a defect in metaxylem development in the stem.

Pleiotropic Factors

Several of the factors mentioned previously have pleiotropic effects involving either different structures within one organ or structures in different organ systems. The most dramatic of these are the teosintoid factors (Tp, Tp2, Cg, tb), which to one degree or another cause plants to become 'primitive' in appearance, and the andromonoecious dwarfs (an, d, d2, d3, d5, D8), characterized by a shortened culm, short, broad leaves, and anthers in the ear. These and other genetically controlled alterations, as well as modifications due to disease, accidents, and environmental factors, have made it possible to define the following groups of homologous structures, based upon customarily used morphological criteria and internal anatomy:

scutellum, coleoptile, leaf
anther, leaf, pistil
tiller, ear branch, spikelet
leaf sheath, husk, glume
leaf blade, flag leaves (on husks)

Three other pleiotropic factors deserve mention. Pygmy (py) causes a number of plant parts to become reduced in size and abnormally proportioned. These plants are about 3/4 normal height and have short, pointed leaves with longitudinal striations of white tissue. The barren stalk factors (ba, ba2) prevent the formation of ear shoots on the culm and lateral branches on the tassel. Brittle stalk (bk2) affects plant morphology indirectly by causing plant parts to become extremely fragile. This property, which is of interest not only as an alteration in cell wall construction but also as a source of easily disrupted tissue, has not yet been explained or exploited.

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47 GENETIC APPROACHES TO MERISTEM ORGANIZATION

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A large number of mutants in corn affecting virtually all aspects of the vegetative form and reproductive structures are known (reviewed by Coe and Neuffer, 1977). The anthocyanin and plastid mutants can now be used routinely to genetically mark the cells of the shoot apical meristem and analyze the ontogeny of the primary plant body through clones. First we shall briefly describe the morphology of the corn plant and then discuss some of the new features of the apical meristem emerging from such studies.

Morphology of the corn plant: Corn is grown as an annual crop. The plants are about two to three meters tall with about 15 to 20 nodes. The stem is segmented, bamboo-like, and each node bears a single, simple leaf. The leaves are arranged in two alternate rows and the primordia of the first five or six leaves are already present in the embryo of the mature, dry kernel. After germination, 10 to 15 additional leaves and male and female inflorescences are produced. Prior to the formation of leaves 6 through 12, the average size of the shoot apex becomes successively larger due to an increase in cell number, and this accounts for a gradual increase in the width of these leaves relative to length (Abbe *et al.*, 1941; Ledin, 1954). The term shoot apex, as used here, refers to the region of the shoot apical meristem distal to the youngest leaf primordium.

The male inflorescence, or tassel, consists of a single central spike and about 10 to 50 lateral spikes. Some of the lower lateral spikes branch further to produce one to four second order spikes. On the axis of lateral spikes, the spikelets are arranged in two rows with a pair of spikelets at each location. Each spikelet has glumes that enclose two male florets. The ear shoot represents a modified axillary branch whose meristem produces about 8 to 14 specialized leaves commonly known as husks and finally terminates in a cob bearing the kernels. In the husks, the leaf blades are either absent or remain short, but the sheaths are well-developed and wrap around the cob. The axis of the ear shoot, commonly known as the shank, is also jointed and distinguishable into nodes and internodes. The female florets are arranged in double rows on the cob.

The vegetative shoot apical meristem: The structure of the apical meristem has been described in terms of cytological zonation pattern using the conventional methods. The shoot apex of young seedlings (72 hours after sowing) shows a single-layered tunica covering the corpus (Popham, 1951; Bonnett, 1953; Ledin, 1954). The cells of the tunica divide anticlinally and periclinal divisions occur at the time of initiation of leaves and florets. No distinct tunica initials have been observed. The cells of the

corpus divide in various planes and a group of three to six larger and centrally located corpus mother cells have been reported (Ledin, 1954). Based on the vacuolation pattern and staining reaction, the corpus is further distinguishable into the subtunica layer, the subcorpus initials, and the rib meristem. The tunica-corpus theory of Schmidt (1924) has been a valuable concept to describe the structure of the shoot apex, but its biological significance is not clear. The layered arrangement of cells in the outer layers may simply reflect the preferred or the predominant orientation of cell division within the meristem.

The structure of the shoot apex has also been studied using a variety of analytical techniques (for a critical review, see Chapter 5 in Steeves and Sussex, 1972), but a unified picture of the development of the primary body from the shoot apical meristem is yet to emerge. The conventional approaches neither tell us about the fate of individual cells nor can we infer the time when a cell or a group of cells at a specific location becomes destined to produce a particular structure.

If the fate of each cell and its cellular progeny (clones) could somehow be followed, then, in principle, the development of the primary body could be described in terms of cell lineages and spatial and temporal interactions between cells. Genetic techniques offer one of the viable approaches to generate and mark the needed clones.

Analysis of the apical meristem through cytochimeras: By employing colchicine-induced periclinal chimeras Satina, Blakeslee and Avery (1940) demonstrated the presence of three independent layers in the shoot apex of Datura. The analysis of leaves and floral apices showed that the derivatives of these layers were present in them (Satina and Blakeslee, 1941). Since then, periclinal cytochimeras have been extensively used, and there is strong evidence to support the existence and functional reality of apical layers in dicotyledonous and monocotyledonous plants (Stewart, 1978; Stewart and Dermen, 1979). The outermost, second and third cell layers shall be referred to as L-I, L-II and L-III. Stewart and Dermen (1979) have suggested that "the ultimate source of new cells in the apical meristem is from one to three initial cells in each apical layer." Their studies in several plants indicate that the initial cells may not be permanent in an absolute sense. In plants with a mericlinal plastid chimera, the sectors measuring 1/4 and 1/3 of the stem circumference were found to extend through several nodes and then these sectors completely disappeared after a period of growth (Bain and Dermen, 1944; Dermen, 1945; Stewart and Dermen, 1970). This type of sectoring pattern would be expected if an initial cell functioned for a while and was then replaced by another initial; the latter could arise from a shifting arrangement of cells at the summit. Whereas, periclinal chimeras have been very useful to establish the clonality between cell layers and their derivatives, the fate of individual cells cannot be followed, nor can we determine if lineages of two or more cells from each layer are present in an organ.

Clonal analysis of meristem organization in corn: The analysis of clones induced at various stages of development is proving very instructive to understand the functional organization of the shoot apical meristem. The basic strategy is to use kernels (mature or at various stages of embryo development) or seedlings that are heterozygous for anthocyanin factors (A/a B/b Pl/pl Bz2/bz2 R-r/r-g) or factors affecting the color of plastids

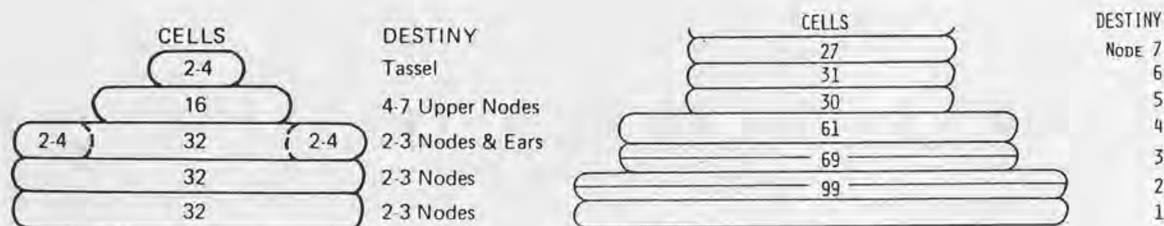
(Wd/wd, Yg2/yg2), to eliminate the dominant allele by x-raying and to then examine the clones (sectors) in various parts of the mature corn plant. In addition to anthocyanin markers, mutations such as Vg, Tu, Ts6, or ra can also be employed to trace the clones in the tassel and ear shoot. The expression of these mutations is cell autonomous in the tassel (Johri and Coe, 1980). A plant heterozygous for the anthocyanin factors develops purple color in virtually all the parts and, depending on the dominant marker lost, pale purple or colorless (i.e., green) sectors are observed against a purple background. Clones originating in the L-I cell layer can be easily distinguished, while those from L-II or L-III can be observed in leaves or husks in the sectioned material. Since any cell of the apical meristem can lose the dominant allele, and if all cells are capable of dividing and proliferating, then we expect a random distribution of sectors in the mature plant. The sectors are in fact found to be randomly distributed and extend through one, two, three or more nodes. Often identical sectoring patterns are observed at the same nodes in several plants. The sectors are scored for their extent (number of nodes affected), width (as a fraction of culm perimeter; sheath, husk or leaf width) and location (in the plant and position relative to midrib in leaf).

The reciprocal of the fraction represented by a sector is expressed as apparent cell number (ACN). ACN is an extremely useful parameter and is an overall estimate of the number of cells giving rise to a particular part of the corn plant. Since the size of each sector is highly variable, it is impractical to describe the proliferation pattern of a cell meaningfully. However, the average proliferation pattern of a group of cells at a given node level can be described. Determining the sector length in terms of "extent" at each node level gives us precisely this information and the proliferation pattern of a group of cells is thus predictable. The analysis of sectors in terms of ACN and extent, first introduced by Coe and Neuffer (1978), has permitted a more incisive analysis of the organization of the shoot apical meristem (Johri and Coe, 1980; 1981).

Steffensen (1968) has described the postembryonic development of the 15 or so upper leaves. The average sector width indicated that the blades in upper leaves develop from half as many cells as the blade in the lower leaves. The proliferation of a cell lineage along the margin was several fold more than that along the midrib. The developing leaf primordium amounted to 2/3 of the circumference of the apex and the gap coincided with the position of the midrib of the next younger leaf. As a consequence of this, a sector along the leaf margin continues in the leaf above adjacent to the midrib. It was observed that the cell divisions are regulated in zones of the apex but not precisely. "Apparently a cell can survive in the upper apex without dividing, while other cells in the same zone can divide more than once" (Steffensen, 1968). The cells in the lower part of the shoot apex usually produce the lower half of the plant, while those at the crest of the apex generate the upper half.

Coe and Neuffer (1978) observed that the sectors extend about 2 or 3 nodes each in the lower 6-8 nodes (nodes 7 through 15) and about 4 to 6 nodes in the upper nodes (nodes 15 through 20). The ACN and extent data show that there are 2 to 4 cells at the top to generate the tassel (Fig. 1), followed by 16 cells destined to become the 4-6 upper nodes (above the ear) and finally three levels of 32-cells-each representing the remaining nodes.

The 32 cells at a given level develop into 2-3 nodes. The ear shoot develops from 2-4 cells which descend as a subset of the 32 cells at that level. These results strongly suggest that functionally, the L-I layer of the shoot apex at the dry kernel stage behaves as if it is organized like a stack of coins or discs. Each disc can be conceived of being represented by a group of cells, such that each cell can proliferate to a variable and different extent, but as a group, the proliferation pattern of cells is fixed to some degree and is predictable.



Figures 1 and 2. Number and destiny of L-I cells in the shoot apical meristem of the corn embryo at the dry kernel stage. Figure 1 (left). Nodes 8 through 20. Figure 2 (right). Nodes 1 through 7 (no sectors were observed in the first internode).

Specification and development of nodes: The majority of sectors start at the base of an internode, continue up through one or more nodes and terminate in a leaf. At the ear-bearing nodes, the sectors start in the bract or husk and end in the leaf situated one or two nodes above. These results confirm the observations of Sharman (1942); the clonal analysis and the developmental anatomy both show that an internode corresponds to the lower half of a developing node (associated with the leaf above), and that the ear shoot represents an axillary bud associated with the leaf above and not with the leaf in whose axil it appears. Thus a leaf, its internode below and the axillary bud constitute a developmental unit.

Though none of the L-I cells have become committed to produce any of the specific nodes 8 through 20, the number of times a cell divides does not appear to be completely random either. At these nodes no sectors were observed to begin in the middle of an internode, suggesting that during the formation of individual nodes a single layer of cells destined to produce the entire internode is produced. A direct evidence for this comes from the distribution of sectors in nodes 1 through 7. In this experiment, the dry kernels were x-rayed and the seedlings were treated with gibberellic acid. This treatment results in an elongation of the lower internodes and sectors can be scored easily (Johri and Coe, 1981). The sectors occupied only part of an internode for nodes 2 and 3, but a complete internode for nodes 4 through 7 (Fig. 2). The sectors in the second internode were microscopic, 4 cells in width and about 16 to 20 cells in length. In the apical meristem of the dry embryo, internodes 2 and 3 are thus represented by at least 2 cell layers, internodes 4 through 7 by a single layer of cells and the remaining nodes, 8 through 20, by groups of 32- or 16-cells in such a way that each group generates 2-3 or more nodes. Groups of cells thus become destined at

successively higher levels and specification of destiny progresses from the base of the plant toward ear-bearing nodes. Likewise, upon x-raying the seedlings 8 or 13 days post-sowing, sectors are found to extend for one internode only up to node 11 and for more than one node above 11th node. Thus at least four nodes differentiate during 8 to 13 days after sowing the kernels. It is not known if the process of specification of destiny of a group of cells is analogous to or identical with the process of cell determination. Whether or not the property of "destiny" of cells is heritable through mitosis remains to be established.

After a node is specified, each nodal initial no longer behaves as an independent component and collectively all the nodal initials become destined to produce a particular single node. The average sector width shows that the lower nodes are specified when there are about 32 L-I cells, while nodes 11 and 12 become determined when there are about 64 L-I cells (Figs. 1 and 3 in Johri and Coe, 1980). Since the size of shoot apex is known to increase gradually during the formation of leaves 6 through 12 (Abbe et al., 1941; Ledin, 1954), the presence of a larger number of cells during the formation of nodes 11 and 12 is not surprising. The number of L-I cells during the specification of nodes 13 through 20 is yet to be investigated.

Sectors measuring about 1/64 of the culm perimeter are still observed to span the entire internode in the lower nodes (internode 4 in Fig. 2), even though these nodes are specified when there are 32 L-I cells. These 32 cells seem to divide generating 64 cells and then cell divisions leading to the lengthening and widening of the internode axis occur.

There is strong evidence that even the lower nodes are initially produced as a group about 7 or 8 days after pollination. In these experiments the developing kernels (168 and 192 hours after pollination) were x-rayed and allowed to mature. In the plants grown from these kernels, the individual sectors starting in the first leaf terminated in the second leaf or in any of the leaves 3 through 7. This observation suggests that a group of nodes is produced first. In some plants sectors were present either only in the first leaf or in leaves 2 to 4. This pattern indicates that individual nodes are generated starting from the first one (the first node is the lowermost one). The most plausible trend indicates that during embryogeny 8 or 16 initials destined to produce a group of nodes are first produced. A single initial cell of this group divides in such a way that it generates a specific nodal initial and an undifferentiated cell. The latter in turn follows the same pattern of cell division and finally all the nodes are formed as shown in Fig. 3.

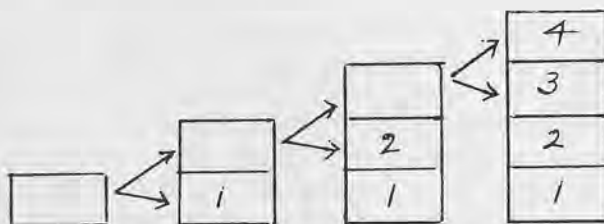


Figure 3. Proposed cell division pattern of a group initial cell. Numbers 1 to 4 refer to the lower four nodes. It is assumed that this initial cell is destined to produce four nodes.

Early embryogeny and establishment of bilateral symmetry: The pattern of sectors in plants obtained from kernels x-rayed either just after fertilization or during the initial phases of embryo development has been investigated to some extent by Stadler (1930), McClintock (cited in Steffensen, 1968) and Steffensen (1968). A more detailed and systematic study is, however, needed in this particular area. The existing observations tend to indicate that the bilateral symmetry of the shoot apex may be established at the first vertical division in the terminal cell of the 3- or 4-celled proembryo. Half green and half albino plants separated at the midrib and continuing into the tassel have been described when kernels were x-rayed at the time of the first vertical division (Steffensen, 1968). Though the two cells usually generate these symmetrical halves, there seems to be nothing that prevents one cell from contributing lineage to the other half. The separation of two halves does not appear to be an absolute determinative event, and topographical parameters may also be involved. In one of the plants where clones had been induced during embryonic development (6 days after pollination), a sector showing loss of Pl and measuring about 1/4 to 1/3 of the culm perimeter, was present on the left of the midrib in leaves 1 through 7 (in this instance the uppermost leaf was counted as 1). It extended below but included part of the blade on the right of the midrib in leaves 9 and 11 (Johri and Coe, 1981). Since this sector crossed the midrib, the two halves separated by the midrib cannot be considered as compartments. The proliferation pattern of the two cells separated at the first vertical division does not seem to be predetermined and spatial location seems to override the lineal continuity in specifying the position of the midrib.

Proliferation of cell lineages in the tassel: Most of the tassels develop from 4±1 cells of the embryo. When a sector includes the central spike, the spike is divided vertically into halves, showing that the subset of cells developing into the central spike comes from two separate cells. An elegant expression of this is observed in -/ra sectors where all the additional branch spikes are arranged only on one half of the central spike. The separation of a tassel into two parts based on clonal boundaries is consistent with the cleavage pattern of the zygote. The two cell lineages continuing into the central spike seem to be derived from the two cells separated by the first vertical division of the 3- or 4-celled proembryo (Coe and Neuffer, 1978). A tassel thus consists of two halves, each of which descends from two cells of the apical meristem. By the time the kernels are mature, the four cells at the top of the meristem have already become destined to produce the tassel because the vast majority of the clones induced at this stage do not extend from the plant body into the tassel. It is not known as yet whether there are genes that specify the collective decision of four cells to produce a tassel. The factors ramosa, tunicate, tassel seed-6 and vestigial alter the tassel morphology by modifying the developmental decision of an individual clone and not of each half or of four cells that generate the tassel. The four cells seem to be derived from the L-I of the apical meristem, and the contribution of L-II or L-III, if any, is not known at this stage. It is conceivable that only cells derived from the embryonic L-I and L-II participate in tassel formation because the gametes have been found to originate from these two cell layers in Chlorophytum (Stewart and Dermen, 1979). All four cell lineages are present at the base of the tassel, but their relative proliferation is highly variable and finally, only two of these extend into the central spike in such a way that only one lineage is derived from each half of the plant.

Proliferation of cell lineages in the ear shoot: The distribution of sectors at nodes 12 through 14 shows that an ear shoot represents the lower part of an internode. The same cell lineage continues along the margins of the leaf sheath above. Normally each nodal initial cell seems to divide into a leaf initial and an internode initial. The derivates of cells generating the sheath margin continue proliferating and give rise to an ear shoot at the base of a developing internode. The organization of the meristem in an ear shoot is similar to the shoot apex of the main axis and lineages of at least two layers can be traced in the husk. The sector width shows that one to three cells of the L-I and a small number of L-II cells participate in the development of the ear shoot. The precise number of L-II cells is unknown, but since the size of L-I and L-II cells is the same, the number of L-II cells is likely to be one to three.

The proliferation of each cell lineage is highly variable in terms of the shape of the clones and the number of husks through which a clone continues in the ear shoot. During ontogeny, periclinal cell divisions seem to occur frequently in L-I and L-II cells with the result that one cell layer gets displaced by the other. One out of five ear sectors extends into the cob. When induced at the dry kernel stage, the clonal boundaries show no relationship to rows of florets even though a pair of rows develops from a single branch primordium. The clonal tissue switches a complete row or often only half a row laterally as determined on the basis of glume color. A row of branch primordia does not originate from the lineage of the same cell through the entire length of the cob. Each row can develop from cells derived from two different but adjacent cell lineages. In many instances the florets at the border showed half-sectored glumes. Unlike the tassel the cob is not divisible into lateral halves based on clonal restriction. The clonal boundary that runs along the midrib of leaves and through the central spike in the tassel is not distinguishable in the ear shoot. This seems to be due to a variable proliferation of cell lineages and also due to the fact that the initials of the ear shoot may occasionally be derived either from the front half or the back half of the proembryo.

Conclusions: In this brief review, we have tried to focus attention on some of the new features of corn development based on the analysis of clones which provide fresh insights and a different framework to interpret the functional organization of the shoot meristem. The clones can be induced by x-raying the mature or developing kernels or a seedling heterozygous for anthocyanin or chlorophyll factors. The sectors in the culm originate from the L-I cell layer and can be scored in terms of ACN (apparent cell number) and extent in the mature plant. The clones induced at the dry kernel stage occur randomly distributed in the corn plant. Therefore cell divisions occur throughout the L-I cell layer and all cells contribute to the development of the primary plant body. No evidence was found for the presence of a group of L-I initials at the distal end of the shoot apex or for the presence of anneau initial zone. These features may be related to the determinate growth pattern in corn.

In the apical meristem (dry kernel stage) only the destiny of cells producing the tassel and the lower 6-7 nodes is observed to be firmly specified. The other cells of the shoot apex behave as uncommitted and each lineage can extend from one to five nodes or so. Functionally, the shoot apical meristem in corn can be visualized to have been organized like a stack of discs, and we describe this organization in terms of four distinct

zones. The lowermost zone (a) consists of three nodes that have already differentiated individually. Their internodes are represented by at least two layers of cells and are in the process of expansion in the vertical direction. The next zone (b) consists of three to four nodes (nodes 4-7), which have also differentiated individually, but their internodes are represented by a single layer of initial cells. The next zone (c) consists of cells that later become nodes 8 through 20, and groups of nodes are represented by layers of cells. The data on extent indicate the presence of three levels of 32 cells each and a single level of 16 cells. The 32 cells at a level generate on an average of 2-3 nodes each, while the 16 cells develop into 4-6 nodes below the tassel. The zone (d) consists of 2-4 cells at the end destined to produce the tassel.

The precise number of nodes generated by a group of 16 or 32 cells is regulated by genetic and environmental factors. The maximum potential is expressed only under optimal growth conditions, and a group may generate only a single node under suboptimal growth conditions (unpublished observations of Johri and Coe). Our model of the shoot apical meristem predicts that all nodes are initially produced as a group and that individual nodes differentiate from the base toward the top. There is strong experimental evidence to support both of these views. The analysis of clones induced at the seedling stage shows that the specification of individual nodes progresses in a time-phased manner starting from the base of the plant and progressing toward the tassel and that initials are laid down a single node at a time. Differentiation of 11 individual nodes was observed in the seedlings 8 to 13 days after sowing. The distribution of clones induced during embryo development strongly suggests that even the first few nodes are produced initially in a group about 7 or 8 days after pollination. All nodes are thus produced initially in groups and the formation of such groups is detectable during early embryogeny.

The axis of bilateral symmetry may be established at the first vertical division in the terminal cell of the 3- or 4-celled proembryo. Lineages of both of the cells separated by that vertical division are present in the shoot apex. The anatomical studies, however, show a lateral origin of the shoot apex on the anterior face of the embryo (Randolph, 1936) and the differentiation of the axis is first recognizable 9 or 10 days after pollination. Based on the sectoring patterns, it appears to us that the shoot apex is terminal in origin and that it differentiates much before it can be recognized morphologically in histological sections. The growth of scutellar tissue could result in shifting the shoot apex to one side and thereby give the impression of a lateral origin.

The shoot apex maintains at least two and possibly more apical cell layers. The derivatives of L-I and L-II layers are distinguishable in leaves and ear shoots. The proliferation of these two layers is much more variable in leaves and ear shoots than in the culm. A noteworthy feature observed repeatedly is the high degree of variability not only in the size of clones, but also in time and extent when cells at similar locations proliferated in different shoot apices. The distribution of clonal boundaries in the leaf, tassel, ear shoot and culm shows that the final fate of a cell is determined late by its spatial location rather than by its lineal descent.

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48 REPEATED EXCISION AND ANALYSIS OF DEVELOPING KERNELS FROM A SINGLE MAIZE EAR

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The full development of a maize kernel requires about 60 days after pollination; this is then followed by a period of dehydration. During the 60 days development, the various endosperm storage proteins are accumulated in large amounts at specific times (Ingle _et al._, 1965). The major storage proteins, the alcohol soluble zeins, are synthesized and deposited between about 15 and 40 days after pollination. At the end of the synthetic period, the amount of zein can be in excess of 12 mg per endosperm accounting for 60 percent of the total protein in the mature kernel (Hansel _et al._, 1973). The time specificity of endosperm protein accumulation and the wide range of mutants available that modify the times and rates of accumulation (Coe and Neuffer, 1977), make the developing maize kernel a particularly interesting developmental system to study.

We describe below a method for preparing kernels for a developmental series from a single maize ear. This method has been applied to the study of the expression of the zein genes during development of the kernels from normal and mutant (opaque-2) maize plants, but it is equally applicable to any of the wide range of maize kernel proteins that may be of developmental interest.

The maize ear comprises 8 to 16 or more rows of kernels arranged on the woody rachis (the cob). This is enclosed within a number of large foliaceous bracts (the husk). Each row may comprise as many as 30 or 40 kernels. In order to study the development of the maize kernels within the husk, we have found that one can dissect the husk longitudinally to expose the row of kernels beneath. The kernels can then be excised without disturbing the adjacent rows and the husks folded back into place and sealed with grafting wax to prevent dehydration. Using this method, we have removed kernels from a single ear every two days for two weeks. Depending upon the number of rows of kernels, it is feasible to make as many as ten samplings without any observable detrimental effect on the development of the remaining kernels. As a control for the normal development of the kernels, protein extracts were made from the excised kernels and compared by SDS-polyacrylamide gel electrophoresis with the patterns of protein extracts made from kernels allowed to develop for the same period in an undisturbed ear. No differences were seen. Furthermore, it is possible to allow the excised kernels to incorporate radioactive amino acids into protein in a type of _in vivo_ assay system. The kernel will continue to incorporate ^{3}H-Leucine into zein for over 24 hours after excision.

For a developmental time series, it is normally possible to remove sufficient kernels at each sampling for several experiments. We have made protein extracts, ^{3}H-Leucine incorporation into isolated kernels, and prepared RNA for _in vitro_ translation assays, Northern type experiments, and several other experiments from single samplings of 10 - 15 kernels at ages from 10 to 40 days after pollination.

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49 CHLOROPHYLL AND CAROTENOID MUTANTS

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The most frequently observed class of mutants in maize is probably the one in which the chlorophyll and/or carotenoid pigments are altered. Undoubtedly, this is due to the many gene loci involved in the development of normal plant pigmentation. Among these are the loci responsible for chlorophyll and carotenoid biosynthesis. Also, there are probably numerous loci necessary for normal chloroplast development. Failure of many of these latter genes to function normally will result in chloroplast structural changes that may interfere with the normal incorporation of plastid pigments. In addition to changes in the nuclear genes controlling normal pigmentation, there also are changes in the plastid DNA that affect the accumulation of chlorophyll and pigments.

By the end of the second decade of this century, papers were appearing on the genetics of "chlorophyll" mutants in maize. Since that time, many reports of pigment deficient mutants have appeared. There has been a rather extensive literature on the genetics of these mutants (The reader is referred to Coe and Neuffer, 1977, for an extensive tabulation of mutants that have been described, their assigned symbols and phenotypes). The genetics of some of these mutants have been worked out, enabling their placement on the linkage map (Again, the reader is referred to Coe and Neuffer, 1977, for this information). The geneticist and plant breeder have probably seen many more pigment-deficient mutants than those described in the literature. Often a worker does not have the inclination or time to work up the genetics for such mutants. Thus, many are observed but few are studied extensively enough for a description of them to appear in the literature. For example, in my present research, I observe hundreds of chlorophyll and/or carotenoid mutants each year but very little is done with most of them except to record that they have occurred.

These mutants have not been utilized to their full potential as tools in the study of pigment biosynthesis, pigment interactions, and the effect of pigment alterations on photosynthesis. These mutants also can be useful in studying the role of pigments in the development of chloroplast structures.

The remainder of this report will be a brief review of studies of a few of these mutants, which might be of interest to plant molecular biologists.

One class of pigment-deficient mutants that has been most thoroughly studied is the white-endosperm-albino mutants. Mutants of this class have white or pale yellow endosperm and give albino seedlings (in a few instances the seedlings can be pale green or full green). A survey of these mutants was published by Robertson (1975). They have been used to establish the genetic control of carotenoid biosynthesis. Mutants are known that accumulate various combinations of phytoene, phytofluene, zetacarotene, neurosporene, and lycopene (see Robertson, Anderson, and Bachmann, 1978, for a review of this work).

Other studies with these mutants have established that they can make protochlorophyllide in the dark and convert it to chlorophyll in the light, and that under dim light conditions they can accumulate chlorophyll. However, plants grown under high light intensities have no chlorophyll. These mutants have been used to study the interaction between carotenoid pigments and chlorophyll. The results of this work support the hypothesis that colored carotenoids are probably involved in protecting chlorophyll from photoautooxidation in corn (see Robertson, Anderson, and Bachmann, 1978, for references to this work).

Many of the white-endosperm-albino mutants are also viviparous. Two groups, one at Texas A and M University (Smith, McDaniel, and Lively, 1978) and the other at Yale University (Robichaud, Wong, and Sussex, 1980), have found these mutants useful in studying abscisic acid synthesis and its relationship to developmental seed dormancy in corn. Several of these viviparous mutants have been shown to have a reduced level of abscisic acid synthesis. Since both carotenoid and abscisic acid biosynthesis are altered in these mutants, there is a possibility that the two biosynthetic pathways are related in some way. The nature of this relationship, if any, has not been defined as yet.

These mutants have also been used to study the relationship between plastid pigment production and chloroplast development (see Robertson, Anderson, and Bachmann, 1978, for a review of this work).

The luteus (yellow seedling) mutants have proved to be useful tools in the study of the genetic control of chlorophyll biosynthesis in corn. Mascia (1978) found three mutants which were defective in the conversion of protoporphyrin IX to Mg-protoporphyrin and two which were defective in the conversion of Mg-protoporphyrin monomethyl ester to protochlorophyllide. The effect of these biosynthetic deficiencies on chloroplast development was also studied (Mascia and Robertson, 1978).

Other classes of plastid pigment mutants have been studied from time to time but most of these studies have been of a rather cursory nature.

It should be mentioned here that some of the photosynthetic mutants described in the next article also have altered plastid pigment phenotypes.

In summary, there is a vast array of chlorophyll and pigment mutants in maize. The molecular basis for the pigment deficiencies in most of these is as yet unknown.

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50 PHOTOSYNTHETIC MUTANTS OF MAIZE

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Some of the more significant questions in plant developmental biology are concerned with elucidating the mechanisms by which the nuclear and organelle genomes and their products interact during organelle development. Maize is particularly well suited for such studies, since many nuclear loci known to affect organelle biogenesis and function have been described in this organism (Coe and Neuffer 1977); furthermore, our knowledge of the physical characteristics and coding properties of maize organelle DNA is advancing rapidly, especially in the case of the chloroplast (Bogorad et al. 1979). In this report I shall briefly describe the selection, characterization, and genetic manipulation of nuclear maize mutants defective in photosynthesis. Some of these topics have been described in more detail in a recent review by Miles (1979). These photosynthetic mutants have proven to be useful in structure-function studies, and several appear to be good candidates for elucidating mechanisms by which the nuclear and chloroplast genomes interact during the assembly of the thylakoid membrane.

Selection of Photosynthetically Impaired Mutants

With the exception of maize, the vast majority of well characterized photosynthetic mutants in flowering plants were originally selected on the basis of alterations in pigment content. While many of these mutants have proven valuable in photosynthetic studies, most of these pigment deficient mutants are decidedly pleiotropic, limiting their usefulness in developmental analysis. Rather than selecting photosynthetic mutants on the basis of altered pigmentation alone, we have employed the technique described by Miles and Daniel (1973) to select photosynthetically blocked mutants by visually screening large populations of seedlings for elevated levels of chlorophyll fluorescence. This technique takes advantage of the fact that most of the quanta absorbed by chlorophyll in functional chloroplasts are converted into chemical energy (via photosynthesis), while the bulk of the absorbed energy is returned as fluorescence ($\lambda > 670$nm) in photosynthetically impaired chloroplasts. We have had success in isolating green photosynthesis mutants exhibiting specific structural and functional alterations using this technique.

For large scale screening procedures, M2 seedlings bearing mutations produced by EMS (ethyl methanesulfonate) treatment of mature pollen grains by M. G. Neuffer (1978) were planted in parallel rows in a greenhouse sand bench and grown to the 2-3 leaf stage. Screening was performed at night using a hand held long wavelength ultraviolet light source (peak emission, 366nm; UVL-56, Ultraviolet Products, San Gabriel, Ca.) to activate chlorophyll fluorescence, and the fluorescence was viewed through a red cutoff filter (complementary to the exciting light, transmitting 50% at 605nm and

and 90% at 683nm; red plexiglass with these characteristics is suitable). A good test of the lamp/filter combination to be used in screening is to infiltrate normal leaves with a photosystem II specific inhibitor such as diuron (DCMU; 3-(3,4-dichlorophenyl)-1,1-dimethylurea, from DuPont) and visually compare the level of fluorescence observed in treated leaves with that observed in untreated controls. For convenience, the red cutoff filters were mounted in the hinged flip up section of a pair of welder's goggles; for eye protection, UV blocking lenses (cut from UV protective goggles; UVC-303, Ultraviolet Products) were fitted to the stationary inner portion of these goggles. Seedlings exhibiting high levels of chlorophyll fluorescence (hcf) were easily distinguished from normal sibs through the red filter.

For optimal efficiency in screening, the seedlings should be viewed on a dark night without interfering light from the moon or nearby street lights. The stray light problem is especially bothersome in the winter when the ground surrounding the greenhouse is snow covered and highly reflective.

Using the fluorescence screening procedure, 50 families segregating high fluorescent seedlings when retested in the laboratory were identified from 1972-1975 (representing about 1.5% of the total families screened, Leto and Miles 1977); the collection now numbers over 100 (Miles, personal communication). Although many of the mutants selected on the basis of elevated levels of chlorophyll fluorescence were pigment deficient, many fully green, highly fluorescent mutants were also recovered.

In addition to the fluorescence screening procedure, a selective technique for identifying photosynthetically deficient mutants has been developed using the photodynamic inhibitor diquat (1,1'-ethylene-2,2'-dipyridylium bromide) as a selective agent (Miles 1976). Diquat kills photosynthesizing cells by mediating the formation of reactive peroxide radicals or H_2O_2 using electrons derived from photosystem I (Moreland 1967). This method should prove useful in selecting photosynthetic mutants from large populations; furthermore, it provides a means for selecting mutants blocked on the oxidizing side of photosystem II. Such mutants are typically low fluorescent and would be missed in a visual fluorescence screen, but could be picked up by screening seedlings which survive diquat treatment.

Growth of Mutants in the Laboratory

Seedlings are routinely grown in small plastic or rubber trays filled with vermiculite (expanded mica) and moistened with half strength Hoagland's solution or a commercial nutrient solution. Planting in parallel rows facilitates subsequent fluorescence screening (carried out in a darkroom). The trays are kept in a locally constructed growth chamber under cool white fluorescent illumination ($200 \mu E/M^2/sec^2$, 16hr photoperiod) at a temperature of 32°C day, 27°C night. Since high fluorescent mutants are poor photosynthesizers nearly all mutants tested to date are seedling lethals, growth to the 3 leaf stage (about 14 days) being presumably supported by kernel storage products. The onset of seedling death is marked by progressive leaf necrosis and wilting, until the entire seedling collapses. While hcf mutants grow vigorously to the three leaf stage, we have found in labeling studies that active synthesis of thylakoid membrane polypeptides ceases several days before the onset of seedling death, and hence it is probably best to harvest seedlings 5-7 days before the anticipated onset of leaf necrosis.

Types of Photosynthesis Mutants Recovered

Following identification of families segregating high fluorescent mutants in the greenhouse, kernels were obtained from each of these families and regrown in the laboratory. Those families continuing to segregate high fluorescent seedlings in the laboratory were increased, and high fluorescent seedlings and wild type sibs compared side by side in a battery of standard photosynthetic tests to determine the site of the functional block in each case (see Miles 1979 for standard methods). On the basis of these tests the hcf mutants recovered to date can be divided into roughly four groups (Miles 1979): (1) Electron transport mutants exhibiting various degrees of inhibition in photosystem II, photosystem I, or both; (2) Mutants exhibiting good rates of whole chain electron transport activity but low rates of cyclic or noncyclic photophosphorylation; (3) Mutants lacking spectrally detectable amounts of thylakoid membrane-bound cytochromes involved in electron transport, accompanied by concomitant blocks in photosystem activity in many cases, and; (4) Mutants exhibiting good rates of linear electron transport and photophosphorylation but poor rates of whole plant CO_2 fixation; these are good candidates for dark reaction mutants. Green and yellow-green mutants were recovered in each of these classes. Thus, mutants involving most portions of the electron transport chain and possibly some of the dark reactions leading to carbon fixation were recovered by the high fluorescence screening technique.

We note that many families segregating high fluorescent mutants in the greenhouse failed to do so when regrown in the laboratory (Leto and Miles 1977). These families should be retested, since they may constitute a source of temperature or light sensitive photosynthetic mutants.

Genetic Analysis of hcf Mutants

Since hcf mutants were derived from treatment of mature pollen grains with EMS (Neuffer 1978, Neuffer and Coe 1978) and since organelle genetic information is not transmitted through the pollen grain in maize, it was expected that all hcf mutants recovered would display Mendelian patterns of inheritance. To date, we have found no evidence to support a maternal pattern of inheritance for any hcf mutation.

With few exceptions the high fluorescent mutants are sufficiently blocked in photosynthesis to prevent homozygous recessive seedlings from surviving in the field even under the most favorable conditions. Following field planting of an F_2 family, plants surviving to flowering are either homozygous dominant (1/3) or heterozygous (2/3) for the hcf locus. In all cases examined thus far we have not been able to reliably distinguish between these two classes in the field. For routine propagation, we self all surviving plants. Problems arise, however, when outcrosses must be performed with plants of known genotype, since this can only be determined by progeny testing. Assuming that an F_2 has been planted, the following standard protocols have proven useful:

General Outcrossing (for propagation, stock maintenance, linkage testing)

It is most convenient to use plants in a family carrying an hcf locus as male. The critical cross is between a plant confirmed to be heterozygous for

the hcf locus and a suitable female (this could be an inbred or hybrid adapted to a particular geographic region, marked stock for linkage testing, etc.); in this instance the genotype of the male plant is determined by selfing and scoring the resultant progeny for the segregation of hcf seedlings. Repeating the outcross 3 times results in a probability of 0.96 that at least one of the selected male plants will be heterozygous, and the probability increases to 0.99 if five replicas are performed.

If it is deemed desirable to use confirmed heterozygous plants as female (in order to preserve the original hcf cytoplasm), these can be identified by sib crossing as described below for Source B plants in allelism tests.

Allelism Testing

In this instance the critical cross is one between heterozygous plants in two families (Source A and Source B) segregating independently selected hcf loci; if the loci in the two sources are allelic, high fluorescent seedlings will segregate in the F1.

Again, heterozygous plants in each source must be identified by progeny testing. This is complicated by the fact that many maize stocks are effectively one eared, so that progeny testing by selfing in both sources is not possible. The following protocol was employed when performing allelism tests among hcf loci (Leto 1978); self one plant in Source A and outcross (as male) to Source B. Test the plant in Source B by crossing (as male) onto three sibs. If repeated three times (i.e. 15 pollinations) the probability of completing the critical cross with confirmed heterozygous plants in each source is about 0.65; seven repetitions (35 pollinations) are needed to reach a probability value of 0.9.

Because of the effort involved in performing allelism tests among seedling lethals, we confined our initial efforts to testing pairs of families segregating mutants sharing a common functional block or mapping to the same chromosome arm (see below). The data, presented in Table 1, indicate only one case of allelism to date. As might be expected in the assembly of a complex organelle, it appears that mutation in one of several independent loci may lead to similar functional lesions in the photosynthetic pathway.

Mapping to Chromosome Arm Using B-A Translocations

Certain races of maize contain supernumary, or B, chromosomes (Longley 1938) which, in contrast to the 10 chromosomes of the transcriptionally active A genome, appear to be genetically inert (Randolph 1941). The B chromosomes can be introduced into standard maize lines by crossing, and many well defined B-A translocations have been isolated following X-ray irradiation of mature pollen or nearly mature tassels from plants carrying both A and B chromosomes. Since the B centromere undergoes a high frequency of nondisjunction at the second division of the microspore, these translocations provide a mechanism for producing sperm nuclei lacking most, or all, of a given A genome chromosome arm. As a consequence, B-A translocations are useful for mapping purposes (reviewed in Beckett 1978; see Beckett, this publication), and a series of well characterized B-A translocations can be used to locate a large number of recessive loci to nuclear chromosome arm in a single generation.

Table 1. Allelism Tests Among Several High Fluorescent Mutants (Leto 1978, Leto and Miles 1979).

Cross	Shared Characteristic	Allelism?	No. Confirming Crosses
+/hcf*-9 X +/hcf*-3	Photosystem II Mutants (see text)	Allelic	3
+/hcf*-19G,YG X +/hcf*-3	As Above	Non-Allelic	3
+/hcf*-9 X +/hcf*-19G,YG	As Above	Non-Allelic	4
+/hcf*-6 X +/hcf*-2	Missing Cyt. f, b_6	Non-Allelic	3
+/hcf*-2 X +/hcf*-38	As Above	Non-Allelic	1
+/hcf*-6 X +/hcf*-38	As Above (a)	Non-Allelic	Several
+/hcf*-15 X +/hcf*	Uncovered by TB-1Sb-2L4464	Non-Allelic	1
+/hcf*X +/hcf*-3	As Above	Non-Allelic	3
+/hcf*-15 X +/hcf*-3	As Above	Non-Allelic	1

(a) C.D. Miles, personal communication

The critical cross in this instance is between a plant carrying a confirmed B-A translocation (tested by crossing to appropriate tester stocks) and a plant known to be heterozygous for the hcf-locus in question (tested by sib crossing). Such testing is laborious when large numbers of mutants are to be mapped; in routine mapping work each of the B-A translocations (comprising a set covering portions of 18 of the 20 chromosome arms) was crossed as male onto three plants in a family segregating the hcf locus to be mapped. This give a probability of 0.96 that at least one of the three plants in the hcf source line will be heterozygous. The resulting F_1 kernels (optimally 100 seed from each cross) were germinated in a sand bench and screened for families segregating high fluorescent seedlings.

Data for the first 12 hcf loci located to chromosome arm are presented in Table 2. It can be seen that hcf loci have been located to several chromosome arms on the nuclear genome. A disproportionate number of loci are located on the long arm of chromosome 1 (1L); Miles (personal communication) has located additional mutants to this chromosome arm. More data needs to be collected to determine whether this concentration of hcf loci on chromosome 1L is fortuitous or whether it represents a true clustering phenomenon. In related studies we found that the rate of light reaction photosynthesis can be modulated as a function of the dosage of the long arm of chromosome 1 in instances where normal alleles were present at all the hcf loci (Leto, Beckett, and Arntzen, unpublished).

Table 2. Arm Location of Several hcf Mutants (Leto 1978, Leto and Miles 1979).

Mutant	Uncovering Translocation	F_1 Segregation WT	hcf	Probable Arm Location (f) (g)
hcf* [a]	TB-1Sb-2L4464	133	33	2L.28 →
hcf*-2[b]	TB-1La	826	149	1L.20-.80
hcf*-3[c]	TB-1Sb	417	83	1S.05-.53
hcf*-12	TB-1La	71	15	1L.20 →
hcf*-13[d]	TB-1La	167	16	1L.20-.46
hcf*-15[e]	TB-1Sb-2L4464	173	21	2L.28 →
hcf*-19YG	TB-3Sb	275	32	3S
hcf*-23	TB-4Sa	82	8	4S.25 →
hcf*-26	TB-6Sa	315	28	6S.50 →
hcf*-34	TB-6Lc	233	34	6L
hcf*-41	TB-1La	143	32	1L.20 →
hcf*-46	TB-3La	77	11	3L.10 →

(a) Not uncovered by known TB-1Sb in 17 crosses
(b) Also uncovered by TB-1La-5S8041
(c) Also uncovered by TB-1Sb-2L4464
(d) Also uncovered by TB-1La-4L4692
(e) Not uncovered by known TB-1Sb in 7 crosses
(f) Decimal numbers refer to cytological distance from centromere (see Beckett 1978)
(g) S = short arm L = long arm (e.g. 1L = long arm of chromosome 1)

An Example Analysis -- Mutants Defective in Photosystem II

At the present time perhaps the most intensively studied high fluorescent maize mutants are a group exhibiting similar functional blocks in photosystem II. Initially four mutants were investigated in parallel, the green mutants hcf*-3 and hcf*-9, and the yellow-green mutants hcf*-19G and hcf*-19YG. Parallel fluorescence, spectroscopic, electrophoretic, and activity assays revealed a striking correlation between the extent of the loss of photosystem II activity, the loss of cytochrome b-559 (a thylakoid membrane bound cytochrome that is both functionally and structurally associated with photosystem II), and the nearly specific loss of a lamellar polypeptide with

an apparent molecular weight of 32,000 (Leto and Miles 1980, Leto et al. submitted) this polypeptide is not the apoprotein of cytochrome b-559, across this series of mutants. Ultrastructural examination of freeze-fractured hcf*-3 thylakoids revealed the specific loss of large particles (multimeric protein aggregates) previously suggested to be the morphological equivalent of photosystem II reaction center complexes (Leto et al. 1980). However, despite these losses several lines of evidence suggest that at least some of the components thought to be constituents of the photosystem II complex are present in hcf*-3 thylakoids (Leto and Miles 1980, Leto et al. submitted). We currently believe that the 32,000 dalton polypeptide is an integral part of the photosystem II complex and may be required for the assembly of functionally active and morphologically distinguishable photosystem II reaction centers.

Allelism tests (Table 1) revealed that hcf*-3 and hcf*-9 are allelic, while neither hcf*-19G or hcf*-19YG are allelic to hcf*-3 (Leto and Miles 1979). Unfortunately the genetic relationship between hcf*-19YG and hcf*-19G remains undetermined (Leto 1978). Using the B-A translocation mapping technique, hcf*-3 has been located to the short arm of chromosome 1 and hcf*-19YG to the short arm of chromosome 3 (Table 2). Thus, we have identified at least two, and possibly three, nuclear loci which are involved in the assembly of the photosystem II complex in thylakoid membranes.

Because the structure and lamellar polypeptide composition of hcf*-3 is now well defined, and because hcf*-3 contains an active photosystem I, we have used these membranes as a model system to test the mechanism by which light energy is trapped and differentially distributed between photosystem I and photosystem II (Leto and Arntzen 1980).

A most intriguing question is the mechanism by which mutation at the nuclear hcf*-3 locus leads to the failure of the 32,000 dalton polypeptide to accumulate in mutant membranes, since the structural gene encoding this polypeptide is located on chloroplast DNA (Bogorad et al. 1979). As revealed by labeling studies, the 32,000 dalton polypeptide is first synthesized as a precursor with an apparent molecular weight of 34,000 - 34,500; a post translational event ("processing") postulated to be under nuclear control results in the modification of the precursor and the appearance of the product (32,000 dalton) polypeptide (Grebanier et al. 1978). In recently completed studies, we found that thylakoids from the nuclear hcf*-3 mutant fail to accumulate either the labeled chloroplast-encoded precursor (34,000 daltons) or product (32,000 dalton) polypeptides (Leto et al. submitted, Leto et al. 1980). The loss of the labeled 34,000 and 32,000 dalton polypeptides is not due to a general inability of hcf*-3 chloroplasts to synthesize or integrate chloroplast encoded polypeptides, since other polypeptides of cytoplasmic origin are synthesized and integrated into hcf*-3 thylakoids. We are pursuing further collaborative studies employing a molecular clone encoding the 34,000 dalton precursor with the hope of determining whether the hcf*-3 locus plays a direct role in the transcription, translation, or turnover of these thylakoid membrane polypeptides. We suggest that such nuclear mutants affecting the appearance of cytoplasmically-encoded proteins are potentially useful tools for probing the interactions between nuclear and chloroplast encoded information during plastid development.

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51 MAIZE BREEDING AND FUTURE GOALS

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Historical

The early white settlers in North America were introduced to maize grown by the Indians in the area. There is little doubt that the crop proved to be an important factor in providing for successful establishment of the early colonies. The maize first grown by the settlers became known as Northern Flints or New England Flints. The crop was dependent upon man for its propagation and as cultivated by the Indians apparently provided an important and stable food supply. As man moved inward from the coast over time the seed carried along with the settlers ultimately was subject to mixing (crossing) with another germplasm complex from southern USA believed to have arisen from Tuxpeno (Mexico) and known largely as Southern Dent or Gourdseed Dent (Anderson and Brown, 1952). Increased vigor and production following these mixtures resulted finally in the development of what has come to be known as Cornbelt dent varieties. These were better yielding types and varied selection pressures (mass selection) gave rise to a large number of open-pollinated varieties as selection for adaptation occurred within a wide range of ecological niches throughout the agricultural regions of the USA up to the early part of this century. Once reasonable adaptation was obtained further gains in productivity from mass selection seemed unobtainable. A shift to variety crosses was initiated for "pollen selection" by Bidwell (1868) and Beal (1876). Variety crosses, however, did not catch on because of differences in variety parents. Each strain of any given variety performed differently in a cross so that there was no consistency in performance of Variety A x Variety B say, when different sources of the parents were used.

The ear-to-row method of breeding (half-sib) was introduced by C. G. Hopkins in 1896 at the Illinois Agricultural Experiment Station (Hopkins 1902). A portion of seed of each of the selected ears was grown to determine the breeding value (yields) in single row plots. By planting remnant seed from the better yielding families it was believed that the performance of the variety would be improved. Hopkins used the system to modify oil and protein content of the corn kernel. The program has continued to this day with striking changes in oil and protein content both high and low (Dudley et al., 1974). Improvement of yield was generally not obtained due mainly to lack of understanding of parentage control and of field plot techniques. Replication was unknown and breeding plots themselves were always planted as part of a larger field of the varieties. A modified ear-to-row (Modified HS) (Lonnquist 1964) has been shown to be highly effective for population improvement (Webel and Lonnquist 1967).

At the turn of the century some investigators began to inbreed corn. C. P. Hartley of the Bureau of Plant Industry did some inbreeding and obtained increased yields from crosses of partially inbred lines (1904). He discontinued the practice, thinking there were better ways of improving corn. The first reported cross yield between inbred lines (S3) was that of Shamel (1905).

In 1905 G. H. Shull began inbreeding corn for a study of inheritance of kernel row number as influenced by self pollination and cross pollination. The resulting crosses among inbred lines led to the suggestion of a new method of corn breeding. His suggestions based on studies made with inbreeding and crossing were published in 1908 and 1909. E. M. East also began inbreeding corn in 1905 at the Illinois College of Agriculture and continued later at the Connecticut Agricultural Experiment Station. Other early contributors to corn improvement through inbreeding and selection were G. N. Collins, H. K. Hayes, M. T. Jenkins, E. G. Montgomery, F. D. Richey, G. F. Sprague, G. N. Stringfield, and H. A. Wallace.

The first attempts to produce hybrids using inbred lines resulted in loss of interest from the point of view of commercial possibilities since the lines were very weak resulting in low yield of poor quality seed. Shull (1911) made the first double cross (cross between two productive single crosses) but Jones (1918) presented evidence on the value of double cross hybrids as a practical means of utilizing the heterosis phenomenon in maize and immediately there was a rapid increase in number of inbreeding programs both public and private. By 1930 use of hybrid seed corn had reached the take-off stage. Commercial seed companies had been formed and many small seed producers were becoming interested in producing hybrid seed. Within the next 15 years the corn acreage in the USA planted to hybrid seed had reached approximately 100 percent.

Hundreds of breeders both public and private were engaged in line development. Sources of inbreeding gradually changed from O. P. varieties to more exotic varieties, advanced generations of hybrids, and backcrosses of various types. Since line improvement is basically a quantitative genetic problem, population improvement schemes were actively employed to provide better sources for inbreeding. Recurrent selection schemes of various types were developed and are being used to successfully provide better sources for the extraction of new inbred lines. Procedures employed for population improvement include mass selection; modified ear-to-row selection (modified half-sib selection); RR(F) recurrent reciprocal full sib selection; and S_1-S_2 evaluation, among others.

The current objectives in breeding are oriented in such a way as to provide increased gene frequencies of those genes contributing to improved performance. If the breeder has any reason to believe the population which he might use to extract superior lines is lacking desirable alleles he will introduce germplasm from logical sources and then use selection procedures designed to increase gene frequency so as to improve chances of selecting better genotypes for future hybrids. It has become obvious that newer lines being used as parents in hybrids are healthier, sturdier, and higher yielding than parent lines of the recent past. This has provided the basis for production of single cross hybrids successfully. Caution in the intercrossing of parent lines for subsequent line extraction is needed. Maintenance of diversity of parentage is important. The use of population improvement (recurrent selection) schemes giving rise to new improved sources for inbreeding must be given high priority.

Future Objectives In Maize Breeding

New hybrids of the future must meet needs required from any expected and unexpected changes in production practices. The most recent changes

have been increased plant densities (more plants/unit area); heavier fertilizer applications; and earlier planting. Important modifications in maize genotypes include (1) ability to undergo more rapid drydown at maturity to reduce the need for artificial drying, (2) greater disease and insect resistance, and (3) higher yield potential. This higher yield potential may be higher yielding capability *per se* and/or greater yield response to higher fertility and density levels. Russell (1974) has demonstrated improved yielding ability of more recently developed hybrids when grown at higher density and fertility levels.

What of the future? Some of the major problems will involve interaction with new races and strains of the pests which inhabit the cropping environment. Provisions must be made to continue a reasonable number of the germplasm collections that have been made for future possible needs. From time to time these collections will be of tremendous importance. Maintenance of a broad range of genetic variability for future potential needs is of utmost importance as we look to the future for all important crop species. The future may not be now but it may well be nearer than we think. We must continue to use all means of preparation for the future at all times! Advances in productivity potential of maize have been made through increasing frequencies of favorable genes scattered widely in the species. It has been a slow but steady process. With appropriate selection techniques the process can be expected to continue for some time.

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52 MAIZE BREEDING AND ITS RAW MATERIAL

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The improvement of maize in the future will become more dependent upon the use of genetic variability that is stored in germplasm banks and from that in its wild relatives, namely teosinte and Tripsacum. Some of the variability that eroded away during recent inbreeding may now be recovered without loss of certain combining abilities by outcrossing to their racial ancestors or their derivatives. Teosinte and Tripsacum germplasms are more suited for special objectives not achievable directly from the races of maize.

1. Experimental Introgression from Teosinte

Teosinte germplasm that has a free gene-flow with maize is part of the maize gene pool and thus, it may be recovered more easily from some of the races of maize for breeding purposes. But, there are four or five blocks of tightly linked genes separating maize from Mexican teosinte that are not readily transferable because of their role in maintaining teosinte's floral integrity. While the key floral traits of maize may jeopardize the survival of teosinte, the reverse of teosinte traits in maize can occur if they are deliberately selected and maintained by man. These essential teosinte segments tend to reduce yield in maize derivatives when they are homozygous. But when they are heterozygous, yields are significantly increased (Sehgal, 1963; Cohen, 1982) and factors for insect and disease resistance may be carried by the segments that have escaped a natural transfer to maize because of their linkage arrangement.

2. Preparation and Use of Tripsacum Germplasm

The special preparatory and maintenance systems to use Tripsacum germplasm in maize breeding are described in greater detail elsewhere (Galinat, 1973). The hybridization is usually difficult, depending on the stocks of maize and Tripsacum, and when hybrid embryos do develop, they usually require embryo culture. About nine months later, the F1 plants usually have a high frequency of sterile megaspores except for any unreduced eggs, common in certain corn stocks (ig gene). Fertile eggs may be consistently produced if the F1 is a tetraploid hybrid such as our cross of 4 n su g maize X 4 n Tripsacum dactyloides var. Florida which eventually yielded Tripsacum chromosome 7. It is marked by the Starchy-sugary locus and has known value in maize improvement (Galinat 1977; Cohen 1982).

By another technique, the sterile diploid hybrid may be made into a fertile amphidiploid by doubling its chromosomes with colchicine treatment such as we did with our WMT maize X T. dactyloides, Kansas hybrid. In the case of the WMT marker gene stock of maize, a recessive marker was on each of the ten chromosomes. This enabled us to identify and extract the Tripsacum chromosomes on the second backcross to WMT maize and eventually to cross-map the maize and Tripsacum homeologs (Galinat, 1973).

The Tripsacum germplasm may be maintained either as interchange chromosomes carrying segmental substitutions from Tripsacum or as extra or "addition" chromosomes. When originally selected during repeated back-crossing to maize, an alien addition chromosome from Tripsacum is univalent (unpaired). This extra chromosome can then be doubled by two different systems to obtain a fully fertile disomic alien - addition line, in which the alien chromosome is bivalent (paired). The disome can be obtained during selfing in which the male and female gametes involved in a fertilization each carry the extra chromosome or as a result of non-disjunction of the extra chromosome and the subsequent inclusion of the unreduced pair in a functional gamete.

Certain of the Tripsacum chromosomes fail to synchronize their meiotic processes with those of maize. They prove difficult if not impossible to manipulate in a maize background. The various Tripsacum chromosomes differ in the degree of their architectural correspondence to maize chromosomes and, thereby, in their capacity for manipulation during maize breeding.

3. The Nature and Purpose of the Germplasm Bank

The germplasm bank is a living repository of genetic material that has been collected from the primitive land races about the world. We have tried to preserve at least some of the primitive raw material that went into the evolution of the uniform modern varieties because emergencies may now arise in which we need that ancestral germplasm for breeding purposes. In the breeding of a relatively few inbred and hybrid lines adapted to the U. S. Corn Belt, we have cannilized evolution and discarded the genetic flexibility for divergence. The process is similar to that which occurred many thousands of years ago during selection leading to an emergence of corn from its wild ancestors. As certain metabolic and structural traits become both fixed and complicated, limits are set upon our ability to either revert or remake them. The discarded ancient types include experiments from the evolutionary by-ways as well as the ancestral steps that went into the ascent of the modern forms. Now with changing systems of agriculture and changing consumer markets, the old building blocks may once again become a cornerstone for adaptability to the service of man.

The use and restructuring of previous systems for new purposes has already been involved in the origin of the maize cob. The cupule evolved stepwise culminating in the teosinte fruit case. But, when its function was transferred from one of enclosing and protecting a grain-bearing spikelet to one of providing structural support to a thickened rachis, the modern cob became possible.

As part of the effort to preserve viable germplasm in seed banks, we need studies of the chemical mechanisms for seed death. For example, if the cause of death is an oxidation of the lipids, the means to inhibit this process is necessary. The storage of seed at temperatures of 0-4° C, at 10 percent moisture levels, and in sealed jars that can build up CO_2 all seem to contribute to longevity. The biochemistry of seed death is currently under study (Priestly et al., 1981).

4. A Preparatory System for the Use of Z. diploperennis

Preliminary steps have already been undertaken by several of the major seed companies in preparation for the use of Z. diploperennis as perhaps the only source of resistance to some new devastating epidemic. Because of the several generations required to transfer any such resistance trait from teosinte to corn, they have developed a "head start" program. According to this system, hybrids between the most important corn inbreds such as Mo17 and B73 are made with Z. diploperennis and then backcrossed to these inbreds and selfed. This material carrying ca 25 percent Z. diploperennis would be held in viable condition in cold storage germplasm banks. When the emergency of an epidemic arose, it would then be taken out of cold storage and screened for the necessary resistance without presenting any time-consuming problems of the initial hybridizations and backcrossing as well as final selfing. Differences between the backcross and selfed progeny might reflect dominant vs. recessive properties of any resistance factors. But, such germplasm developed by seed companies is privately owned and not available for public use by Experiment Station researchers.

While it is possible that this pre-breeding strategy to bring the raw Z. diploperennis germplasm into a usable status could result in some loss, yet, if the stored sample is relatively large, perhaps 5,000 kernels, it should be possible to screen out and capture the essential resistance factors.

As Z. diploperennis may be propagated by seed that has been inbred in the greenhouses of corn breeders, genetic erosion may cause some of its original resistance factors to be lost or "wear off." The hybrids should be made with the original stocks.

5. Exotic Germplasm as Raw Material

The enhancement of a given genetic system may result from the introduction of active duplicate systems from exotic germplasm. Autopolyploids have been regarded as storehouses that accumulate and conceal recessive genes. Upon the production of polyhaploids (parthenogenic diploids), these recessives are released to the process of evolutionary divergence. But, on the diploid level, we may also have duplicate loci. There is a redundancy with duplicate instructions because only one of them is necessary to control a critical function. Thus, the redundant set is allowed to vary into various recessive states that would have been rejected if solitary. Meanwhile a different so-called exotic race with the same horizontally duplicate loci may have developed the alternative locus as its functional instructions for achieving fitness.

When the two races with their alternative or so-called "horizontal" systems are hybridized, the subsequent segregations explode into a new diversity of types. The recessive genes once hidden horizontally may segregate into the double recessive condition and, thereby, are released for a try-out against evolutionary fitness. But, the same segregation may also yield the double dominant condition that results in complementary gene action for the synthesis of a greater product - perhaps a larger kernel or a longer ear. The double heterozygote for these dominants may have a similar beneficial effect that is recognized as a component of heterosis. Thus, the gradual synthetic evolution depends on genetic drift, divergence and then reconvergence. When we erode everything down to single systems thru inbreeding, we allow evolution to grind to a halt. The solution is to preserve

the various races of maize and relatives of maize with their probable alternative systems of doing things so that we may tap into this resource for the raw material to bring about continued maize improvement.

Finally, the high frequency of duplicate loci in maize as well as a few possible repeated sequences suggest that the double systems evolved with an active development function in which one replicate has a regulatory action or else an amplifying effect upon the genetic signals from the other. In any case, active instead of passive duplicate systems would give a greater end product for progressive evolution or domestic advancement.

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53 WILD RELATIVES OF THE MAIZE GENE POOL

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Maize is unique among the grasses because it possesses its flowers, male and female, in different positions on the same plant, a system that structurally promotes outcrossing (Galinat, 1977; Mangelsdorf, 1974; Weatherwax, 1954). The male flowers are borne terminally in the tassel and the female flowers are found as the familiar ear in a lateral position half way down the plant. Only the teosintes, the closest relatives of maize, possess a similar separation of the flowers.

Both the teosintes and maize are highly variable, outcrossing, wind-pollinated taxa. In outward appearance, stalk, leaves, and tassel, both taxa are so similar that the most reliable distinguishing character separating the two is the pistillate fruit, a distichous spike (two ranked) in teosinte, and a polystichous structure (the many rowed ear) in corn. This difference is controlled by two or three major structural genes and many modifiers. The seeds of maize are retained on the cob at maturity while the seeds of teosinte are dispersed as rachis-segments from the disarticulating spike and on maturity fall to the ground. This ability to disperse seed, well protected by a hard encasing lower glume, distinguishes the teosintes as wild taxa (Wilkes, 1966).

There are approximately 200 recognized races of maize, (Zea mays L. 2n=20) all of them known only in the cultivated state. There are three taxa of teosinte, Zea mexicana (Schrader) O. Knutze, 2n=20, the annual diploid of wide distribution in Mexico and Guatemala; Zea perennis (Hitchcock) Reeves and Mangelsdorf 2n=40, the tetraploid perennial form now extinct, or extremely limited in its distribution to a few clones in its type locality, Cuidad Guzman, Jalisco in Central Mexico, but widely grown in experimental gardens of maize geneticists; and the recently discovered Zea diploperennis Iltis, Doebley & Guzman, 2n=20, the diploid perennial form found in a single locality, El Chante in Jalisco, the most primitive of the teosinte forms and possibly the ancestor of the other two forms (Wilkes, 1979). Alternative taxonomy to that used here can be found in the literature (de Wet, Harlan and Grant, 1971; Doebley and Iltis, 1980; Iltis and Doebley, 1980; Smith, Goodman and Lester, 1981) but in terms of indicating gene pools they all break down to comparable groups. All three taxa of teosinte hybridize easily with maize and the F_1 hybrid from diploid parents is both robust and fertile, although cryptic genetic differences do exist as shown by the mutagenic effect of hybridizing maize and teosinte (Mangelsdorf, 1958).

Annual teosinte is recognized to have contributed significantly to the racial variation of maize in Mexico through introgressive hybridization (Welhausen et al., 1952; Wilkes, 1977). There are six recognized races of annual teosinte, four of which occur in Mexico (Nobogame, Central Plateau, Chalco, and Balsas), and two in Guatemala (Huehuetenango and Guatamala). In the Mexican States of Jalisco, Guanajuato and Michoacan teosinte is a wild, sometimes weedy, plant found mostly along stone fences, bordering maize fields - not because it has invaded the maize fields as a weed but because it is making a last stand on this narrow strip of untilled soil. In

other localities such as Chalco in the Edo de Mexico it has successfully invaded the maize field proper and survives as a maize-mimic. The largest and least maize-like of the Mexican teosintes is found in the mountains (800 to 1900m.) of the Rio de las Balsas in Guerrero and Michoacan.

In Guatemala there are two populations, Jutiapa (race Guatemala) in southern Guatemala which is most like Z. diploperennis and members of the related genus Tripsacum in that it tillers profusely at the base, has a tendency toward perennialism and lacks a central spike in the tassel. The other race (Huehuetenango) in the north of Guatemala is more closely related to Mexican races of teosinte than to the Jutiapa population. The major structural genes separating maize and annual teosinte are less than five but there are numerous modifying factors. An estimate would be about 10% of the genes are unique to teosinte, while 90% are held in common with maize. The cytoplasm of maize and the teosintes differ and teosinte offers good potential for the improvement of disease resistance in maize to viral and mycoplasmal diseases (Nault and Delong, 1980; Nault and Findley, 1981). Seed of teosinte is readily available from several researchers actively working with the plant and from the USDA gene bank, Experiment, Georgia 30212.

Beyond the primary gene pool of maize and the teosintes lies a secondary source of transferable genes by the usual breeding process in the genus Tripasacum (Culter and Anderson, 1941; deWet, Gray and Harlan, 1976). The genus Tripsacum, all perennials, has assumed increasing significance in maize genetics since the hybridization of maize with a Kansas clone of Tripsacum dacyloides, first reported by Mangelsdorf and Reeves in 1931. Not only did Mangelsdorf and Reeves cross Tripsacum with maize making an intergeneric hybrid, they then crossed the hybrid with annual teosinte to produce a trigeneric hybrid. Teosinte is now recognized to be cogeneric with maize and even in the "trigeneric hybrid" the teosinte chromosomes paired with the maize chromosomes and not the Tripsacum chromosomes, which underwent elimination. Research of the last 50 years has produced almost all combinations of maize-Tripsacum hybrids but a teosinte-Tripsacum hybrid has proved very elusive, maybe hybrids with the newly discovered Z. diploperennis might break the genetic barrier that exist between teosinte and Tripsacum spp.

Eight of the 13 to 15 recognized species of Tripsacum (deWet, Timothy, Hilu and Fletcher 1981; Wilkes 1972) are native to Mexico and Guatemala, and the ninth T. floridanum Porter ex Vassy, 2n=36 is native to the tip of Florida, the rest are found in South America. The centers of variation for these perennial grasses are found along the western escarpment of Mexico and in the mid-elevations of the eastern Cordillera of South America. The habitat preferences of Tripsacum ssp in Mexico are nearly identical to those of teosinte, seasonally dry, summer rains, an elevation of about 1500 meters and limestone soils. Tripsacum spp in South America and the United States are adapted to less dry habitats.

Tripsacum is usually placed in the tribe Maydeae along with Zea (maize and teosinte) and these two genera are the only New World members of this group of grasses. Tripsacum does show certain morphological resemblances to members of the tribe Andropogoneae particularly to the genus Manisuris (Chandravandana and Galinat, 1976). The only significant difference between Tripsacum and Manisuris is that the latter has perfect flowers while Tripsacum has both male and female spikelets which are borne distinctly, but

which, unlike those of maize and teosinte are on the same inflorescence. Recent biochemical systematics have looked at the evolution of Zea and Tripsacum relative to the genera Coix, Andropogon, Bothricochloa, Elyonurus, Dichanthium, and Manisuris (Timothy, Hu, and Levings III, 1981; Smith & Lester, 1980; Levings III, Timothy and Hu, 1976).

Evolution by polyploidy has been the mode in the genus, again unlike maize and teosinte, which have followed an introgressive hybridization mode at the diploid level. The diploid forms of Tripsacum are all morphologically distinct and allopatric in their distribution. The polyploid forms are not always easily distinguishable on either a morphological or a geographic basis and there is considerable evidence to indicate that they hybridize readily with both other polyploids and the diploids in the field (Randolph, 1970; Rao and Galinat, 1974; Rao and Galinat, 1977; Tantravahi, 1971). The sexually sterile Tripsacum andersonii Gray 2n=64 and Tripsacum dactyloides var meridonale deWet and Timothy 2n=36, 72 and also 2n =36 + 10 of maize; of South America are thought to be the product of hybridization with maize (deWet et al., 1981).

At the present time only the broad evolutionary relationships in the genus Tripsacum are known. It is hypothesized that from the presumed center of origin and present center of diversity on the western escarpment of Mexico (1000-1500 meters) where the two Mexican diploids T. zopilotense Hernandez-X and Randolph, 2n=36 and T. maizar Hernandez-X and Randolph, 2n=36 occur; the two divergent diploids moved both northward into what is now the United States and that these taxa gave rise to T. dactyloides L., 2n=36, 72; and to the south to give rise to South American forms of T. dactyloides and T. australe Cutler and Anderson, 2n=36. In addition the following taxa are also known from South America T. cundinamarce deWet and Timothy, 2n=36, and T. peruvium deWet and Timothy, 2n=72, 90, 108. Evidence of hybridization is present in all these taxa. Similarly, the tetraploid complex of Central Mexico, T. lanceolatum Rupr. ex Foun., 2n=72 and T. pilosum Scrib and Merrill, 2n=72, are both of complex ancestry, as are the tetraploids of Southern Mexico and Guatemala T. laxum Wash. and T. latrilofium Hitchcock. In addition T. fasciculatum Trin ex Ascherson, and T. bravum Gray are named Mexican species.

Experiments have established that exchanges can and do occur between maize and Tripsacum chromosomes (Galinat, 1977) and postulated naturally occuring hybrids with polyploid Tripsacum spp. have been cited for South America. The evidence to date from Mexico and Central America is that naturally occuring hybridization of maize has been limited to teosinte. It was with T. dactyloides (2n=72) that Mangelsdorf and Reeves first successfully hybridized maize with Tripsacum. Since then maize has been hybridized successfully with most of the species at both the diploid and tetraploid level. Studies of the hybrids have indicated that certain segments of Tripsacum chromosomes can be substituted for corresponding segments in maize chromosomes and the plants remain both viable and fertile. Galinat has mapped more than 50 homologous loci on the chromosomes of these two genera using an ingenious technique (Galinat, 1974). Recently T. dactyloides has been used to transfer to maize a major gene locus conditioning resistance to the rust, Puccinia sorghi (Bergquist, 1981). The potential to use Tripsacum in somatic hybrids and as a source of physiology genes and for disease resistance is tremendous. For example, the strong root system of some of the Tripsacum taxa might radically change the corn

plant of tomorrow and the hairy leaf surface might offer protection from plant juice sucking insects, notably those that transmit viruses.

The accumulated information on maize-Tripsacum hybrids and their derivatives indicates that the respective genetic architecture of maize (2n=20) and Tripsacum (2n=36, plus polyploids), while quite different, are more similar than their karyotypes would suggest. Because most species are tropical and frost sensitive a genetic garden has been established at the Fairchild Tropical Garden, Coral Gables, Florida, which contains perennial plantings of all the species and some if not much of the variation in the genus. This living garden of collections made in the last twenty years represents a gene bank for research and future plant breeding with maize and is available to the user community. The potential to exploit the genus Tripsacum and the teosintes in the breeding of maize is still in the beginning stages.

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54 SYSTEMATICS OF ZEA AND THE SELECTION OF EXPERIMENTAL MATERIAL

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Those who choose to study maize often do not know what a difficult taxon they are dealing with. Not only is the variation in the species very great, but its genetic composition is intimately linked to several related species. If the researcher wishes to not just describe a new view of one kind of maize, but to expand the study to prove its utility in new applications, he must somehow select from this broad array a representative set of materials. An understanding of the systematics of *Zea* will make the selection procedure much easier and improve testing of the reliability of a technique under many different genetic and environmental conditions (Batra *et al.*, 1978).

Maize varies more than any other crop species, by close to an order of magnitude in ear length, kernel length, plant height, etc. It is an important crop from the mouth of the Amazon to well above Lake Titicaca (3812 m elevation). Principal components analysis of maize data shows that numerous independent dimensions can be easily described, and each needs to be accounted for in a careful systematic study. Kernel color and texture, cupule width and depth, glume length and venation are some of the obvious characters differentiating types of maize, and chromosome knobs, coleoptile isoenzymes and pest resistances also provide good discrimination. If more than a few of the independent dimensions are omitted, because of economy or unfamiliarity, distortions in the systematic relationships are to be expected. Many an argument has been caused by a differing selection of characters.

Much of the variation in maize has been caused or maintained by introgression from teosinte. There is good documentation of F_1 and advanced generation hybrids in maize fields in many parts of Mexico and Guatemala (Randolph, 1976; Smith *et al.*, 1981; Wilkes, 1967, 1977), hybrids which are usually distinguishable by at least several of the many characters which separate maize from teosinte. Mexican archaeological remains of maize, those less than 4000 years old, often show signs of introgression from teosinte--very deep, long cupules, hard glumes, etc. (Galinat, 1977; Sehgal, 1963; Wilkes, 1977). Remains dating 5000-3000 B.C. have shallow, very small cupules and very thin glumes, and they show little shift to larger (over 50 mm long) types (Bird, 1979; Mangelsdorf, MacNeish and Galinat, 1967). At each of the sites spanning these periods, increase in variation and ear size comes after the appearance of teosintoid traits, seeming to indicate that teosinte had a large role in the evolution of modern maize races and the high yields of some races.

Teosinte also shows signs of ongoing introgression from maize. Within populations of the three diploid teosinte species, specimens may often be found with one or more traits evidently coming from maize, and races and populations of *Zea mexicana* can be ranked by a maizoid scale (Smith *et al.*, 1981; Wilkes, 1967, 1977). Most samples of *Z. luxurians* seem to indicate some maize introgression, evidenced by occasional wide cupules, more triangular fruit cases, non-red silks, several ranks in the tassel, longer central tassel spikes, etc. Seed samples of the Honduras population of *Z.*

luxurians often have two spikelets per cupule and other fruit-case traits indicating maize introgression, and the plants grown from them are maizoid. Seed of Z. diploperennis yields a variable array of plants. Because of these and many other observations, several people have proposed that at least Z. mexicana is a product of hybridization between an early maize and one of the species in the Section Luxuriantes (Bird, 1979; Kempton and Popenoe, 1937; Longley, 1937; Wilkes, 1979).

The taxonomy of teosinte and maize has undergone considerable change in the past few years. Doebley and Iltis (1980 a, b) have divided Zea into two sections, Zea and Luxuriantes. The latter contains Z. luxurians (Durieu) Bird (Wilkes' race Guatemala), Z. diploperennis Iltis, Doebley and Guzman, and Z. perennis (Hitchc.) Reeves and Mangelsdorf, and Section Zea has one broad species including maize and annual teosinte (except that in SE Guatemala); the division is based on tassel morphology, but other information substantiates it. They also divided Z. mays into ssp. mays (maize), ssp. parviglumis (races Huehuetenango and Balsas) and ssp. mexicana (three Mexican races). However, there are many differences separating all maize from all Mexican annual teosinte (Galinat, 1978) and few separating ssp. mexicana from ssp. parviglumis. Furthermore, ssp. parviglumis is cytogenetically a mixture (see below). The maintenance of Z. mexicana as a species including all annual teosinte except race Guatemala and its division into races still seems best (Bird, 1978; Smith et al., 1981; Wilkes, 1967, 1977). A careful, integrated analysis of a large and varied body of data, including studies of plant, ear and tassel morphology, chromosome knobs, isozymes, and whatever other features can be surveyed, is needed.

The classification of maize, though once a formalized scheme with var. everta, var. indurata, etc. (Tapley, 1934), is best left informal for the present, using an incomplete hierarchy based on races (land races). These can be flexibly changed to include or exclude populations without need for formal definitions and renaming. There is, therefore, some confusion over terms, and it may be best eventually to formalize at least some of the system. Early efforts at definition of maize races were greatly augmented by the long-term studies by the Rockefeller Foundation staff in Mexico and Colombia, associates such as Edgar Anderson, Hugh Cutler and Paul Mangelsdorf, and scientists of the Mexican, Peruvian and many other governments (Brown and Goodman, 1977; Mangelsdorf, 1974). Most of the maize of Latin America was described in eleven books, ten of them published by the NAS-NCR (summarized by Hernandez, 1973). There have been subsequent revisions and additions, and the work has been extended to Europe. Using a definition of race which lumps some of those described, there are about 240 published Latin American races, some known in detail, some occurring in several countries, some barely mentioned. Many races remain to be discovered or described. About twenty races might be unique to Canada and the United States (Brown and Goodman, 1977; Tapley, 1934). There is an unknown number in the Old World, but one can guess at 40 additional races. Of course, there are many geographical and morphological subraces.

This array of about 300 races cannot yet be analyzed as a body, but several discussions have treated a majority simultaneously. Each analysis featured different character sets. When eight ear characters were used in principal components analysis followed by cluster analysis based on the principal components, nineteen complexes of Latin American races were defined as very unique and cohesive (Bird and Goodman, 1978). Many small

or intermediate groupings were omitted, and Central American and Lesser Antillean races were excluded for lack of some data. Brown and Goodman (1977) placed the races of eight New World regions into 54 groups, eighteen of which have only one member. Another analysis, based in part on geographic data, defined 14 broad groups (Goodman and Bird, 1977). A great many races in these three studies remained unclassified or do not fit the groups well because of multiple or uncertain relationships.

An entirely different approach is provided by studying patterns of positions, sizes and frequencies of chromosome knobs (Kato, 1976; Kato and Blumenschein, 1967; Longley and Kato, 1965; McClintock, 1978; McClintock et al., 1981). Here teosinte and maize have been given equal attention, so interesting comparisons are possible. There has been little effort in these studies to group races; but trends were emphasized. Paths of race migration or influence were plotted, and enormous amounts of data, less subjective and less influenced by the environment, were provided. No quantitative analyses of these data had been reported until recently (Bird, 1980b; Smith and Goodman, 1981; Smith et al., in press), with teosinte clearly receiving the emphasis. Wilkes' races are generally recognizable in knob patterns, but Balsas can be split into two or three parts, and a new grouping, of populations in western Mexico state and eastern Michoacan, is evident. Z. luxurians is very different, having thirteen terminal knobs, ten of which are lacking in Z. mexicana and Z. mays. Race Huehuetenango of northwest Guatemala, though showing all Z. luxurians' knobs, has three terminal knobs which are common in Z. mexicana plus two unique to Huehuetenango. Z. diploperennis and Z. perennis have small heterochromatic regions terminal on many chromosomes (Longley, 1941; Pasupuleti and Galinat, 1981), in large part fitting the Section Luxuriantes pattern.

Another area of advance in Zea systematics is the use of biochemical analyses of coleoptile isoenzymes, cytoplasmic DNA, seed proteins, etc. (Goodman and Stuber, 1980; Smith and Lester, 1980; Timothy et al., 1979). Here, as with the knob studies, there is less environmental influence on the characters than in morphological studies, and many characters can be measured relatively cheaply and reliably. Many grass genera are separable by seed proteins, analyzed by several techniques (Smith and Lester, 1980). Z. luxurians does not seem to have any seed proteins in common with Tripsacum which the other species of Zea do not have, but the populations used are Florida and Honduras, seemingly maizoid compared to populations from Jutiapa, Guatemala. Even given these maizoid tendencies, they seem to be separated from all the maize and Z. mexicana materials tested, by at least several electrophoretogram bands, while the latter two species are identical (Smith and Lester, 1980). Cytoplasmic DNA, both mitochondrial and chloroplast, serves to separate the two sections of Zea, and to subdivide each roughly as expected (Timothy et al., 1979). Though almost identical in cytoplasmic DNA and very similar in two digests of mitochondrial DNA, Z. luxurians and Z. perennis differ significantly in one digest (Timothy et al., 1979). Huehuetenango and NW Balsas differ from the other Z. mexicana tested in one digest, and Nobogame differs in another. The maize tested differs from most Z. mexicana in at least the last two digests.

The results of Goodman and Stuber (1980) indicate that perhaps our most complete information on maize-teosinte relationships and racial differences will come from analysis of coleoptile isoenzyme frequencies. Using isoenzymes controlled by 226 alleles at 23 loci, they can distinguish most US

inbred lines, an unexpected degree of definition. Their information on races of maize and teosinte is eagerly awaited.

Selecting materials

Two lists are included (Tables 1 and 2) which are attempts to sample the variation of maize and teosinte as efficiently as possible. They are ordered so that the most useful samples are at the top. It would be best to use all, but if they prove too many, the top three or more from each list would suffice. Not only should they be used to test new techniques, but the morphological studies previously carried out on so many materials should be repeated with all these materials, in several environments.

It is a difficult process to settle on a representative list of only 12 maize races. One cannot merely go across the major branches of a dendrogram and select one race from each because the systematics of maize is more a multidimensional network in which a point, race, interacts with almost any other point if transported into the same locale. The major barriers are differences in adaptation to heat -- some need considerable heat to mature, others are killed by that heat -- and differences in susceptibility to pests. Here we (Major Goodman, personal communication) have somewhat favored United States and Mexican materials, and only a few high altitude types are included because of the problem of adaptation to the usual United States conditions. The best place to grow these outdoors would be in central coastal California where all would grow at least fairly well, but not all of us are so fortunate to have these ideal conditions. It is assumed for this listing that popcorns and other small-eared races are conservative, maintaining ancient combinations of alleles selected before regional differences were blurred by long-range communication and consequent hybridization and replacement. Also there should be less variation per locus in small types -- popcorns suffer much less from inbreeding depression than do Corn Belt Dents (Mangelsdorf, 1974; p. 220). Therefore the smaller materials chosen may be easier to understand while representing many of the components which make up the higher-yielding races. Most of the races selected were once probably involved in the evolution of several more productive races; Ladyfinger and Pororo are fairly uninfluential types. Reid's Yellow Dent and Coroico are end products of complex interactions.

If one were interested in expanding the list, the following races might be selected: Uchukilla (Bolivia), Tuxpeño (Mexico), Chulpi (Ecuador), Pima-Papago (United States), Puya (Venezuela), Confite Morocho (Peru), Zapalote Chico (Mexico), Cuzco (Peru), Jala (Mexico), Cateto (Brazil), Sabanero (Colombia), Confite Puneño (Bolivia), and Huandango (Ecuador).

Reid's Yellow Dent represents the most common experimental type of maize, the Corn Belt Dent race complex. Its ear is fairly large, typical of the complex, and, when inbred, it reveals a complex genetic composition (Wallace and Brown, 1956).

Ladyfinger, also from the midwest United States, has small ears and kernels. Its cupules are very small, as unlike the product of hybridization with teosinte as can be found (Bird, unpubl.).

Coroico has a remarkable ear structure combining very long cupules and rachis segments with broad, floury kernels in many rows. This combination

forces pairs of rows to compress into a single "row", interlocking. It seems to have evolved from races now found largely in Colombia (Bird, 1980a; Ramirez et al., 1960).

Palomero Toluqueño has a unique morphology -- bulging, pubescent leaf sheaths, weak root system, few tassel branches and a many-rowed, almost pyramidal ear -- shared with several other races in the Mexican Mesa Central (Wellhausen et al., 1952).

Pollo has very short ears with few rows and lies at one end of a complex trend toward a very large-eared race, Montana (Roberts et al., 1957).

Dzit-bacal has eight rows of thin, dent kernels and a narrow, flexible cob (Wellhausen et al., 1957). Its chromosome knob number is very high, at positions typical of many, possibly all Caribbean races.

Huayleño represents a set of race complexes dominating the Peruvian, Bolivian and Chilean highlands. The kernels, in many colors, are usually very soft and elongate. Two chromosome knobs are typical of Andean maize (Grobman et al., 1961).

Pororo combines a Ladyfinger-like ear with prolificacy and the Caribbean knob pattern (Ramírez et al., 1960).

Longfellow has a long, 8-rowed ear and very few chromosome knobs. It is closely related to one of the two major components of the Corn Belt Dents (Brown and Anderson, 1947).

Nal-tel, though a small flint, seems close to white and purple popcorns of Venezuela (Wellhausen et al., 1952).

Pira Naranja is unique in its very many kernels per row and orange endosperm (Roberts et al., 1957). Probably it was an important contributor to Caribbean flints.

Cariaco represents a variable set of generally purple, yellow or white flours which are found from Mexico to Paraguay. Trends connect it to many of the complexes listed (Roberts et al., 1957).

Palomero Toluqueño, Pollo and Huayleño are highland races that will suffer in hot regimes.

The teosinte list (Table 2) contains all four species and four of the Z. mexicana races, omitting populations from in or near Michoacan, the races NW Balsas and Central Plateau and the population or race from western Mexico-eastern Michoacan. Z. luxurians and SE Balsas are represented by relatively pure forms; it might be interesting to study the more maizoid samples of these taxa, especially the Florida and Honduras races of Z. luxurians which so often represent that race in studies, to see if they have a broad range of introgressed traits. The collections representing each race were chosen because of their typical form, their use in previous research and their availability.

Table 1. Collection identification, distribution and racial complex for twelve maize races, ordered by general utility. With the identitification there is a code for the storage center where it is available, C for CIMMYT, I for ICA, P for PCIM and PI number if at the USDA North Central center, the addresses of which are in an accompanying article (Bird 1982). The race distribution is limited to the country of origin -- the race may extend to other countries. Most of the racial complex names are further described in Bird and Goodman (1978) or Bird (1980).

Race	Collection Identification	Race Location	Racial Complex
Reid's Yellow Dent	PI 213719	Illinois, United States	Corn Belt Dents
Ladyfinger	PI 217407	Iowa, United States	Midwest small popcorn
Coroico	Bolivia 1063 (C,I)	lowland Bolivia	Amazonian Interlocked Flours
Palomero Toluqueño	Mexico 5 (C)	central highland Mexico	Mexican conical popcorn
Pollo	Cundinamarca 401 (I)	highland Colombia	North Andean small flint
Dzit-bacal	Guatemala 322 (C)	lowland SE Guatemala	Mesoamerican Elongate Dents
Huayleño	Ancash 180 =Peru 752 (P)	central highland Peru	Altiplano Small Flours
Pororo	Bolivia 806 (I)	lowland Bolivia	Paraguayan Elongate Popcorns
Longfellow	PI 217408	Ontario, Canada	Northern Flints
Nal-Tel	Yucatan 7 (C)	southern lowland Mexico	Caribbean Rotund Popcorns
Pira Naranja	Nariño 521 (C,I)	southern Colombia	Colombian elongate popcorn
Cariaco	Cordoba 338 (C,I)	lowland Colombia	Tropical Lowland Flours

Table 2. Collection identification and distribution for seven teosinte races, ordered by general utility. All are available from the USDA Southern Center and one is at CIMMYT, addresses in the accompanying article (Bird 1982).

Species - Race	Collection ID	Race Location
Z. luxurians - Jutiapa	PI 306616	southeast Guatemala
Z. mexicana - SE Balsas (El Salado)	PI 384061	central Guerrero, Mexico
Z. mexicana - Chalco	PI 331782 =K 67-3 (C)	southeast Mexico, Mexico
Z. diploperennis (diploid perennial)	PI 441929	southwest Jalisco, Mexico
Z. mexicana - Huehuetenango	PI 343233	northwest Guatemala
Z. perennis (tetraploid perennial)	TL76B (C)	south Jalisco, Mexico
Z. mexicana - Nobogame	PI 343249	southwest Chihuahua, Mexico

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55 MAIZE AND TEOSINTE GERMPLASM BANKS

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For over fifty years maize breeders and their associates have collected and maintained maize and teosinte germplasm for use in improving maize stocks, especially in Latin America. Gene pools and composites formed from these collections continue to be used to develop new varieties which are spreading around the world, increasing yields and decreasing susceptibility to pests (Hallauer, 1978; Rockefeller Foundation, 1966 pp. 100-125). Tens of thousands of original collections and the lines, composites and varieties developed from them are available in many germplasm banks (Brown, 1975). This is cause for celebration, but there is also cause for concern (Committee on Genetic Vulnerability of Major Crops, 1972). The success of the breeding programs puts indigenous races in danger, while the germplasm collections have not fully sampled the variability available, some of which may prove vital to future needs.

During the 1920s and 1930s expeditions from several countries collected maize in many areas, but their efforts have not left a permanent source of germplasm for present use. In 1943 the Rockefeller Foundation established a joint program with the Mexican government to improve yields of maize in Mexico and the Caribbean region. Collecting began that year. By 1950 it was evident that progress was being made in breeding, in large part due to the collecting efforts and careful study of the materials (Wellhausen et al., 1952), so in the early 1950's similar programs were established in Colombia and Brazil (Brieger et al., 1958; Roberts et al., 1957). Collections from the Andean countries, Venezuela to Chile, were brought to the Colombian center, where most, except for Peru, are available today (below). Materials from Eastern South America, Guyana to Argentina, were collected by the staff of the Brazilian program. The United States and Canada have never been systematically collected, but government agencies and individuals have put together a fair set of samples (Skrdla et al., 1978; Timothy and Goodman, 1979). Collections were made in Asia, Africa and Europe in a more piecemeal way. The European Association for Research on Plant Breeding (EUCARPIA) maintains a germplasm bank. Collecting is increasing in Europe (de Bakker, 1981). Large sets of Turkish and Yugoslavian collections and small sets from China, Czechoslovakia, Ethiopia, Israel, Russia and South Africa are maintained by the United States Department of Agriculture (USDA) (Skrdla et al., 1978). The Centro Internacional de Mejoramiento de Maiz y Trigo (CIMMYT) has many collections from Afghanistan, Angola, Austria, Ethiopia, Israel and Uganda.

Once at the centers, the collections were increased, observed and incorporated into breeding programs where appropriate. Identification of the entries was standardized. For Latin America, collections were given a number preceded by the country of origin, or in the cases of Mexico, Colombia and Peru, by the state of origin. Groups (below) were named similarly: Panama Group 86 or Jalisco Gp 49B. In the United States, Plant Introduction (PI) numbers have been assigned. The ears originally collected and ears from the increase/observation plots were used to cluster the collections

morphologically. Field data were checked for consistency of the clusters, leading to further clustering and checking. Many volumes of data on plants, ears and tassels were accumulated, but very little has been published. Summaries are available in the many books on races which were written (see Timothy and Goodman, 1979, for the list).

As a way to reduce the work of ongoing survey of available germplasm, especially where a great many collections were made of one race in one area, seed of similar collections were lumped to form "groups". These were narrowly defined at the Mexican center, and were more broadly defined at the Brazilian center. Racial and other composites in Mexico and Colombia were broader still (Timothy and Goodman, 1979; Gutiérrez, 1974; Wellhausen, 1978).

Teosinte has not been collected and maintained in the same systematic manner. Individuals, especially H.G. Wilkes, T.A. Kato, and G.W. Beadle, have sent collections to the Southern Regional Plant Introduction Station (1977) and to CIMMYT, but entry identification and description is haphazard. Much needs to be done, especially with Zea luxurians and Z. perennis (Bird, 1982; Galinat, 1972; Wilkes, 1972).

Knowing what materials are in the banks is a problem. The Committee on Preservation of Indigenous Strains of Maize (1954-55) published a two-volume, mimeographed list of the 10,992 collections then available at the three centers. Most of these entries are still available, but many collections have been made since, material collected earlier has been increased for distribution, and there has been some loss. No other listing has been published except occasionally by the USDA (Skrdla et al., 1978; Southern Regional Plant Introduction Station, 1977) and by Illinois Foundation Seeds, Inc. (Henderson, 1972). The Mexican program was reorganized in 1960 becoming the Instituto Nacional de Investigaciones Agricolas (INIA) and CIMMYT, both northeast of Mexico City. Most of the collections used for the early studies of Mexican, Central American and Caribbean races went to INIA, and CIMMYT started a new collecting program. The Brazilian center sent all its collections to the U.S. National Seed Storage Laboratory in Ft. Collins, CO, and from there they went to CIMMYT where they are available today, having been increased during the 1969-1974 renovation of that bank's materials (Gutiérrez, 1974). The Programa Cooperativa de Investigaciones en Maíz (PCIM) maintains the Peruvian collections. Because of poor adaptation, erratic funding from international agencies, and some major accidents, the Bolivian and Chilean germplasm has not fared well (Timothy and Goodman, 1979), but the Venezuelan, Colombian and Ecuadorian germplasm at the Instituto Colombiano Agropecuario (ICA) is in good condition (M.M. Goodman, pers. comm.). Some races are not now available because of these problems, and germination and servicing of seed requests is variable. Also, no one center has samples of a majority of the races, or even of all the racial complexes. Certainly there is a paucity of representation of the races in public hands in the United States.

There was some hope that the Information Sciences/Genetic Resources Program (IS/GR) of the University of Colorado, funded by the International Board for Plant Genetic Resources, would produce catalogues for the various banks, standardize nomenclature, and make much of the accumulated, unpublished data more accessible (Vincent et al., 1978). Without publishing one catalogue, that program was disbanded in 1980. How available their data

files are remains a serious question. They did, however, publish a guide to the characters used by the various banks to describe their entries (IS/GR, 1977), and a draft version of a CIMMYT catalogue (with numerous errors and false entries) was offset printed.

Discussion

This is not the place to record all the problems of the past, but there is need for some consideration of what might be done to improve the situation. There are five areas of special need.

1) Lists of entries in New and Old World public germplasm banks need to be published, with information on collecting site, species and racial identification, and synonyms such as original identification number or temporary labels used in other publications.

2) The germination status of bank entries needs to be checked on a scheduled basis, and where germination is dropping, the entries need to be increased from material kept in long-term, very cold storage.

3) After obtaining the lists in (1), above, and further analyzing available data and materials, there should be more collecting of those races or subraces poorly represented or those areas poorly sampled.

4) Collections typical of each race and subrace and collections used in important studies need to be increased and made available in all of the major germplasm banks. The accompanying article (Bird, 1982) is a start toward recommending standard material for a wide range of studies.

5) Some of the gene pools and high-yielding varieties from around the world need to be regularly entered into the germplasm maintenance system because they also provide important combinations of alleles which may be lost in time.

Money spent on germplasm conservation is well spent. Millions of dollars spent on radiation or other treatments to produce mutations gain little compared to one field trial surveying the world's races for pertinent traits. CIMMYT (1981) and many other centers have developed gene pools, and these have proved basic to breeding new, high-yielding varieties of maize. However, local programs need to check original germplasm as well, looking for pest resistance or whatever. Long-term goals of increasing the functions of maize, such as alcohol production, or of developing semi-perennial forms for fodder depend on even more basic sampling and crossing of species and races. Much botanical, cytogenetic and biochemical study of present germplasm is needed.

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see also: de Bakkar (1981) and Henderson (1972).

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56 MAIZE BREEDING AND FUTURE GOALS: MODIFIED "HARD-ENDOSPERM" OPAQUE-2 MAIZE

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Before 1964, most attention on protein nutritional quality improvement in maize was concerned with increasing protein levels via increased nitrogen fertilization applications, maintaining protein levels as yields increased, and seeking higher lysine collections or racial composites via whole kernel lysine analyses with either microbiological assays or the automatic amino acid analyzers that had recently become available.

In July, 1964, the long known genetic marker opaque-2 was announced to profoundly affect maize protein composition and hence amino acid content (Mertz, et al., 1964). It was the first discovery of a single, homozygous recessive endosperm protein control gene that reversed the "increased nitrogen fertilizer--increased prolamin and lowered maize nutritional quality" relationship that had, by this time, become almost dogma.

This bit of fresh air in the cereal nutritional area, touted to be the most important agricultural discovery for the next 25 years, was short lived. Yield estimates from the earliest opaque-2 hybrids were approximately 15 percent below comparable normal genotypes primarily because closely-linked genes from the popcorn background source adversely influenced field corn yields via low test weight. Despite improvements that lowered yield losses to 0 to 5 percent, opaque-2 maize carried a stigma not readily removed from the farmer-growers mind.

Secondary problems in the post-harvest handling, storage, and processing of opaque-2 occurred also. The soft, floury phenotype and improved nutrition was conducive also to improved mold growth during storage. Soft kernels were mechanically damaged during binning and out-loading which increased susceptibility of those kernels to moisture, insects, and molds. Opaque-2 maize was overly dusty and tended to produce flour instead of the more desirable grits during dry-milling. Starch yields were lower in wet-milling than for normal, non-opaque types (Mertz and Nelson, 1966).

The final blow was dealt by the marketplace. Grain handlers needed a rapid lysine assay to permit binning of high lysine maize separately from non-opaque types and to pay an appropriate premium for lysine. Not only did a rapid lysine assay not exist but separate bins were not available at local elevators where grain identity preservation must begin. Consequently, the farmer-grower, paying more for opaque-2 seed (compensation for new varietal development), took a small yield loss and received no premium or other market incentives for the loss. Even if he wished to feed his own high lysine grain to swine or other animals, he couldn't store at the local elevator for lack of identity and had to provide on-farm storage.

Yet, the tantalizing promise remained. With the same inputs of fertilizer, sunshine, water, and soil, a maize crop of approximately 3.5 to 4 times better protein nutritional quality could be obtained with opaque-2 maize.

Early on, the soft, floury kernel phenotype was changed to a more crystalline normal type with variable allelic doses and with a double mutant opaque-2/floury-2 gene combination (Bates, 1966; Nelson, 1966) although agronomic quality was lacking. Maintenance of heterozygous opaque-2 hybrids was not a practical way to overcome seed density and appearance problems. Double mutants have shown limited promise (Paez, 1973; Brown, 1975) although preliminary examination of various double mutant types still reveals reduced starch contents (Glover et al., 1975) and thus reduced test weight, yield, and processing desirability. The most useful opaque-2 maize kernel improvement has been through modifier genes.

Modifier genes, non-identified quantitative genes, may be accumulated to change the endosperm phenotype from floury to crystalline. Such a "background effect" is generally non-predictable. CIMMYT (1968-69) has been the leader in opaque-2 modification research emphasizing a two-phase chemical lysine screening with intensive selection for agronomic type. Vasal (1975) has reported on a series of studies to modify kernel vitreousness. This has been a particularly effective way to convert advanced opaque-2 genotypes back to more acceptable non-opaque types.

Ortega (1980) reviewed studies to determine how modification occurs in the protein fractions but found none concerning breeding progress. Reserve seed from two CIMMYT composites subsequently was planted, maintained under uniform environmental conditions, and harvested for kernel characteristic and protein extraction studies. Modified hard endosperm opaque-2 (HEo2) populations were heterozygous with respect to kernel density and vitreousness. Protein values were intermediate between opaque-2 and non-opaque phenotypes. Endosperm lysine of HEo2 types ranged from 2.5 to 3.0 percent on a protein basis. Modification was a gradual process, but by selection cycle 4, it was approximately 80 percent complete. This is very rapid compared to developing agronomic quality of a mutant type.

One may conclude that conversion of advanced open-pollinated maize composites offers the most rapid improvement of maize protein nutritional quality. It is farmer-grower oriented, however, because it doesn't involve proprietary inbred lines, and thus does not offer agribusiness incentive for seed development. Specific grain identity will be even more critical in market channels because the floury phenotypic marker will be absent. Some market demands will have been met, however, and the other needed technology will undoubtedly drop in place.

Tremendous strides have been made in the past 18 years. Seven years remain to fulfill original high expectations of opaque-2 maize. It may be tight, but modified genotypes may well prove the key to opaque-2 success.

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57 GENETIC DIVERSITY OF MAIZE: DISEASE RESISTANCE

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Maize is a cross-pollinated plant that has evolved or been selected by man into thousands of varieties or races each having within it a great deal of genetic variability. However, the crop is grown in commerical agriculture as hybrids obtained by the systematic crossing of two to four inbred lines. Suitable crosses between selected inbred lines result in heterosis (hybrid vigor). In the U.S. Corn Belt, most hybrids are single-crosses obtained by the crossing of two inbred lines. Inbred lines are obtained by self-pollinating plants for six to eight generations, at which time they become phenotypically uniform and nearly homozygous.

Most inbreds in the U.S. Corn Belt are obtained by selfing open-pollinated varieties, hybrids, or composites all of a single maize race derived from crosses between Southern Dents and Northern Flints. Over 500,000 inbreds are developed each year by public and private maize breeders and the best inbreds are tested in over 200,000 hybrid combinations for grain yield and numerous other traits. Those giving high stable yields and having the necessary disease resistance and other traits are advanced through more performance testing to seed production by commerical seed firms and offered for sale to farmers. Farmers select the most superior hybrids based on farm performance and continue to demand seed of them. Thousands of land varieties and races of maize have been collected and are maintained in germplasm banks. These have been sampled by plant breeders. The incorporation of exotic germplasm into U.S. maize hybrids so far has not led to more heterosis but new alleles for disease and insect resistance have been found.

Maize in the U.S. is exposed to many diseases caused by numerous pathogenic fungi, bacteria, viruses, mycoplasma, and nematodes. Seedling diseases reduce seed germination and plant stands. Some bacterial and viral diseases weaken or kill plants in the early vegetative phases of growth. Most maize diseases occur after mid-season and during the reproductive phase of growth. Several important leaf diseases reduce the photosynthetic area and compete with the ear for photosynthates. The fungal leaf diseases can develop in epidemic proportions as did Southern leaf blight in 1970 (Hooker, 1974) and spread over large areas causing severe losses in yield. Stalk and root rots kill plants prior to maturity. They also weaken stalks so additional losses occur at harvest. In the last 20 years, eight new maize diseases have occurred in the U.S.

There are several diseases, important elsewhere in the world, that do not occur in the U.S. Genetic resistance is the primary means of disease control in maize. Resistance to the pathogen may range from partial to nearly complete and breeding for resistance is part of all maize breeding programs.

A wide array of interacting genetic systems conditions resistance to maize pathogens. These include one or a few alleles, several alleles acting in an additive manner, and extra-chromosomal systems. Only a few of the

known systems (Hooker, 1978a) are discussed as examples in this report. The genotype of the pathogen can be of extreme importance in maize disease resistance. Some alleles for resistance in the plant function against all isolates of a particular pathogen while, in contrast, other alleles condition good resistance against some biotypes but little or no resistance against other biotypes of the same pathogen.

Monogenic inheritance is known in maize to several pathogens. The dominant genes Ht, Ht2, Ht3, and HtN have been identified for resistance to the fungus Helminthosporium turcicum the cause of Northern leaf blight (Gevers, 1975; Hooker, 1977; Hooker and Tsung, 1980). Over 34 discoveries of genes at the Ht locus on chromosome 2 have been made (Hooker, 1978b; Perkins and Hooker, 1981). These occur in maize from many areas of the world and in teosinte. Gene Ht2 was located in inbred NN14 from Australia and HtN from the Mexican variety Pepetilla. Dominant gene resistances have been shown to H. carbonum, the fungus causing Helminthosporium leaf spot, to Erwinia stewartii, the bacterium causing bacterial leaf blight and wilt, and to wheat streak mosaic. A hypersensitive infection-type form of resistance to common rust, caused by Puccinia sorghi, is inherited as single dominant alleles at five loci (Hagan and Hooker, 1965; Wilkinson and Hooker, 1968). Rp3 is on chromosome 3, Rp4 is on chromosome 4, and loci Rp1, Rp5, and Rp6 are closely linked and are on the distal end of chromosome 10. Genes Rpp, Rpp2, Rpp9, Rpp10, and Rpp11 have been identified for resistance to Puccinia polysora, the cause of Southern rust. Some alleles are fully dominant, wherease others are incompletely dominant. Resistance to H. maydis, the cause of Southern leaf blight, is due to the recessive gene rhm (Smith and Hooker, 1973).

Gene Rp3 conditions dominant gene resistance to P. sorghi biotype 901aba but recessive resistance to biotype 933a (Hooker and Saxena, 1967). This apparent reversal of dominance may be due to dosage effect where one dose of the allele in the heterozygote is adequate to condition resistance against biotype 901aba but not against biotype 933a. Another possibility is a dominant and a recessive gene closely linked together. Using a flanking marker and a test-cross population of 7800 plants, it was impossible to show any cross-over between the two putative genes. If two genes do occur, they are probably less than 0.02 map units apart.

While resistance to rust (P. sorghi) is conditioned by single dominant genes, there are "alleles" at Rp1 and at Rp3. This concept of allelism is the classical one where the genes have an identical location on the chromosome but show a phenotypic difference in reaction to an array of naturally occuring biotypes of P. sorghi. Resistance in the heterozygote against any P. sorghi biotype is always dominant to susceptibility, but the alleles do not fall into a linear dominance relationship. The alleles were detected by empirical screening of maize from many areas of the world. Resistance at the five Rp loci was found in maize from Argentina, Australia, Ethiopia, Guatemala, Kenya, Mexico, Peru, South Africa, Turkey, Uruguay, Yugoslavia, and the U.S. (Hooker, 1969).

Two or more maize stocks may each have a gene that conditions the same resistant or susceptible reactions to hundreds of P. sorghi biotypes but subsequently be differentiated into different alleles by the use of more biotypes. In addition, more intensive investigations have now shown that there are closely linked genes at what was first presumed to be a single

locus. Because of these facts, any listing of sources of resistance and the "alleles" they contain or any listing of "alleles" at the same locus must both be considered as tentative.

A region of about 3 chromosome map units of the terminal end of maize chromosome 10 is involved in the expression of rust resistance. Situated here is Rp1, flanked on each side by Rp5 and Rp6 and linked to Rpp 9 (for resistance to P. polysora) by 1.16 map units. Rp1 is linked to Rp5 by 1.1 map units and to Rp6 by 2.1 by map units. Data also show that the Rp1 locus is about 0.4 map units long and contains within it a series of functional genes that can be differentiated by recombination values ranging from 0.10 to 0.37 percent (Saxena and Hooker, 1968). This region may have evolved by repeated duplication and subsequent variation of the duplications from the original gene. It is suspected that other maize chromosome regions that condition resistance to other pathogens of maize may show a similar type of genetic complexity.

Polygenic inheritance of resistance to maize disease is common. It has been amply demonstrated for resistance to Northern leaf blight (Hughes and Hooker, 1971), Southern leaf blight (Lim and Hooker, 1976), corn rust (Hooker, 1969), stalk rot caused by Diplodia maydis (Kappelman and Thompson, 1966), and other diseases (Hooker, 1978a). In polygenic inheritance, numerous genes are involved and each gives a similar effect on disease reaction. Resistance is in degree rather than in kind of infection. Individual genes cannot be distinguished and segregation is continuous, ranging from the most extreme levels of resistance to susceptibility. Gene action is usually additive although in maize hybrids, a heterotic effect is noted. The diversity of this type of disease reaction within maize germplasm is tremendous. Special statistical techniques and experimental designs are needed to study quantitative inheritance. In maize, quantitative disease reaction is usually highly heritable and can be selected and stabilized by plant breeding. It tends to be effective against all biotypes of individual pathogens and is extremely valuable in the development of disease resistant hybrids for commercial agriculture.

Extrachromosomal inheritance of disease reaction, while rare, is of major importance in maize (Hooker, 1974). The cytoplasm is the primary factor in resistance H. maydis race T, the cause of the Southern leaf blight epidemic of 1970 and to Phyllosticta sp., the cause of yellow leaf blight. Both of these fungi produce a pathotoxin that interacts with mitochondrial membranes of cms-T cytoplasm resulting in their breakdown. Prior to 1970, cms-T cytoplasm was widely used to produce hybrid maize seed because this cytoplasm does not allow pollen production in the absence of nuclear gene factors for fertility restoration. In cross-pollinated plant species, cytoplasmic male-sterility is needed or is very useful in the production of hybrid seed. Normal cytoplasm and cms-C or cms-S cytoplasms for male sterility are resistant to H. maydis race T and to yellow leaf blight.

The gene-for-gene relationship prevails in several maize-pathogen systems. In this relationship, for each gene conditioning resistance in the maize plant there is a corresponding gene in the pathogen which may occur either in the allelic form for avirulence (inability to infect a plant having a particular gene for resistance) or for virulence (ability to infect a plant having the gene for resistance). Resistance (lack of disease) occurs when any pair of corresponding genes are in the allelic form for resistance in the

plant and for avirulence in the pathogen. All other combinations of corresponding genes result in disease development. What has just been described is often referred to as "third order interaction" between genes. First order interaction is between alleles at one locus in one organism (dominance) and second order interaction is between alleles at two loci in the same organism (epistasis). Now, there is also possible a "fourth order interaction", and this occurs when the maize plant has two or more genes for resistance and it is interacting with the pathogen. Here, the maize plant is resistant when any one or more of the corresponding genes in the pathogen are for avirulence. Gene Ht in maize is widely functional throughout the world in conditioning resistance to *H. turcicum*. Virulence in *H. turcicum* to maize gene Ht was detected in Hawaii in 1972 (Lim *et al.*, 1974) and has now also been found in the U.S. Corn Belt. Virulence in the pathogen is inherited as a single gene. Maize plants having Ht2 are resistant to this new biotype. Virulence in *H. maydis* race T to plants having cms-T cytoplasm for male sterility is also inherited as a single gene (Hooker, 1974). The effectiveness of major genes for resistance in maize when the crop is grown in agriculture depends upon the frequency of alleles for virulence and their combination in the pathogen population to which the plant is exposed. A diversity of genes used in combination would appear to offer the most protection.

Different genes in the maize plant can also interact with each other to give a greater degree of resistance expression. The monogenic and polygenic systems interact to enhance the expression of each system (Hooker and Kim, 1973). Genes Ht and Ht2, when in the same plant, condition a higher degree of resistance than that conditioned by either gene alone (Hooker, 1977). There are numerous other examples (Hooker, 1978a).

The physiological basis for plant resistance is sometimes known. For example, maize plants having gene Ht show small chlorotic lesions with limited fungus sporulation when infected with an avirulent biotype of *H. turcicum*. Susceptible plants have large lesions without chlorosis and copious sporulation within the lesion. Two phenolic compounds are formed in plants having gene Ht when infected with pathogenic but avirulent biotypes of *H. turcicum*. These compounds inhibit the growth of the pathogen and are not detected in susceptible plants (Lim *et al.*, 1970). In other diseases where toxins are involved, resistant plants do not provide suitable sites for toxin interaction.

The American farmer is highly efficient in maize production. A major portion of his success is because maize breeders and private seed companies have provided him with a succession of superior hybrids having disease resistance, high yield potential, and other desirable traits. The maize germplasm used in U.S. Corn Belt hybrids is the most elite in the world. However, the genetic base of U.S. maize is narrow and that deployed on farms at any one time is a highly selected sample of the total available. In contrast, the genetic base of pathogens that cause disease is unrestricted and is free to come into evolutionary balance in agriculture.

Man has little control over pathogen evolution or the environment, but he does determine what maize germplasm is used in breeding and how this germplasm is utilized in agriculture. Losses due to maize diseases are largely preventable and this responsibility has been assumed by the maize breeder and the seed industry. Regions in the U.S. where resistance to specific diseases is needed are now largely known although new diseases

continue to appear and current diseases sometimes spread into new areas. There is a diversity of known genes and cytoplasms for disease resistance and more are revealed through research each year. The availability of a broad spectrum of resistance to different pathogens provides more alternatives to the informed breeder and seedsman. As new resistances are discovered, they are incorporated into elite germplasm, and their relative usefulness in agriculture determined. Genetic studies of resistance leads to a greater understanding of germplasm and its intelligent usage. However, such studies are not essential to plant breeding. It is known that some forms of resistance are more useful than are others. Widespread testing is a powerful tool in the identification of broadly functional forms of resistance to disease. Successful maize breeders make a special effort to ensure that plant populations used as sources of new inbred lines contain a high level and diversity of resistance types. Susceptibility to important diseases is avoided in inbred line and hybrid development.

Finally, strong breeding programs and a strong seed industry make possible the rapid utilization in American agriculture of the most elite maize germplasm available. Use of hybrids of diverse parentage, and hybrid diversity in dimensions of both time and space reduce the risk presented by the widespread usage of a few elite inbred lines. Genetic diversity does not assure disease resistance, only some protection against the unexpected. With winter production facilities and foundation seed reserves, rapid shifts from risky genetic or cytoplasmic bases are now possible, should the need arise and be adequately demonstrated.

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58 PLANT REGENERATION IN TISSUE CULTURES OF MAIZE

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Tissue cultures capable of plant regeneration may be efficiently initiated from immature embryos of maize and more recently from several other sporophytic tissues including immature tassels and ears (Green and Phillips, 1975; Freeling et al. 1976; Green 1977; Molnar et al. 1980; Rhodes et al. 1982). The morphology of these cultures is typically one of a complex association of shoot and root meristems as well as less differentiated tissues (Springer et al. 1979). This morphology has been diagnostic of the capacity to regenerate plants of maize with the exception of a new culture type to be discussed below.

The various sporophytic tissues which can be used to initiate regenerable tissue cultures have their individual experimental advantages. Immature embryos are available in large numbers from a single pollination and they develop in relative synchrony on the same ear. Consequently they can be used effectively to compare genetic differences or experimental treatments among sibs. Immature tassels, on the other hand, are obtained from donor plants which are usually 6 to 7-weeks-old. This allows time for the expression of certain genetic markers which permit the identification of cytogenetically altered plants, such as haploids, monosomics, or plants with deletions, prior to initiation of cultures.

Tissue Culture Initiation

To initiate cultures from immature embryos, the husked intact ear is sterilized in 2.5% sodium hypochlorite plus a small amount of detergent for 20 min. and then rinsed three times in sterile water. Each rinse should be for a minimum of 5 minutes. Using sterile instruments in a sterile environment, such as a laminar air flow hood, the crown of each kernel is cut off and a rounded-tip spatula is used to scoop out the endosperm. Embryos up to 2 mm in length usually remain associated with the endosperm and can be seen with the unaided eye and removed from the endosperm with the tip of the spatula. The embryo is placed on the culture medium with the embryonic axis against the medium. This embryo orientation permits extensive proliferation in the scutellum and minimizes germination and further development of the embryonic axis. Up to ten embryos fit easily in a 100 x 25 mm Petri dish containing 50 ml of medium. Masking tape or Parafilm strips provide a good seal for the dishes.

Immature tassels for tissue culture studies are typically obtained from 6 to 7-week-old field or greenhouse grown plants. The tassels in these plants are usually 2 to 3 cm in length and are encased in many layers of young leaves. Sterilization of the tassel is not necessary if the leaves surrounding it are carefully removed in a sterile environment. The entire tassel is cut into 1 to 2 mm thick cross sections and all these pieces are immediately transferred to a Petri dish containing 50 ml of culture medium to minimize desiccation. Orientation of the pieces on the medium does not appear to be critical.

Although several growth medium formulations will support the growth of regenerable tissue cultures of maize, the most frequently used media are MS (Murashige and Skoog, 1962) and N6 (Chu et al. 1975, Chu 1978). A particularly critical ingredient in the medium is the auxin which must be of sufficient potency and concentration to suppress germination of immature embryos and root formation in immature tassel sections during the initiation of the tissue cultures. 2,4-dichlorophenoxy acetic acid (2,4-D) at 0.5 to 1 mg/l has been used with the greatest success as an auxin for tissue culture propagation in maize.

After three to four weeks of incubation at 25-28°C under low fluorescent light (1.5 W/m^2), regenerable cultures from immature embryos or tassels can be identified by the presence of scutellar-like bodies. Vigorous cultures will be light green or yellow and may have small leaves developing among the scutellar-like bodies. The growth and regenerability of these cultures can be maintained for several years by transferring pieces about 5 mm in diameter to fresh medium every 3 to 4 weeks.

Plant Regeneration

To regenerate plants from these cultures the auxin (2,4-D) must be greatly lowered or omitted from the medium. Depending on the degree of organization in the culture it may be useful to lower the auxin in steps via successive transfers or to remove it entirely in one transfer. Shoots which emerge are transferred to 250 ml Erlenmeyer flasks containing 75 ml of medium without 2,4-D and incubated under bright fluorescent light (8-10 W/m^2; 16/8 hr photoperiod). When an adequate root system has developed, the plant is transferred to 2 inch plastic pots containing a 1:1 mixture of sterile soil and vermiculite. It is critical at this stage to thoroughly rinse all the growth medium off the roots before transplanting the plant into soil. The plants should be kept in a humid, brightly-lighted environment for 2 to 4 weeks until established, before transplanting to a greenhouse or field. Regenerated plants often have more vigor and are more likely to produce morphologically normal tassels and ears if the various steps in regeneration and establishment are carried out in as short a time as possible. Pollination of regenerated plants is accomplished by selfing or crossing with other plants to produce the desired kernels and progeny. Seeds from regenerated plants are generally grown into plants using standard cultivation practices.

Genetic Analysis

Cytogenetic analysis of plants regenerated from diploid maize cultures, eight-months-old or less, has indicated a high degree of chromosomal stability (Green et al. 1977; McCoy and Phillips, 1982). The instability which does occur usually involves missing or broken chromosomes. Abnormal plants may have sectored tassels such that one or more of the tassel branches exhibit partial pollen sterility and one or more of the branches have normal fertility. Meiotic analysis of pollen mother cells in young tassels has identified a total of 9 cytologically abnormal plants among 277 examined (Green et al. 1977; Edallo et al. 1981; McCoy and Phillips, 1982). The limited data available on the chromosome constitution of plants regenerated from older tissue cultures indicates a much higher frequency of abnormalities. Eleven plants regenerated from three-year-old cultures all possessed the same phenotype (i.e. oppositely arranged leaves and ca. 100 cm

tall) at maturity (Green et al. 1977). Three plants were analyzed cytologically and each plant contained a broken chromosome 6, deficient for the distal third of the long arm. This is an indication that cytological abnormalities may be increased in older cultures.

An interesting aspect of recent studies is that tissue culturing induces considerable variation in the genetic makeup of cells and that this variability is recovered in the progeny of regenerated plants. A high frequency of spontaneous mutations with simple Mendelian inheritance has been observed in R2 progenies (Edallo et al. 1981). The type of endosperm and seedling mutants found were similar to spontaneous mutants described in maize. A high rate of spontaneous mutation makes these cultures of particular interest in selection experiments.

Mutants have been selected from maize tissue cultures in two separate experiments. Selection for resistance to H. maydis race T pathotoxin in diploid, Texas male sterile cultures resulted in the recovery of resistance which was transmitted to regenerated plants and progeny (Gengenbach et al. 1977). This selection also changed male sterility to fertility. Both resistance to the disease and fertility were inherited as maternal traits. Selection for resistance to lysine plus threonine in diploid cultures also produced resistance which was recovered in the progeny of regenerated plants (Hibberd and Green, 1982). This lysine plus threonine mutant, Ltr*-19, overproduces threonine in large quantities in the seed and is inherited as a dominant gene.

Somatic Embryogenesis

A major new type of maize tissue culture has been developed recently which is distinguished from those discussed previously by its friability, rapid growth, and capacity to regenerate plants by somatic embryogenesis (Green, 1982). These cultures are light yellow and appear undifferentiated in that little organization is visible to the eye. Closer examination by light and scanning electron microscopy, however, reveals somatic embryos which follow very closely the known developmental sequence for zygotic embryos (Randolph, 1936).

These embryogenic cultures are initiated directly from the scutellum of immature embryos or as spontaneous sectors growing from established organogenic tissue cultures of the type discussed earlier in this paper. They can be initiated and maintained on both MS and N6 medium containing 0.5 to 1.0 mg/l 2,4-D. The cultures are first noticed as small friable regions which are light yellow. These cultures may not show visible organization initially but frequently within 2 weeks of their appearance, microscopic examination reveals embryos at globular or slightly more advanced stages of development. Once established these cultures exhibit a rapid growth rate and must be subcultured every 2 weeks. They are maintained by transferring 4 to 5 pieces of callus, each 5 to 10 mm in diameter, to 100 x 25 mm Petri dishes containing 50 ml of fresh medium.

The established cultures exhibit a high degree of embryogenic activity; frequently hundreds of embryos per callus are visible. They are first seen as small globular structures and their continued development is evidenced by differentiation of the suspensor, scutellum and embryonic axis tissues. Embryo development up to the coleoptilar stage (Abbe and Stein, 1954) progresses quite normally on either MS or N6 media containing 2% sucrose

and 0.5 to 1.0 mg/l 2,4-D. Development beyond this stage is very abnormal unless the callus is transferred to medium lacking 2,4-D but with 6% sucrose to increase the osmolarity of the medium. Maturation proceeds rapidly on this medium and after 10 to 14 days embryos have developed which are similar in size to those found in seeds. Their most prominent features are a well-developed scutellum and embryonic axis. These embryos germinate rapidly when transferred to medium lacking hormones and 2% sucrose. Shoot and root development occurs simultaneously and the young plants are then grown under conditions described in the Plant Regeneration section.

These friable embryogenic callus cultures are appropriate for the initiation of suspension cultures which are grown in liquid MS medium containing 2,4-D and aerated on a gyrotory shaker. The resulting cultures are well-dispersed and composed of cell aggregates ranging from about 2 mm in diameter down to single cells. Embryo development rarely proceeds as far as the globular stage in these cultures. When these suspensions are plated on agar-solidifed MS medium, active embryogenesis is observed in the resulting callus cultures.

Other Important Factors

The genotype of the donor tissue substantially influences the ease with which tissue cultures of maize are obtained and the duration of regenerability. When using immature embryos, some genotypes (i.e. A188, WF9, and ND203) produce regenerable cultures very efficiently while similar cultures are initiated with great difficulty from other genotypes, such as W23 and A632. Regenerable cultures have been initiated from immature embryos of 70% of the approximately 40 different genotypes we have examined, without modification of the standard cultural regime. Modifications in the growth medium have improved the performance of some more difficult genotypes. Typical modifications include adjustments in the 2,4-D concentration and alternative macro-and micro-nutrient formulations of the medium. Characteristic differences among genotypes are also noted when cultures are initiated from immature tassels. Growth conditions can and should be optimized to produce the most desirable cultures from specific genotypes.

The developmental stage of the donor tissue is an important factor in the initiation of regenerable tissue cultures. As immature embryos become more fully developed, they rapidly lose the ability to initiate regenerable cultures (Green, 1977). Embryos 3 mm or longer are generally ineffective for culture initiation. For most genotypes, embryos which are 1 to 2 mm long most reliably produce the desired cultures. Immature tassels more frequently produce regenerable cultures when they are 1 to 3 cm long.

Incubation conditions used for maize tissue cultures are very similar to those for cultures of other species. The growth rate of maize cultures increases with temperature up to 30°C. The most frequently used temperature for incubation is 28°C. Temperature control in growth rooms and chambers should be as uniform as possible to minimize the condensation of water from the growth medium onto the lids and walls of Petri dishes and other containers. The greater the fluctuation in temperature, the greater the problem of condensation. Severe condensation can interfere with culture growth as well as increase the chances of microbial contamination. Humidity control in growth rooms and incubators is generally not necessary if dishes and flasks are sealed properly with tape, parafilm, or aluminum foil. These

seals are also important in preventing microbial contamination of the cultures.

Although not dependent on light, growth of the cultures is often improved and regeneration yields more vigorous plants when they are grown in the light. Typical light sources include standard Cool-White, Grow-Lux, or Agro-Lite fluorescent fixures. These provide light intensities up to 1.5 W/m^2 which is more than adequate for the cultures. Typical photoperiods are 12-16 hours of light per day.

Cultures should be subcultured as frequently as necessary to maintain vigorous growth. The actual time interval is influenced by the type of culture, volume of culture medium, and the amount of inoculum used for the subculture. Cultures with rapid growth rates, such as the friable embryogenic lines, are transferred every two weeks while slower growing cultures are transferred about every four weeks. Careful management of the subculture and incubation conditions is especially important for the maintenance of the regenerability of cultures. The potential to regenerate numerous plants from tissue cultures of maize can be maintained for several years if necessary.

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59 MAIZE AND CEREAL PROTOPLASTS -- FACTS AND PERSPECTIVES

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For numerous reasons which are best elucidated in the articles of this special publication on maize, Zea mays has become a model plant species. A tremendous knowledge has accumulated over the years on its anatomy and developmental biochemistry and physiology of healthy and diseased plants and, more recently, on the molecular biology of this important cereal crop plant. This knowledge, however, glaringly contrasts with our apparent inability, at present, to handle and manipulate maize -- and other cereal -- tissue, cell and protoplast cultures in as efficient a way as we do with the well known dicot model plant species, Nicotiana, Petunia, and Datura.

With a special emphasis on maize, this article shall serve as a review and an assessment of what cereal protoplasts in general and maize protoplasts in particular have contributed to our understanding. And by looking at the present state and today's limitations we may more clearly see the challenges and needs of tomorrow's cereal protoplast research.

Protoplast isolation

Leaves provide large and sufficiently homogeneous populations of differentiated cells for protoplast isolation and leaves can be removed from the plant body without sacrificing what might be a unique and irreplaceable individual. Both with dicot and with cereal plant species, leaf mesophyll cells have therefore been one of the most convenient and most commonly used sources of protoplasts. We have usually employed macerozyme and cellulase in a one-step isolation procedure (Lorz and Potrykus, 1976) but mixtures of other commercial enzymes (hemicellulase, xylanase, pectolyase, cellulysin, driselase and others) have also been used with good results. To facilitate cell wall degradation the leaves are either stripped of their lower epidermis (Brenneman and Galston, 1975; Okuno and Furusawa, 1977) or cut in fine diagonal sections (Lorz and Potrykus, 1976). Using these procedures large quantities of protoplasts can be routinely isolated from all cereals (Evans et al., 1972; Kanai and Edwards, 1973; Potrykus et al., 1976; Okuno and Furusawa, 1977; Chin and Scott, 1979; Lai and Liu, 1980) both from leaf sheath and leaf blade and also from etiolated leaf cells (Harms and Potrykus, 1978a; Hampp and Ziegler, 1980).

Young maize plants with their solid voluminous stem provide another source for the bulk isolation of protoplasts from internodal pith parenchyma (Potrykus et al., 1977). Because of their low buoyant density (Harms and Potrykus, 1978a) stem protoplasts are preferably isolated in low density media, i.e. $CaCl_2$ solution, in which they can be sedimented by centrifugation. An alternative or additional purification method for maize stem protoplasts is by floatation on dense media, i.e. iso-osmolal sucrose solution.

Vasil and Vasil (1974), Taylor and Hall (1976), Lin (1980) and Senn and Pilet (1980) have isolated protoplasts from maize roots. Their limited quantity and the variety of cell types make root protoplasts a suitable system for morphological and physiological studies of differentiation rather than suggesting their use for protoplast culture and somatic cell genetics.

While the commonly used enzymes do not degrade pollen cell walls, they are active at the earliest stages of microspore development. Deka *et al.* (1977) and Potrykus (pers. commun.) have used pollen tetrads of several plant species, including maize, as a source of haploid protoplasts.

Suspension cultures, as reported for maize (Oswald *et al.*, 1977; Potrykus *et al.*, 1977, 1979a; Shannon and Liu, 1977; Sheridan *via* Green, 1977; Sanchez de Jimenez *et al.*, 1978; Brar *et al.*, 1979; Polikarpochkina *et al.*, 1979; McCormick, 1980; see also W. F. Sheridan, this publication) and other cereal species (see Thomas *et al.*, 1979) have frequently been used for protoplast isolation. At present, there appear to exist four distinct types of cereal suspension cultures whose characteristics deserve careful consideration for their great influence on protoplast isolation and culture.

Most of these liquid cultures are highly heterogeneous mixtures of proliferating root meristems and loose non-dividing cells of various ploidy states (King *et al.*, 1978). As their sole morphogenetic response these usually slow growing and short-lived cultures may sporadically form roots. Protoplasts thereof are hardly isolated from the dividing meristem but rather from the terminally differentiated cortical and cap cells which apparently are no longer competent to divide and proliferate.

Rarely with cereals have suspension cultures been established which show the characteristics of dedifferentiated, unorganized, dividing cell populations ("cell lines"; King, 1980) of dicot species. In cereals, the most typical cell line type cultures are the *Triticum monococcum* suspension culture of Gamborg and Eveleigh (1968) and a stem protoplast-derived suspension culture of *Zea mays* (Potrykus et al., 1977). These finely dipersed, fast growing ($t_d = 23$ h for the maize suspension), but karyologically abnormal cultures give very high yields of protoplasts (Potrykus et al., 1979). However, as they completely lack the ability to regenerate plants, protoplasts from these lines will be of little use in cereal somatic cell genetics.

Recently, Vasil and Vasil (1980) and Lu and Vasil (1981) have succeeded in establishing embryogenic suspension cultures of *Pennisetum americanum* and *Panicum maximum*, respectively. Embryos and whole plants have been regenerated from these heterogeneous meristemoid containing cultures. Similar embryogenic suspensions have now been established with *Oryza sativa* (Wakizuka and Potrykus, in.prep.) but attempts with maize, sorghum and wheat have only yielded non-embryogenic rooting-type cultures (Brettell, Potrykus, King, pers. commun.). At least a portion of protoplasts from these cultures, derived from highly vacuolated and densely cytoplasmic cells, eventually may retain totipotency and give plants back upon culture, as has been described for *Pennisetum americanum* (Vasil and Vasil, 1980).

A new type of a cereal suspension culture - friable, homogeneous, rapidly proliferating and embryogenic - has been initiated with maize (Green pers. commun.) but its potential as a source of totipotent maize protoplasts remains to be tested. (See Green and Rhodes, this publication).

Uses of maize protoplasts for physical, physiological and plant pathological studies

The ease with which protoplasts can be isolated from a wide range of tissues, organs and cultured cells and their lack of a rigid cell wall make them an attractive experimental system for an increasing number of research areas including physical, physiological and plant pathological studies.

Density gradient centrifugation of maize protoplasts has revealed tissue-specific differences in their buoyant densities (Harms and Potrykus, 1978a) which hold potential for the enrichment of fused protoplasts from fusion mixtures (Harms and Potrykus, 1978b). Karyoplasts and cytoplasts, produced upon high speed centrifugation in iso-osmolal density gradients (Lorz and Potrykus, 1980), can be useful in studying the metabolic activities of enucleated cytoplasm or the genesis of vacuoles. Electrophoretic mobilities of maize root (Senn, 1980) and mesophyll protoplasts (Halim and Pearce, 1980) have been investigated to separate protoplast types and in attempting physical selection of fusion products.

As a consequence of their nudity, protoplasts provide free access to and across the plasmalemma membrane thus facilitating studies for which the cellulose wall previously had been an unwanted barrier. Ion uptake has been studied with maize mesophyll and root protoplasts by Taylor and Hall (1976) and Lin (1980). Paczkowski et al. (1980) have observed changing amino acid uptake and incorporation characteristics with maize cell line protoplasts as they regenerate into cell clusters. RNA synthesis in protoplasts from gibberellin-sensitive maize seedlings was enhanced by cyclic AMP in a way similar to the effect of gibberellic acid (Tarantowicz-Marek and Kleczkowski, 1975).

Because of their lack of cell walls, protoplasts have greatly improved the isolation of structurally and functionally intact cell organelles, i.e. chloroplasts (Rathnam and Edwards, 1976a; Horvath et al., 1978; Day et al., 1981) and plasma membranes (Perlin and Spanswick, 1980; Lin, 1981).

Enzymatic digestion of maize leaves releases protoplasts from the mesophyll cells but not from the strands of bundle sheath cells (Kanai and Edwards 1973). Mesophyll protoplasts and bundle sheath cells, (which are easily separated by density centrifugation), as well as their isolated chloroplasts, have widely been used for photosynthetic studies (see Edwards et al., 1976) and to investigate the intercellular and subcellular localization of enzymes involved in C_3 and C_4 photosynthetic pathways (Gutierrez et al., 1974; Edwards et al., 1976; Matsumoto et al., 1977), in sugar metabolism (Herbert et al., 1979; Usuda and Edwards, 1980), nitrate assimilation (Rathnam and Edwards, 1976b) and protein catabolism (Lin and Wittenback, 1981).

Maize protoplasts carrying T, C, and S male-sterile and TRf (fertility restored) and N cytoplasms have been employed to compare the cellular and subcellular effects and mode of action of Helminthosporium maydis race T toxin on resistant and susceptible maize lines (Pelcher et al., 1975; Earle et al., 1978; York et al., 1980; Gregory et al., 1980; see also E. Earle this publication).

From the few works mentioned above (and even more so from many other works involving dicot and other cereal protoplasts) it appears that protoplasts are being utilized as an experimental system of great value and potential for many physiological studies.

Genetic modification of cereals

Genetic modification of cereals through manipulation of protoplasts has not yet proceeded beyond the very initial stages mainly due to the limitations of cereal protoplast culture discussed below.

Fusion of maize and barley mesophyll protoplasts with protoplasts from carrot and soybean suspensions has led to the formation of hybrid cells (Kao *et al.*, 1974; Dudits *et al.*, 1976) but their development ceased after a few divisions, as was the case with fusion products of sorghum cell culture and maize mesophyll protoplasts (Brar *et al.*, 1980). Soybean protoplasts resistant to *Helminthosporium maydis* race T toxin have been reported to confer resistance to the products of fusion with susceptible T cytoplasm maize protoplasts (Earle and Gracen, 1979). Blue-green algae (Burgoon and Bottino, 1976), isolated wheat nuclei (Lorz and Potrykus, 1978) and isolated metaphase chromosomes (Szabados *et al.*, 1981) have been transferred into maize protoplasts by means of polyethylene glycol treatment similar to protoplast fusion.

All genetic modification approaches, if aimed at improving crop plants via single cell technologies, will require totipotent protoplasts or single cells as recipient materials. Of similar importance is the availability of protoplast cultures exhbiting maximum viability and vigorous responsiveness leading to a high frequency of cell division and colony formation for all genetic manipulation (fusion, organelle and DNA uptake) can be expected to result in a (transient, at least) distortion of cellular metabolism and to affect protoplast viability. The potential for high division response could counteract these effects and thus greatly enhance the chances for recovering genetically modified cells.

Cereal protoplast culture: state ...

Cereal protoplast culture is still in its infancy. Despite numerous misleading reports (see references in Potrykus, 1980), mesophyll protoplasts of all cereal species so far have resisted all attempts to culture them, and figures of cell division and colony formation may have been subject to misinterpretation (Potrykus, 1980). Potrykus *et al.* (1976) have reported on extensive screening of culture media, genotypes and other factors which, however, could not establish conditions reproducibly inducing cereal mesophyll protoplasts to divide. Mitotic figures in cultured cereal mesophyll protoplasts, as occasionally observed with oat (Kaur-Sawhney *et al.*, 1980) and with maize and barley after fusion with protoplasts from carrot and soybean suspensions (Kao *et al.*, 1974; Dudits *et al.*, 1976), could not dispel the notion that cereal mesophyll cells, unlike their dicot counterparts, may be irreversibly arrested and no longer competent to respond to culturing.

It is not surprising that protoplasts from cultured cereal cells were expected to more readily embark upon division in culture; colony formation from this sort of protoplasts has been reported for *Saccharum officinarum* (Maretzki and Nickell, 1973), *Bromus inermis* (Kao *et al.*, 1973), *Hordeum*

vulgare (Koblitz, 1976), Oryza sativa (Deka and Sen, 1976; Cai et al., 1978), Triticum monococcum (Dudits, 1976), Pennisetum americanum (Vasil and Vasil, 1979), Zea mays (Potrykus et al., 1979a) and Sorghum bicolor (Brar et al., 1979). It is interesting to note that extensive variation of the culture conditions did not improve the division response of protoplasts, even in the case of the fast growing maize cell line beyond what appeared to be a threshold of 30% (Potrykus et al., 1979a). Similarly, Brar et al. (1979) have found all their attempts inadequate to induce sustained divisions in protoplasts cultures from their Black Mexican Sweet suspension. None of the above protoplast-derived callus material could be induced to regenerate plants.

The only example of sustained division in protoplast cultures from a cereal plant tissue are stem protoplasts of maize (Potrykus et al., 1977) which, in a single experiment, gave rise to callus and a cell line type suspension culture. Again, these cultures completely lacked morphogenetic potential.

From the situation outlined above it is evident that the cereal protoplast problem is two-fold:

(1) the choice of totipotent protoplasts competent to embark upon sustained division at a high frequency, and

(2) development of conditions enabling cereal cells to express totipotency upon morphogenetic induction.

In assessing the present state of cereal cell culture King et al. (1978), Thomas et al. (1979), Potrykus (1980) and Vasil (1981) have aptly stressed these points in saying how important and urgent it is to establish cereal single cell systems with full totipotency before any genetic modification can be seriously considered as a potential tool to creat novel plants that may be of agronomic significance. Clearly, more basic work is needed to solve these problems.

... and perspectives

Recently, Vasil and Vasil (1980) have established embryogenic suspension cultures of Pennisetum americanum and protoplasts thereof have divided to produce callus cultures from which embryos and whole plants have been regenerated. This first example of totipotent protoplasts from a cereal species --although the true single cell origin has been a matter of discussion and the division frequency, plating efficiency and plant formation may still be low for genetic modification experiments -- has renewed confidence as to finally being able to culture what have been the most recalcitrant protoplasts.

Subsequently, embryogenic suspension cultures have also been established in Panicum maximum (Lu and Vasil, 1981) and Oryza sativa (Wakizuka and Potrykus, in prep.) and there is preliminary information (Vasil, 1981) that protoplasts from the Panicum culture have been cultured to give embryogenic callus and whole plants, while protoplast culture in the case of rice has as yet failed to produce morphogenetic or embryogenic callus (Potrykus, pers.commun.).

Stimulated by the reports on successful suspension and protoplast culture in the primitive cereal species, Pennisetum and Panicum, increasing efforts are being made in several laboratories to establish embryogenic suspension cultures of the highly bred modern cereal cultivars as potential sources of totipotent protoplasts -- as yet with limited success. Such cultures, once initiated, would probably face the inbuilt shortcomings of suspension cultures, increasing karyological abnormalities and declining morphogenetic potential. Using such cultures would also require the isolation with high efficiency of competent protoplasts from the dividing totipotent cells of embryoid and meristemoid clusters. For their use in genetic modification studies the true protoplast and single cell origin of the regenerated plants would need to be confirmed.

From ongoing work with maize, rice, wheat and sorghum genotypes (Brettel, King, Potrykus, Wakizuka, Wernicke, pers.commun.) it is evident that tissue culture response and the ability to form morphogenetic and embryogenic cultures under certain cultural conditions is highly dependent upon the genotype. Tedious genotype screening is therefore necessary. This genotypic variation of response certainly represents a serious limitation to practical crop improvement employing single cell technologies whenever the genetic modification of a specific cultivar is desired.

To improve the division response and the plating efficiency of cereal protoplast cultures it might be necessary to work out more favourable culture media by employing large scale screening techniques like the multiple-drop-array (MDA) technique of Potrykus et al. (1979b). However, extensive variation of culture media with protoplasts from maize stem (Potrykus et al., 1977) and maize suspension cultures (Potrykus et al., 1979a) did not provide substantial evidence that the culture medium composition should be a major key to cereal protoplast culture, except when specific, as yet unknown, triggers are missing. But such findings may not be generalized.

Instability and rapid senescence has been reported of oat mesophyll protoplasts (Brenneman and Galston, 1975) and treatment with senescence retardants like dibasic amino acids, polyamines and kinetin has been suggested as a potential means for oat protoplast improvement (Kaur-Sawhney et al, 1977; Galston et al., 1978). Cereal protoplasts, cereal mesophyll protoplasts in particular, might well require as yet unknown factors in addition to those provided by the culture media in use so far. Such triggers might come from pretreatments, use of conditioned culture media, coculture with other protoplasts, and feeder or nurse cultures.

Callus, somatic embryo and whole plant formation has recently been obtained from cultured leaf segments of Sorghum bicolor (Wernicke and Brettell, 1980, Panicum maximum (Lu and Vasil, 1981a) and Pennisetum purpureum (Haydu and Vasil, 1981). The significance of these reports lies in the fact that they have clearly demonstrated the totipotency of cereal leaf cells which was expressed in a transient way at certain stages of leaf maturation. Additional support that cereal cells may indeed not be terminally differentiated, arrested and completely unresponsive comes from the tumorous proliferations that are induced by the fungus, Ustilago maydis, on various parts of the corn plant.

The recent encouraging achievements reviewed above have stimulated old and new activities in the field of cereal tissue and protoplast culture

which hopefully will soon bear fruit to resolve the most urgent problems of cereal protoplast culture for their efficient and beneficial use in cereal somatic cell genetics and genetic modification.

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60 BLACK MEXICAN SWEET CORN: ITS USE FOR TISSUE CULTURES

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ORIGIN

Black Mexican sweet corn (BMS) is an old standard variety that has been commercially available in New England for over one hundred years. Despite its name it is believed to have originated in upper New York State (Taply et al. 1934, Galinant 1971). Its source is not certain, but Tapley et al. (1934) have suggested that it was derived from the "black puckers" sweet corn of the Iroquios. Galinant (personal communication) has noted that the inclusion of the term Mexican is likely to have been adopted to simply indicate that it was a new or exotic variety when first introduced commercially.

It was pointed out by East (1909) that all sweet corns are not similar and that some are derived from floury dent corn varieties and others from flint corn varieties. The fact that BMS possesses all of the traits characteristic of flint corn, including tillering, long, expanded husks, kernel row numbers in the eight to twelve range, and differs only in its sugary trait resulted in East concluding that BMS originated from one of the northern flints by its mutating from the dominant starchy to the recessive sugary condition. The standard northern flints include Parker's flint, Longfellow flint, Wilbur's flint, and Gaspe flint, among others (Brown and Anderson 1947). It is of some interest that among the many strains of maize tested in my laboratory (see below) the only other strain observed to form suspension cultures besides BMS was Parker's flint. How closely related these two varieties may be is not known but both have their origins in the same area of the country, have similar growth habits and plant characteristics and may be closely related.

ESTABLISHING SUSPENSION CULTURES

By 1970 I had initiated a program of maize tissue culture research at the University of Missouri in Columbia. It was difficult to establish and maintain callus cultures from the original genetic stock that was first evaluated, and a screening of genotypes was undertaken. Eventually, among 35 tested, several strains were found to respond well when mature kernels were germinated under aseptic conditions and stem sections in the region of the first node above the hypocotyl were placed on the medium of Linsmaier and Skoog containing 2 to 4 mg/l of 2,4-D. A callus formed on the surface of one or more of the thin transverse sections, and this could be subsequently subcultured on the same medium (Sheridan 1975a). Most of the 35 strains tested produced some type of callus, but in many cases the subsequent growth of the callus was quite slow.

When the callus cultures of these strains were tested for growth in liquid medium of the same composition on a reciprocal shaker most of them grew as large clumps and did not form satisfactory suspension cultures.

The exception was Black Mexican sweet corn. Its callus was lighter colored and more friable than that produced by the other strains and, when placed in liquid medium formed a rather well dispersed suspension culture. These results were presented at a Cell Biology meeting and published in abstract form (Sheridan 1975b) as well as being described at the Annual Allerton Maize Genetics Conference. Subsequently, in preparation for spending an academic year abroad I transferred the BMS cultures to C. E. Green's laboratory where they were grown and where additional cultures were initiated (see Green 1977 for a photograph of these cultures and a description of them).

The capacity of BMS callus to form suspension cultures does not demonstrate a requirement for the presence of the sugary trait (su1 su1). This is evident from the success of Potrykus *et al*. (1977) in establishing a suspension culture from a non-sugary strain of maize as well as by the success in my laboratory in establishing suspension cultures with Parker's flint.

USES OF BMS SUSPENSION CULTURES

Suspension cultures of BMS have been used in the study of homoserine dyhydrogenase (Walter *et al*. 1979) and for the isolation of protoplasts and subsequent establishment of callus cultures from the protoplasts (Chourey and Zurawski 1981). See also Brar *et al*. (1979) for the use of BMS suspension cultures in the study of maize protoplasts.

Suspension cultures of BMS are well suited for studying the dynamics of the cell cycle as well as for studies seeking to develop a single cell culture system of maize. A cell cycle time of 27 hours has been observed (Wang *et al*. 1982, cited with permission). In these cultures 28 percent of the cells were actively dividing (proceeding through the cell cycle) with a doubling time of 62 hours. The same research group achieved a partial synchronization by using either hydroxyurea plus colchicine or colchicine alone to produce a 2.0 to 2.5 fold increase in the mitotic index of these cultures (Mi *et al*. 1982, cited with permission).

Considerable progress has been made in the development of a maize single cell culture system by the use of a feeder layer of BMS suspension culture cells (Jane Adam Smith, personal communication). Individual BMS cells and small clumps displayed a 50 percent plating efficiency when plated on Millipore filters (Cat. No. AA BG 047) which were placed over a suspension culture. Suspension cultures of other species were not suitable for cross feeding. The highest plating efficiency was observed when cells were collected from cultures in early logarithmic growth phase.

FUTURE USES AND DIRECTIONS

A major goal in the development of plant tissue culture systems is the capacity to regenerate plants from cultured cells or tissues. The scutellar tissue culture system of Green and Phillips (1975) is the most successful tissue culture system presently available for maize (see article by Green and Rhodes in this publication). It was observed by these workers that the BMS strain, when carrying the recessive alleles for the waxy locus as well as for the sugary locus (wx1 wx1, su1 su1) readily regenerated plants from scutellar derived cultures. Although the common form of this strain (carrying the non-waxy Wx1 Wx1 but sugary su1 su1 traits) has not shown

much of a regenerative response when immature embryos were cultured (C.E. Green, personal communication; W. F. Sheridan, unpublished results), the report by Sachs et al. (this publication) indicates that in their laboratory the common form of BMS has readily regenerated. It is likely that modification of the medium components, particularly the 2.4-D level, may result in an increased plant regeneration frequency in cultures derived from embryos of the common form of BMS.

There are indications that BMS may be well suited for anther culture. The data supporting this suggestion are included in the report on maize anther culture by Nitsch et al. (1982). They observed that four hybrids where BMS was a parent (Illinois High Oil x BMS, W23 x BMS, Illinois High Oil/BMS x W23/BMS, and BMS x Lai-Pin-Pai/Golden 113) responded when anther cultured and yielded haploid plants. It should be noted that the anther culture success rate was low and that in a recent report on maize anther culture where BMS, along with several other strains was tested, it failed to respond (Brettell et al. 1981). In the latter report the success rate was also low (for further details see the accompanying article on maize anther culture in this publication).

Because of its capacity for growth as a suspension culture the BMS strain has been selected for construction of maize DNA libraries. This work is underway in the laboratories of J. Messing (personal communication) E. Sheldon (see article in this publication) and W. J. Peacock (see Sachs et al this publication).

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61 ANTHER CULTURE OF MAIZE

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Introduction

Androgenesis is the formation of an embryo whose genomic constitution is derived solely from the male gametophyte. This situation may be obtained at a low frequency in maize by using certain special stocks as the male parent. Anther culturing is important because it can result in the direct formation of embryos from microspores. Furthermore, success at anther culturing of maize should eventually lead to the development of a system for culturing of free microspores. This would provide a system wherein free cells with a haploid chromosome constitution could be cultured in liquid medium with the eventual formation of embryos and mature plants. Such a culture system should be of considerable value in fundamental studies in developmental and molecular biology as well as genetic engineering of maize.

Historical

Androgenesis via anther culturing was first reported by Guha and Maheshwari (1964, 1966) with Datura and this technique was subsequently extended to other solanaceous species (Bourgin and Nitsch 1967, Nitsch and Nitsch 1969, Sunderland and Wicks 1971). Although some success was obtained with barley (Clapham 1973), rice (Niizeki and Oono 1968), rye (Thomas and Wenzel 1975, Wenzel et al. 1975, 1976) and wheat (Picard and DeBuyser 1973) its success rate with cereals in general was quite low and particularly so with maize (Clapham 1977).

Maize remained a very difficult subject for anther culturing throughout the first half of the 1970s but a limited amount of success was obtained in several Chinese laboratories during the latter half of the decade. The way was paved for this advance by the development of the N6 medium for anther culture of rice (Chu et al. 1975). This medium which contains a higher ratio of nitrate to ammonium ion, among other differences, than the medium of Murashige and Skoog (1962) (MS) was found to be superior to the MS medium for maize anther culturing (Chu 1978). It was also found that 12% sucrose was the optimal level for this purpose.

A success rate of about 1% of the anthers cultured responding was obtained by using either the N6 or the somewhat similar "Yu-pei" medium (Ku et al. 1978) and several genotypes were cultured resulting in the formation of calluses or embryos, some of which grew into mature plants (Chu 1978, Miao et al. 1978; Ku et al. 1978; Institute of Maize 1979).

Present Situation

Recent research involving collaboration between the laboratories of C. Nitsch, M. G. Neuffer and my laboratory have led to a modest improvement in the success rate, in some cases as high as 10 percent, with maize anther

cultures (Nitsch et al. 1982). An analysis of our observations as well as those reported by the Chinese workers indicates that, so far, four factors have been identified that are important for maize anther culturing. A consideration that only one or two microspores out of two or three thousand or more per anther responds to culturing by growing into a callus or embryo and that, at best this happens in only one out of every ten anthers on the average, indicates that other important factors remain to be identified. Nevertheless, it is clear that even the limited success reported to date depends on selection of: (1) the proper genotypes, (2) suitable stage of microspore development, (3) a culture medium of appropriate composition, (4) proper pretreatment and culture temperature.

Genotype specificity

The Chinese workers have examined a wide range of genotypes for their responses to anther culturing. It was reported by Miao et al. (1978) that "In 1975 only 9 out of 159 sets of material inoculated produced callus or embryoid." Subsequently, when 32 F1 strains, produced by crossing a high responding strain with a low responding strain, were tested, 28 of the F1 strains produced callus or embryos when anther cultured. More than eighty strains of maize were evaluated for anther culture response by Ku et al. (1978). Although they provided no detailed report of the differences in response, they noted that there was a relationship between induction frequency and genotype. Their data on the effect of various hormones was obtained with the highly responding strain Lai-pin-pai.

Two strains, King Hwang-9 and King Hwang-13 obtained from China because of their high response capacity have been found responsive by Y. C. Ting (personal communication) as well as by R. Brittel and I. Potrykus (personal communication). In addition, the two Chinese strains Golden 113 and Lai-pin-pai have been responsive (Nitsch et al. 1982). Several hybrid combinations involving Illinois High Oil, Alexander's High Oil, Black Mexican Sweet, and W23 have also responded in culture (Nitsch et al. 1982) but these results do not indicate whether or not the individual strains would be responsive in culture.

The results to date suggest that the capacity to respond in anther culture is a dominant trait but they do not illuminate whether or not this response is under single gene or multigene control. The low frequency of response observed with even the relatively high responding strains makes a genetic analysis of this trait a technically difficult undertaking.

Stage of microspore development

The optimum stage of development is when the microspores are uninucleate and the nucleus is located in the center of the microspore. At this stage the tassel is still not visible and is enclosed by the uppermost leaves. If a portion of the tassel is visible when one looks down into the whorl of leaves from a position straight above the plant, then the tassel is too old. To collect the tassel the stem is cut well below the uppermost leaves and the upper portion of the plant is brought to the laboratory. The stem is carefully opened by splitting it along its length taking care not to damage the tassel which should be removed intact and kept in a moist environment.

The stage of microspore development can be checked cytologically by preparing slides from anthers removed from different locations on the tassel. Two features of tassel development should be kept in mind: florets mature sooner on the middle of the spikes than at the ends and each spikelet contains two flowers with the upper flower's anthers being larger and two or three days more advanced than the anthers of the lower flower. It is convenient to draw a diagram of the tassel, sample both flowers of spikelets from various locations, and note on the diagram the development stage of the microspores from the different locations and then decide which portions of the tassels to use for anther culturing.

The staining of microspores is somewhat difficult because of their dense walls. It is possible to stain their nuclei with aceto-carmine but a superior technique for determining the developmental stage of microspores is to mount them in a staining solution consisting of 50 mg Trypan blue (Color Index No. 23850) in 100 ml distilled water. The dye acts as a vital stain and is of value for examining microspores from cultured anthers, as well, since living microspores exclude the dye to a large extent and their cytoplasm remains nearly colorless while dead cells take the dye up readily and appear dark colored. The Trypan blue is particularly helpful in determining whether the microspores are uninucleate or have divided to become binucleate since, in living cells, the nuclei stain a light blue and the nucleolus is especially prominent since it stains even darker. The cells can be examined immediately after the slide is prepared and the developmental stage may be quickly determined.

Culture medium

At the present state-of-the-art, the medium of choice for culturing anthers of maize is the N6 medium (Chu et al. 1975, Chu 1978). In addition to mineral salts the original medium contained glycine (2 mg/l), thiamine -HCl (1.0 mg/l), pyridoxine -HCl (0.5 mg/l), nicotinic acid (0.5 mg/l), 5% sucrose and 0.8% agarose. Subsequently it was reported that an elevated sucrose level of 15% (Chu 1978) or 12% (Ku et al. 1978, Miao et al. 1978) was optimal and that the inclusion of activated charcoal (5 g/l), and casein hydrolysate (500 mg/l) were of benefit (Miao et al. 1978, Chu 1978, Ku et al. 1978). However, except for the antiauxin triiodobenzoic acid (TIBA) plant growth regulators were not found by the Chinese workers to be of benefit.

Although it is superior to the MS medium, the use of the N6 medium has not been marked with a high success rate with maize anthers (Thomas et al. 1979, Brettel et al. 1981, personal communications from I. Potrykus and C. E. Green). In the recent study of Brettel et al. (1981) it was reported that embryos were obtained at a low frequency from three genotypes (Seneca 60 and two unnamed Chinese genotypes) while 10 genotypes failed to respond. Out of about 22,000 anthers plated, 22 responded and 53 embryos were obtained of which 14 developed into plants.

A success rate of 3 to 10% was observed in some experiments by Nitsch et al. (1982) using the N6 medium with some modifications (see Table 1). These included the use of 6% sucrose and the addition of proline to the medium. Proline was included because it is present as the free amino acid with the highest concentration in developing zygotic maize embryos (Sheridan and Neuffer, submitted). The formation of one or more embryos from the

microspores of three to 10 anthers per hundred plated is inadequate to use this technique for most purposes. It may very well be that the induction medium is essentially satisfactory but that other manipulations such as pre-treatments, and shifts to media of different composition (i.e., sucrose level, etc.) will be more important for future increases in the anther culture success rate.

The medium recommended by Nitsch et al. (1982) is described in Table 1. The mineral salts, sucrose, charcoal and agarose are sterilized by autoclaving while the other constituents are filter sterilized and then mixed with the partially cooled autoclaved materials. It is convenient to dispense the medium at about 5 ml per dish into 50 mm diameter tight lidded, sterile plastic culture dishes (Falcon #1006). Prior to use the dishes of media should be kept enclosed in polyethylene wrap or plastic bags and will store satisfactorily at room temperature for 20 or 30 days, possibly longer.

Table 1. N6 Medium for Anther Culture of Maize

Components	mg/l	Components	mg/l
$(NH_4)_2SO_4$	463	Glycine	2.0
KNO_3	2830	Thiamine HCl	1.0
KH_2PO_4	400	Pyridoxine HCl	0.5
$MgSO_4.7H_2O$	185	Nicotinic acid	0.5
$CaCl_2.2H_2O$	166	Sucrose	60,000
$MnSO_4.4H_2O$	4.4	Agarose	6000
$ZnSO_4.7H_2O$	1.5	Casein hydrolysate	500
H_3BO_3	1.6	L-proline	100
KI	0.8	Activated charcoal	5000
Iron: 27.85 mg of $FeSO_4.7H_2O$		TIBA	0.1
and 37.25 mg Na_2EDTA*		pH	5.8

* Add 10 ml per liter of medium of a solution made by dissolving 2.785 g. $FeSO_4.7H_2O$ in 500 ml H_2O and mixing with 500 ml H_2O containing 3.725g Na_2-EDTA.

Stress treatments

It has been widely observed that the success rate of anther cultures is increased when detached inflorences or plated anthers are exposed to a cold treatment. This was first reported by Nitsch and Norreel (1972, 1973) and

has subsequently been observed to be effective not only with dicots (Nitsch 1974, 1975, 1977, Sunderland 1978, Sunderland and Roberts 1979) but also with cereals including rice (Genovesi and Magill 1979), barley (Sunderland and Evans 1980) and maize (Nitsch *et al*. 1982).

A second and important type of stress that contributes to anther culture success is osmotic stress. One form of this is to simply cut a flowering spike and allow it to stand in water for a couple of days prior to removing its anthers, a technique used with barley (Wilson 1977, Wilson *et al*. 1978). The more frequent form is to plate anthers on a high sucrose (6 to 12%) containing medium. The osmotic stress produced by the sucrose appears to be especially important for anther culture success with cereals (See Reinert and Bajaj 1977, and Clapham 1977 for reviews). In rice Chen (1978) reported that 9% sucrose was most stimulatory while with maize Ku *et al*. (1978) tested 6, 9, 12, 15 and 18% and found 12% to be the optimal level. Recently, it has been observed (Nitsch *et al*. 1982) that a 6% sucrose level is optimal with regard to obtaining embryos and minimizing callus growth from microspores in cultured maize anthers.

The recommendation of Nitsch *et al*. (1982) is to wrap the detached tassel and surrounding leaves in foil and incubate at 14°C for one week prior to plating of anthers, or alternatively the anthers may be given the cold treatment in the dark during the first week following plating. After the 14°C treatment, the cultures are placed at 20°C for 7 to 10 days and then transferred to 25 to 27°C.

Future Directions

In order to fully utilize maize for studies in plant molecular biology it is essential that it become possible to manipulate maize at the cellular level by *in vitro* culture techniques that allow the eventual recovery of the complete, fertile plant. The eventual goal of maize anther culture research should be the development of a culture system wherein free microspores produce embryos at a high frequency and these embryos can be readily doubled in chromosome number and grown into mature plants at or near a 100% success rate. A microspore culture system has been reported for tobacco (Nitsch 1974a, 1974b, 1975, 1977) and this technique has been extended to potato (Weatherhead and Henshaw 1979). It should be noted that Nitsch (1977) reported the formation of embryoids at a low frequency when maize microspores were cultured in a liquid medium but that they ceased development at an early stage of embryogenesis.

The development of a maize anther culture system with a high success rate and the eventual achievement of an efficient microspore culture system will depend upon a greater understanding of the phenomena crucial to the success of these systems and the development of techniques and culture media producing these phenomena. The fundamental event in the production of embryos from microspores is the change in developmental pathway so that a microspore shifts from its normal destiny of becoming the mature male gametophyte, the pollen grain, to a new sporophytic pathway toward embryo formation. When the microspore is induced to take the latter pathway, it divides into a proembryonic structure which can develop into an androgenic haploid embryo or become disorganized in its growth and form a haploid callus. In the former case a plant can appear by germination of the embryo while in the latter case a plant can regenerate from the callus either via organogenesis or somatic embryogenesis.

It appears evident that the simple manipulation of the auxin to cytokinin ratio of the culture medium is not a sufficient action to induce or control androgenesis. There has been no paradigm published for anther culturing that provides a theoretical framework for its experimental analysis. Future studies of the molecular events producing the shifts in developmental pathways during somatic embyrogenesis should provide the basis for such a testable model.

It will be especially valuable to obtain a developmental profile for each of the classes of plant growth regulators present in microspores prior to and during induction and embryo formation. Analyses of the levels of the growth regulators released into the culture medium will also be helpful since such data will provide insight into the bases for the beneficial effects of conditioned media.

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62 MAIZE ENDOSPERM CULTURES

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The successful *in vitro* culture of maize (*Zea mays* L.) endosperm tissue was first reported in a talk by LaRue (1949). Straus and LaRue (1954) later detailed the establishment of cultures from two sweet corn varieties. Cultures originally produced from 'Black Mexican Sweet' (Straus 1958) were used in many subsequent studies (Farrar and Ganugapati 1970; Graebe and Novelli 1966; and Straus 1960). The culture medium used by Straus and LaRue (1954) contained 0.5 percent yeast extract as an organic nitrogen source. This was later replaced by asparagine (0.2 percent in a chemically defined medium (Straus 1960). Straus' (1960) medium has since been most commonly used for the growth of maize endosperm callus cultures. Farrar and Ganugapati (1970) studied the growth of maize endosperm callus on solidified media in which asparagine was replaced with different ammonium salts. Ammonium salts of succinate and citrate supported active growth, but ammonium salts of acetate, oxalacetate, and oxoglutarate were toxic. Ammonium nitrate at low (0.05 percent) concentrations supported slow callus growth but was inhibitory at higher concentrations. Graebe and Novelli (1966) used a slightly modified Straus' medium for the large-scale production of maize endosperm tissue in suspension culture.

Straus and LaRue (1954) and Tamaoki and Ullstrup (1958), although successful in culturing maize endosperm from sweet corn, were unable to establish continuous cultures derived from starchy maize endosperm. Tabata and Motoyoshi (1965) reported heredity control of callus formation in maize endosperm. They successfully grew endosperm cultures derived from a cross between two starchy lines, S41 x S42. They also reported active growth of the S42 inbred but only the hybrid was maintained in culture for several years. After many unsuccessful attempts to culture endosperm from the inbreds Oh43 and W64A on several different media, Shannon and Batey (1973) took endosperm explants from 18 diverse inbred and 5 hybrid lines and determined their growth on Straus' (1960) defined medium modified to contain 2 g/l asparagine and 2 g/l yeast extract. Explants from two starchy inbreds, A636 and R168 and two hybrids, P3369A and Px610 grew actively and were maintained in culture for several years. The inbred A636 continued vigorous growth and was used for several studies (Boyer and Shannon 1974; Chu and Shannon 1975; Shannon and Liu 1977). Thus, maize endosperm from starchy and several endosperm mutants can be successfully grown *in vitro* and in the next section, the procedures used in my laboratory will be reviewed.

Procedures for Establishment and In Vitro Growth of Maize Endosperm

In our original attempts to establish maize endosperm cultures, we used explants from kernels 7 to 12 days post-pollination (Shannon and Batey, 1973). We were most successful using endosperm explants from 10-day-old kernels. Explants from older kernels generally did not proliferate but rather enlarged, filled with starch, and died. The ear was first surface

sterilized for 5 minutes in a 10 percent solution of commercial sodium hypochlorite NaClO bleach followed by two rinses in sterile water. The pericarp and aleurone at the top of the kernel were removed and the endosperm was scooped out with a micro spoon-spatula. Four explants were placed in each culture tube on 5 ml slants of Straus' completely defined medium (Straus, 1960) modified to contain 2 g/l yeast extract in addition to the asparagine. Explants were transferred to fresh media after 21 days in culture and subculturing was continued on a 21-day schedule, thereafter. Actively growing stock cultures of A636 were maintained on this solid medium for many years. The cultures were grown in an unlighted chamber at about 29°C.

Prior to the initiation of liquid cultures, about six explants of actively-growing tissue were placed in 250-ml Erlenmeyer flasks on 50 ml of solidifed Straus medium. After 14 to 21 days of growth, all the tissue in a flask was transferred to a 250-ml Erlenmeyer flask containing 90 ml of Straus' defined medium, as modified by Graebe and Novelli (1966). This liquid contained 3 percent sucrose. Stock solution cultures were transferred on a 21-day schedule and each flask, containing 90 ml of medium, was inoculated with approximately 2.4 g (a rounded teaspoon full) fresh weight of tissue. We found that smaller tissue inoculations could be used but the lag period prior to active growth was considerably extended. Maize endosperm tissue growing in liquid Straus' medium was used for studies of cell division and chromosome constitution (Boyer and Shannon 1974) and starch synthesis (Chu and Shannon 1975) and the reader is referred to these papers for details on the experimental procedures.

To be an effective system for metabolic studies, a cell culture should: a) grow in a completely defined medium containing a minimum number of organic additives, and b) grow as a suspension of single cells or very small clusters of cells. The cultures derived from the inbred A636 and maintained on Straus' medium grew as small clumps of tissue (approximately 3 mm in diameter or less). To produce cultures more suitable for metabolic studies, Shannon and Liu (1977) studied the growth of established A636 cultures in four basic media having various modifications of the organic constituents. The basic media tested were the B-5 medium of Gamborg, Miller and Ojima (1968), RPM medium of Nesius, Uchytil and Fletcher (1972), RM-1964 medium of Linsmaier and Skoog (1965) and the standard Straus medium as used by Graebe and Novelli (1966). This study (Shannon and Liu 1977) clearly showed that maize endosperm derived from the starchy inbred A636 could be grown in a simple medium containing only the inorganic salts of the Linsmaier and Skoog (1965) medium, sucrose, and thiamine (Table 1). This tissue had an absolute requirement for thiamine, but grew equally well in the presence or absence of the other vitamins and myoinositol. Auxins and kinetin appeared to reduce the early rate of growth but had little or no effect on the final weight of the tissue or degree of tissue aggregation. Although tissue growth in the simplified medium was equal to that in the standard Straus' medium, growth could be increased over 30 percent by adding 2 g/l of asparagine. In the simplified medium containing asparagine, maximum tissue fresh weight was obtained 12 days after subculture. The tissue consisted of uniformly small cell clusters, which were loosely packed and friable. These rapidly-growing cultures accumulated little or no starch.

Based on the media study of Shannon and Liu (1977), I would recommend that a solidified (7 g/l of Noble Agar) medium containing the Linsmaier and Skoog (1965) inorganic salts plus asparagine, thiamine, and sucrose be

used for initiation and maintenance of the cultures. Cultures growing on this medium will probably need to be subcultured every 12 days. Alternatively, more slowly growing maintenance callus with a longer time between transfers can be obtained by growing the tissue on the above medium containing a reduced amount of NH_4NO_3 and/or asparagine or by using Straus' medium. For metabolic studies, I would recommend that the tissue be grown as a suspension in the simplified medium given in Table 1 plus asparagine. The ability of maize endosperm to grow rapidly as a suspension of relatively small cell clumps in a simple defined medium free of added growth regulators should make this tissue a useful tool for a wide variety of metabolic and developmental studies.

Table 1. Simplified Medium for Growth of Maize Endosperm in Suspension Culture.*

Component	Amount mg/l	Component	Amount mg/l
NH_4NO_3	1650.0	$CoCl_2.6H_2O$	0.025
KNO_3	1900.0	$KH_2PO_4.H_2O$	170.0
$MgSO_4.7H_2O$	370.0	H_3BO_3	6.2
$MnSO_4.H_2O$	16.9	$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.6	$FeSO_4.7H_2O$	27.8
$CuSO_4.5H_2O$	0.025	$Na_2EDTA.2H_2O$	37.3
$CaCl_2.2H_2O$	440.0	Thiamine.HCl	0.4
KI	0.83	Sucrose	30000.0

* The pH of the medium is adjusted to 5.6. More active growth is obtained by also adding 2 g/l of asparagine. The appropriate quantity of asparagine should be dissolved in hot water (2 g in 100 ml of water) and then add it to the medium prior to adjusting the pH and bringing the medium to volume.

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63 SOMATIC CELL GENETICS OF MAIZE: *IN VIVO* AND *IN VITRO* EXPRESSIONS OF MAIZE MUTANTS

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A wealth of information is now available on the isolation and expression of single gene mutants in higher plants, particularly in maize where the application of the paraffin oil method for pollen treatment (Coe 1966) allows the isolation of chemically induced mutants with a frequency as high as 41 percent (Neuffer 1978). Less information is available on the isolation and characterization of mutants in cell culture. This applies particularly to corn where difficulties are encountered in establishing cell suspension cultures, regenerating protoplasts and inducing embryogenic calli (King *et al.*, 1978). What is completely lacking is the information on a comparative analysis of mutant expression in the cell vs. the whole organism level.

Different expressions of a mutant in cell culture as compared to that at the plant level might indicate the organizational level at which the mutant is operating. Two approaches are feasible for a comparative study of the mutants: (i) *in vitro* isolation and *in vivo* expression; and (ii) *in vivo* isolation and *in vitro* expression.

The former allows the screening of millions of cells and the application of stringent criteria of selection, while the latter results in the immediate obtaining of mutant plants without the inconvenience of chimeric mutants if pollen rather than seed mutagenesis is applied.

(i) In vitro isolation and in vivo expression

In vivo expression of mutants isolated in culture is a prerequisite for the validity of the *in vitro* selection in terms of plant improvement. This prerequisite, however, is not always fulfilled (Maliga, 1980). Lack of expression at the plant level as well as lack of sexual transmission of the character selected in culture are often observed. Possible explanations for lack of expression of the selected property at the plant level include epigenetic changes, chimeric origin of the mutant or selection against mutant cells if calli rather than single cells are used for mutagenesis and the existence of major differences between the organismic vs. the cultured cell's metabolism.

So far in maize very few cell variants have been isolated *in vitro* to make the approach feasible. In one case selection at the cell level proved successful. Plants resistent to *Helminthosporium maydis* race T were obtained following selection for *H. maydis* pathotoxin resistence in tissue culture of susceptible, Texas male-sterile (T) cytoplasm maize Gengenbach *et al.* (1977). This resistance was cotransmitted with male fertility and resistance to *Phyllosticta maydis* pathotoxin. Similar cases of apparent pleiotropism have been reported in *N. tabacum* (Chaleff and Keil, 1981) while selecting for resistance to picloram but in neither case has the basis of the association been elucidated. While considering the *in vitro* vs. *in vivo* gene

expression, it should also be kept in mind that many cases have been reported of induced variation associated with tissue culture (Skirvin, 1978).

Maize plants regenerated from morphogenic embryo cultures show a dramatic increase in mutation frequency when compared to that of plants grown from seed (Edallo et al., 1981). It might well be that part of such a genetic variability is pre-existing in the differentiated cells of the explanted tissue while the remaining part is due to in vitro multiplication (Barbier and Dulieu, 1980).

In future research projects more emphasis should be given to the search for biochemical mutants expressed at the cell level, including auxotrophs, analogue resistant and temperature-sensitive mutants that appear to be among the most promising ones for metabolic studies at the cell and plant level.

(ii) In vivo isolation and in vitro expression

Many genetic markers expressed at the plant level appear well suited for an analysis of their expression at the cell level in culture conditions. Most promising appear those whose expression in culture is easily predictable and biochemical mutants whose gene product or metabolic route affected are known. An example of the former is the group of complimentary genes responsible for anthocyanin production in the outer layer of the endosperm (the aleurone) and in plant tissues while among biochemical mutants, some well suited to this analysis are the gibberellin sensitive dwarf mutants (Phinney, 1960), the structural gene for the enzyme UPD glucose: flavonoid 3-O-glucosyltransferase (Dooner and Nelson, 1977; Larson and Coe 1977) where phenotypic changes in pigment composition can be correlated to changes in enzyme activity and the adh mutant which may be distinguished from wild type in culture by its resistance to allyl alcohol or to its sensitivity to acetaldehyde (Dhaliwal and King, 1979) and the proline-requiring mutants (Gavazzi et al., 1975).

Some preliminary data on the expression in culture of genes responsible for anthocyanin synthesis in maize, obtained in callus cultures from immature endosperms, suggest that pigment production in culture is the result of activation of a gene system whose expression is normally confined to the mature plant tissues (Gavazzi and Racchi, 1981).

Another important aspect of these studies is gene regulation, i.e., how regulatory processes are affected by altering the differentiation pattern of the organism. Regulatory systems particularly suited to an analysis of their in vitro vs. in vivo expression are those known as controlling elements and paramutation. Controlling elements are transposable chromosome elements that affect the functioning of various loci (McClintock, 1950). Their association with a gene alters the timing, cell specificities and the level of activity of a functioning gene. They are often organized in a two-element system interacting in the modulation of gene expression. Genetic markers generally employed to detect their presence are those affecting pigment synthesis in the outer layer of the endosperm (aleurone).

The endosperm cultures approach to their study would allow laboratory manipulations of the elements not feasible under normal conditions and would possibly lead to the isolation of their product. This approach has been first

adopted by Gorman and Peterson (1978) by means of endosperm cultures obtained from immature tissues (8-12 days post-pollination). The first results indicate that interaction of defined controlling element components take place in cultured tissues. Further work should be aimed at elucidating the mechanisms of action of these elements in *in vitro* conditions.

Paramutation, first described by Brink (1956), is a heritable change resulting from an interaction between alleles in the somatic cells of appropriate heterozygotes. Paramutation studies with the *R* locus in maize, which conditions pigmentation of various parts of the plant and aleurone, have shown that the critical events which lead to paramutation occur in the somatic tissues of the plant. The phenomenon has been mainly studied in the aleurone tissue. The feasibility of growing and manipulating this tissue under strictly controlled conditions as in endosperm culture will allow a series of experiments that might help to elucidate the basic mechanism underlying paramutation.

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