Instituto Juan March
de Estudios e Investigaciones

4

CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

The Past and the Future of Zea Mays

Organized by

B. Burr, L. Herrera-Estrella and P. Puigdoménech

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J. L. Bennetzen
S. P. Briggs
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I N D E X

P R O G R A M M E

THE PAST AND THE FUTURE OF *Zea mays*

Monday, May 11th

Chairman: B. Burr.

- Welcome.

J. Doebley - Genetic Analysis of the Morphological Evolution of Maize.

D.A.Hoisington - Molecular Approaches to Understanding Resistance to Tropical Insect Pests.

B. Burr - Gene Mapping in Maize.

J.L.Bennetzen - Parallel Studies of Genome Composition and Organization in Maize and Sorghum.

V. Szabo - Mapping of the Key Traits Distinguishing Maize and Teosinte Using RFLP Markers.

J. Kermicle - Crossing Barriers within Zea.

L. Herrera-Estrella - Evaluation of Open-Pollinated Maize Cultivars Using RFLP'S.

Tuesday, May 12th

Chairman: P. Puigdomènech.

H. Saedler - Transposable Elements in Zea mays.

H.K. Dooner - Analysis of Recombination and Transposition in Maize Using the Bronze Locus and the Transposable Element Activator.

G. Gavazzi - Interaction between Members of the R Family.

M. Motto - Regulatory Genes Affecting Maize Seed Storage Protein Synthesis.

A. Viotti - Molecular and Functional Analysis of Different Mutations at the Opaque-2 Locus of Zea mays.

T. Nelson - Gene Expression Patterns and Tissue Development in C_4 Leaves.

S. Hake - The Role of Homeobox Genes in Maize Development.

Wednesday, May 13th

Chairman: L. Herrera-Estrella.

P. Arruda - *Sequence Analysis of Prolamin Genes from Maize, Coix and Sorghum Reveals Highly Conserved Protein Structure and Regulatory Elements.*

P.Puigdoménech - *Functional and Evolutionary Analysis of Genes Coding for Proline Rich Proteins from Maize and Related Species.*

C. Gigot - *Structural and Functional Analysis of Maize Histone Gene Promoters.*

S.P. Briggs - *Cloning and Characterization of HM1, a Gene for Leaf Blight and Ear Mold Resistance.*

G. Neuhaus - *Genetic Engineered Cell Lineages in Maize Using the Microinjection Approach.*

M. Fromm - *Transgenic Maize and Commercial Applications.*

Short Oral Presentation *J. Escudero: Agrobacterium-Mediated DNA Transfer to Meristematic Tissue of Maize Immature Embryos.*

Round Table on "Prospects for New Transformation Techniques in Maize".

INTRODUCTION

P. Puigdoménech

Since the origin of photosynthetic organisms, plant species have been born and have died but they have rarely had such an interesting life as maize is having. It is a species built by man but it has a dark and discussed origin, it has been adored as an atribute of gods but it has extensively been manipulated by peasants and breeders from all over the world for their profit. Now, cultivation of maize is essential worldwide as a source of protein, carbohydrates and lipids, and it is also intensively cultivated for very precise applications. In some countries sweetcorn is a delicatessens and children from all countries are fond of popcorn and have drinks full of corn syrup. There is probably no other higher organism which such a versatility in its applications and in its genetics. The transformation of maize by molecular methods appears feasible and, in some industrial laboratories, it is becoming routine. The future of maize may be from now on in the hands of genetic engineers.

The idea of a meeting on the Past and the Future of *Zea mays* comes from a reflection on the properties and present knowledge of such a fascinating species. On the one hand, data on genetic analysis of maize and its suposed ancestor, teosinte, have allowed to dissect the genetic changes that lead from a wild, perennial, poorly productive species to the major crop that we know now. One the other hand, maize transgenic plants are not a surprise any more. It seemed therefore interesting to bring together people working in such distant fields that rarely meet, specially if we introduce the distance across the Atlantic. And it was found interesting to mix people with the idea of discussing whether anything could be deduced from the study of the past to the prospects of the future.

Of course, maize has a number of unique features that makes it a model species for crop plants in a variety of directions. The genetic map of maize is among the most complete ones in plants and it can now be compared with data coming from related species such as sorghum or Coix. Data on mechanisms that take part in the

fluidity of a large genome such as maize are now available thanks to the extensive work carried out on transposible elements and on recombinatory phenomenons. Maize development can now be analyzed at the molecular level and homeotic genes have been isolated and characterized. An increasing number of genes have been cloned (they have to be measured in hundreds) and a large collection of probes and promoters are now being analyzed structurally and functionally. The genes being cloned include genes regulating the expression of other genes like those coding for storage proteins, and genes coding for resistance towards a pest or a pathogen. Finally, transformation methodologies are increasingly efficient. Genes can now be introduced in maize by protoplast transformation, by microinjection, by particle bombardment and the action of Agrobacterium and viruses are being explored. The transformation of immature embryos partially digested with the enzymes used for the preparation of protoplasts seems to be specially efficient.

The workshop seemed to come at a very special moment for the study of maize. The data on its past are becoming clear and plausible pathways for the appearance of the cultivated maize have been published. And the applications of molecular techniques are a reality. The interest of seed industries in these subjects is evident. Scientists from several large companies were present in the meeting. The prospects for transgenic maize seeds in the market are not very distant. Therefore a future at least as interesting as its present and as fascinating as its past may be expected for *Zea mays*.

MONDAY MAY 11th

John Doebley
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St. Paul, MN 55108 USA

GENETIC ANALYSIS OF THE MORPHOLOGICAL EVOLUTION OF MAIZE

The combined use of molecular markers and quantitative genetic models provides a powerful means for dissecting the genetic basis of of complex traits. Specifically, molecular marker loci can be used as tags to locate morphological trait loci and investigate their effects on the phenotype. This approach was used to investigate the genetic basis of the dramatic morphological differences between maize (Zea mays ssp. mays) and its probable progenitor, teosinte (Z. mays ssp. parviglumis). Results from two F_2 populations indicate that the key traits differentiating maize and teosinte are each under multigenic control, although for several traits, such as number of ranks of cupules, the data are consistent with a mode of inheritance that would involve a single major locus plus several modifiers. For other traits, such as presence/absence of the pedicellate spikelet, the data from both populations indicate multigenic inheritance with no single locus having a dramatically larger effect than the others. Most of the variation for the dramatic differences in inflorescence morphology between maize and teosinte is explained by the same five restricted regions of the genome in both F_2 populations. For one of these regions, the teosinte chromosome segment has been back-crossed into maize. This segment appears to contain a single major locus (tga1, teosinte glume architecture) that controls glume development including its size, orientation and degree of induration. The phenotype of tga1 appears to be strongly affected by genetic background. Another of these regions encompasses a previously described gene, tb1 (teosinte branched), and the effects of this region on inflorescence architecture are similar to the known effects of tb1. The data suggest that the differences between teosinte and maize involve, in part, developmental modifications that enable (1) secondary inflorescences, which are programmed to develop into tassels (male) in teosinte, to become ears (female) in maize, and (2) the expression of male secondary sex traits on a female background in maize. Similar changes were likely involved in the origin of maize.

MOLECULAR APPROACHES TO UNDERSTANDING RESISTANCE TO TROPICAL INSECT PESTS

D.A. Hoisington, D.C. Jewell, J.A. Deutsch and J.A. Mihm
The International Maize and Wheat Improvement Center (CIMMYT, Mexico

According to CIMMYT data on the major maize production environments in 29 countries with a maize area of 400,000 ha or more, approximately 30 million hectares, out of a total of 55 million, are seriously affected by insect pests. The availability of maize germplasm resistant to these pests combined with good agronomic performance will allow developing countries to sustain their current rates of productivity while at the same time practicing sound agriculture. Through the years, the maize program at CIMMYT has addressed this problem by utilizing traditional breeding methodology coupled with artificial infestations to develop maize varieties with improved resistance to insect pests. The newer technology involving the application of molecular markers has the potential to make the development of improved germplasm with both good agronomic performance as well as adequate levels of host plant resistance, faster and more efficient.

Restriction fragment length polymorphisms (RFLPs) are not fundamentally different from other genetic markers; however, they have the advantage of being co-dominant, available in large numbers, highly polymorphic and are detectable in all germplasm. The development of a project directed towards the understanding of a quantitatively inherited trait such as insect resistance requires a team effort involving a breeder, entomologist, molecular geneticist and biometrician. Several approaches are also required in order to fully understand the effect and importance of each quantitative trait locus (QTL). These include the analysis of several types of segregating populations (F2s, recombinant inbreds, etc.), several populations within each type, advanced populations (both selected and unselected) and inbred lines. The final proof of marker-trait associations is achieved through the use of the correlated molecular markers to successfully drive selection in a population for the trait of interest. If successful, it is expected that RFLPs (and perhaps other, newer types of molecular markers) will result in a cost savings and gain in efficiency in the incorporation of resistance into elite germplasm.

The approaches being utilized at CIMMYT for the location and verification of chromosomal regions controlling the resistance in maize to attack by the first generation Southwestern corn borer (*Diatraea grandiosella*) include the molecular and field analysis of a range of inbred materials, F5 lines derived from a single cross between a resistant and susceptible parent, and four F2 mapping populations involving crosses between two resistant and two susceptible inbreds. The results of these studies support the origin of generalized resistance from Antigua germplasm and suggest that important chromosomal regions controlling resistance are located on chromosomes 1L, 2, 3L, 5L, 9S and 10L. The results also confirm that the resistance is polygenic, primarily additive in gene action, and that there is a large genotype by environment interaction.

Gene Mapping in Maize

B. Burr, F.A. Burr, and E. Matz
Brookhaven National Laboratory
Upton, NY, USA

Recombinant inbred (RI) strains provide an excellent mechanism for single gene mapping and for identifying genetic factors affecting quantitatively inherited traits. One of the most important characteristics of a family of homozygous RI strains is that they constitute an essentially permanent population in which segregation is complete. This means that they can be replicated in different laboratories so that many geneticists can work with the same population and its associated shared database. The current database based on two RI families of 48 and 41 members contains 715 mapped loci. If a molecular geneticist wants to map a newly cloned gene, he or she begins by determining which restriction enzyme shows polymorphism between the parental inbreds that gave rise to the RIs. The RI DNAs are digested with the same enzyme and probed with the gene. The resulting allele distribution is compared with the database using a computer program that reports distance from linked loci. In this way a new gene can be mapped with only two Southern blotting experiments.

RIs should also be ideal populations in which to map genetic factors associated with quantitative inheritance. The ability to replicate individual genotypes means that an accurate estimate of the genotypic component of variation can be obtained. Additionally, a dense genetic map should permit an accurate placement of these markers. We have concentrated on two model systems – anthocyanin pigmentation and telomere length. The latter property is highly polymorphic in maize, although it has no apparent phenotypic effect, and is under multigenic control. A goal of this work is to demonstrate that genetic factors that are identified as affecting these traits are essentially wild-type alleles that vary in their level of expression.

Parallel Studies of Genome Composition and Organization in Maize and Sorghum

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Department of Biological Sciences
Purdue University

We are interested in sorghum (*Sorghum bicolor*) as a model organism for the study of maize (*Zea mays*) for three major reasons. First, sorghum is the most closely related to maize of any major crop species, thereby making many of the technologies developed for use in maize easily adaptable for analysis of sorghum. Second, sorghum is an unusually hardy plant with abiotic and biotic stress resistances which may be useful in the improvement of maize. Third, the smaller genome size of sorghum may make it more amenable than maize to map-based molecular technologies, including chromosome walking.

We have begun our analyses of the sorghum genome by generating a genetic map through the use of maize restriction fragment length polymorphism (RFLP) probes. To date, we have used 250 fragments of low copy number maize DNA as probes in gel blot hybridization analyses of two distantly related *S. bicolor* lines. All but four of these probes yielded one or more bands of hybridization under the standard high stringency conditions employed. Of these, 115 were polymorphic between these two parents, and 104 have been mapped. The map now contains eleven linkage groups, with 77 linked markers, and covering about 525 cM. Given our population size, the 27 unlinked markers that we have found represent at least an additional 700 cM. Hence, the genetic map of sorghum is at least 1200 cM in this cross. So, despite having a genome that is physically three to four fold smaller than that of maize, sorghum has a genetic map of very similar dimensions.

In general, the genetic maps of sorghum are both qualitatively and quantitatively very similar. Maize and sorghum have a similar linear order of genes, but we have detected a few major chromosomal rearrangements that differentiate the species. Most of the duplicated probes in maize are also duplicated in sorghum, and often in the same approximate map positions. Hence, these data suggest that the duplications of the maize and sorghum genomes are likely to have occurred prior to the divergence of these two species.

Although gene number/type, sequence homology, and basic chromosomal structure have not diverged much between these two species, analyses of repetitive DNAs yields a much lower level of sequence conservation. Fourteen classes of highly repetitive maize DNA were used as probes of sorghum DNA; only ribosomal DNA and one other repetitive sequence hybridized to sorghum DNA at either high or low stringency. The middle repetitive transposable elements *Ac*, *Spm*, and *MuA* from maize hybridized weakly to sorghum. We are now cloning these elements from sorghum for comparison to their homologues in maize.

Currently, we are attempting to place additional RFLP, morphological, isozyme, and quantitative trait loci on the sorghum map. In a limited diversity study using RFLP probes, we have found that *Sorghum bicolor* is much less variable than *Zea mays*. This may be partly due to a lower level or activity of transposable elements in sorghum than in maize, although we have identified a probable autonomous transposable element in candystripe sorghum.

We have cloned a number of genes from sorghum, using maize DNA as probes. We have cloned sorghum rDNA and homologues of the maize *B*, *P*, and *R* loci. These loci will be compared at the sequence level. We also have collaborated with Dr. Keith Edwards at ICI Seeds in the molecular cloning of over 300 kb of contiguous DNA in and around the *Adh1* locus of maize. We are analyzing the structural and functional components of this stretch of DNA, and plan to compare it to the homeologous region in sorghum.

These combined experiments will provide answers regarding the modes in which sequences and their chromosomal arrangement have varied during the evolutionary descent of these two closely related species. In addition, we hope this study will provide insights into the degree to which sorghum might be helpful in the map-based analysis and isolation of important maize genes.

VERONIQUE SZABO

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MAPPING OF THE KEY TRAITS DISTINGUISHING MAIZE AND TEOSINTE UNSING RFLP MARKERS

Segregation of key traits distinguishing maize and teosinte were analyzed in three F_2 and three backcross populations between the maize inbred T232 and *Zea mays* ssp. *parviglumis*. These traits are: paired *vs.* single female spikelets, two-ranked *vs.* many-ranked ears and non-indurated *vs.* indurated glumes. Other traits studied were inclination of the kernels towards the rachis, distichous *vs.* polystichous central staminate spike and, the number of tillers. 50 RFLP markers covering the genome were initially used to map these traits, where upon other linked markers were used to refine the map. All traits, except paired spikelets with two genes, showed a simple mode of inheritance. These major genes were found in four chromosomal regions. The placement of most of these genes do not agree with previous authors. Possible reasons for these discrepancies will be discussed. These results support previous suggestions that maize is differentiated from teosinte by as few as five major gene changes; although, the additional contribution of modifier genes must be taken into account for the complete transformation of one to the other. For the most part, recombination was normal in these populations; however, in eight regions there was reduced recombination relative to the values reported for the same regions within maize.

Crossing Barriers within *Zea*

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As a wind pollinated species, maize needs means of preventing indiscriminant hybridization. One efficient means is pollen rejection by silks. Although the basis for the failure of interspecific pollen to function is not directly amenable to genetic analysis, an array of factors affecting crossing within maize and between closely related taxa is known. Those factors involving specific interaction between pollen and silk genotype include the pollen preference (or "gametophyte factor"), systems *Ga*1, *Ga*2, *Ga*4, and *Ga*8. In a cross where all the pollen derives from one heterozygous plant, the effect of these factors is to distort the transmission of linked loci; when pollen from different plants are competing, selection affects the entire genome. In the genetic background of many popcorn varieties and meso-American maize races, *Ga*1 prevents fertilization by pollen of North American dent strains. Similarly, a crossing barrier complex isolated from ssp. *mexicana* teosinte serves to reject the pollen of all maize strains and could be a factor in isolating teosinte and maize in sympatric populations. Infrequent crossover products involving the teosinte complex retain only the pollen function. The putative reciprocal crossover class has not been identified. Neither the basis for recognition nor the nature of the rejection response is known for any of these systems.

EVALUATION OF OPEN-POLLINATED MAIZE CULTIVARS USING RFLP'S.

Azucena Mendoza[1], Mireya Sanchez[1], Martha Betancourt[1], Rosalia Maciel[1], David Jewell[2], James Deutsch[2], Luis Herrera-Estrella[1] and June Simpson[1].
1-CINVESTAV, Irapuato, Gto. México. 2- CIMMYT, El Batan, México.

Open-pollinated maize cultivars offer several advantages over maize hybrids when grown under marginal conditions in developing countries. They are adaptable to widely differing growth conditions, need little investment in terms of fertilizers and pesticides and allow small farmers to produce their own seed rather than buy costly hybrids annually. In fact under these conditions open-pollinated cultivars (OPC'S) give equivalent or better yields than hybrids. Excluding, China, Brazil and Argentina (developing countries where most hybrids are grown), 84% of all maize grown in developing countries is grown as OPC's

Although offering many advantages, the mechanisms governing disease resistance and differences in yield etc. are poorly understood in OPC's. In addition evaluation, manipulation and follow up of useful characteristics in breeding programmes is extremely complicated in such heterogeneous populations. With the advent of RFLP technology however it should be possible to evaluate in general terms the amount of polymorphism and heterozygosity in these types of populations and to attempt to relate this information to useful characteristics found within a population. This information could then be used to monitor these characteristics in breeding programmes involving OPC's.

With these aims a study of two related OPC's differing significantly in yield was carried out in order to determine the effectiveness of RFLP's in characterising OPC's. The two OPC's used were derived from Tuxpeño germplasm from CIMMYT gene pool 21, classified as tropical late white dent. A total of 80 different loci were analysed dispersed throughout the maize genome with 8 loci analysed per chromosome.

One of the problems of studying populations of this type is to determine what size of sample to use in order to have a faithful representation of the population as a whole. We determined that sample sizes of 100 plants are adequate since they would only fail to include very rare alleles which are most probably not important in determining the overall characteristics of the population. Results also showed high levels of polymorphism ranging from 2 to 15 alleles per locus. Differences in numbers of alleles detected for each cultivar were not significant, however types and frequencies of alleles were significantly different. The level and type of heterozygotes

was also determined for each cultivar. Overall levels of heterozygosity were shown to be very similar although some individual loci showed substantial differences in heterozygosity levels between the two cultivars. Types of heterozygote also differed greatly between the two populations. These results indicate that RFLP's will be useful in evaluating and manipulating OPC's. In addition the basis for the significant difference in yield between the two varieties is probably based on the type and frequency of alleles fixed in each population and in the type of heterozygotes formed rather than due to overall levels of heterozygosity thought to underlie the phenomenon of hybrid vigour often exploited in maize hybrids.

TUESDAY MAY 12th

H. SAEDLER

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Transposable elements in Zea mays

A multitude of transposable elements seem to fall into three basic categories which might be distinguished by their mode of transposition:

a) excision and reintegration: Ac/Ds and En/Spm might be candidates
b) replicatory: Mu might belong into here
c) retrotransposons: Bs1 and Cin 4 seem to be representatives

While elements of categories a and b not only affect genes and gene expression by virtue of integration, they also can affect gene structure and function if excised by leaving a footprint behind. This could be instrumental in evolutionary processes. Retrotransposons seem to lack this latter property. Hence their contribution to evolutionary processes seems to be limited by virtue of their insertion close or into a locus and thus affecting gene function. In any case retroelements could serve as a molecular tool not only for gene cloning but also as a marker in time or geographic location in the evolution of corn lines, mainly because once integrated at a given site they are fixed to that chromosomal position and subsequntly distributed to the progeny.

ANALYSIS OF RECOMBINATION AND TRANSPOSITION IN MAIZE USING THE *BRONZE* LOCUS AND THE TRANSPOSABLE ELEMENT *ACTIVATOR*

Hugo K. Dooner, Alemu Belachew, Diane Burgess, George Chuck, James English, Susan Harding and Edward Ralston. DNA Plant Technology Corp. Oakland, CA 94608, USA.

Because it confers a seed color phenotype and it is flanked by two easily scored markers that affect endosperm traits, the *BRONZE* (*BZ*) locus is one of the best reporter genes in maize to study the occurrence of infrequent events, such as those associated with intragenic recombination and transposition. We have used *BZ* in combination with the transposable element *ACTIVATOR* (*Ac*) to gain new information on such events. We have found that: (1) Recombination per kilobase (kb) inside of *BZ* is much higher than the average for the maize genome, but drops off sharply in the repetitive DNA region immediately proximal to *BZ*. Results from other genes suggest that this may be a general phenomenenon in maize. (2) *Ac* transposition from the *BZ* locus occurs preferentially to sites linked genetically to *BZ* in *9S* and is both bidirectional and nonpolar. (3) *Ac* transposition to sites that are genetically unlinked to *BZ* is nonrandom: *9L* is highly preferred and, among chromosomes other than *9*, certain ones are preferred over others.

Interaction between members of the *R* family

Giuseppe Gavazzi, Gabriella Consonni, Chiara Tonelli
Dipartimento di Genetica e di Biologia dei Microrganismi - Università degli Studi di Milano, Via Celoria 26, 20133 Milano Italy.

R is a regulatory gene involved in the control of anthocyanin biosynthesis in maize.
R respond to many exogenous and endogenous signals. The response is monitored by changes in colour, its intensity,distribution and tissue specificity. Development is the result of differential gene activity in response to signals. So studying *R* might help to unravel the much more complex phenomena of morphogenesis and development.
After a brief introduction on the genetics of the *R* gene family and its single components, data will be presented on the response of members of these family to irradiation with light of different quality. Attention will then be given to a particular case of interaction between members of the *R* family, disclosing an unexpected kind of communication between different modules of the family. The phenomenon, named reversed paramutation, exhibits a striking dependence on the pairing of two target gene copies and it implies the ability of one copy to communicate with the other; communication meaning a signal that one gene is sending so that the target of the signal, presumably the promoter of the other gene, allows its gene to be activacted in tissues that are of competence of the first one.

REGULATORY GENES AFFECTING MAIZE SEED STORAGE PROTEIN SYNTHESIS

Mario Motto, Massimo Maddaloni, Hans Hartings, Carlotta Balconi, Eduardo Rizzi, Adriano Marocco, Enzo Martegani[1], Isabella Mauri[1], Francesco Salamini[2], Stephan Lohmer[2] and Richard Thompson[2], Istituto Sperimentale Cerealicoltura, Bergamo, Italy; 1) Dip. Fisiologia e Biochimica Generali, Milano; 2) Max-Planck-Institut, Köln, Germany

We have investigated the effect of three dominant mutations fl2, De-B30, and Mc which reduce zein level in the endosperm. It is shown that both b-70s resemble heat shock proteins in that they bind ATP, cross react with HSP antibodies and has N-terminal sequence homology to chaperon-like HSP70. The physical similarity of b-70 to known molecular chaperone proteins and its association with abnormal accumulation in endosperm mutants may reflect a biological function to mediate protein folding and assemby in maize endosperm. Out of the recessive mutants o2, o6 and o7, the locus O2 play a central role in regulating the expression of certain members of the zein gene family and the expression of an abundant cytosolic albumin, b-32. The protein encoded by O2 is a member of the bZip family of eukaryotic transcription factors. By expressing the O2 gene product (O2-protein) in tobacco leaf protoplasts, one can trans-activate 22 kDa zein and b-32 promoters, demonstrating that O2 protein acts as a transcriptional activator. DNA binding analyses indicate that O2 recognizes a target site that is present in promoter of 22-kDa zein and b-32 genes. Additional experiments show that co-expression of b-32 in tobacco protoplasts potentiates the trans-activation of target promoters by O2. This indicated that b-32 appears to increase in a non-specific manner the translation of reporter genes expressed in this assay and therefore presumably interacts with some component of the translation machinery. It was shown that the presence of uORFs of the major coding sequence of the O2 locus reduces trans-activation by O2 probably by interferring in the translation of O2 mRNA. A functional homology between the GCN4 protein and the O2 product has been obtained through expression of O2 cDNA in the yeast S.cerevisiae. The regulation of zein synthesis in endosperm cells by amino acid supply will be also presented and discussed.

MOLECULAR AND FUNCTIONAL ANALYSIS OF DIFFERENT MUTATIONS AT THE OPAQUE-2 LOCUS OF ZEA MAYS

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CNR Istituto Biosintesi Vegetali - Via Bassini 15, Milano - Italy

The transcriptional regulation of zein genes and the synthesis of zein polypeptides that lead to the formation of protein-bodies, involve several steps both at the nuclear and cytoplasmic level. Mutations at the O2, O7 and fl2 loci have been shown to differentially affect both zein gene transcription and protein body formation.
The opaque-2 locus encodes for a DNA-binding protein belonging to the b-Zip family of transcriptional activators found in mammalian, yeast and plant cells like jun, fos, GCN4 and TGA1.
The activation of transcription follows the binding to short target sequences present in a subset of zein genes belonging to the high molecular weight (H) and usually absent in the low molecular weight (L) classes.
Different wild-type or mutant alleles have been characterised by Southern and Northern analysis using both O2 and zein probes.
The different alleles can be grouped according to the hybridization patterns obtained probing the O2 cDNA to EcoRI and SacI digested DNAs.
On the other hand, Northern analysis carried out on total RNA from maize endosperm of wild-type and O2 alleles either with H and L zein or O2 probes, reveals level and type of zein and O2 transcripts characteristic of each mutation.
By Western analysis the O2 antibody reveals in all the wild-types examined a double band with an apparent molecular weight of about 68-69 kD.
However, the different o2 alleles show in some cases faster migrating O2 polypeptides or absence of any O2 product.
The overall results clearly indicate a different molecular nature of the various O2 mutations and suggest different molecular mechanisms in the regulation of the expression of the H and L zein genes mediated by the O2 polypeptides.

Gene Expression Patterns and Tissue Development in C4 Leaves
Timothy Nelson, Yale University

During the development of plant organs, cells of varying clonal history often arrive at the same differentiated fate. In the leaves of plants capable of C4 carbon fixation, two major photosynthetic cell types—bundle sheath and mesophyll—differentiate as the leaf vascular system is established. Several studies (Dengler lab, Nelson lab, Freeling lab) have demonstrated the variable cellular ontogeny of BS and M cells of C4 leaves. This abstract summarizes our current view of the differentiation of these cell types. Current experimental data suggests that bundle sheath (BS) and mesophyll (M) cells must interpret positional information distributed around each vein to correctly express cell-specific genes. Light plays a crucial role in the generation or interpretation of this positional information.

In C4 plants, photosynthetic BS and M cells differentiate in the context of Kranz leaf anatomy to carry out the 2-cell metabolic cooperation called C4 carbon fixation. In maize, the C4 cycle requires the compartmentalization of three enzymes in M cells (PEPCase, NADP-MDH, and PPdK) and two enzymes in BS cells (RuBPCase and NADP-malic enzyme). Our strategy has been to use the C4 biochemical system to obtain tools to study the development of the two interacting cell types.

Markers characteristic of BS and M cells appear at a time in development which is coincident with the development of veins. We have shown by immunolocalization, in situ hybridization, and RNA blot analysis that C4 genes are expressed early in leaf development, at the time of vein initiation. Cell position relative to veins is a critical factor in differentiation of BS and M cells. In situ hybridization experiments revealed with high resolution that BS- and M-specific genes are turned on in precursor cells in radial patterns centered at veins—BS genes near and M genes far from the center. Cells beyond a critical radius differentiate in a default non-C4 state. Genes permitting C3 (non-C4) carbon fixation are activated before those for the C4 pathway.

A simple model accounts for existing data on spatial control of BS and M cell differentiation: A vein-centered effector ("A") informs precursor (ground) cells of their radial distance from the nearest vein. Cells with high A turn on BS-specific genes; cells with lower A turn on M-specific C4 genes. In the absence of A, cells follow a default pathway for differentiation, which results in a non-C4 photosynthetic cell. This vein-origin information system would be an efficient way to maintain local control over leaf cells, since veins are progressively initiated to subdivide the leaf as cell number increases.

Several pieces of information support this model:

1) In *argentia* mutants, BS and M cells are retarded in development until all veins have been initiated. Their subsequent differentiation follows the pattern in which the local vein was initiated.
2) Several maize leaf-like organs (e.g., husk leaves) exhibit greater spacing between veins and thus greater interveinal cell number (up to 20). Local control over C4 gene expression extends only a few cells distant from a vein. Beyond this, M cells differentiate as default non-C4 photosynthetic cells.
3) Consistent with these gene expression patterns, husk leaves include C4-fixation regions adjacent to veins and C3 regions more distant, as distinguished by physiological methods.

4) The distance over which veins influence C3 vs C4 differentiation depends on illumination level. With strong illumination, the C4 pattern extends a greater distance from veins than in low illumination. The C3 default pattern of gene expression occurs if light is absent during development.

Our current studies focus on the establishment of the Kranz pattern early in the leaf primordium and on the activation of individual C4 genes during the establishment of this pattern.

The Role of Homeobox Genes in Maize Development.

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The isolation and study of homeobox genes in animal systems has led to a detailed understanding of molecular events that regulate animal development. Members of homeobox gene families often act in concert to specify particular developmental processes. We discovered a family of homeobox genes in maize by transposon tagging the *Kn1* mutation. Approximately ten homeobox genes have been isolated that are related to *Kn1* in the homeobox, but differ in sequence outside. We mapped the genes to chromosome position and analyzed expression patterns for some. Analysis of the *Kn1* mutations in maize and overexpression of the *Kn1* gene in tobacco allow us to make predictions of *Kn1*'s function, and to speculate on the role of plant homeobox genes in general.

WEDNESDAY MAY 13th

Sequence analysis of prolamin genes from Maize, Coix and Sorghum reveals highly conserved protein structure and regulatory elements.

Adilson Leite[1], Jose A. Yunes[1], Laura M. M. Ottoboni[1], Maria Luiza P.N. Targon[1], Marcio J. da Silva, Gonçalo A. de Souza Filho[1] and Paulo Arruda[1,2*].

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The prolamins are the major seed storage proteins of maize, *Coix* and Sorghum. In all these three cereals this protein fraction accounts for more than 60% of the total protein and is constituted by a size and charge heterogeneous family of polypeptides, deposited in protein bodies within the rough endoplasmic reticulum of endosperm cells. The prolamins of maize, *Coix* and Sorghum are named zeins, coixins and kafirins respectively. They can be classified as α, β, γ and δ prolamins according to their solubility characteristics. The four prolamins classes can be found in maize while *Coix* and Sorghum presents only α and γ prolamins. Alpha and γ-prolamins are the predominant protein classes in all the three cereals. The α-prolamins accounts for more than 70% of total prolamins in maize, *Coix* and Sorghum. They are constituted by a large heterogeneous family of polypeptides grouped into the 19 and 22 kD size classes in maize, 17, 25 and 27 kD in *Coix* and 25 kD in Sorghum. The high MW α-prolamins represent the largest prolamin multigenic families as they are encoded by a family of ~120 genes in maize, ~20 genes in Sorghum and ~50 genes in *Coix*.

Several cDNA and/or genomic clones encoding the prolamin genes of *Coix* and Sorghum were isolated and sequenced. We did not find, either by Southern hybridization and nucleotide sequencing, sequences corresponding to the 19 kD α-zein in both *Coix* and Sorghum. However the protein sequences of the 25 kD α-coixin and 25 kD α-kafirin were highly homologous to the 22 kD α-zeins and for this reason these proteins were named 22 kD-like α-prolamins. The alignment of amino acid sequences of the 22Kd-like α-prolamins from *Coix*, maize and Sorghum revealed a highly conserved protein structure. The proteins consists of a N-terminal tail, containing the signal peptide, followed by ten tandem repeats and a short C-terminal tail. The difference between the 22Kd-like α-prolamin and the 19Kd α-zein lies in the fact that the 19Kd protein is exactly one repeat motif shorter than the 22 Kd proteins. Repeat motifs 3, 5 and 7 are closely related one to another as are the repeats 4, 6 and 8. This could be the result of two intragenic duplications from an ancestral pair of repeats, respectively the forbears of the current repeats 3, 5 and 7 and repeats 4,6 and 8.

The analysis of regulatory sequences of the 22 kD-like α-prolamins of *Coix*, maize and Sorghum, showed the characteristic eukaryotic TATA, CATC boxes and the ~-300 prolamin box at conserved regions in the three species. The first 13 base pairs of the prolamin box is completely conserved in *Coix*, maize and Sorghum 22 kD-like α-prolamin genes, but it is less homologous in the 19 kD α-zein gene. Most important was the identification of putative *Opaque-2*-like boxes in both 22Kd-like α-coixin and 22Kd-like α-kafirin genes. Homologous sequences to the *Opaque-2* boxes GATC(G)A and

TCCTCATGAA of the pML1 zein promoter, were found in the 22 kD-like α-coixin and α-kafirin promoters. The presence of *Opaque-2*-like sequences in the *Coix* and sorghum genome was confirmed by Southern hybridizations using a fragment of a maize *Opaque-2* cDNA clone as a probe. The results points to the presence of one and more than one gene homologous to the maize *Opaque-2* gene in the *Coix* and sorghum genome respectively and suggest that the 22Kd- α-prolamins of *Coix*, maize and Sorghum shares similar transcription regulation.

The α-coixin of 17 kD is a minor prolamin constituent. It can be classified as α-coixin based on solubility properties, but cDNA cloning and sequencing revealed that it is a sulfur-rich protein highly homologous to the 15 kD β-zein.

The γ-prolamins appears on SDS-PAGE as a single polypeptide of 27 kD in Sorghum and 22 kD in *Coix* while in maize there are two γ-zeins, one of 27 kD and one of 16 kD. In all three cereals γ-prolamins are encoded by only one or two genes. The alignment of the amino acid sequences of the γ-prolamins of *Coix*, maize and Sorghum showed that the proteins are highly homologous. They are constituted by a N terminal tail containing a signal peptide and tandem repeats composed of PPPHVL. The differences in the MW among γ-prolamins appears to be due to variations in the number of PPPHVL repeats.

Sequence analysis of the regulatory region of the γ-zein and γ-kafirin genes revealed homologous regulatory boxes. These regulatory sequences should interact with conserved transacting factors in *Coix*, maize and Sorghum, since similar levels of transient expression were observed when a construct containing the γ-kafirin promoter fused to the GUS reporter gene, was bombarded to immature endosperms of the three species.

Taken all the results together, we suggest that the structural and regulatory genes involved in the expression of prolamin genes in *Coix*, maize and Sorghum, originated from a common ancestor and that variations were introduced in the structural and regulatory sequences after species separation.

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FUNCTIONAL AND EVOLUTIONARY ANALYSIS OF GENES CODING FOR PROLINE-RICH PROTEINS FROM MAIZE AND RELATED SPECIES. Lluis RUIZ-AVILA, Matilde JOSE, Denis TAGU, Regina RAZ, Marcelo MENOSSI, José Antonio MARTINEZ-IZQUIERDO and Pere PUIGDOMENECH. Departament de Genètica Molecular. CID-CSIC. Jordi Girona, 18. 08034 Barcelona. España.

Proline-rich proteins are well known components of plant cell wall. The probably have a structural function but they also take part in the defense reactions against fungal infection and wounding. The best known proline-rich proteins are hydroxyproline-rich glycoproteins (HRGP), also called extensins in dicotyledonous species, proline-rich proteins (PRP) from soybean and a number of othe similar proteins that include some nodulins and proteins having a proline-rich region in part of their sequence (hybrid PRPs or HyPRP). The structure, expression and promoter analysis of the HRGP and HyPRP genes from maize and related species have been studied.

Maize HRGPs have been characterized in maize at protein, cDNA and genomic level (1,2). Its mRNA is accumulated in organs of the plant rich in dividing cells and it may be induced by wounding (3), by *in situ* hybridization the mRNA is mainly observed in provascular cells (2). A tissue specificity has also been observed in defined tissues of immature embryos (4). In these tissues it is possible to observe that in scutellum the HRGP gene expression cannot be detected both at protein and mRNA levels. By polarization microscopy it is possible to compare the location of HRGP mRNA accumulation with the distribution of cells at different stages of cell wall formation appearing as an early event in cell differentiation (5). The gene coding for a protein having a hybrid proline-rich and hydrophobic sequence has also been cloned and it shows a pattern of expression specific of immature embryo and complementary to HRGP (6).

The HRGP seems to be encoded in cereals (maize, sorghum or rice) by a simple family of genes, probably by a single gene (2). Comparison of sequence between related species allows to define a conserved zone in the 5' flanking region (7) that contains sites hypersensitive to nucleases (8). The promoter activity of this region has been studied by microbombarding and by transformation in heterologous systems. The results of these experiments will be presented.

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8. Nuclease sensitivity of a maize HRGP gene in chromatin and in naked DNA, M.P. Vallés, J. Bernués, F. Azorín & P. Puigdomènech. Plant Sci. (1991) 78, 225-230.

Structural and functional analysis of Maize histone gene promoters

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In maize the histone H3 and H4 multigene families are organized into 8 to 10 subfamilies. Genes belonging to 3 H3 and 2 H4 multigene subfamilies show very similar developmental and organ specificity but are expressed at different levels. The amounts of specific mRNAs increase in parallel to the DNA synthesis during germination. In the adult plant the levels are highest in organs with high mitotic activity but also significant in non proliferating tissues.

The structure of the chromatin of the promoter region of the H4C7 gene has been studied by PCR-mediated *in vivo* foot-printing. Foot-printing experiments were performed using proliferating and non-proliferating BMS cell-suspension and nuclei isolated from germinating embryos. Sequences protected against and accessible to either dimethylsulfate (DMS) or DNase I could be precisely localized. The protected regions, supposed to interact with transacting factors, correspond to the major consensus motifs found in the histone gene promoters. Detailed foot-printing of the ACGTCA motif was compared to results obtained with the same motif present in other plant gene promoters.

The promoter regions of different H3 and H4 genes were fused to the GUS (glucuronidase synthase) gene used as a reporter gene as transcriptional fusions. Two of the 3 promoters are able to drive the expression of GUS in tobacco protoplasts during transient expression assays but failed to show any replication-dependent induction. These experiments allowed to determine regions within the promoter with regulatory functions.

Transgenic tobacco plants were regenerated from the protoplasts transformed with the same constructions by direct gene transfer. On the other hand, transgenic *Arabidopsis* were generated by transferring the same chimaeric genes via *Agrobacterium tumefaciens*. The GUS activity was revealed by histochemical tests in different organs of the transgenic tobacco and *Arabidopsis* at different developmental stages. The activity was shown to correlate with meristematic tissues in both plants. We deduce that the maize histone gene promoters can drive tissue-specific expression in dicots.

Protoplasts were prepared from transgenic *Arabidopsis* and tested for the GUS activity driven by histone gene promoter in complete medium and under conditions blocking the DNA synthesis. The results showed that the maize promoters can initiate a basal constitutive expression and an enhanced replication-dependent activity during the S phase of the cell cycle, thus confirming the maize histone gene promoters being functional in dicotyledonous plants.

Cloning and characterization of HM1, a gene for leaf blight and ear mold resistance.

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Disease caused by Cochliobolus carbonum race 1 can destroy a crop by either killing the plants or by molding the ears. It is unlikely that maize could be widely grown without genetic resistance to this pathogen. Resistance is conferred by functionally duplicate loci, HM1 and HM2, located on chromosomes 1 and 9, respectively. HM1 can be fully dominant (resistant) throughout the life and tissues of the plant whereas HM2 is semi-dominant and acts primarily from the time of flowering through maturity. Certain alleles of HM1 are similar to HM2. These genes are effective only against race 1 of C. carbonum and have no other known phenotype.

Race 1 is distinguished by the production of a compatibility factor called HC-toxin. This molecule permits the fungus to colonize hm1 hm2 maize; it accounts for both pathogenicity and specificity. Dose-dependent inhibition of root growth is a convenient and quantitative assay for HC-toxin, hence, the designation as a toxin. There is no evidence that HC-toxin kills host cells in advance of the growing hyphae. Histological studies show that colonization is normal for a fungal pathogen, with the growing tips of the hyphae being well advanced beyond damaged leaf tissues. Hm1 causes maize to be 100-fold less sensitive to HC-toxin.

HC-toxin reductase (HCTR) can be extracted from resistant maize seedlings but not from susceptible seedlings; the enzyme requires NADPH as a co-factor. The biological activity of HC-toxin is abolished by the enzyme. Genetic tests show that HM1 controls the activity of HCTR. Therefore, HCTR activity is the mechanism of this race-specific disease resistance.

We have cloned HM1 by transposon tagging. Insertions of Mu1, Mu3, Spm/En, or a small uncharacterized element all prevent the accumulation of a 1.3 kb Hm1-specific transcript. This transcript is produced constitutively in low abundance. A 1.6 kb cDNA has been cloned that corresponds to the Hm1-transcript. The cDNA clone contains splice concensus sites that indicate the presence of an unprocessed 286 bp intron. Removal of the putative intron eliminates 2 stop codons in the open reading frame (one is spliced out and the other is lost due to a frameshift), alters the size of the clone to match the observed transcript, and extends the region of homology between HM1 and related genes. The cDNA is very homologous to part of the dihydroflavanol-4 reductases from Antirrhinum and Petunia and to A1 from maize. The region of homology is similar to the concensus peptide sequence that has been identified as the NADPH or NADH binding domain of reductases and dehydrogenases. The homology with NADPH-dependent enzymes indicates that HM1 encodes HCTR.

Genetic engineered cell lineages in maize using the microinjection approach.

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The capacity to introduce exogenous DNA into meristematic cells would lead to substantial improvement in the field of transformation of recalcitrant species such as cereals. In this plants genotype-specificity and regeneration capacity is still the limiting factor for transformation via protoplasts or biolistic approach.
In the present study we are using microinjection to introduce exogenous DNA into individual cells of the shoot apical meristem of maize. Microinjection has been chosen because it allows to introduce DNA into defined cells under optical control. Under these conditions individual cells in different cell layers can be manipulated, and subsequently followed during plant development using visible marker genes.
For this purpose an appropriate cultivation system of isolated shoot apical meristems was developed. In order to follow up the fate of the transformed cells and to define which cells in the meristem are responsible for the development of the generative tissue we are using constructions carrying the LC - gene (gift from S. Wessler) under the control of different promoters. Lc is a regulatory gene controlling the anthocyanin biosynthetic pathway and therefore its expression results in anthocyanin production. The plasmids were tested in a maize protoplast system. Considering the possibility of this system - cell lineage and visible marker expression- we will discuss our approach and data obtained so far.

TRANSGENIC MAIZE AND COMMERCIAL APPLICATIONS

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The corn biotechnology team at Monsanto is working on producing genetically engineered corn plants that will control feeding damage from the European Corn Borer (*Ostrinia nubilalis*). One method to control corn borer is to express the Bacillus thuringiensis (Bt) gene in the plants. As background to this work, the gene transfer method and selectable marker system for producing the transgenic plants will be described. A summary of the gene exprossion data and inheritance data in transgenic corn will also be presented, followed by a description of the insect control of the Bt expressing corn plants.

SHORT ORAL PRESENTATION

AGROBACTERIUM-MEDIATED DNA TRANSFER TO MERISTEMATIC TISSUE OF MAIZE IMMATURE EMBRYOS

Jesús Escudero, Gunther Neuhaus and Barbara Hohn

Agrobacterium mediated DNA transfer to maize (Zea mays) has been achieved using the technique known as agroinfection. This approach uses maize streak virus (MSV) as part of the bacterial DNA transferred to the plant cell (T-DNA). Maize immature embryos have been shown to be competent to agroinfection, the plant developmental stage and genotype being the two main factors affecting the susceptibility to the viral infection (Schlappi, M. and B. Hohn, The Plant Cell, 4:7, 1992). We have used this method to investigate whether particular meristematic cells can be targeted with Agrobacterium and still be functional, which would mean that the bacterial interaction with the plant can also take place intracellular. Zygotic maize embryos were microinjected at early developmental stages with Agrobacterium tumefaciens into a single cell of the apical meristem. The observed efficiency ranges between 10-30% of plants showing viral symptoms using inocula of about 20 bacteria per plant cell microinjected. The process seems to be absolutely dependent on the plant genotype and responds to induction by phenolic compounds as acetosyringone. Preliminary results will be presented on the association of this DNA transfer with the plant meristematic tissue.

POSTERS

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GENETIC STUDIES OF IN VITRO ANDROGENETIC DEVELOPMENT WITH CORN

Screening large number of ancestries made clear that genetic variations exist for in vitro androgenesis response with maize. In the early part of this work haploid plants were generated and progenies were derived after spontaneous chromosome doubling.

The androgenetic process produces lines with a very high androgenetic capability and there was a high level of expression in hybrid structures (percentage of responsive anthers) including elite lines which showed little androgenetic aptitude. It was also possible to obtain hybrid structures revealing a very high heterosis effect. The androgenetic anther percentage response of the DH5 x DH7 hybrid for example was often as high as 75 %.

This first step genetic analysis was continued with a more precise study including a characterisatiuon of DH5 x DH7 hybrid. It is clear that androgenesis itself does not select for high response levels, it was possible to obtain both, lines with the high heterotic value of the hybrids and also lines with very little potential. This suggests there are complementary genetic systems in the parental lines which act together. The regeneration potential of androgenetic embryos was evaluated for these lines. These experiments did not reveal any strong genetic correlation between the two characters and it was easy to recombine a high level of androgenetic anther response with a high capability of plant regeneration from androgenetic embryos.

This sample of recombinant lines indicates that a small number of genes or a cluster of genes differenciate both parental lines from androgenetic traits. A more precise identification a localisation of genes involved would simplify back-cross experiments to material with greater agronomic potential.

Keith Edwards, Helen Thompson, Antoine de Saizieu, Mark Eyers and Keith Rufener, I.C.I. Seeds.

THE USE OF YAC LIBRARIES IN THE MAPPING OF THE MAIZE GENOME

The recent developments in Yeast Artificial Chromosome (YAC) systems capable of cloning DNA fragments several hundred kilobase in size, opens up the possibility of isolating and characterising agronomically important sequences via chromosome walking. Using a maize YAC library containing 79,000 clones with an average insert size of 145 kb we have begun to isolate and characterise various single copy and repeat sequences from throughout the genome. Using this approach we have so far shown that YACs are capable of cloning DNA fragments which cannot be cloned in prokaryotic based systems. This strategy will allow us to build up a catalogue of sequences which should ultimately lead to the ordering of the entire library.

Using the library to isolate single copy sequences we have shown that despite the large physical/genetic distance ratios observed within the maize genome, single YACs are capable of linking markers which can be separated on the genetic map.

We will present data on the construction of the library together with results on the characterisation of individual YAC clones.

GENETIC ANALYSES OF DOUBLED HAPLOID LINES IN MAIZE: ITS POTENTIAL USE IN GENE MAPPING.

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The segregation and recombination of a common set of 94 RFLP markers was compared in an F_2 population and an anther culture (AC) derived population. One hundred F_2 individuals and 98 doubled haploid (DH) lines were obtained from the cross R6 (a proprietary line, not responsive to anther culture) x DH.89.1 (a line very responsive to anther culture). Significant deviations from the expected Mendelian segregation ratios for pooled data were observed only for the DH population. The differential transmission of alleles strongly favored the responsive parent (66 of the 69 markers which showed disturbed segregation). There was considerable heterogeneity among the markers showing a skewed segregation towards DH.89.1. This set of markers could be divided into 2 homogeneous subgroups, one comprising 59 markers (% DH.89.1: 70), the other comprising 7 markers (% DH.89.1: 84). Despite these single factor disturbed segregations, MAPMAKER program could be used to construct a linkage map with the DH population. The comparison with the F_2 linkage map demonstrated the consistency of the 2 maps for 97% of the length covered by the DH linkage map. These results are discussed in relation to the use of DH lines in maize breeding and RFLP mapping.

ADH1 in *Avena*: Phylogenetic analysis

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The possibility of sequencing proteins and nucleic acids has provided a powerful tool to determinate the evolutionary history of organisms. In order to make this possible it is necessary to have an evolutionary model on which to base specific predictions. The neutralist model provides a theoretical frame in which nucleotide substitutions are accumulated at a constant rate during long periods of time. In this way, these macromolecules become a molecular clock, and there exists a linear relationship between the number of differences between sequences and the time elapsed since these sequences diverged from a common ancestor. This fact allows the inferring of the real-time phylogeny of the molecules analyzed if an independent way of calibrating the molecular clock is available.

Different proteins and genes exhibit different substitution rates. And as a result the choice of the correct molecule is of great importance in phylogenetic studies. The sequence of the molecule should have the information needed to discriminate among alternative phylogenies.

The sequence analysis has also made the study of the evolution of the genes themselves possible. Processes as gene conversion, duplications, unequal crossing-over, etc. are detected in these kinds of works.

In the work we are carrying out, the main goal is not as much the construction of the phylogenetic relationships among some plant species, as the study of the effects that polyploidy has on evolutionary rates. Genus *Avena* has been the election because it includes diploid, tetraploid and hexaploid species.

We have focused on gene *adh1* because it is a single-copy gene that evolves as a molecular clock in other related grasses (maize, pearl millet), and its sequence contains enough information to discern among several alternative hypothesis in cereals. The analysis of maize *adh1* alleles is a good example of the utilization of sequence information in tracking down the origins of genic variation: It has been possible to estimate their times of appearance and the occurrence of processes of gene conversion between them.

Avena adh1 gene has been analyzed in two diploid species, *A. hirtula* and *A. longiglumis.* The sequencing was performed from products obtained by PCR. These included from the exon 2 to the exon 6. The sequence shows a high degree of conservation in exons, a fact that has made their identification possible. The position of introns is also conserved among species, their sequences have greatly diverged, though, with several additions/deletions along them.

We have used the relative rate test proposed by Wu and Li to check to see if *adh1* evolves as a molecular clock in oats. The results indicate that the rate of substitutions is constant for all the species analyzed only in the third base of the codons. Consequently, the phylogenetic analysis has been made using these bases preferentially.

Comparing the sequence of the allele *1F* of maize with the sequence of *adh1* of *A. hirtula*, a synonymous substitution rate of 5.97×10^{-9}, and a non-synonymous substitution rate of 2.13×10^{-10} has been obtained. The substitution rate in introns is 6.80×10^{-9}.

Different phylogenetic analysis has been worked out using several methods: parsimony, distance matrix and likelihood methods. All the results have yielded the same branching order which agree with the evolutionary position that classic taxonomy has proposed for *Avena*, more related to *Hordeum* than to *Zea* or *Pennisetum*.

Between *A. hirtula* and *A. longiglumis* the similarity has been very high, with just seven nucleotide substitutions in exon sequences , all of them synonymous, and 20 in intron regions. These few changes point out the recent divergence of these species from their common ancestor.

DETECTION OF THE PROTEIN ENCODED BY TRANSPOSABLE ELEMENT Ac *IN SITU*

Manfred Heinlein

By using five antisera raised against different epitopes of the Ac-protein a signal has been obtained in endosperm nuclei of Ac-containing endosperm, which is absent in endosperm-nuclei devoid of Ac. The signal is not seen if various preimmunesera are taken as primary antibody. If an antibody is used which have been incubated with its specific antigen prior to its application the sensitivity of the detection is much reduced. The signal appears as large rod-like complexes, about 2 µm long and 0.2 µm wide. With decreasing Ac-dosage the complexes become less detectable. The visibility varies between endosperms containing different Ac-bearing alleles. In nuclei double-stained for DNA and Ac-protein, the complexes in some cases appear to be perpendicular oriented relative to DNA. The inner endosperm nuclei are endopolyploid at later stages of development and cytological observations suggest that the endochromosomes are at least partially polytene. Since the Ac-protein binds to DNA the complexes therefore might be interpreted as protein bands spanning the associated endochromosomes at specific sites.

THE DIFFERENCES IN THE *TRANS*-EFFECTS OF THE Ac-ELEMENTS PRESENT IN wx-m7 AND wx-m9Ac ARE NOT DUE TO THE GENETIC BACKGROUND

Manfred Heinlein and Peter Starlinger

In a previous communication (Maydica 36 (1991): 309-316), we showed that different isolates of the maize transposable element Ac located at different insertion sites gave rise to distinguishable variegation patterns for the excision of a non-autonomous Ac derivative from the bz-m2(DI) allele. These differences were seen as different frequencies and sector sizes of pigmented areas on *bronze* kernels.

In the case of the wx-m7- and the wx-m9Ac-alleles, these differences could not be ascribed to differences in DNA sequence, as both alleles had been found to be identical. Long-range position effects were also excluded, since the two Ac insertions were located within the same gene (same orientation). In the experiments, it could not be decided, whether the differences were due to modification of the DNA-sequence, e.g. by methylation, to position effects manifested at different sites within the same gene, or to the presence of modifier genes differing between the maize lines employed. To analyse this further, we crossed plants which were heterozygous for wx-m7 and wx-m9Ac against a bz-m2(DI)wx tester strain. In the progeny, the waxy-allele specific *Bronze* variegation phenotype and the Wx reversion phenotype segregated with the original waxy-mutable allele (tested by PCR). This indicates, that that the allele-specific Ac-action is either due to the waxy-mutable alleles themselves or to an allelic difference of a tightly linked gene.

FREIBURG, GERMANY; University of Freiburg

Winfried Hetz, Michael Schwall and Günter Feix

Identification of a mutant deficient in secondary root formation

In an attempt to generate mutants with a defect in an agronomically important trait, we crossed a flint inbred (obtained from W. G. Pollmer / Hohenheim, Germany) with an EN carrying dent inbred (obtained from P. Peterson / Ames, USA). In the segregating F2 generation we recovered among several other aberrant phenotypes a plant with a severe deficiency in the formation of lateral roots. Furthermore, this plant does not form any secondary or crown roots and depends for its growth and survival completely on the primary root which remains viable and functional up to maturity of the plant. The plants show a reduced vigour and need special care like intense watering and a supporting stick.
Genetic analysis of this plant material indicated that the observed aberrant phenotype is caused by a recessive mutation. The mutant appears to be different from the rootless mutant described previously (Jenkins, M.T. (1930) J. Hered. **21**, 79-80) which was provided to us by the Maize Genetics Stock Center.
Investigations are now in progress on whether the mutation is caused by the insertion of an EN element and would hence allow the isolation of the affected gene.

MEMBERS OF A FAMILY OF REPETITIVE DNA FROM THE GENUS *ZEA*, HAVE CHARACTERISTICS OF RETROELEMENTS. **Rosa Aledo, Carlos M. Vicient, Amparo Monfort, Regina Raz, Pere Puigdomènech and José A. Martínez-Izquierdo.**
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The isolation of a new family of repetitive DNA sequences from the teosinte *Zea diploperennis*, 253 bp long on average, termed ZEAR (1), has allowed to find a putative new family of retroelements. The short repetitive elements are interspersed in the genome and they show the same genomic organization pattern and similar copy number in all the *Zea* species examined, except for *Zea perennis*. The constancy in genomic pattern and copy number suggest that a mechanism of gene conversion have occurred among those repetitive sequences during the evolution of the *Zea* species.

ZEAR elements have some features resembling those of coding sequences. In fact, when sequencing flanking regions, an ORF including ZEAR is revealed. This ORF codes for a protein of 157 residues, rich in glycine, serine and aspartic and glutamic acids. The ORF is flanked by a TATA box and three putative polyadenylation sites. The ZEAR probe hybridizes with RNA extracted from different tissues of maize and from teosinte, indicating that these repeats or similar ones are present in transcribed sequences. In fact, one cDNA, 1809 bp long, fished out with a ZEAR probe from a maize cDNA library, contains the ORF corresponding to the genomic one, and shows 77% similarity over 1.9 kbp with the teosinte genomic sequence.

Experiments of Southern hybridization and genomic reconstruction (copy number estimation) have shown that those repeats are part of larger repetitive elements, referred as to ZRT (*Zea* retroelement), that probably belong to the superfamily of retroelements, as several indications suggest: 1) The large ZRT members are also interspersed in the genome and present in all the maize chromosomes, as shown by *in situ* hybridization. 2) As mentioned above ZEAR and flanking sequences are present in relatively long RNA transcripts. 3) Upstream the region corresponding to the cDNA, there is a sequence similar to defective *cin1* maize elements. 4) Downstream the cDNA there are nine 81 bp long tandem repeats, like in the retroposon TOC1 of *Chlamydomonas reinhardtii*. 5) Further downstream these repeats there are ORFs with similarities to polymerases of viruses and retroelements. These results will be discussed in relation to the similarities observed with retroelements such as retrotransposons or retroposons and retro- or pararetro-viruses.

1.- Raz, R., Puigdomènech, P. & Martínez-Izquierdo, J.A. (1991). A new family of repetitive nucleotide sequences is restricted to the genus *Zea*. Gene 105, 151-158.

Use of Molecular Markers for Estimating QTL Effects in a Simulation Study.

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Use of molecular markers in populations under selection has been proposed to estimate effects of quantitative traits loci (QTL). However, further information is required about the magnitude of the QTL effects that can be estimated in relation to the genetic variance, the heritability, the marker mapping density of the genome and the size of the experiment. Monte Carlo datasets of selfed families from backcrosses of the F_1 to both parents were generated. The multivariate stepwise regression with an F-value corresponding to $\alpha = 0.005$ was applied to the phenotypic means. An expression for the ratio of the phenotypic variance to the square of the additive effect as a function of the number of progenies, the heritability and the marker mapping density was derived. Simulation results agreed fairly well with predictions based on developed theory. Additive effects smaller than one-fourth the standard phenotypic deviation were unbiasedly estimated most of the situations. Dominance effects were estimated in a lower frequency and biased upward. Reduction of the environmental error variance and increase in the marker mapping density augmented the power of the test. It is suggested that evaluation of 500 selfed backcross families in replicate trials would be able to estimate unlinked QTL's in moderately complex traits. Estimates of linked QTL effects will need better and larger experiments.

CLONING AND SEQUENCING OF 2,3-BISPHOSPHOGLYCERATE-INDEPENDENT PHOSPHOGLYCERATE MUTASE GENE FROM MAIZE.

Pablo Pérez de la Ossa, Xavier Graña and Fernando Climent.

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Phosphoglycerate mutase (PGAM) catalyses the isomerization of monophosphoglycerates in the glycolisis/gluconeogenesis pathway.There are two types of phosphoglycerate mutases: one that requires 2,3-bisphosphoglycerate as a cofactor (PGAM-d) and the other that does not (PGAM-i). The two types also differ in their structure, reaction mecanism and kinetic properties. In maize, PGAM has a maximun level of expression during the second day of the seed germination. In order to study the molecular bases of the PGAM-i expression, the PGAM-i gene has been cloned from lambda EMBL-3 genomic library from *Zea mays* 7-day old seedlings screened with the full length cDNA. Two clones were isolated and one, 14 Kb, was selected. Deletions were performed and sequenced. Four exons (161 bp, 137 bp, 158 bp and 649 bp respectively) and four introns (1220 bp, 659 bp, 80 bp and 218 bp respectively) have been sequenced. Different poliadenilation signals in the 3' region of the gene have been found.

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MOLECULAR ANALYSIS OF MAIZE B-CHROMOSOMES WITH A VIEW TO DETERMINING THEIR ORIGIN

An attempt was made to isolate unique B-chromosome DNA sequences in order to investigate their DNA sequence composition. An examination of restriction enzyme digests of rye plants with and without B-chromosomes led to the discovery of unique repetitive B-chromosome sequences (Sandery et al 1990). The same approach was adopted in an attempt to isolate similar sequences on the maize B-chromosome but none were found. Partial genomic libraries were constructed from a 10B maize plant and the clones were screened by Southern hybridisation to identify B-chromosome correlated sequences. DNA sequences common to the A + B-chromosomes and sequences common to the B-chromosomes only were detected but there were no unique B-chromosome sequences. These results have been supported by in-situ hybridisation. We have strong evidence to suggest that the B-chromosomes in maize are derived from the A-chromosome set.

EVALUATION OF OPEN-POLLINATED MAIZE CULTIVARS USING RFLP'S.

Azucena Mendoza[1], Mireya Sanchez[1], Martha Betancourt[1], Rosalia Maciel[1], David Jewell[2], James Deutsch[2], Luis Herrera-Estrella[1] and June Simpson[1].
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Open-pollinated maize cultivars offer several advantages over maize hybrids when grown under marginal conditions in developing countries. They are adaptable to widely differing growth conditions, need little investment in terms of fertilizers and pesticides and allow small farmers to produce their own seed rather than buy costly hybrids annually. In fact under these conditions open-pollinated cultivars (OPC'S) give equivalent or better yields than hybrids. Excluding, China, Brazil and Argentina (developing countries where most hybrids are grown), 84% of all maize grown in developing countries is grown as OPC's.

Although offering many advantages, the mechanisms governing disease resistance and differences in yield etc. are poorly understood in OPC's. In addition evaluation, manipulation and follow up of useful characteristics in breeding programmes is extremely complicated in such heterogeneous populations. With the advent of RFLP technology however it should be possible to evaluate in general terms the amount of polymorphism and heterozygosity in these types of populations and to attempt to relate this information to useful characteristics found within a population. This information could then be used to monitor these characteristics in breeding programmes involving OPC's.

With these aims a study of two related OPC's differing significantly in yield was carried out in order to determine the effectiveness of RFLP's in characterising OPC's. The two OPC's used were derived from Tuxpeño germplasm from CIMMYT gene pool 21, classified as tropical late white dent. A total of 80 different loci were analysed dispersed throughout the maize genome with 8 loci analysed per chromosome.

One of the problems of studying populations of this type is to determine what size of sample to use in order to have a faithful representation of the population as a whole. We determined that sample sizes of 100 plants are adequate since they would only fail to include very rare alleles which are most probably not important in determining the overall characteristics of the population. Results also showed high levels of polymorphism ranging from 2 to 15 alleles per locus. Differences in numbers of alleles detected for each cultivar were not significant, however types and frequencies of alleles were significantly different. The level and type of heterozygotes

was also determined for each cultivar. Overall levels of heterozygosity were shown to be very similar although some individual loci showed substantial differences in heterozygosity levels between the two cultivars. Types of heterozygote also differed greatly between the two populations.
These results indicate that RFLP's will be useful in evaluating and manipulating OPC's. In addition the basis for the significant difference in yield between the two varieties is probably based on the type and frequency of alleles fixed in each population and in the type of heterozygotes formed rather than due to overall levels of heterozygosity thought to underlie the phenomenon of hybrid vigour often exploited in maize hybrids.

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ISOLATION OF HOMEOBOX GENES INVOLVED IN MAIZE EMBRYOGENESIS

We isolated a new maize homeobox gene by screening a lambda gt11 expression library with the 26 bp Shrunken feedback control element. *Zmhox1a (Zea mays homeobox)* is an unidentified maize gene mapping to the tip of the long arm of chromosome 6. *Zmhox1a* is a member of a new class of maize homeobox genes, with its homeodomain only distantly related to the *Knotted* gene product. The 3.1 kb *Zmhox1a* transcript is detected in different maize tissues and encodes a nuclear protein of 112 or 115 kd size. Presence of the *Zmhox1a* mRNA and absense of the protein in roots indicates a posttranscriptional control mechanism. The acidic character of the known first 279 amino acids at the amino-terminus implies a transcriptional activator function. In the carboxy-terminal part the *Zmhox1a* protein is related to the human Oct2 transcription factor, homology to the POU specific domain is restricted to the POU-B subdomain. DNA-binding of the Zmhox1a homeodomain has been confirmed in DNase I footprinting experiments to three sites flanking the TATA-box of the Shrunken promoter.

To obtain a collection of different maize homeoboxes, we used the homeobox of *Zmhox1a* for a screening under low stringency conditions. We isolated nearly 90 clones, which have been closer analysed and classified at present. The hybridisation and sequence data provide evidence that they will fall into nearly ten different classes of related homeoboxes.

One of the isolated genes, *Zmhox1b*, is closely related to *Zmhox1a* over the entire protein coding region and presumably maps at the same position within the genome. Another one *(Zmhox2a)* contains two complete DNA-binding homeodomains and shows an extended similarity to the *Drosophila paired* segmentation gene. The mRNA is 6 kb in size and found at rather high level in the maize embryo.

The next question to be answered will be: is there any function of these homeobox genes in the embryogeny of maize?
Therefore we isolated embryos of eight different developmental stages, beginning with the "Transition Phase", about ten to twelve days after pollination, until "Stage 6", the mature embryo. At present we are constructing cDNA libraries of all these stages which are screened with a set of distantly related homeoboxes of maize. The next steps will be expression studies by Northern analysis and *in situ* hybridisation.

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Loss and Gain of Opaque-2 Transactivation Via Single Site Mutations of Zein Promoters

By utilizing a homologous transient expression system, we have demonstrated that the product of the *Opaque-2* (*O2*) gene, Opaque-2 protein (O2) confers a positive transactivation on a 22-kDa zein promoter. This *trans*-acting function of the O2 protein is mediated via its sequence-specific binding to a *cis* element (the O2 target site) present in the 22-kDa zein promoter. A multimer of a 32-bp promoter fragment containing this O2 target site confers transactivation by the O2 protein. Both the palindromic ACGT core and adjacent nucleotides of the O2 target sequence are essential for O2 binding. A single nucleotide substitution in the O2 target sequence not only abolishes the O2 binding *in vitro*, but also its response to transactivation by the O2 protein *in vivo*. We have also demonstrated that an amino acid domain including the bZIP motif is essential for the *trans*-acting function of the O2 protein. Similar but not identical O2 target sequence motifs can be found in the promoters of zein genes of different molecular weight classes. Conversion of such a motif in a heterologous zein promoter to an exact O2 target sequence by site-directed mutagenesis is sufficient to increase the binding affinity of the O2 protein *in vitro* and to confer transactivation by O2 *in vivo*.

LIST OF INVITED SPEAKERS

Workshop on

THE PAST THE FUTURE OF Zea mays

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Organized by V. Conejero and L. C. Van Loon. Lectures by L. C. Van Loon, R. Fraser, J. F. Antoniw, M. Legrand, Y. Ohashi, F. Meins, T. Boller, V. Conejero, C. A. Ryan, D. F. Klessig, J. F. Bol, A. Leyva and F. García-Olmedo.

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259 **Workshop on Transcription and Replication of Negative Strand RNA Viruses.**
Organized by D. Kolakofsky and J. Ortín. Lectures by A. K. Banerjee, M. A. Billeter, P. Collins, M. T. Franze-Fernández, A. J. Hay, A. Ishihama, D. Kolakofsky, R. M. Krug, J. A. Melero, S. A. Moyer, J. Ortín, P. Palese, R. G. Paterson, A. Portela, M. Schubert, D. F. Summers, N. Tordo and G. W. Wertz.

260 **Lecture Course Molecular Biology of the Rhizobium-Legume Symbiosis.**
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261 **Workshop The Regulation of Translation in Animal Virus-Infected Cells.**
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