IAEA-TECDOC-1010

Application of DNA based marker mutations for improvement of cereals and other sexually reproduced crop plants

Proceedings of a final Research Co-ordination Meeting
organized by the
Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture
and held in Vienna, 4–8 November 1996

INTERNATIONAL ATOMIC ENERGY AGENCY IAEA

The IAEA does not normally maintain stocks of reports in this series. However, microfiche copies of these reports can be obtained from

INIS Clearinghouse
International Atomic Energy Agency
Wagramerstrasse 5
P.O. Box 100
A-1400 Vienna, Austria

Orders should be accompanied by prepayment of Austrian Schillings 100,– in the form of a cheque or in the form of IAEA microfiche service coupons which may be ordered separately from the INIS Clearinghouse.

The originating Section of this publication in the IAEA was:

Plant Breeding and Genetics Section
Joint FAO/IAEA Division
International Atomic Energy Agency
Wagramer Strasse 5
P.O. Box 100
A-1400 Vienna, Austria

APPLICATION OF DNA BASED MARKER MUTATIONS FOR IMPROVEMENT OF CEREALS AND OTHER SEXUALLY REPRODUCED CROP PLANTS
IAEA, VIENNA, 1998
IAEA-TECDOC-1010
ISSN 1011–4289

Printed by the IAEA in Austria
March 1998

FOREWORD

Since the beginning of crop domestication, visual markers have played a role in plant selection for improved yield, adaptation and quality. However, the number of such markers is limited and they are based on phenotype and not genotype which often makes visual marker-based selection difficult. Fifteen years ago a new set of markers was developed which, for the first time, allowed the practical detection of genetic variation in the DNA, the molecule carrying the genetic information. These molecular markers offered the opportunity to analyse the genetic basis of changes in plants caused by induced mutations, whether through ionizing radiation or chemicals. Perhaps the greatest potential for application of these new markers may be in the developing countries, for local and under-investigated crops with little genetic information.

The Co-ordinated Research Programme (CRP) on the Application of DNA Based Marker Mutations for Improvement of Cereals and Other Sexually Reproduced Crop Plants represents the first of three CRPs dealing with the application of molecular markers to mutations and plant breeding and was implemented between 1992 and 1996. A second companion CRP entitled Use of Novel DNA Fingerprinting Techniques for the Detection and Characterization of Genetic Variation in Vegetatively Propagated Crops devoted to the application of molecular markers in vegetatively propagated crops species was implemented between 1993 and 1997. One positive consequence of these two CRPs has been the implementation of a third CRP entitled Radioactively Labeled DNA Probes for Crop Improvement, which began in 1995 and aims to provide enabling technologies, in the form of probes and primers, to laboratories in developing countries. The rapid development of molecular marker technologies has also resulted in a dramatic increase in requests from developing Member States for technical co-operation projects utilizing molecular markers to improve local varieties for biotic and abiotic stresses and other traits of relevance. With the intensified use of induced mutations in genetic studies, it will be important to continue the important work of understanding induced mutations at the molecular level.

Evidence of the progress made in implementing molecular marker technologies in laboratories around the world is presented in this publication, which contains the results presented by the participants at the fourth and final Research Co-ordination Meeting of the CRP held in Vienna, 4–8 November 1996. The FAO and the IAEA wish to express their sincere appreciation to the participants of the meeting for their work during the project period resulting in the summary and scientific reports presented in this publication.

The officers responsible for this publication are E. Weck and A. Ashri of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

EDITORIAL NOTE

In preparing this publication for press, staff of the IAEA have made up the pages from the original manuscripts as submitted by the authors. The views expressed do not necessarily reflect those of the IAEA, the governments of the nominating Member States or the nominating organizations.

Throughout the text names of Member States are retained as they were when the text was compiled.

The use of particular designations of countries or territories does not imply any judgement by the publisher, the IAEA, as to the legal status of such countries or territories, of their authorities and institutions or of the delimitation of their boundaries.

The mention of names of specific companies or products (whether or not indicated as registered) does not imply any intention to infringe proprietary rights, nor should it be construed as an endorsement or recommendation on the part of the IAEA.

The authors are responsible for having obtained the necessary permission for the IAEA to reproduce, translate or use material from sources already protected by copyrights.

CONTENTS

Summary of the Co-ordinated Research Programme 1

Investigation of the somaclonal and mutagen induced variability in barley by the application of protein and DNA markers 21
A. Atanassov, E. Todorovska, A. Trifonova, M. Petrova, E. Marinova, M. Gramatikova, D. Valcheva, S. Zaprianov, N. Mersinkov

Searching for RFLP markers to identify genes for aluminum tolerance in maize 33
E. Paiva, M.A. Lopes, S.N. Parentoni, P.R. Martins, G.A. Torres

Determination of molecular markers associated with anthesis-silking interval in maize 41
J. Simpson

Incorporation of conventional genetic markers and RAPD markers into an RFLP based map in maize 47
E.H. Coe, Jr., M.D. McMullen, M. Polacco, G.L. Davis, S. Chao

Tagging of blast resistance gene(s) to DNA markers and marker-assisted selection (MAS) in rice improvement 55
J.Y. Zhuang, J. Lu, H.R. Qian, H.X. Lin, K.L. Zheng

RAPD tagging of a salt tolerance gene in rice 63
H. Ding, G. Zhang, Y. Guo, S. Chen, S. Chen

Rice genome mapping and its application in rice genetics and breeding 67
M.Y. Eun, Y.G. Cho, J.H. Hahn, U.H. Yoon, B.Y. Yi, T.Y. Chung

Detection of genetic variability in basmati and non-basmati rice varieties and their radiation induced mutants through Random Amplified Polymorphic DNA (RAPD) 85
S. Farooq, N. Iqbal, M. Arif

Application of DNA RFLP procedures in interspecific gene transfer: The *Lr19* translocation of wheat 97
R. Prins, G.F. Marais, A.S. Marais, Z.A. Pretorius, B.J.H. Janse

Microsatellites in wheat and their applications 103
P. Stephenson, G.J. Bryan, J. Kirby, M.D. Gale

Advances in sorghum genetic mapping with implications for sorghum improvement . . 111
M. Lee

Comparative genome analysis and resistance gene mapping in grain legumes 119
N.D. Young

List of Participants 125

SUMMARY OF THE CO-ORDINATED RESEARCH PROGRAMME

1. BACKGROUND

The use of genetic markers to enhance the efficiency of crop improvement, by both conventional and mutation breeding, has long been recognized. In the early nineties, molecular marker-mediated systems were in use in a few of the more advanced wheat, barley, maize and rice breeding programmes and were proving very useful. Therefore, it was deemed very important to expand the use of these marker systems to include developing countries and their crops. In fact, it was expected that the impact of marker assisted selection would be greater in less advanced breeding programmes. In order to achieve this impact, it was clear that it would be necessary to transfer technology from the most advanced laboratories to laboratories in developing countries using induced mutations in their breeding programmes. For this reason, in 1992, a CRP entitled The Application of DNA Based Marker Mutations for Improvement of Cereals and Other Sexually Reproduced Crop Plants was initiated.

At the inception of this CRP, DNA-based genetic maps had been developed for a range of crop plant species. These maps were mainly based on restriction fragment length polymorphism (RFLP) markers. By the time the CRP was implemented, newer marker systems based on the polymerase chain reaction (PCR), including randomly amplified polymorphic DNA (RAPD) markers, were also becoming available. Since this time, additional marker systems have been developed and have been incorporated into the research of this CRP. This is evidenced in this summary and the reports which follow.

Independent of the specific system selected for application in plant breeding, molecular markers provide a host of new opportunities for improving breeding efficiency. Specific applications include analysis of breeding systems, screening of segregating populations, purity assessment, genetic distance measurements between potential parent varieties, and improved efficiency of backcross programmes. Levels of heterozygosity can be accurately measured and employed for optimizing F_1 hybrid programmes. Knowledge of marker linkages facilitates the efficient introgression of chromosome segments carrying economically important alleles from wild relatives to cultivated crops. The development of detailed genetic maps also allows marker assisted juvenile selection, analysis and selection for genetic regions responsible for quantitative traits, and the possibility of employing DNA markers as entry points for DNA-walking to linked genes with a view to their cloning for transformation of other genotypes or species.

The goals of this CRP were to develop ways to accelerate plant breeding programmes and to make them more efficient, particularly those employing induced mutations. More specifically:

- to foster the development of bilateral links between breeders and molecular geneticists for development of appropriate genetic stocks and transfer of techniques for the use of markers in local breeding programmes;
- to establish the nature of spontaneous and irradiation induced DNA polymorphism with a view to exploitation by targeted probes and primers;
- to facilitate information exchange to enhance the awareness of breeders, particularly in developing countries, of the possibilities provided by DNA marker-mediated systems and provide advice on the availability of appropriate DNA probes and PCR primers and their use;
- to investigate the relative efficacy of radioactive and non-radioactive detection systems.

During the period of this CRP, the development and application of molecular marker techniques in plant breeding and genetics programmes around the world has been tremendous. This has resulted in an enormous increase in basic knowledge which has created the demand for increasing numbers of induced mutations to aid in the understanding of basic plant biology processes which have direct implications for practical applications in plant breeding. The challenges for the future remain the practical and cost effective deployment of this technology to solve problems of importance in Member States. The renewed growth in the use of induced mutations, when combined with molecular genetics, offers powerful tools for designing the solutions for ever more effective plant breeding in the future.

2. SPECIFIC RESULTS OF THIS CRP

This CRP has laid the foundation for a better understanding of induced mutations at the molecular level. Population analyses have established marker associations for traits of agronomic interest allowing marker assisted selection with radioactive and non-radioactive probes. A brief summary of the achievements of the participants during the duration of this CRP follows.

Atannasov, Bulgaria (7233/RB): Irradiation in barley proved important for regeneration and may have created specific DNA-level alterations, as measured by ribosomal DNA markers. Molecular markers, including protein, RFLPs, and RAPDs, were used to characterise Bulgarian barley cultivars and the their regenerants.

Paiva, Brazil (6998/RB): RFLP markers were compared with information about aluminum tolerance in maize. These results suggest that on chromosome 8 in maize there is a region related to aluminum tolerance.

Simpson, Mexico (7071/RB): Maize lines contrasting in anthesis-silking interval (ASI), a trait strongly linked to drought tolerance, have been analyzed under different water stress conditions in the field and with molecular markers. Correlation of markers with field data has revealed molecular markers strongly associated with flowering time and yield.

Davis, USA (7007/CF): Terminal radiation-induced chromosome deficiencies, were used to map terminal markers in maize. Integration of classical genetic markers, in particular mutants, onto the maize RFLP map will provide the tools necessary to further our understanding of plant development and of complex traits. Placement of mutants and cDNAs into bins (short regions of each chromosome) using a standard or core set of markers provides a necessary resource for the identification of gene functions in maize.

Zhuang, CPR (6999/RB): Two, or more, blast resistance genes in rice were associated with DNA markers in two F_3 populations. One resistance gene was located in the vicinity of RG81 in both populations. Crosses were made between elite varieties and blast resistance donors to develop populations for DNA marker-assisted selection of blast resistance. In addition, blast resistance donors and 48 varieties currently in wide use in rice breeding were analysed for DNA polymorphisms to produce a database for future MAS program.

Chen, CPR (7070/RB): A salt tolerant rice mutant was obtained through EMS induction followed by selection *in vitro*. A molecular marker was identified whose genetic distance from a salt tolerance gene is about 16.4 cM.

Eun, Rep. of Korea (7001/CF): The isozyme marker, *EstI-2,* and two RFLP markers, *RG109* and *RG220*, were linked at a distance of less than 1 cM with the semidwarf (*sd-1*) locus on chromosome 1 in rice. These markers could be used for precise *in vitro* selection of individuals carrying the semidwarf gene using single seeds or leaf tissue of very young seedlings, before this character is fully expressed in the mature plant. Appropriate application of marker-assisted selection, using *EstI-2* and RFLP markers for the semidwarf character, in combination with other markers linked to genes of agronomic importance in rice, holds promise for improving the efficiency of breeding, and the high-resolution genetic and physical mapping near *sd-1*, aimed at ultimately cloning this valuable gene.

Farooq, Pakistan (7002/RB): A population survey identified RAPD marker differences between Basmati and non-Basmati rice types.

Prins, R. South Africa (7003/CF): Gamma radiation was used to characterize and fine-map regions of a leaf rust resistance chromosome segment introduced into wheat.

Gale/Stephenson, UK (7004/TC): A landmark report showed marker comparisons which recognized the similarity of the cereal genomes (synteny) offering the intriguing possibility of mapping or cloning a gene in one species as a path to mapping or cloning the gene in related genera. The development of microsatellite markers from wheat has resulted in 150 primer pairs which have been tested for genetic polymorphism using a panel of ten wheat varieties. The microsatellites show high levels of genetic polymorphism and an average 3.5 alleles per locus with an average polymorphism information content (PIC) value of 0.5.

Lee, USA (7008/CF): A genetic map based on restriction fragment length polymorphisms was constructed in sorghum. Comparison of sorghum and maize RFLP maps on the basis of common sets of DNA probes revealed a high degree of conservation as reflected by homology, copy number, and collinearity. Examples of conserved and rearranged locus orders were observed. The same population was used to map genetic factors (mutants and quantitative trait loci, QTL) for several traits including vegetative and reproductive morphology, maturity, insect and disease resistance. Of the six QTLs detected for plant height in sorghum (an important character for sorghum adaptation in temperate latitudes for grain production) four seemed to be orthologous in maize. RFLP data seem to portray genetic relationships more accurately than the methods based exclusively on the coancestry coefficient. Thirty-two SSR loci have been mapped throughout the sorghum genome.

Young, USA (7009/CF): Multiple generations and populations were used to map soybean cyst nematode resistance. Using DNA markers, genome organization and several important disease resistance genes have been analyzed in mungbean, cowpea, common bean, and soybean. There is significant conservation of DNA marker order, though the conserved blocks in soybean are much shorter than in the others. DNA mapping results also indicate that a gene for seed weight may be conserved between mungbean and cowpea. Using the linkage maps, genes that control bruchid and powdery mildew resistance in mungbean, aphid resistance in cowpea, and cyst nematode resistance in soybean have all been mapped and characterized. For some of these traits resistance was found to be oligogenic as evidenced by DNA mapping.

The high quality of the research results achieved in this CRP suggests that it has succesfully linked scientists in developing and developed countries. This approach should be continued in order to ensure that scientists in developing countries actively participate in the latest molecular genetic developments and use them for the benefit of their agriculture, farmers and food security.

3. MARKERS, MAPPING, AND GENETICS

The use of DNA markers has had an enormous impact on understanding the basic biology underlying the breeding of sexually reproduced crops. Since their introduction in the early 1980s, a variety of DNA markers have been developed, some hybridization-based and some PCR-based. The use of DNA markers has made it possible to: (a) "fingerprint" genotypes precisely, (b) verify F_1 hybrids, (c) estimate genetic distance and forecast heterotic groups, (d) select the best individuals for breeding, (e) to discover breaks of undesirable linkages flanking genes of interest and recover the recurrent parent's genotype, (f) undertake genetic analysis for QTL, and (g) clone genes of economic importance. It is certain that DNA markers will radically alter the practice of plant breeding in the years to come.

3.1. Types of molecular markers

The most commonly used DNA markers are RFLPs and RAPDs. RFLPs were the first to be developed and they remain widely employed today. However, the RFLP procedure is time-consuming and requires radioactivity or complex visualization procedures. RAPDs, which are PCR-based markers, are much simpler to use, but are not co-dominant, are less predictably portable from one population to another, and also suffer from difficulties with reproducibility. The practical application of these marker systems in breeding situations may also be limited by the inadequate frequency of polymorphic loci. Another marker technology is known as Variable Number of Tandem Repeats (VNTRs) or "microsatellites". They have many desirable features, including the use of a PCR reaction instead of a blotting procedure, show co-dominant inheritance usually, and have genome specificity in polyploids. However, they are expensive and time consuming to develop because generation of DNA sequence information is required. Consequently, it will be a long time before maps of microsatellites are developed even for the most widely cultivated crops.

Yet another new marker technique has recently become popular, namely Amplified Fragment Length Polymorphism (AFLP) [1]. The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. Although expensive and technically more demanding than the previously mentioned techniques, AFLP generally produces many informative bands per sample. AFLP offers special promise in identifying markers tightly linked to deletion mutations induced with some mutagens [2] and in genomic regions tagged by bulked segregant analysis of nearly isogenic lines. The newest type of DNA marker, the "biochip", which will most likely open many new possibilities in research and application, is still in the development stages.

3.2. Considerations and limitations in the use of molecular markers

In deciding which marker system is best for a given application, several key factors should be considered. Among the most important are: the use of radioactive compounds, the number of samples to be processed, the need to transfer the data from one population to another (or one species to another), the level of molecular polymorphism differences between the parents, the amount of tissue sample available, and what sorts of genomic resources are already available in the crop systems. The information is summarized in Table I for the most widely used marker types.

There are certain limitations for some or all types of molecular markers. DNA isolation and purification remain a time consuming process, especially for large numbers of samples. Any marker system that requires acrylamide gel electrophoresis is technically challenging. Managing the data generated by a molecular marker project is also very

demanding. Capturing, proofreading, and organizing molecular marker data requires careful planning and execution. This is complicated further when the data are shared among different laboratories with distinct mapping or breeding populations, or different crop species.

3.3. Types of genetic markers

Integration of linkage information derived from various types of markers has significantly improved the resolution of crop genome structure and created opportunities for improved interpretation of the genetic basis of crop improvement. Prior to the availability of DNA markers, the available maps provided few opportunities for identifying suitable sources of germplasm with genetic variation for traits desired by plant breeders. Likewise, the maps based solely on mutations identified by alleles with large phenotypic effects and cytological markers, have created very few opportunities for geneticists and molecular biologists to assess the agricultural significance of certain genes; much less, devise directed strategies for locating and characterizing genes presumed to be highly significant. Currently, considerable efforts are devoted to integration of crop genetic maps and to relate genetic data derived from DNA markers, mutations, cytogenetic markers, and QTL. This resource will provide, for the first time, the essential context for objective dialogue between plant breeding and many branches of basic science.

One of the initial stages of recent map integration has involved cytological and DNA markers. Where adequate cytogenetic stocks have been available, combining the information with DNA markers has significantly enhanced our knowledge of genome organization for crops such as tomato and potato [3], maize [4], wheat [5], and barley and rye [6]. These investigations have revealed patterns of genome duplication, recombination, and cytogenetic/genetic distances along the chromosomes. This information is essential for efficient deployment of a wide spectrum of genetic technologies from targeted cloning of important genes through introgression of useful genes from exotic germ plasm into cultivated crops [7].

Another phase of map integration involves DNA markers and structural mutations. Reports of genetic linkage between DNA markers and deletions have increased at a seemingly exponential rate. To the extent that such alleles have been used in breeding programmes, these reports comprise a considerable array of expanded opportunities for using markers as indirect selection criteria. An area of potentially more pervasive significance for crop improvement has been integrative mapping of partly-sequenced cDNA clones in crops such as maize [8]. Maps rich in deletions provide many opportunities for matching mutants, collected and characterised over several decades, with specific DNA molecules (across taxa). Eventually, this process will provide a basis for determining the biophysical basis of genetic variation and phenotypic expression for many traits. To the extent that functions and DNA sequences have been conserved across plant taxa [9], integrated maps and their markers may represent a very important plant genetic resource for crop breeding.

The third, and least complete, phase of integrative mapping involves polygenes or QTL [10]. Despite the inherent ambiguities of the process, QTL mapping provides vital information for basic and applied aspects of crop improvement. QTL mapping allows a pangenomic assessment of gene location and action for potentially any phenotype (i.e. trait).

TABLE I. COMPARISON OF DIFFERENT MARKER SYSTEMS

	RFLP	RAPD	Microsatellites	SCARS/CAPS	AFLP
PRINCIPLE	Endonuclease restriction Southern blotting Hybridization	DNA amplification with random primers	PCR of simple sequence repeats	Polymorphic PCR products or endonuclease restriction of PCR products	Endonuclease restriction followed by selective PCR amplification
TECHNOLOGY					
Type of polymorphism	Single base changes Insertions Deletions	Single base changes Insertions Deletions	Changes in repeat length	Single base changes Insertions Deletions	Single base changes Insertions Deletions
Genomic abundance	High	Very high	Medium	High	Very high
Level of polymorphism	Medium	Medium	High	Medium	Very high*
Dominance	Co dominant	Dominant	Co dominant	Codominant	Dominant/Co dominant
Amount of DNA required	2-10ug	10-25ng	50 100ng	50 100ng	500ug*
Sequence information required?	No	No	Yes	Yes	No
Radioactive detection required?	Yes/no	No	No/yes	No	Yes
Gel system	Agarose	Agarose	Acrylamide/agarose	Agarose	Acrylamide
IMPLEMENTATION					
Development costs	Medium	Low	High	Medium/high	Medium/High
Start up costs	Medium/High	Low	High	High	Medium/High
Portability Lab/Crop	High/High	Medium/Nil	High/Low	High/Low	High/Nil
SUITABLE APPLICATIONS	Comparative Mapping Framework mapping	Varietal /hybrid identification Marker assisted selection	Framework/region specific mapping Fingerprinting Marker assisted selection	Framework mapping Marker assisted selection Can be converted to allele specific probes	Fingerprinting Very fast mapping Region specific marker saturation

* Because this technique leads to multiple infomative DNA bands per sample, the generalizations in the table are based on results typically achieved with an entire sample (rather than individual bands)

Several aspects of QTL mapping make this approach especially powerful for adding important genes and regions to maps. First, DNA markers provide relatively comprehensive coverage of the genome. Second, the choice of mapping parents is extensive so adequate DNA polymorphism may be detected. Therefore, important genetic regions may be added to maps in a directed manner, depending on the ability to assess genetic diversity and variation for the trait(s). Unlike mapping deletions, this approach may provide a more sensitive survey of the genome because it does not require fortuitous observation and recovery of alleles with highly qualitative effects on the trait(s) of interest.

Analysis of nearly isogenic lines and subsequent QTL mapping in maize have identified regions with major effects on components of maturity (e.g. days to flowering and number of internodes) [11, 12]; and tissue culture response [13] on chromosomes 8 and 9, respectively. Of course the identified QTLs cannot usually be located on the maps with the same precision and accuracy as other markers. However, the limitations created by the positional ambiguities may often be insignificant relative to the value of identifying the controlling chromosome regions for the first time.

The benefits of integrated maps have been realized in a few investigations. Mapping with chromosome translocations has provided independent verification of QTLs in maize for resistance to an insect [14], a virus [15], and a fungal pathogen [16]. Comparisons of genetic positions of QTLs and loci defined by previously identified mutations [17] have supported the hypothesis that quantitative and qualitative genetic variation may often originate from alleles at common loci [18]. Positive tests of allelism between a QTL and a mutant allele [19] and sequence analysis of alleles at the *Sh* locus [20] in maize have provided additional evidence in support of this hypothesis. In these situations, the supporting evidence was gathered in a very directed manner due to the availability of an integrated map.

3.4. Development of integrated maps

Genetic maps have previously provided few advantages to plant breeding and crop improvement programmes even for crops with relatively well-developed maps, such as maize and tomato. The primary problems with the practical utilization of genetic maps have been the lack of informative markers in the germ plasm used by breeders, the predominant types of markers used to create maps (chromosomal markers), the poor integration of maps each based on a different type of marker, and the polyploid nature of many crop genomes. The current and future generations of maps alleviate these problems in significant ways.

The advent of DNA markers has enhanced the relevance of genetic maps for plant breeding and improved the prospects of using linkage information as an important element in crop improvement schemes. The maps have provided a new source of information and raw materials (genes) for plant breeding as well as an impetus for modifying some plant breeding procedures.

As better genetic maps have been constructed using molecular markers, some with thousands of loci, the need for better genomic informatic resources has grown. Recently, genome databases for many major crop systems (see below) have been developed and most can be accessed through the World Wide Web. A suitable site for accessing these databases can be found on the USDA-National Agricultural Library Web page (address: http://probe.nalusda.gov:8300/plant/index.html). The following list includes examples of the databases included and the crops they cover:

AAtDB--*Arabidopsis*
Alfagenes--alfalfa (*Medicago sativa*)
BeanGenes--*Phaseolus* and *Vigna*
ChlamyDB--*Chlamydomonas reinhardtii*
CoolGenes--cool season food legumes
CottonDB--*Gossypium hirsutum*
GrainGenes--wheat, barley, rye and relatives
MaizeDB--maize
MilletGenes--pearl millet
PathoGenes--fungal pathogens of small-grain cereals
RiceGenes--rice
SolGenes--Solanaceae
SorghumDB--*Sorghum bicolor*
SoyBase--soybeans
TreeGenes--forest trees

3.5. Comparative mapping

The use of common sets of DNA probes to detect and map homologous sequences across sexually isolated species has revealed a surprisingly high degree of conservation in terms of copy number and homology of low copy probes, linkage, and locus order. Recognition of the considerable conservation of these features within groups of plants such as rice, wheat, and maize [21]; sorghum and maize [22]; wheat, barley, and rye [7]; tomato, potato, and pepper [3, 23]; *Arabidopsis* and *Brassica* [24] has inspired the suggestion of considering such groups as single genetic systems [9, 25].

This concept should have considerable merit and mutual advantages for breeders and geneticists. Often, the genome size of one member of the group is several fold smaller than that of other members. The smaller genome size should accelerate positional cloning of some orthologous genes [26]. For example, a rust resistance gene has been cloned in barley through parallel chromosome landing in rice. Once the gene in the source species has been cloned and sequenced, this information may be used to quickly isolate the orthologous gene in the target species as demonstrated by the isolation of the gene for chalcone flavanone isomerase in maize using sequence information from *Petunia*, snapdragon, and bean [27]. Recently, Resistance Gene Analogs (RGAs) have been isolated from soybean by using DNA sequence data of resistance genes isolated from several unrelated dicots. However, the repertoire and number of mapped and characterized genes may vary considerably between members of a group (e.g. tomato vs. potato, maize vs. sorghum). In these instances, map information from the "gene rich" species may provide important clues about a map region's genetic content in the "gene poor" species and vice versa. Comparisons of locus order and distribution of recombination events may also elucidate barriers and suggest strategies to incorporate germ plasm in wide crosses [7]. For plant-breeding programs, this information represents an opportunity for a considerable and directed expansion and improved definition of various crops' gene pools.

Comparative mapping of DNA clones has provided the basis for parallel investigations of other markers. For example, a region containing a locus that conditions the absence of ligules has been conserved in rice, wheat and maize [21]. Similar inspections of linkage data of other taxa should reveal many other examples such as the parallel linkage between genes for resistance to leaf rust (*Puccinia* spp.) and prolamines in oats, wheat, and maize [28]. Recently, the pattern of conserved linkage and function has been extended to include QTLs.

The initial report of orthologous QTLs noted that the RFLP loci with the greatest effects on seed weight in mung bean and cowpea were detected by the same clones [29]. In a similar manner, comparative mapping in maize and sorghum has revealed three putatively orthologous regions for plant height [30]. In sorghum, each region has a major effect on that trait and on a unique suite of other traits (e.g. tillering, panicle dimensions, leaf length and width) much like some of the *dw* loci in sorghum. Interestingly, plant height mutants at maize genetic loci in related regions have pleiotropic effects on some of the same combinations of traits as the sorghum QTLs and the candidate *dw* loci.

An essential resource for comparative studies will be the organization and integration of information about mutants, physical or cytological events, QTLs, and molecular markers into defined sections of the chromosomes, both within and across species. For example, information is currently catalogued in the Maize Genome Database by 20 cM intervals for approximately 5000 variants in maize with future links to other species envisioned.

3.6. Molecular analysis of induced mutations

The application of X rays and other physical mutagens to plant breeding in the past seventy years has been influential in increasing variability for practical breeding applications. The combination of new molecular technologies including, molecular markers, subtraction cloning methods and differential display analysis, with induced mutagenesis promises to radically improve our fundamental understanding of plant genetics and physiology. Practical approaches, using all available technologies, are crucial to meeting the increasing demands for food production around the globe.

Induced mutations are important in plant breeding especially when the existing genetic variation in the adapted germplasm is limited, when a single or a few genes are involved in the trait, when it is easy to screen for the trait and large populations can be tested, and when one is attempting to obtain a desirable trait without disrupting other traits. These considerations can give a selective advantage to the use of induced mutations for achieving plant breeding goals.

A broad range of plant traits have been successfully mutated through the use of radiations; some were induced by chemicals, mainly ethyl methane-sulphonate (EMS), N-methyl-N-nitroso urea (MNH), N-ethyl-N-nitroso urea (ENH), ethyleneimine (EI), and ethylene oxide (EO). Traits improved in released varieties of various crops include growth habit, earliness, short stature, lodging resistance, stem stiffness, oil composition; leaf, flower and fruit characters, and disease resistance.

Induced mutations can be useful in gene cloning approaches by offering an additional source of variability for locating genes of interest. Chemical mutagens tend to produce small alterations from point mutations up 50 bp deletions, whereas physical mutagens (radiations) tend to produce larger ones. Physical mutagens have been shown by molecular evidence to produce deletions from 17 bp up to 20 cM in length. In addition to simple deletions, physical mutagens are capable of creating more complex inversions, translocations and other alterations. The creation of large deletions may facilitate gene isolation from stocks containing radiation-induced mutations via subtractive hybridization or representational differences analysis.

The possibility of finding induced mutations directly via RFLP, PCR or DNA fingerprint screening has been investigated by a number of groups. For example, nine

independent mutations in downy mildew resistance genes (*Dm*) were induced with fast neutrons in lettuce [2]. A linear order of deletion breakpoints and molecular markers was established along the chromosome. The region surrounding *Dm3* was stable through many generations of breeding and the deletion breakpoints provided greater genetic resolution than meiotic recombinants. There was no evidence at the molecular marker level of chromosomal rearrangements associated with these deletions. On the other hand, a study in *Arabidopsis* utilized radiation-induced mutations in two genes, chalcone flavanone isomerase (*CHI*) and dihydroflavonol 4-reductase (*DFR*): one *CHI* allele, generated by fast neutron irradiation contained a 1.4 kb inversion within the gene as well as a 272 bp insertion adjacent to an inversion which was transferred from 38 cM away on the same chromosome; a *DFR* allele, induced by X-rays, contained a 72 bp deletion and a 7.4 kb deletion which flank a 2.8 cM inversion. These complicated alterations associated with physical mutagens suggest that some caution may be required before cloning genes known only by phenotype.

The cellular mechanisms that contribute to these types of rearrangement are still poorly understood. Mutations involving large deletions have only rarely been identified in plants. Even in other organisms, only limited analysis of these types of lesions has been performed thus far. The molecular biological tools available today make it possible to isolate induced mutations at known gene loci in a relatively short period of time and to facilitate improved recovery of desired induced mutants.

3.7. Benefits for crop improvement

The benefits of comparative and integrated maps for plant breeding programs are substantial for the short and long terms; they may be summarized as follows: 1) to the extent that mutations are utilized by breeders, integrated maps increase opportunities for indirect selection methods; 2) the ability to share genetic information between sexually isolated species should accelerate isolation of targeted genes; 3) the definition of crop gene pools should become broader and more precise for specific genes; 4) understanding of the biological basis of complex traits should improve by providing a common language for various branches of biology; 5) important genes may be localized by a variety of increasingly complementary methods for potential use in transgenesis projects; and 6) an element of objective hypothesis testing has become available for plant breeding.

There is so much to learn about so many shared sequences that comparative and integrative mapping is easily justified. However, this approach has some limitations. One limitation may relate to the observation that 10-20% of the low copy DNA clones from one species seem to be specific, or at least much more homologous to the source species [22, 29]. That could represent a substantial number of genes. At least some of those genes might confer unique or neomorphic functions in the source species. Undoubtedly there will be many examples of species-specific low copy sequences that turn out to be very important genes.

3.8. Map-based cloning

In the long-run, the most powerful use of molecular markers in plant breeding may be the ability to clone genes known only by phenotype. In the past, cloning such genes was difficult or impossible. With the advent of molecular marker technology, transposon or T-DNA tagging [31-33], and large scale sequencing projects, important genes have now become accessible to molecular cloning. Briefly, molecular markers provide the essential starting points for physical isolation of genomic regions containing a gene of interest. Often, the same

efforts that are involved in tagging a gene as part of a marker-assisted selection program have side-benefits in uncovering the starting points for genomic isolation. In crops where transposon or T-DNA mutagenesis are not feasible, physically induced variants combined with molecular marker analysis provide an important alternative to clone genes [2]. As new technologies emerge, such as differential display, they may be used in conjunction with current technologies to speed gene isolation [34, 35]. As economically important genes are cloned, the capacity to understand their basic function, modify genes, create new phenotypes, and transfer them to unrelated species will become more feasible.

Earlier strategies of gene cloning relied upon knowing the gene product (RNA or protein) in order to isolate cDNAs and genes of interest. This meant that genes for many important traits such as disease resistance or developmental control, for which nothing was known at the molecular level, were impossible to isolate. Now with the availability of numerous public databanks containing sequence information from a variety of organisms, assessment of consensus motifs in orthologous or homologous gene sequences is possible and functionality can often be deduced by comparison of these motifs. Recent evidence suggests that a candidate gene approach may be useful in associating functions with phenotypes [36]. Many of the original cloning strategies were restricted to a single crop. Knowledge of collinearity across genomes of related organisms has opened the possibility of cross-species cloning strategies facilitated by comparative molecular maps.

The four necessary components of any map-based cloning strategy are: a genetic mapping resource, a physical mapping resource, an economical, high-throughput screening technique, and appropriate assays for complementation. Genetic mapping resources include high-density molecular maps, comparative maps, and sequenced cDNA (EST) libraries. Many techniques are available for developing high-density molecular maps as discussed previously. Choice of technique is dependent on a combination of biological and economic factors including laboratory infrastructure, genome size, level of polymorphism, and availability of marker systems. Anchor or core marker sets for each species are being used to determine relative alignments of molecular maps. These alignments will facilitate sharing of EST information across species as discussed above. Physical mapping resources include libraries in bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs), and cosmids, or chromosome-specific libraries, and radiation-induced deletion lines. Most assays for complementation require some form of transformation. However, efficient transformation systems which are suitable for this purpose have been developed so far for some major crops, but not for all.

3.8.1. Tagging strategies

Types of cloning strategies such as transposon tagging, that directly mark sites, rely on efficient transposon activity in the species of interest. They have worked well in maize and tomato, but this approach may be inapplicable generally. *Agrobacterium* mediated transformation is being utilized in a number of crop species but its applicability is limited to crops where efficient transformation and regeneration, and efficient means of screening for the desired mutants are available. In recalcitrant species, e.g. many legumes, this strategy may be impractical. Newer techniques, such as differential display, which do not require direct marking of genes can be utilized in species where transformation, regeneration, or transposons are not available. When utilized in materials containing small deletions such as those generated by some forms of physical mutagenesis, differential display offers a potential tool for focusing on the genomic region of interest.

Projects orientated towards map-based or site-tagged cloning require substantial financial support in addition to a sophisticated laboratory set up. Therefore, the goals are often beyond the specific use of cloned genes in breeding or even genetic engineering strategies; they are aimed at answering basic biological questions as those regarding the nature of the events occurring during the infection process, the basis of resistance and indeed of avirulence or the processes which occur at the molecular level during symbiosis between the plant and nitrogen fixing bacteria, problems difficult to investigate by other means. The costs involved in time and resources demonstrate that groups undertaking such work are very committed to the study of a particular system or to the isolation of agronomically very important genes.

3.8.2. Transgenic approaches

The end result of identification of genes of economic interest is their application in production of commercial materials. Transformation utilizing genes of economic interest is a reality for many crops. Molecular marker technology provides the foundation for isolation of genes involved in quantitative, costly, or difficult to score traits. The bottleneck in organisms which have a good transformation system in place are appropriate genes of agronomic interest. Limitations to widespread use of transformation in crop improvement include lack of efficient transformation systems for many organisms and a means to transform multiple gene traits. One forthcoming possibility for transformation of multiple gene traits is the use of plant artificial chromosomes. An additional concern for transgenic plants is whether adequate guidelines for release of genetically altered organisms are in place. A potential use of physical mutagenesis is to delete unwanted selective markers in the later stages of transgenic line evaluations.

4. PRACTICAL MARKER APPLICATIONS

The potential utility of DNA markers has been recognized and established in many plant breeding programs. Indeed, the primary obstacles to widespread use of markers are the lack of facile marker systems and the resources required to support their use. The advantages provided by markers are many but they depend on numerous variables such as crop biology, trait(s) undergoing selection, resources, and type of cultivar. However, it has become clear that markers should be considered as an option under many circumstances and aspects of crop improvement.

4.1. Diagnostics and DNA fingerprinting

Monoclonal and polyclonal antibodies, which assess the presence of proteins, have been used routinely for strain identification of viruses. This has assisted artificial selection by verifying the presence of the virus in the absence of symptoms in the host plant. It helps in the assessment of the homogeneity and stability of the pathogen population, introduction of new strains and purity of inoculum. However, there are limitations to extending these techniques to other pathogens (fungi, bacteria, nematodes) and parasitic plants, e.g. *Striga*, *Orobanche* and *Cuscuta*. The development of PCR based DNA markers would ameliorate this situation and make important contributions to crop improvement by clarifying the genetic architecture and repertoire of the pathogen populations.

One of the simplest and most widely applicable uses of DNA markers in plants is DNA fingerprinting. Depending on the resolution required, selected RFLPs or RAPD markers may be very suitable as "fingerprinting" markers. Microsatellites and AFLPs are very well suited for fingerprinting because of their high level of polymorphism. An obvious

fingerprinting application would be to ensure that an accession in a breeding program is uniform genetically and that crosses presumed to be made with this accession were, indeed, carried out with the correct material. Eventually, DNA fingerprinting may also play an important role in developing "core" sets of accessions for screening, as maintained in germplasm collections.

Fingerprinting can also be used to identify and monitor germplasm after it has been released or commercialized. Seed companies and national cultivar registration agencies have an interest in DNA fingerprinting because the technology can be used to protect intellectual property, establish identity, and assess purity. The same principles may be used to determine the contributions of raw germplasm (obtained from developing countries) in breeding programmes in developed countries. Fingerprinting can also be used in the grain trade to prevent adulteration, e.g. blending in rice shipments.

4.2. Verifying crosses and parentage

This application of DNA markers, which is very similar to the one mentioned in the section of DNA fingerprinting, can and does have an enormous practical impact. Many breeding programs begin with a cross between dissimilar parents. Sometimes, visual markers are available to ensure that the presumed F_1 is, indeed, the result of a cross and not a self. When visual markers are not available, DNA markers offer a simple and convenient method to ascertain that a presumed F_1 is indeed such, and not a self or an unintended outcross.

4.3. Genetic distance, heterotic groups and heterosis

In crop species where hybrids are used, DNA markers may enhance breeders' abilities to select the best combinations of parents. DNA markers can estimate combinations of parents that are genetically most dissimilar from one another (which might indicate potential hybrid vigor). Moreover, DNA markers that definitely place accessions into a specific heterotic group could be used to assign unknown genotypes without the need for extensive field trials. Such information would be extremely valuable in the organization of breeding programmes intended to produce hybrid cultivars for crops previously unexposed to hybrid breeding schemes [37].

4.4. Marker-assisted selection

Along with the use of markers to recover recurrent parent genotype, marker-assisted selection (MAS) is probably the broadest and most far-reaching application of DNA markers in plant breeding. Whenever a trait is difficult or expensive to assess, markers can potentially simplify and expedite the selection process. Moreover, the use of markers offers the opportunity to select individuals that carry the best possible genotype. This is based on the ability to select positively for the trait of interest (selection at two tightly and flanking DNA markers) and to select against marker alleles linked to undesirable loci.

In the simplest case, marker-assisted backcross breeding might be targeted on a single locus trait (such as a disease resistance or stress tolerance character) coming from a wild relative. In fact, almost any transgene undergoes MAS because the most elite germplasm is usually not yet available or amenable to cell culture regimes used to produce transgenic plants. Typically, a suitable segregating population would be generated and DNA extracted from all individuals. Markers flanking the gene of interest would then be tested and used to identify individuals that have retained the target gene. This would be followed by a marker

analysis of polymorphic loci throughout the remainder of the genome for those individuals that most resemble the cultivated parent. In the process, rare recombination events around the gene of interest would be uncovered, potentially eliminating undesirable trait loci that might be "dragged" along in a backcross program. These markers can also be useful for predicting the adult phenotype at the seedling stage [38], thus reducing population sizes and costs. In any case, the goal would be to develop a new, and superior, cultivar faster and more efficiently than by conventional breeding alone. A similar, but expanded strategy could be utilized to pyramid together many marked genes into a single cultivar. This approach has been deployed to develop cultivars for several crops [37].

There is a potential 'downside' to MAS backcross breeding that routinely invokes selection against presumably unwanted regions of the donor genome. It has been well established that the donor parents often contribute favourable alleles for traits other than the one being transferred to the recurrent parent [10, 37]. In such cases, MAS could produce inferior cultivars relative to those produced by backcrossing (BC) without MAS. At the outset of the BC program, it will be important to determine the necessity of maximizing the recovery of the recurrent parent genome, as is the case when maintaining many quality factors in the final product, e.g. rice, malting barley, soybeans.

A modified form of MAS BC might be more appropriate for most other circumstances. The modification involves MAS BC only for the marked single gene, or a few QTL, and more relaxed selection, not using markers in other regions of map, for that population. Such a scheme would then allow breeders more opportunities to select on those unmarked regions [37].

Often, traits of interest must first be mapped and analyzed with DNA markers before marker-assisted selection can begin. Mapping is often a time-consuming and difficult process. Occasionally DNA fingerprinting sets of consanguineous lineages may substitute for conventional cosegregation analysis. Even when a trait is well-characterized in terms of markers in one population, it is not entirely clear how transferable this type of information will be to other breeding populations. Interactions with the environment and genetic background continue to be important in plant breeding. If a new mapping initiative needs to be mounted for each new set of parents, marker-assisted breeding will be more expensive and more difficult than anticipated.

4.5. Mapping QTLs

While many important traits in plant breeding are controlled by single major loci, the majority of agriculturally important characters are multigenic, strongly influenced by the environment and expensive to evaluate directly. Before the advent of DNA markers, such traits could only be analyzed and manipulated by statistical analyses of the phenotypes. Of course, enormous progress has been made by this approach, but the inability to describe and select for specific QTLs may limit the breeders' ability to make progress in the future.

With DNA markers, complex traits have been resolved into discrete QTLs and modified through MAS [37]. The individual effects of QTLs, their interactions, and their usefulness in breeding can all be evaluated. Then, once QTLs of interest are well-defined in terms of DNA markers, the strategy of marker-assisted selection described earlier can be implemented. Traits as genetically complex as yield or nutritional value are amenable to MAS of favourable QTLs. However, it should be understood that genetic inferences may be limited to specific reference populations because of genetic heterogeneity for the trait(s), epistatic

gene action (vastly underestimated by present mapping programs), and unknown genetic constitution among potential parents at the QTL 'regions'.

It has been recognized that QTL mapping may be a very deceptive enterprise because one should expect to detect evidence of putative QTL merely due to random chance [17, 37]. QTL detection is affected by several factors such as the sample size of the mapping population, quality of the trait data, marker density of the map, sampling of the environment, and sampling schemes of the population (e.g. tails vs. unselected progeny). Mapping algorithms, and user awareness have advanced from single point analysis, to interval mapping (MAPMAKER-QTL), to composite interval mapping (e.g. QTL Cartographer, PLABQTL) so that QTL detection has become more precise and reliable provided the raw data were of high quality. Wisely used, all mapping methods may produce reliable information suitable for MAS.

Implementation of this approach in plant breeding does however, require relatively sophisticated statistical techniques, routine access to DNA marker information and additional human resources skilled in DNA technology, mapping methods, interpretation of statistics, and proper experimental design.

4.6. Cultivar development

Tools of molecular biology and mutagenesis have been used efficiently for the development of superior and novel cultivars in various economic plants including maize, soybean, cotton, squash, tomato, and rapeseed. All of these were available by 1996. Given the rather recent development of the array of enabling technologies, it should be appreciated that adoption and implementation have been very rapid. Many other cultivars will be developed through these methods for traits such as quality (starch properties, oil quality, protein quality), disease resistance, insect resistance, and exploitation of heterosis (through engineered nuclear male sterility). These methods not only facilitate the production of novel cultivars, but also the production of cultivars in far less time.

However, many challenges remain to be met to deal with complex, multigenic, characters such as drought-, cold- and salt-tolerance, and yield. Considerable progress has been made using DNA markers to tag agronomically valuable genes in many crop species and thus the foundation has been established for using those markers and mutations for more rapid cultivar development, also in species largely ignored by the private sector. Virtually all phases of plant breeding i.e. parents' selection, prediction of progeny performance, progeny selection and varietal identification, have been affected by these tools. The prospects are very good for markers to positively affect the rate of genetic gain. The critical component then, will be maintenance and creation of genetic variation through the various methods.

4.7. Overall perspectives

DNA markers have already revolutionized the genetic analysis of agriculturally important traits. Still, there has been a great deal of effort to utilize marker technology in plant breeding, while practical examples of success remain few. In part this may be due to the fact that economically important traits really are complex (perhaps more genetically complex than expected). However, the biggest constraint to the use of DNA markers in plant breeding remains their relatively high cost and complexity. Classical breeders are accustomed to screening thousands of plants during a growing season and selecting the best ones for advancement in a matter of weeks. Numbers like these are still out of the question with DNA

marker technology in most developing countries. The development of faster DNA extraction methods, highly polymorphic microsatellite markers, multiplexed analysis (which can assay many marker loci at once), and rapid data entry into computer software programs may eventually produce the increase in throughput necessary to enable DNA markers to reach their potential in plant breeding.

5. RECOMMENDATIONS

5.1. Mutants and molecular markers

It is recommended that the development of new marker methodologies for the genetic improvement of seed propagated species and their adaptation to the needs of plant breeders in developing countries should be continued, especially the following:

1. Induction of more mutants; at present the ability to understand and exploit genetic variation and the relationships among mutants, molecules, and phenotypes is just beginning to attain the power necessary for their analysis in complex biological systems in general, and in plants in particular.

2. Comparison of the uses of radioactive and non-radioactive methods for molecular markers and DNA sequencing.

3. Identification of genetic regions responsible for traits of breeding importance and comparisons across species.

4. Selection for desirable traits, in breeding materials of interest, through the marker associations observed in the initial populations and parental analyses.

5.2. Markers and crop improvement

DNA markers are very useful in many situations in crop improvement, in sexually- and asexually-propagated plants. The application of molecular markers should be expanded, as follows:

1. Markers are an important tool for plant breeding programmes; when used judiciously they can expedite genetic gain and make breeding projects more efficient and reliable.

2. More applications and opportunities are envisioned as breeders become more familiar with the various marker systems, with their appropriate applications and with other breeders' experience.

3. More markers are needed (to cover map gaps, loss or low polymorphism) and better systems are required for use in plant breeding programmes (better, low cost, high throughput, non-gel systems).

5.3. Future activities

1. Projects aimed at developing publicly available resources of molecular markers and constructs for the benefit of all countries should be emphasized.

2. Future projects should take advantage of the resources available in other species by comparative map alignments, in order to enhance efficiency and reduce costs.

3. Coordination and sharing of information and resources among researchers and groups should be a priority of future projects.

4. Goals, strategies, possible collaboration and costs should be carefully evaluated before initiating a map-based cloning project.

5. Appropriate strategies for map-based cloning should be determined individually by availability of resources and expertise.

5.4. Training and education needs

It should be recognized that some aspects of developing and using DNA marker methods and MAS are recent and rather dynamic. Therefore, there is a clear need for training and education at a various of levels and in different techniques. Thus, most plant breeders today have received their most advanced education prior to the development of technologies that have enabled MAS.

Furthermore, application of DNA markers in developing countries is hampered by distance from laboratories developing the techniques and information, difficulties in exchanging materials and importing chemicals, lack of adequate equipment, insufficient staff and limited budgets.

Therefore, training programmes which emphasize judicious design, analysis and interpretation of projects involving molecular markers, QTL mapping and the subsequent decisions if and when to use such information in MAS are much needed.

REFERENCES

[1] VOS, P., HOGERS, R., BLEEKER, M., REIJANS, M., VAN DE LEE, T., HORNES, M., FRIJTERS, A., POT, J., PELEMAN, J., KUIPER, M., ZABEAU, M., AFLP: a new technique for DNA fingerprinting, Nucleic Acids Res. 23 (1995) 4407-4414.

[2] ANDERSON, P.A., OKUBARA, P.A., ARROYO-GARCIA, R., MEYERS, B.C., MICHELMORE, R.W., Molecular analysis of irradiation-induced and spontaneous deletion mutants at a disease resistance locus in *Lactuca sativa*, Mol. Gen. Genet. 251 (1996) 316-325.

[3] TANKSLEY, S.D., GANAL, M.W., PRINCE, J.P., de VICENTE, M.C., BONIERBALE, M.W., BROUN, P., FULTON, T.M., GIOBANNONI, J.J., GRANDILLO, S., MARTIN, G.B., MESSEGEUR, R., MILLER, J.C., MILLER, L., PATTERSON, A.H., PINEDA, O., ROEDER, M.S., WING, R.A., WU, W., YOUNG, N.D., High density molecular linkage maps of the tomato and potato genomes, Genetics 132 (1992) 1141-1160.

[4] WEBER, D., HELENTJARIS, T., Mapping RFLP loci in maize using B-A translocations, Genetics 121 (1989) 583-590.

[5] WERNER, J.E., ENDO, T.R., GILL, B.S., Toward a cytogenetically based physical map of the wheat genome, Proc. Natl. Acad. Sci. USA 89 (1992) 11307-11311.

[6] DEVOS, K.M., MILLAN, T., GALE, M.D., Comparative RFLP maps of the homeologous group-2 chromosomes of wheat, rye and barley, Theor. Appl. Genet. 85 (1993) 784-92.

[7] DEVOS, K.M., ATKINSON, M.D., CHINOY, C.N., FRANCIS, H.A., HARCOURT, R.L., KOEBNER, R.M.D., LIU, C.J., MASOJC, P., XIE, D.X., GALE, M.D., Chromosomal rearrangements in the rye genome relative to that of wheat, Theor. Appl. Genet. 85 (1993) 673-680.

[8] CHAO, S., BAYSDORFER, C., HEREDIA-DIAZ, O., MUSKET, T., XU, G., COE, E.H., Jr., RFLP mapping of partially sequenced leaf cDNA clones in maize, Theor. Appl. Genet. 88 (1994) 717-21.

[9] HELENTJARIS, T., Implications for conserved genomic structure among plant species, Proc. Natl. Acad. Sci. USA 90 (1993) 8308-8309.

[10] TANKSLEY, S.D., Mapping polygenes, Ann. Rev. Genet. 27 (1993) 205-233.

[11] KIM, T.S., PHILLIPS, R.L., EUN, M.Y., "Identification of genomic regions controlling maturity in maize (*Zea mays* L.)", Crop Production and Improvement Technology in Asia, Korean Society of Crop Science, Rep. of Korea (1993) 379-397.

[12] PHILLIPS, R.L., KIM, T.-S., KAEPPLER, S.M., PARENTONI, S.N., SHAVER, D.L., STUCKER, R.E., OPENSHAW, S.J., "Genetic dissection of maturity using RFLPs", Proc. 47th Annual Corn and Sorghum Industry Research Conference (WILKINSON, D., Ed.) Am. Seed Trade Assoc., Washington, DC (1992) 135-150.

[13] ARMSTRONG, C.L., ROMERO-SEVERSON, J., HODGES, T.K., Improved tissue culture response of elite maize inbreds through backcross breeding, and identification of chromosomal regions responsible for regeneration by RFLP analysis, Theor. Appl. Genet. 84 (1992) 755-762.

[14] SCHOEN, C.C., LEE, M., MELCHINGER, A.E., GUTHRIE, W.D., WOODMAN, W.L., Mapping and characterisation of quantitative trait loci affecting resistance against second generation European corn borer in maize with the aid of RFLPs, Heredity 70 (1993) 648-659.

[15] McMULLEN, M.D., LOUIE, R., The linkage of molecular markers to a gene controlling the symptom response in maize to maize dwarf mosaic virus, Mol. Plant-Microbe Interact. 2 (1989) 309-314.

[16] FREYMARK, P.J., LEE, M., WOODMAN, W.L., MARTINSON, C.A., Quantitative and qualitative trait loci affecting host plant response to *Exserohilum turcicum* in maize (*Zea mays* L.), Theor. Appl. Genet. 87 (1993) 527-544.

[17] BEAVIS, W.D., GRANT, D., ALBERTSEN, M., FINCHER, R., Quantitative trait loci for plant height in four maize populations and their associations with qualitative genetic loci, Theor. Appl. Genet. 83 (1991) 141-145.

[18] ROBERTSON, D.S., A possible technique for isolating genic DNA for quantitative traits in plants, J. Theor. Biol. 117 (1985) 1-10.

[19] DOEBLEY, J., STEC, A., Teosinte branched1 and the origin of maize, Maize Genet. Coop. Newsl. 68 (1994) 88-89.

[20] ALREFAI, R., OROZCO, B., ROCHEFORD, T., Detection and sequencing of the transposable element ILS-1 in the Illinois long-term selection maize strains, Plant Physiol. 106 (1994) 803-804.

[21] AHN, S., ANDERSON, J.A., SORRELLS, M.E., TANKSLEY, S.D., Homeologous relationships in rice, wheat and maize chromosomes, Mol. Gen. Genet. 241 (1993) 483-900.

[22] PEREIRA, M.G., LEE, M., BRAMEL-COX, P., WOODMAN, W., DOEBLEY, J., WHITKUS, R., Construction of an RFLP map in sorghum and comparative mapping in maize, Genome 37 (1994) 236-43.

[23] TANKSLEY, S.D., BERNATZKY, R., LAPITAN, N.L., PRINCE, J.P., Conservation of gene repertoire but not gene order in pepper and tomato, Proc. Natl. Acad. Sci. USA 85 (1988) 6419-6423.

[24] TEUTONICO, R.A., OSBORN, T.C., Mapping of RFLP and quantitative trait loci in *Brassica rapa* and comparison to the linkage maps of *B. napus*, *B. oleracea*, and *Arabidopsis thaliana*, Theor. Appl. Genet. 89 (1994) 885-893.

[25] BENNETZEN, J.L., FREELING, M., Grasses as a single genetic system: genome composition, colinearity and compatibility, Trends Genet. 9 (1993) 259-61.

[26] MOORE, G., FOOTE, T., HELENTJARIS, T., DEVOS, K.M., KURATA, N., GALE, M.D., Was there a single ancestral cereal chromosome?,Trend Genet. 11 (1995) 81-82.

[27] GROTEWALD, E., PETERSON, T., Isolation and characterization of a maize gene encoding chalcone flavanone isomerase, Mol. Gen. Genet. 242 (1994) 1-8.

[28] RAYAPATI, P.J., PORTYANKO, V.A., LEE, M., Placement of loci for avenins and resistance to *Puccinia coronata* to a common linkage group in *Avena strigosa*. Genome 37 (1994) 900-903.

[29] FATOKUN, C.A., MENANCIO-HAUTEA, D.I., DANESH, D., YOUNG, N.D., Evidence for orthologous seed weight genes in cowpea and mung bean based on RFLP mapping, Genetics 132 (1992) 841-846.

[30] PEREIRA, M.G., LEE, M., Identification of genomic regions affecting plant height in sorghum and maize, Theor. Appl. Genet. 90 (1995) 380-388.

[31] JONES, D.A, THOMAS, C.M., HAMMOND-KOSACK, K.E., BALINT-KURTI, P.J., JONES, J.D.G., Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging, Science 266 (1994) 789-792.

[32] FELDMANN, K.A., "The utility of insertion mutagenesis in *Arabidopsis* for crop improvement", FAO/IAEA Int. Symp. Use of Induced Mutations and Molecular Techniques for Crop Improvement, IAEA, Vienna (1995).

[33] WALDEN, R., HARLING, H., MIKLASHEVICS, E., LUBENOW, H., SCHELL, J., "Activation tagging and the molecular dissection of complex biological pathways", FAO/IAEA Int. Symp. Use of Induced Mutations and Molecular Techniques for Crop Improvement, IAEA, Vienna (1995).

[34] LIANG, P., PARDEE, A.B., Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction, Science 257 (1992) 967-971.

[35] LIANG, P., AVERBOUKH, L., PARDEE, A.B., Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucl. Acids Res. 21 (1993) 3275-3279.

[36] BYRNE, P.F., McMULLEN, M.D., SNOOK, M.E., MUSKET, T., THEURI, J.M., WIDSTROM, N.W., WISEMAN, B.R., COE, E.H., Jr., Quantitative trait loci and metabolic pathways: Genetic control of the concentration of maysin, a corn earworm resistance factor, in maize silks, Proc. Natl. Acad. Sci. USA 93 (1996) 8820-8825.

[37] LEE, M., "Integrated genetic maps of DNA markers and mutations and their utility for improvement of sorghum and other grasses", FAO/IAEA Int. Symp. Use of Induced Mutations and Molecular Techniques for Crop Improvement, IAEA, Vienna (1995).

[38] CHO, Y.G., EUN, M.Y., KIM, Y.K., CHUNG, T.Y., CHAE, Y.A., The semi-dwarf gene, *sd-1*, of rice (*Oryza sativa* L.) II. Molecular mapping and marker-assisted selection, Theor. Appl. Genet. 89 (1994) 54-59.

INVESTIGATION OF THE SOMACLONAL AND MUTAGEN INDUCED VARIABILITY IN BARLEY BY THE APPLICATION OF PROTEIN AND DNA MARKERS

A. ATANASSOV, E. TODOROVSKA, A. TRIFONOVA, M. PETROVA
Institute of Genetic Engineering,
Kostinbrod, Bulgaria

E. MARINOVA
Norman Borlaug Research Centre,
Kostinbrod, Bulgaria

M. GRAMATIKOVA, D. VALCHEVA, S. ZAPRIANOV, N. MERSINKOV
Institute of Barley,
Karnobat, Bulgaria

Abstract

Barley, *Hordeum vulgare* L., is one of the most important crop species for Bulgaria The characterisation of the genetic pool is of great necessity for the Bulgarian barley breeding programme which is directed toward improving quantitative and qualitative traits.

The regenerants from four Bulgarian varieties Ruen, Jubiley, Obzor (two-rowed, brewery) and Karnobat (six-rowed, forage) have been obtained by using a previously developed regeneration procedure [1] The mutagenic agents, gamma rays and NaN_3, were applied to the initial material from cultivar Ruen The influence of the mutagens on callus formation and plant regeneration was observed A dose of 200 Gy was considered as stimulating regeneration The mutation frequency increased but the spectrum of genetic changes was similar to the control In order to assess genetic variability among the regenerated plants, multiple analytic tools have been employed Field evaluation of the individual regenerants and those derived after mutagen treatment was carried out. A number of plants with valuable agronomic performance traits have been selected

Molecular markers [protein, restriction fragment length polymorphisms (RFLP) and randomly amplified polymorphic DNA (RAPD)] have been applied to characterise the Bulgarian barley cultivars and their regenerants. The changes in DNA loci coding for 26S, 5.8S and 18S rRNA repeats, C hordein locus and mitochondrial DNA organisation have been investigated. The potential for ribosomal DNA length polymorphism in Bulgarian barley cultivars appear to be limited to three different repeat lengths (10 2, 9 5 and 9 0kb) and three plant rDNA phenotypes. Polymorphism was not observed in ribosomal DNA repeat units in somaclonal variants Variation concerning C hordein electrophoretic pattern was observed in one line from cultivar Jubiley Analysis of the HorI locus reveals RFLPs in sequences coding for C hordeins in this line Mitochondrial molecular markers are convenient for detection of DNA polymorphisms in the variant germplasm as well as for the somaclonal variants derived from it. Two lines from Ruen revealed polymorphic bands after hybridisation with mitochondrial DNA probe RAPD assays have been carried out by using 20 different 10-mer primers Heritable polymorphism in several tissue culture derived (TCD) lines was observed RAPD assay is a sensitive and representative approach to distinguish the variability created by tissue culture and mutagenesis

1. INTRODUCTION

Plant tissue culture instability has been documented in several cereal plant species. Somaclonal variation has been studied intensively also in barley [2-5]. The results obtained till now are contradictory. No significant morphological variation was detected in tested tissue culture derived (TCD) lines [6] or only small variation was observed when TCD lines from four barley cultivars were tested in three locations [7]. On the other hand, in a study of 18 cultivars and breeding lines morphological variation was observed [8].

Mutagen treatment of *in vitro* plants increases variability in cereals [9, 10]. The application of mutagens (gamma rays and NaN_3) influences callus formation and the rate of regeneration [Karadimova, personal communication].

Molecular markers [isozymes, restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD)] have been applied to study the genetic changes in nuclear, mitochondrial and chloroplast genomes. Molecular studies on somaclonal variation in wheat and barley [2, 3] have demonstrated that the nucleolus organizer (NOR) loci, coding for rRNA and the electrophoretic pattern of storage proteins could be useful for assessing somaclonal variation.

The plant mitochondrial genome has been proven to be a proper model for investigation of somaclonal variation. Mitochondrial DNA rearrangements in regenerated wheat plants has been reported [11]. A relatively high genetic stability was observed among the 50 regenerated *H. marinum* plants tested [4].

The RAPD technique utilises arbitrary 10 -mer oligonucleotide sequences as primers [12]. Primers hybridise to two nearby sites in the template DNA that are complementary to the primer sequence. Deletions or insertions in the amplified regions or base changes altering primer binding sites will result in polymorphisms. RAPD markers have been applied widely for cultivar identification in barley *H. spontaneum* [13] and for analysing the genetic stability of tissue - cultured plants [14].

In this study we assessed the induced variability in barley tissue culture derived lines and those derived from *in vitro* mutagenesis. The aim was to distinguish reproducibly the level of variation between the donor material and the induced genetic changes at the level of regenerant progeny by utilisation of protein, RFLP and RAPD methods.

2. MATERIALS AND METHODS

2.1. Plant material

The regenerants from four Bulgarian varieties Ruen, Jubiley, Obzor (two-rowed, brewery) and Karnobat (six-rowed, forage) have been obtained by using a previously developed regeneration procedure [1]. Mutagenic agents, gamma rays (100 and 200Gy) and NaN_3 ($x_1 = 5 \times 10^{-4}$M and $x_2 = 5 \times 10^{-3}$M) were applied to the initial seed material from cultivar (cv.) Ruen. Vigorous, green R_0 regenerants were propagated and advanced to the R_5 generation. Plants were evaluated in R_3-R_5 generations. A number of TCD lines originating from individual regenerants have been evaluated for their agronomically valuable characteristics:12 lines from cv. Karnobat, 21 lines from cv. Jubiley, 21 lines from cv. Ruen and 5 lines from cv. Obzor. The following number of plants were evaluated in the *in vitro* mutagenesis experiment: regenerants (R)-25; regenerants after treatment with 100 Gy (R-10)-36; regenerants after treatment 200 Gy (R-20)-30; regenerants after treatment with NaN_3x_1 concentration (R-x_1)-29; and regenerants treated with NaN_3x_2 concentration (R-x_2)-26.

2.2. Hordein assay

Single seeds (20) from ten independent spikes per line were used for hordein extraction. Fractionation of hordeins was achieved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to [15].

2.3. DNA extraction, endonuclease digestion and Southern blot hybridisation

DNA was extracted according to Saghai-Maroof et al. [16], with some modifications. The DNA was digested with the following restriction enzymes: *Eco*RI, *Eco*RI and *Bam*HI, *Sac*I, *Taq*I, and *Hind*III.

2.4. RAPD assay

Twenty different 10-mer primers (kit A, OPERON) were used for the amplification of genomic DNA from 23 plants which originated from single seeds of each line. PCRs were carried out in a 20µl reaction solution containing 15 ng of genomic DNA template, 7.5 pmol of each primer, 200mM of each of dATP, dCTP, dGTP and dTTP;1x PCR buffer (Pharmacia) and 0.8 units Taq DNA polymerase (Boehringer Mannheim). The amplification was performed in a Hybaid Omnigene Thermal Cycler.

The PCR conditions for RAPD analysis were as follows: for pre-denaturation 1 min at 94°C followed by 35 cycles of polymerisation reaction each consisting of a denaturation step for 5 sec at 94°C, an annealing step for 30 sec at 36°C and an extension step for 1 min at 72°C. The last cycle was followed by a pause of 10 min at 72°C to ensure that primer extension reaction proceeded to completion. Amplified DNA fragments were run in 2.0% agarose gels (in 1xTAE running buffer). Each gel was stained with ethidium bromide (0.5 mg/ml), visualised under illumination with UV light and photographed.

3. RESULTS

3.1. Field evaluation

Most of the observed morphological abnormalities in the primary regenerants are likely to be epigenetic and disappear in the progeny generations. Sterile, chlorotic and very weak plants were eliminated in the R_0 generation. Fasciation, shortened internodes, and seed malformations were already reported [1]. Plants were advanced and propagated to R_3 generation. A range of deviations has been observed. They concern agronomically important traits such as: grain yield, lodging, height, days to heading, percentage of plump kernels, test weight and cold tolerance.

TABLE I. CLONES USED FOR ASSESSMENT OF THE NUCLEAR AND MITOCHONDRIAL GENOME ORGANISATION OF *H. vulgare* REGENERATED PLANTS

Probe	Plasmid designation	Contents of plasmid	Vector	Cloning site	Sequence origin
Nuclear	pTA71	18S, 5.8S and 26S and nontranscribed spacer of rDNA	pUC18	EcoRI	wheat
	pTA 630	5S rRNA	pBR322	BamHI	wheat
	pCp387	cDNA of C hordein	pUC18	HindIII	barley
Mitochondrial	ATP A	subunit A of ATPase	pBN121	HindIII	sunflower

The variation between TCD lines is significant and strongly genotype dependent. Final evaluation shows that three TCD lines from Jubiley have increased grain yield, one possesses a shorter stem and one heads two days earlier. Four TCD lines from cv. Ruen show better yield, one is earlier, one has a shorter stem and the other is taller (Table II).

TABLE II. FIELD TESTING OF SC_3 PROGENY OF SELECTED BARLEY TISSUE CULTURE DERIVED LINES

Cultivar	Selected lines No.	Grain yield, t/ha	Heading date	Height cm.	Protein content mg/g	Weight /1000 g	Plump kernels %
Ruen	Control	6.51	13.05	85	12.80	46.0	85.6
	19	6.28	11.05	80	12.62	48.5**	93.0***
	34	6.95**	09.05	80	12.88	45.0	89.5**
	78	7.01	08.05	78	11.93*	40.5	82.6
	103	7.23***	10.04	75***	13.30	45.5	90.9***
	81	6.39*	12.05	75***	13.38	46	87.5*
	89	6.81*	13.05	70*	12.31	46.5	88.6**
Obzor	Control	6.92	16.05	75	12.30	38.5	77.8
	309	6.30	13.05	95***	12.50	40.3*	82.8**
	313	6.80	15.05	98***	12.88	39.8	80.5**
Karnobat	Control	6.72	06.05	104	12.30	33.4	
	39	6.11	08.05	96	13.40	35.0	
	44	6.05	07.05	98	13.50	36.0**	
Jubiley	Control	5.30	08.05	100	12.50	50.0	80.2
	60	5.77**	05.05	94	11.40	52.0*	85.4***
	70	5.90**	08.08	104	12.50	54.0*	91.2***
	57	5.95***	04.05	95	10.30*	52.0*	83.2**

*, **, *** = significant at the 0.05, 0.01 and 0.001 levels, respectively.

3.2. Analysis of ribosomal DNA spacer length polymorphism

The *Eco*RI restriction enzyme defines two main rDNA repeat unit lengths, 9.0 and 10.2 kb, detected in the both control material (cv. Ruen, cv. Jubiley) and their TCD lines and 9.5 and 10.2 kb in cv. Obzor, cv. Karnobat and their TCD lines (Fig. 1).

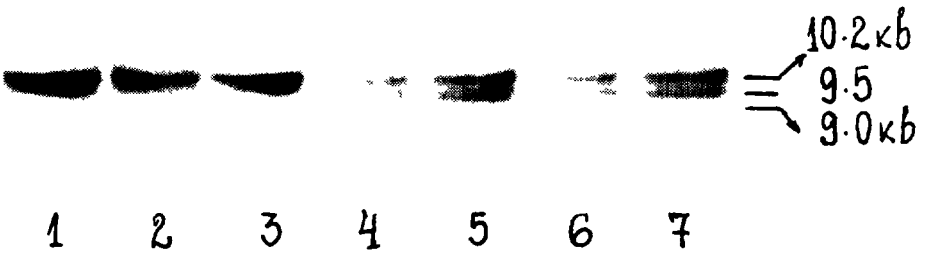


Fig. 1. Southern blot hybridisation analysis of EcoRI digested barley genomic DNA with probe pTA 71. Lane 1 - cv. Obsor; lane 2 - cv. Karnobat; lane 3 - som. 44; lane 4 - cv. Ruen; lane 5 - som. 89; lane 6 - cv. Jubiley; lane 7 - som. 70.

Double *Eco*RI-*Bam*HI digestion of barley DNA results in a range of fragments hybridising with clone pTA 71. No variation in the fragment length or relative intensity of hybridisation bands of *Bam*HI sites in rDNA units was observed among the regenerated plants from cv. Karnobat, cv. Jubiley, cv. Ruen and cv. Obzor (Fig. 2).

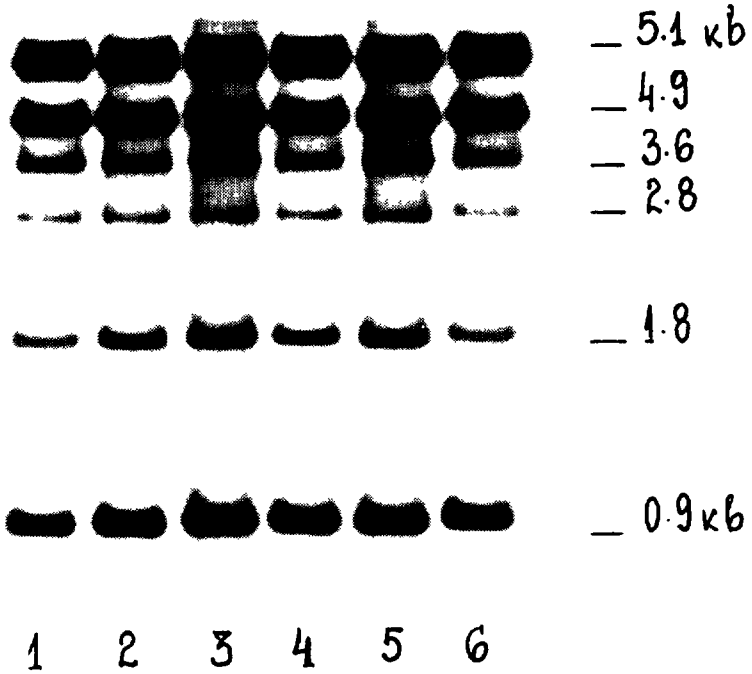


Fig. 2. EcoRI-BamHI pattern of genomic DNA hybridised with 9.0 kb. fragment of clone pTA 71. Line 1 - cv. Obzor; line 2 - som. 309; line 3 - som. 313; line 4 - cv. Karnobat; line 5 - som. 44; line 6 - som. 39.

In vitro mutagen treatment influences the efficiency of callus formation and plant regeneration (data not shown). Mutation frequency was increased but the spectrum of genetic changes was similar to the control (data not shown). Several mutant lines have been selected in R_3 progeny, showing valuable agronomic performance (Table III).

TABLE III. SELECTED LINES AFTER TISSUE CULTURE AND MUTAGEN TREATMENT

Selected lines	Stem height cm	Tillers No.	Internode No.	Spike length cm	Seed number/ spike	Seed weight/ spike	Yield t/ha
Control	98	15.0	4.8	8.9	23.0	1.19	5.86
126 R	95.7	19.0	5.6	9.8	26.2	1.29	5.80
157 R	93.5	18.5	5.9*	10.3**	25.9	1.68**	6.10
139-R-10	90.0**	30.0***	5.5	10.9**	30.9**	1.71***	6.40
152-R-20	92.0*	19.8	5.6	9.8	25.8	1.76***	5.86
153-R-20	90.0**	25.5**	5.7*	9.0	25.2	1.49	5.89
145-R-20	94.0	30.0***	5.5	10.9**	26.3	1.48	5.73
12-5-(-X1	87,0***	12.3	5.1	10.1**	26.0	1.5	6.90
166-2-R-x1	90.0**	19.75	5.2	10.4**	27.7	1.31	5.53
307-R-x2	92.0*	26.9**	5.3	9.8	26.8	1.5	6.70

*, **, *** = significant at the 0.05, 0.01 and 0.001 levels, respectively.

3.3. Eletrophoretic pattern of storage protein by SDS-PAGE and organisation of C hordein coding sequences

The tested B C hordein patterns of somaclones originating from cvs. Ruen, Karnobat and Obzor were similar to those shown by the donor plants. Additional bands in C and B hordein patterns, respectively, in clones 70-3 and 70-4 derived from cv Jubiley were observed.

The rearrangements in gene sequences coding for C hordeins have been analysed by using DNA probe pCp387. Uniformity in hybridisation of *Hind*III DNA patterns of all investigated TCD plants, except plants showing polymorphism at the protein level, was obtained. (Fig. 3). An additional band (2.8kb) was detected in comparison with cv. Jubiley's profile. The results obtained imply that the polymorphism in C hordein pattern in the investigated plants of TCD line 70R (cv. Jubiley) is probably due to alteration in the gene sequences coding for C hordein.

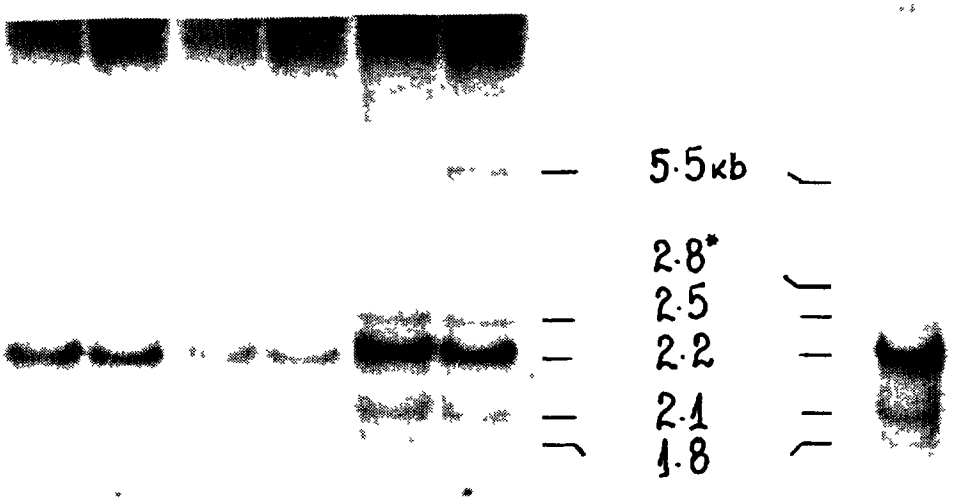


Fig. 3. *Southern blot hybridisation analysis of HindIII digested DNA probed with C hordein Coding sequences (pCp387). Lines 1,2 - som. 57; lines 3,4 - cv. Jubiley; lines 5,6 - som. 70-1, som. 70-2; line 7 - som. 70-3.*

3.4. Mitochondrial DNA organisation

The mitochondrial coding sequences among cereals are highly conserved. Therefore we have used the sunflower mitochondrial gene coding for the α subunit of the ATP-ase complex as a heterologous probe to characterise the organisation of some mtDNA sequences on Southern blots of total DNA. This allows screening of regenerated plants without the need to purify mitochondrial DNA. Hybridisation of *Hind*III digested DNA with the sunflower ATP-α probe revealed two main (12kb and 9.7kb) bands and a few fragments with low intensity in both the cultivars and somaclones. Variant bands (5.1kb, 4.6kb, and 3.2kb) with low intensity were obtained only in *Hind*III DNA patterns of somaclones 81 and 89 of cv. Ruen (Fig. 4).

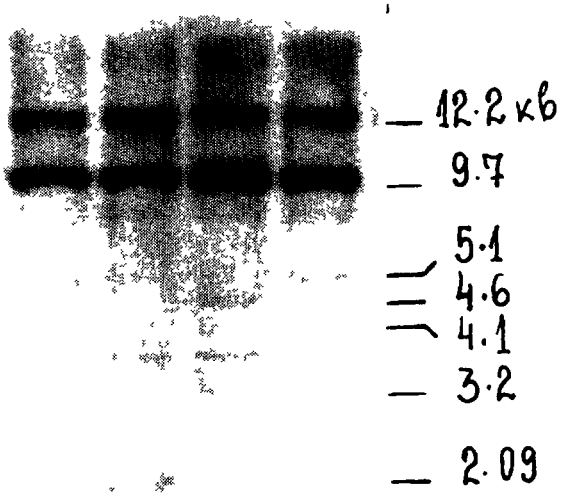


Fig. 4. Southern blot hybridisation analysis of HindIII digested barley genomic DNA with mitochondrial probe ATP-A. Lane 1 - cv. Ruen; lane 2 - som. 78; lane 3 - som. 81; lane 4 - som. 89.

3.5. RAPD analysis of tissue culture derived lines

A few primers providing DNA polymorphism in somaclones were selected. The amplification was repeated twice by using the DNA templates from the same lines of the next generation (RC_5) in order to investigate the reproducibility of the generated polymorphisms in RAPD profiles of these somaclones. Our study shows that primers OPA 08 and OPA 17 produce reproducible polymorphic bands in RAPD profiles (Figs 5 and 6).

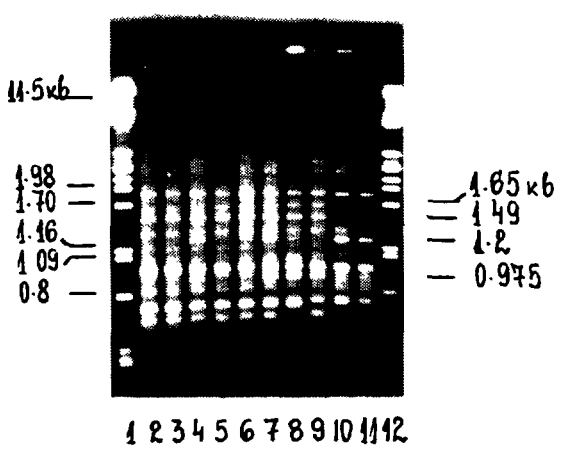


Fig. 5. RAPD patterns generated with primer OPA 08. Lane 1 - marker λ^{PstI}; lane 2 - cv. Ruen; lane 3 - som. 103; lane 4 - som. 78; lane 5 - som. 34; lane 6 - som. 19; lane 7 - cv. Obzor; lane 8 - cv. 468; lane 9 - cv. Karnobat; lane 10 - som. 44; lane 11 - som. 39; lane 12 - marker λ^{PstI}.

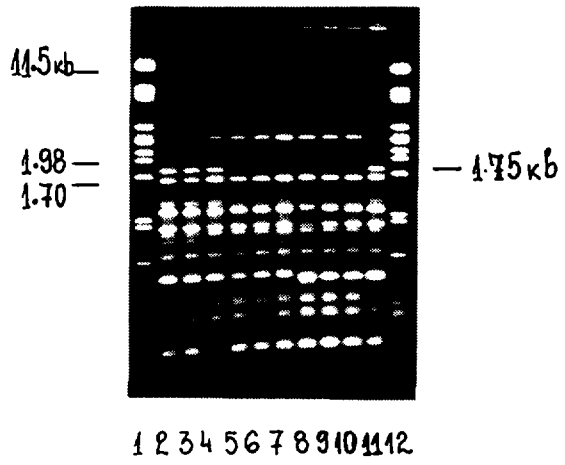


Fig. 6. RAPD patterns generated with primer OPA 17. Lane 1 - marker λ^{PstI}; lane 2 - cv. Ruen; lane 3 - som. 103; lane 4 - som. 78; lane 5 - som. 34; lane 6 - som. 19; lane 7 - cv. Obzor; lane 8 - cv. 468; lane 9 - cv. Karnobat; lane 10 - som. 44; lane 11 - som. 39; lane 12 - marker λ^{PstI}.

Somaclones 39 and 44 derived from cv. Karnobat are characterised with an additional band (1.2 kb) and three missing fragments (1.65 kb, 1.49 kb, and 0.975 kb) in RAPD profiles generated with OPA O8 when compared to the control material. Two additional bands (1.2 kb and 0.975 kb) were observed in somaclone 78 comparing to cv. Ruen and the other somaclones 103, 34, 19.

Primer OPA 17 produced polymorphism in somaclones 34 and 19 (a 1.75 kb fragment) which is not observed in the other two somaclones 103 and 78. An additional band with the same length was present in the RAPD pattern of somaclone 39 in comparison to the control (cv. Karnobat).

Few out of 10 tested primers (OPERON KitA) showed polymorphism in RAPD profiles in several mutant lines. Lines 95 (*in vivo* NaN_3 10^{-3}M) and 307 (*in vitro* NaN_3 10^{-3}M) reveal the absence of DNA fragments in RAPD profiles generated with primer OPA 01 in comparison to the control material and other mutant lines (Fig. 7).

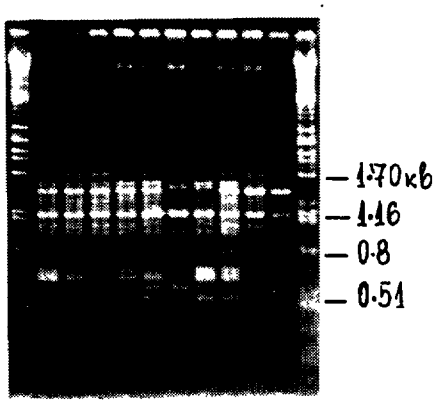


Fig. 7. RAPD patterns generated with primer OPA 01. Line 1 - marker λ^{PstI}; line 2 - cv. Ruen; line 3 - som. 90 (in vivo 10 kRad); line 4 - som. 139 (in vitro 10 kRad); line 5 - som. 78 (in vivo 20 kRad); line 6 - som. 152 (in vitro 20 kRad); line 7 - 87 (in vivo NaN3 105); line 8 -12-5 (in vitro Na N3 105); line 9 - 215 (in vivo NaN3 103); line 11 - 307 (in vitro Na N3 103); line 12 - marker λ^{PstI}.

4. DISCUSSION

The presented data suggest that *in vitro* cultivation induces variability and that the majority of barley plants recovered by callus culture possess both negative and positive changes. While a lower degree of heritable variation among regenerants was reported by Karp et al. [17] our results confirm previous findings in barley [5, 8]. The application of mutagenic agents affects the rate of regeneration. A stimulating effect of 200 Gy gamma ray irradiation was observed. Gross genetic changes were not found in the regenerants. Probably a strong diplontic selection takes place during plant regeneration.

Field tests show that some of the deviations disappear in later progenies. The yield differs over years and it is difficult to use it as a test for detecting somaclonal variation. Probably *in vitro* regeneration influences some characteristics associated with stress factors which are reflected in productivity. Somaclonal variation is not the result of gross genetic changes and field evaluation is not precise enough to detect deviations.

Molecular markers have been used for the estimation of genetic variability induced after regeneration via callus culture in barley [3, 4]. Our data suggest that by using clone pTA71 carrying genes for 26S, 18S and 5.8S rRNA any qualitative or quantitative differences were not detected in the non-transcribed spacer of rDNA of parental and regenerated barley plants. Similar results were reported for wheat by Rode et al. [18]. The results demonstrate relatively high genetic stability in respect to highly repetitive domain of the ribosomal genes.

The *in vitro* culture manipulations may induce qualitative and quantitative variation in the mitochondrial DNA of calli and cell suspensions as described by Shirzadegan et al. [19]. In our investigation two TCD lines had some changes in mtDNA. Those variations in hybridisation patterns could be attributed to somaclonal variation. It is assumed that the alterations occurred in noncoding sequences of the mitochondrial genome [4] and the rearrangements which occur in barley mitochondrial DNA during the tissue culture are rarely transferred to the progenies of the regenerants [3]. In our case the variation was detected in RC_3 progeny which indicates the stable heritable nature of the changes in the barley genome as a result of tissue culture regeneration.

RFLP analysis of the Hor1 locus was performed as a complement to the protein analysis. The observed differences in mobility and number of fragments corresponding to C and B hordeins in some somaclones were not dependent on the changes in the organisation of the coding sequences [2]. Breiman et al. [3] interpreted the lack of relationship between protein and RFLP as a result of post-transcriptional events affecting the level of expression of hordeins or of the rearrangements occurring outside the coding sequences. In our case the observed variation in C hordein SDS-PAGE patterns in one TCD line of cv. Jubiley was confirmed by using RFLP analysis.

Recently, Song and Henry [13] applied RAPD markers to assess the genetic variation within and between wild barley populations (*H. spontaneum*). To our knowledge, RAPD analysis has not yet been reported for the detection of somaclonal variation in barley. We succeeded in finding several polymorphisms in TCD barley lines by using 10-mer primers. They were stable and were inherited in the progeny.

In our studies, 15 families out of 64 TCD lines have been analysed by molecular markers for differentiating the genetic variation induced by tissue culture. Selection of the lines was made on the basis of field evaluation. This could be one of the reasons for finding

only limited variation on the basis of DNA analyses. The second reason could be that the number of the molecular markers used may not be sufficient to screen for somaclonal variation unless sufficient probes are used to saturate the entire genome [20].

The results described here show that somaclonal variation is induced by tissue culture in barley by using a particular regeneration procedure (mutagenic effect of 2,4D - data not shown) and genotypes which may affect the genetic stability in both positive and negative directions. The selected TCD lines show valuable agronomic changes. Tissue culture may induce rearrangements in any part of the entire plant genome. In this respect RAPD markers showing heritable polymorphism are more representative than the limited number of the applied RFLP probes use for detection of somaclonal variation in barley.

REFERENCES

[1] VITANOVA, Z., VITANOV, V., TRIFONOVA, A., SAVOVA, D., ATANASSOV, A., Effect of 2,4-D precultivation on regeneration capacity of cultivated barley, Plant Cell Rpt. 14 (1995) 437-441.

[2] KARP, A., STEEL, S.H., PARMAR, S., JONES, M.G.K., SHEWRY, P.R., BREYMAN, A., Relative stability among barley plants regenerated from cultured immature embryos, Genome 29 (1987) 405-412.

[3] BREIMAN, A., ROTEM-ABARBANELL, D., KARP, A., SHASKIN H., Heritable somaclonal variation in wild barley (*Hordeum spontaneum*), Theor. Appl. Genet. 74 (1987) 104-112.

[4] SHIMRON-ABARBANELL, D., BREIMAN, A., Comprehensive molecular characterization of tissue-culture-derived *Hordeum marinum* plants, Theor. Appl. Genet. 83 (1991) 71-80.

[5] BREGITZER, P., POULSON, M., Agronomic performance of barley lines derived from tissue culture, Crop Sci. 35 (1995) 1144-1148.

[6] LUCKETT, D.J., ROSE, D., KNIGHTS, E., Paucity of somaclonal variation from immature embryo culture of barley, Australian J. Agric. Res. 40 (1989) 1155-1159.

[7] BAILLIE, A.M.R., ROSSNAGEL, B.G., KARTHA, K.K., Field evaluation of barley (*Hordeum vulgare*) genotypes derived from tissue culture, Can. J. Plant Sci. 72 (1992) 725-733.

[8] ULLRICH, S.E., EDMISTON, J.M., KLEINHOFS, A., KUDRNA, D.S., MAATOUGUI, M.E.H., Evaluation of Somaclonal variation in barley, Cereal Res. Commun. 19 (1991) 254-260.

[9] GALIBA, G., SUTKA, J., Frost resistance of somaclones derived from *Triticum æstivum* L. winter wheat calli, Plant Breeding 102 (1989) 101-104.

[10] MALUSZYNSKI, M., SZAREJKO, I., MADAJEWSKI, R., FUGLEWICZ, A., KUCHARSKA, M., Semi-dwarf mutants and heterosis in barley. I. The use of barley sd-mutants for hybrid breeding, In: *Semi-Dwarf Cereal Mutants and Their Use in Cross-Breeding III*, Vienna: IAEA-TECDOC 455 (1988) 193-206.

[11] KARADIMOVA, M. FILEV, K., DJAMBOVA, G., Calusogenesis and plant regeneration from gamma-irradiated cultures of durum wheat (*Triticum durum*). Second International Symposium. Experimental Mutagenesis in Plants. Plovdiv, Bulgaria (1987) 265-268.

[12] HARTMANN, C., RECIPON, H., JUBIER, M., VALON, C., DELCHER-BESIN, E., HENRY, Y., DE-BUSER, J., LEJEUNE, B., RODE, A., Mitochondrial DNA variability in a single wheat regenerant involves a rare recombination event across a short repeat, Curr. Genet 25 (1994) 456-464.

[13] WILLIAMS, J.G.K., KUBELIK, A.R., LIVAK, K.J., RAFALSKI, J.A., TINGEY, S.V., DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18 (1990) 6531-6535.

[14] SONG, W., HENRY, R., Molecular analysis of the DNA polymorphism of wild barley (*H.spontaneum*) germplasm using the polymerase chain reaction, Genet. Res. and Crop Evol. 42 (1995) 273-281.

[15] TAYLOR, P.W.J., GEIJSKES, H.L., KO., T.A., FRASER, R.J., HENRY, BIRCH, R.G., Sensitivity of random amplified polymorphic DNA analysis to detect genetic changes in sugarcane during tissue culture. Theor. Appl. Genet. 90 (1995) 1169-1173.

[16] SMITH, D.B., PAYNE, P.I., A procedure for the routine determination of electrophoretic band patterns of barley and malt endosperm proteins, J. Natl. Inst. Agric. Bot. 16 (1984) 487-498.

[17] SAGHAI-MAROOF, M.A., SOLIMAN, K.M., JORGENSEN, R.A., ALLARD, R.W., Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics, Proc. Natl. Acad. Sci. USA 81 (1984) 8014-8018.

[18] KARP, A., Somaclonal variation as a tool for crop improvement, Euphytica 85 (1995) 1-3.

[19] RODE, A., HARTMANN, C., BENSLIMANE, A., PECARD, E., QUETIER, F., Gametoclonal variation detected in the nuclear ribosomal DNA from double haploid lines of spring wheat, Theor. Appl. Genet. 74 (1987) 31-37.

[20] SHIRZADEGAN, M., CHRISTEY, M., EARLE, E.D., PALMER, J.D., Rearrangements, amplification and assortment of mitochondrial DNA molecules in cultured cells of *Brassica campestris*, Theor. Appl. Genet. 77 (1989) 17-25.

SEARCHING FOR RFLP MARKERS TO IDENTIFY GENES FOR ALUMINUM TOLERANCE IN MAIZE

E. PAIVA, M.A. LOPES, S.N. PARENTONI
Centro Nacional de Pesquisa de Milho e Sorgo (CNPMS/EBRAPA),
Sete Lagoas, MG, Brazil

P.R. MARTINS
CNPMS / CNPq / RHAE,
Sete Lagoas, MG, Brazil

G.A. TORRES
Universidade Federal de Lavras,
Lavras, MG, Brazil

Abstract

The objective of this study was to identify restriction fragment length polymorphism (RFLP) markers linked to Quantitative Trait Loci (QTL) that control aluminum (Al) tolerance in maize The strategy used was bulked segregant analysis (BSA) and the genetic materials utilized were the F_2, F_3 and F_4 populations derived from a cross between the Al-susceptible inbred line L53 and Al-tolerant inbred line L1327 The populations were evaluated in a nutrient solution containing a toxic concentration of Al (6 ppm) and relative seminal root length (RSRL) was used as a phenotypic measure of tolerance The RSRL frequency distribution of the F_2 population was continuous, but skewed towards Al-susceptible individuals Seedlings of the F_2 population with the highest and lowest RSRL values were transplanted to the field and subsequently selfed to obtain F_3 and F_4 families The efficiency of the phenotypic index for selection was found to be greater when mean values were used instead of individual RSRL values F_3 and F_4 families were then evaluated in nutrient solution to identify those that were not segregating Based on average RSRL values, F_3 and F_4 families were chosen to construct the bulks One hundred and thirteen probes, with an average interval of 30 cM, covering the 10 maize chromosomes were tested for their ability to discriminate the parental lines. Fifty four of these probes were polymorphic with 46 showing codominance These probes were hybridized with DNA from two F_3 contrasting bulks and three probes on chromosome 8 were found to be able distinguish the F_3 contrasting bulks on the basis of band position and intensity DNA of families from the F_3 bulks hybridized with these probes showed the presence of heterozygous individuals These three selected probes were also hybridized with DNA from F_2 individuals Two of them showed a significant regression coefficient with the character However, each of these probes explained only about 10% of the phenotypic variance observed in 70 F_2 individuals One of the probes UMC 103 was hybridized with DNA from 168 F_4 families and the regression analysis of RFLP data showed a significant regression coefficient with a determination coefficient of 4 7% These results suggest that on chromosome 8 in maize there is a region related to aluminum tolerance

1. INTRODUCTION

A major constraint to maize production in the tropics is the excessive acidity in the soils In Brazil, acid savannas with low cation exchange capacity and high saturation of toxic aluminum cover 205 million ha. of which 112 million ha are suitable for agricultural production [1]. In most of this area, deficiencies of P, Ca, Mg and Zn are common and saturation of toxic Al and fixation of P by soil particles are usually high [2].

Although technology for topsoil acidity correction is widely used in the tropics, there is no easy way to remove the effects of toxic aluminum in the subsoil. Therefore, to exploit the soil in aluminum-rich areas it is important to develop breeding programs aimed at generating aluminum tolerant cultivars [3]. Maize breeders have identified genetic variability for aluminum tolerance and germplasm suitable for selection is available [4-7].

Several techniques based on evaluations under field conditions and in nutrient solutions have been developed to screen for aluminum tolerance in maize [8]. Although maize breeders have traditionally relied on evaluations in soils with high aluminum saturation, the use of nutrient solutions with high aluminum concentrations has proven to be an effective way to complement field tests. The nutrient solution technique is rapid, allows screening of many progenies in small areas and allows better control of environmental variations, which is more difficult to achieve under field conditions. Among the several criteria utilized to evaluate maize aluminum tolerance in nutrient solutions, seminal root growth seems to be the most reliable [7, 9, 11]. Using this criterion, several authors have shown that the trait is quantitatively inherited, with predominanly additive genetic effects [6, 7, 11]. However, due to its high heritability, the character is expected to be controlled by a small number of genes [12].

Quantitative trait loci (QTL) controlling several important agronomic characters have been successfully studied through the use of molecular markers, utilizing techniques such as Restriction Fragment Length Polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) [13-16]. However, the commonly used strategies to map QTLs are laborious, time consuming and require large number of individuals. Bulked segregant analysis (BSA) was developed as an efficient technique to score molecular markers to specific regions of the genome [17]. This methodology has been shown to be very efficient in studying traits controlled by a small number of genes, and may also be utilized to study major QTLs with large effects on the phenotype [18]. Considering the evidence that aluminum tolerance in maize is a quantitatively inherited trait possibly controlled by a small number of genes, the objective of this work was to identify RFLP markers linked to QTLs affecting this trait by using bulked segregant analysis.

2. MATERIALS AND METHODS

2.1. Phenotypic evaluation

The genetic materials utilized in this study consisted of F_2, F_3 and F_4 populations derived from a cross between the maize inbred lines, L53 and L1327, developed by the maize breeding program of the National Maize and Sorghum Research Center, CNPMS/EMBRAPA, Brazil. These two lines have undergone more than ten generations of selfing. The aluminum susceptible line (inbred L53) was derived from the open pollinated variety CMS11 (pool 21) and the aluminum tolerant parent (inbred L1327) was derived from Cateto Colômbia.

The seeds were germinated for seven days in rolled paper towels moistened with tap water. After measurements of the initial lengths of the seminal roots (ISRL), the seedlings were transferred to plastic plates (49 seedlings per plate) and grown in a greenhouse for seven days in eight liters of aerated nutrient solution containing 6 ppm of aluminum in the form of $KAl(SO_4)_2$ [7]. On each plate there were three seedlings from each of the parental inbred lines. During harvesting, final seminal root length (FRSL) was measured and the plants transferred back to the nutrient solution. ISRL and FSRL were used to calculate the index value for relative seminal root length (RSRL), where RSRL = (FSRL - ISRL)/ISRL.

In order to check the efficiency of the phenotypic index (RSRL), four inbred lines, two Al-tolerant and two Al-susceptible were evaluated in nutrient solution containing toxic aluminum.

The seedlings with the greatest RSRL values (Al-tolerant) and the seedlings with the smallest RSRL values (Al-susceptible) were transplanted from the nutrient solution to field conditions, grown and then selfed to obtain F_3 families. F_3 and F_4 families were also grown in nutrient solution using the same procedure describe above. The objective was to identify F_3 and F_4 families breeding true for the selected trait (Al-susceptibility at one end of the distribution and Al-tolerance at the other end) for bulking and to eliminate the heterozygous ones.

To obtain a phenotypic index based on average values of RSRL, 168 F_4 families were also evaluated in nutrient solution.

2.2. RFLP detection

The set of 113 RFLP markers used in this study corresponds to *PstI*-digested genomic DNA cloned into pUC19 plasmids, obtained from David Hoisington (International Maize and Wheat Improvement Center, CIMMYT, Mexico) and from Theresa Musket (University of Missouri, Columbia). These markers have been previously shown to identify polymorphism in maize and are available with a linkage map data as a public set of maize RFLP probes. One hundred and thirteen probes were selected at an average interval of 30 cM in such a way as to cover all the maize genome. These probes were then tested for their ability to identify RFLPs between the parental inbred lines (L53 and L1327) when their DNAs were digested with *Eco*RI, *Bam*HI or *Hind*III.

The probes were labeled via amplification by the polymerase chain reaction using digoxigenin-11-dUTP (Boehringer Mannheim).

DNA was purified from lyophilized leaf tissue using the method described by Saghai-Maroof [19]. The DNA was quantified visually on 0.8% agarose gels by comparison with standards of known concentration, and dissolved again to a concentration of 1µg/µl in TE (10mM Tris pH 8.0; 1mM EDTA). Approximately 30µg of genomic DNA was digested with each of the three restriction enzymes using 2.5U of enzyme per µg of DNA for 18 hours at 37°C. Digested DNA was loaded onto 0.8% agarose gels prepared with 1x TAE buffer (40mM Tris acetate pH 8.0; 10mM EDTA) and electrophoresed overnight at 50V.

2.3. Bulk analysis

To construct the bulks, DNA was pooled from individuals from each selected F_3 or F_4 family. The DNAs were extracted, quantified and bulked in a such way that in each bulk all individuals were equally represented.

Fifty four RFLP markers previously chosen for their ability to distinguish the parental inbred lines and their F_1s were used to identify polymorphism between the contrasting bulks.

3. RESULTS AND DISCUSSION

3.1. Phenotypic response to Al stress

The RSRL value frequency distribution obtained for the F_2 population is shown in Figure 1. The mean RSRL values of the parental inbred lines (L53 and L1327) were 0.15 ± 0.005 and 0.75 ± 0.028 respectively. The F_2 population showed an average RSRL of 0.43 ± 0.006 and variance of 0.058. Figure 1 shows a continuous distribution, which is typical of

quantitatively inherited traits, with a tendency toward more susceptible individuals. However, the recovery of parental phenotypes suggest that the aluminum tolerance may not be a complex trait and is in agreement with past evidence that tolerance to aluminum is a quantitatively inherited trait controlled by a small number of genes [12].

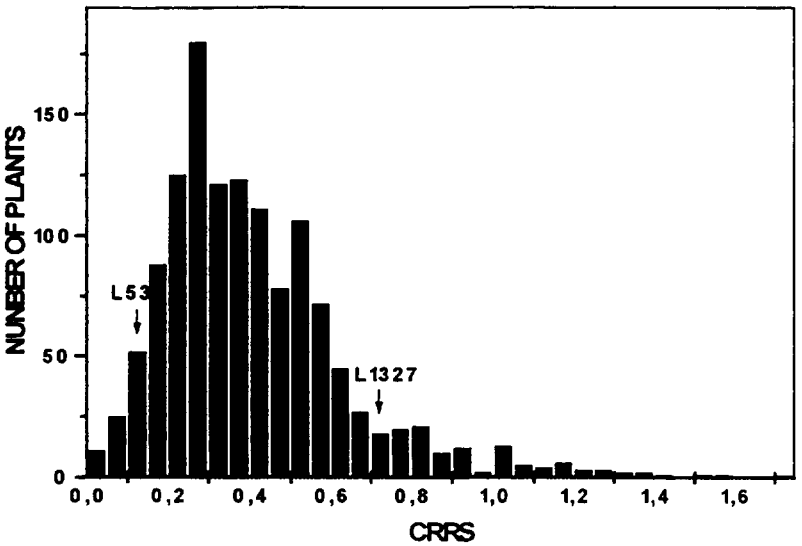


Fig. 1. Histogram of RSRL values frequency distribution obtained from the F_2 population grown in nutrient solution containing toxic concentration of aluminum. 95% confidence intervals are: 0.15 ± 0.01 (L53) and 0.75 ± 0.06 (L1327).

In order to check the efficiency of the phenotypic index (RSRL) four inbred lines, two Al-tolerant and two Al-susceptible, were evaluated in the nutrient solution containing toxic aluminum. The phenotypic index obtained from the tolerant and susceptible inbred lines showed that the index efficiency was greater when mean values were used instead of individuals RSRL values (Fig. 2).

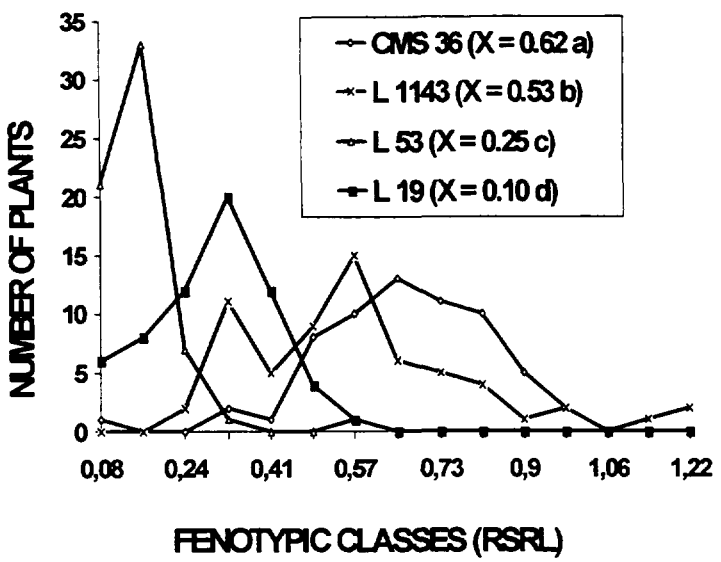


Fig. 2. Frequency distribution of RSRL values of Al-susceptible inbred lines (L19 and L53) and Al-tolerant inbred lines (L1143 and CMS36). Means followed by the same letters are not statistically different at 5% significance level by the Tukey test.

3.2. RFLP analysis

Of the 113 selected probes, 54 were able to distinguish the parental lines and their F_1, with 46 probes showing codominance and eight showing a more complex pattern. Some probes were polymorphic with more than one enzyme, thus a total of 73 RFLP marker loci were identified. This number of polymorphic probes was not sufficient to provide the desired genome coverage since they were not uniformly distributed along the 10 maize chromosomes.

From the 54 markers used to analyze the contrasting bulks three markers on chromosome 8 (UMC48, UMC103 and CSU 155) revealed polymorphisms between them, as a result of differences in band position and intensity as shown in Figure 3.

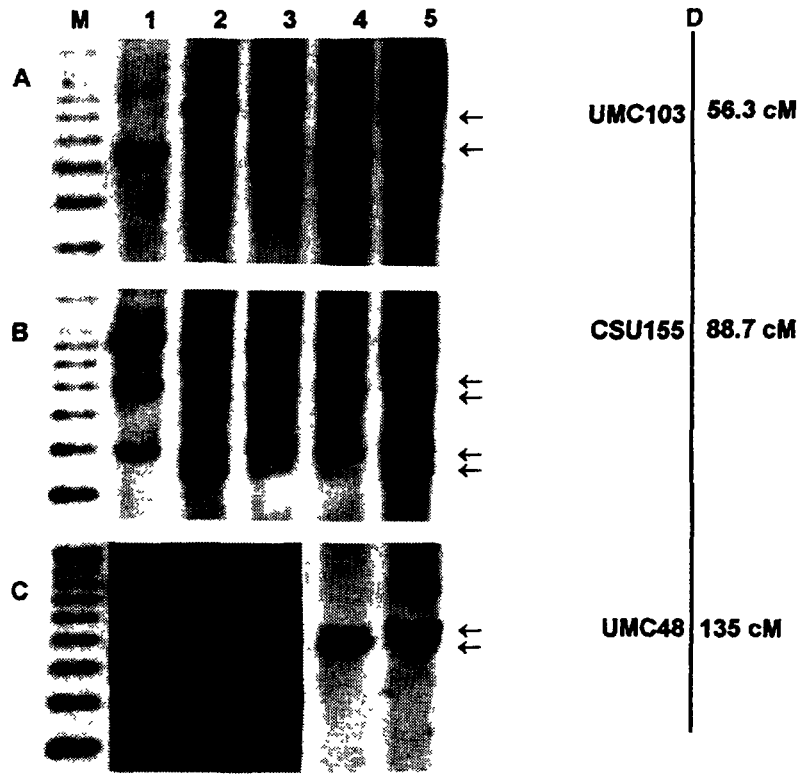


Fig. 3. Southern blots of parental and bulked DNA samples digested with EcoRI and probed with UMC103 (A), CSU155 (B) and UMC48 (C). Lane 1, L53 (Al-susceptible parent); lane 2, L1327 (Al-tolerant parent); lane 3, F_1; lane 4 (Al-susceptible bulk); lane 5 (Al-tolerant bulk), lane M molecular weight marker. (←) Arrows indicate the polymorphic regions. (D) Schematic representaiton of polymorphic markers location on chromosome 8 [20].

Hybridization of DNA from individual members of the F_3 bulks with the 3 polymorphic markers showed that about 50% of the members in each bulk were heterozygous as shown in Figure 4. In the Al-susceptible bulk, the individuals 1 and 4 were consistently homozygous for the 3 markers, the individuals 3, 5 and 6 were heterozygous and the individual 4 varied. In the Al-tolerant bulk, individual 7 did not present a good resolution for interpretation and therefore was not used. Individuals 9 and 10 were consistently homozygous; individual 11 was heterozygous for the three markers and the individual 8 varied from one marker to another.

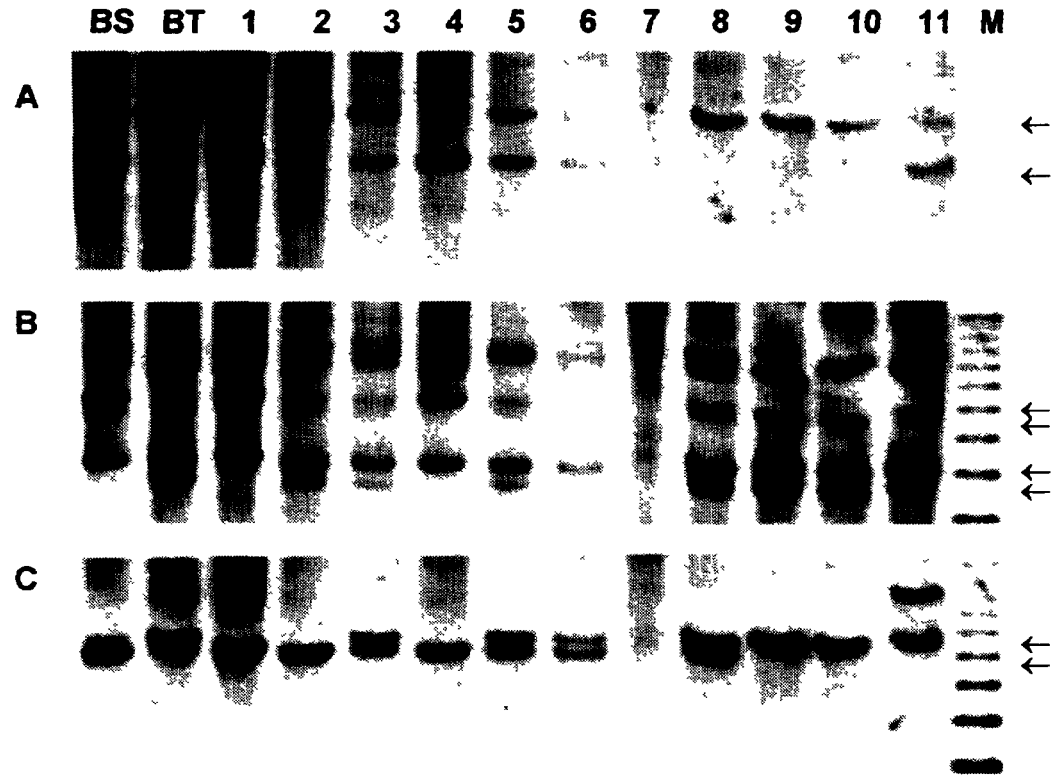


Fig. 4. Southern blots of bulked DNA samples and individual members of the bulks digested with EcoRI and probed with UMC103 (A), CSU155 (B) and UMC48 (C). SB, Al-susceptible bulk; TB, Al-tolerant bulk; 1 to 6, Al-susceptible individuals; 7 to 11, Al-tolerant individuals; M, molecular weight marker. (←) Arrows indicate the polymorphic regions.

Explanations for the presence of heterozygous individuals in the bulks could be that the phenotypic evaluation has not been efficient in identifying contrasting homozygous individuals because of the trait complexity, failures in the methodology or both. Other possibilities are the occurrence of recombination between the QTL(s) and the region where the markers are located.

To confirm that these three probes were linked to the character, an RFLP analysis of 70 F_2 individuals was carried out as shown in Figure 5. Two of the selected probes showed a significant regression coefficient with the character. However, the determination coefficient for each probe was low, explaining only about 10% of the phenotypic variance observed in the 70 F_2 individuals evaluated.

A further analysis using the probe UMC 103 hybridized with DNA of 168 F_4 families, digested with *Eco*RI, showed a significant regression coefficient (P=0.5%) and a determination coefficient of 4.7%.

Although the F_3 and F_4 bulks identified heterozygous individuals, as shown in Figures 4 and 6, the regression analysis of F_2 individuals and F_4 families showed that the UMC 103 marker on chromosome 8 is associated with the trait. These results agree with the findings of Reiter *et al.* [14] who reported that there is a region on chromosome 8 that is highly correlated with root growth when maize is subjected to low-phosphorus stress.

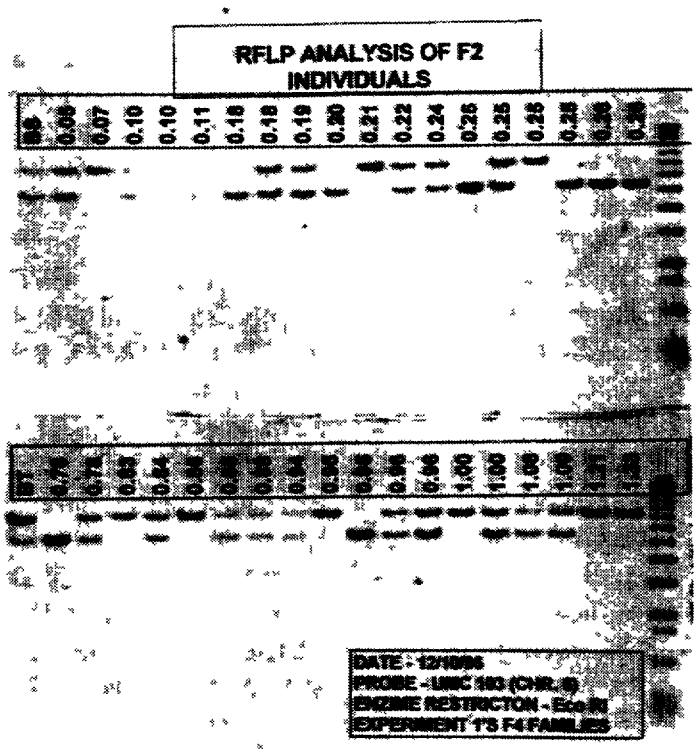


Fig. 5 Southern blots of bulked DNA from Al-susceptible (BS) and Al-tolerant (BT) R_3 families and from 18 F_2 individuals with lowest and highest RSRL values. The DNA was digested with EcoRI and probed UMC103. Numbers above the lanes refer to RSRL values of the F_2 individuals.

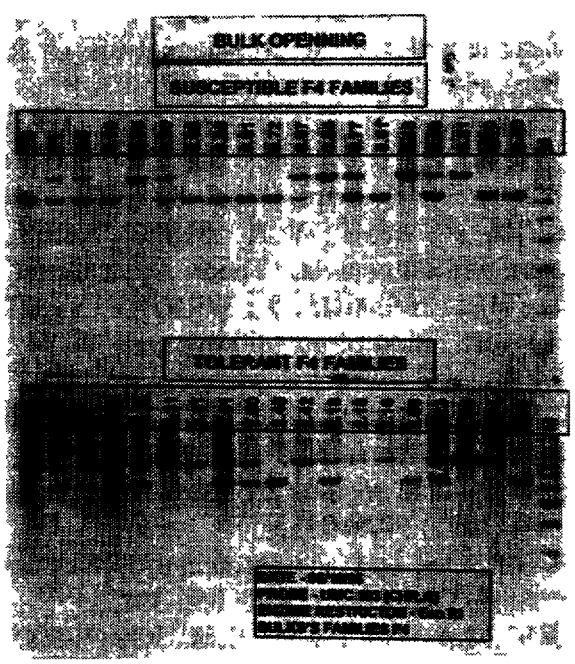


Fig. 6 Southern blots of DNA from parentals genotypes, (Susceptible - PS, Tolerant - PT), bulked DNA from F_4 families (Susceptible bulk - BS and Tolerant Bulk - BT) and DNA from 16 F_4 families with lowest RSRL means and 16 with highest RSRL means. DNA was digested with EcoRI and probed with UMC103. Numbers above the lanes refer to RSRL mean values of the F_4 families.

REFERENCES

[1] OLMOS, I.J.L., CAMARGO, M.N., Ocorrência de Al tóxico nos solos do Brasil, sua caracterização e distribuição. Ciênc. e Cult. 28 (1976) 171-180.

[2] LOPES, A.S., COX, F.R.A., Survey of the fertility status of surface soil under "cerrado" vegetation in Brazil. Soil Soc. Am. J. 41 (1977) 743-747.

[3] GOEDERT, W.J., LOBATO, E., WAGNER, E., Potencial agrícola do cerrado brasileiro. *Pesq. Agrop. Bras.* 15 (1980) 1-17.

[4] RHUE, R.D., GROGAN, C.O., STOCKMEYER, E.W., EVERETT, H.L., Genetic control of aluminum tolerance in corn. Crop. Sci. 18 (1978) 1063-1067.

[5] FURLANI, P.R., LIMA, M., MIRANDA, L.T., MIRANDA, L.E.C., SAWASAKI, E., MAGNAVACA, R., Avaliação de linhagens, materiais comerciais e duas populações de milho para tolerância ao alumínio. *Pesq. Agrop. Bras.* 21 (1986) 655-660.

[6] LOPES, M.A., MAGNAVACA, R., BAHIA FILHO, A.F.C., GAMA, E.E.G., Avaliação de populações de milho e seus cruzamentos para tolerância a toxidez de alumínio em solução nutritiva. *Pesq. Agrop. Bras.* 22 (1987) 257-263.

[7] MAGNAVACA, R., *Genetic variability and inheritance of aluminum tolerance in maize.* Doctoral thesis, University of Nebraska, Lincoln, NE (1982).

[8] HOWELER, R.H., Identifying plants adaptable to low pH conditions. In: *Plant Soil Interactions at Low pH* (Wright, R.J., ed.). Netherlands (1991) pp. 885-904.

[9] RHUE, R.D., GROGAN, C.O., Screening corn for Al tolerance using different Ca and Mg concentrations. *Agron. J.* 69 (1977) 775-760.

[10] GARCIA Jr., O., DA SILVA, W.J., MASSEI, M.A.S., An efficient method for screening maize inbreds for aluminum tolerance. *Maydica.* 24 (1979) 75-82.

[11] SAWASAKI, E., FURLANI, P.R., Genética da tolerância à toxidez do alumínio em linhagens de milho cateto. *Bragantia.* 46 (1987) 269-278.

[12] PRIOLI, A.J., Análise genética da tolerância a toxidez do alumínio em milho (*Zea mays* L.). Doctoral thesis, Universidade de Campinas, Campinas, SP. (1987).

[13] NIENHUIS, J., HELENTJARIS, T., SLOCUM, M., RUGGERO, B., SCHAEFER, A., Restriction fragment length polymorphism analysis of loci associated with insect resistance in tomato. Crop. Sci. 27 (1987) 797-803.

[14] REITER, R.S., COORS, J.G., SUSSMAN, M.R., GABELMAN, W.H., Genetic analysis of tolerance to low-phosphorus stress in maize using restriction fragment length polymorphisms. Theor. Appl. Genet. 82 (1991) 561-568.

[15] DIERS, B.W., KEIM, P., FEHR, W.R., SHOEMAKER, R.C., RFLP analysis of soybean seed protein and oil content. Theor. Appl. Genet. 83 (1992) 608-612.

[16] EDWARDS, M.D., HELENTJARIS, T., WRIGHT, S., STUBER, C.W., Molecular-marker-facilitated investigation of quantitative trait loci in maize. Theo. Appl. Genet. 83 (1992) 765-774.

[17] MICHELMORE, R.W., PARAN, I., KESSELI, R.V., Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA. 88 (1991) 9828-9832.

[18] WANG, G.L., PATERSON, A.H., Assessment of DNA pooling strategies for mapping of QTLs. Theor. Appl. Genet. 88 (1994) 355-361.

[19] SAGHAI-MAROOF, M.A., SOLIMAN, K.M., JORGENSEN, R.A., ALLARD, R.W., Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA. 81 (1984) 8014-8018.

[20] CHAO, S., BAYSDORFER, C., HEREDIA-DIAZ, O., MUSKET, T., XU, G., COE, E.H., RFLP mapping of partially sequenced leaf cDNA clones in maize. Theor. Appl. Genet. 88 (1994) 717-721.

DETERMINATION OF MOLECULAR MARKERS ASSOCIATED WITH ANTHESIS-SILKING INTERVAL IN MAIZE

J. SIMPSON
Centro de Investigaciones y Estudios Avanzados (CINVESTAV),
Irapuato, Mexico

Abstract

Maize lines contrasting in anthesis-silking interval (ASI), a trait strongly linked to drought tolerance, have been analyzed under different water stress conditions in the field and with molecular markers. Correlation of marker and field data has revealed molecular markers strongly associated with flowering and yield traits.

1. INTRODUCTION

Drought is a severe problem in Mexico, where around 83% of the country is classified as arid or semi-arid [1]. Half the surface area depends on a growing season of less than 90 days for annual crops [2]. Drought affects more than 7 million hectares sown with maize, which represents almost 88% of the total area of this cereal grown annually in Mexico. Losses mainly due to water shortage have made it necessary for Mexico to import about 3 million tons of maize annually.

During the growing season, maize requires between 500-800 mm of rainfall depending mainly upon the particular climate, soil and method of management. These values are considered optimal in order to obtain acceptable yields under irrigation. Yields vary from about 6,000 kg/ha to 10,600 kg/ha, suggesting that approximately 750 kg of water are required for each kg of grain produced. However, when irregularities occur in the distribution of water easily obtained by the plant during the growth cycle, severe reductions in grain yield are observed. Many reports indicate that flowering is the most sensitive stage for water shortage [3, 4, 5]. If only 83% of necessary water is provided during flowering, losses of up to 43% can be produced. Reduction in yield under drought stress is due to a combination of effects the direct result of which is the production of reduced numbers of grains per ear [3, 4, 6, 7, 8, 9, 10]. Due to low water potential, growth of stigmas is inhibited, producing asynchrony between anthesis and silking and consequently low levels of pollination.

Another phenomenon associated with low water potentials, is the inhibition of grain filling leading to abortion of grain production. This was thought to be due to lack of transport of photosynthate to the grains but there is also evidence that there is a general lack of photosynthate under drought stress conditions and that transport is not the only effect. This suggests that the early stages of grain filling are dependent on a continuous supply of carbohydrates. Recently it has been shown that the number of grains is closely related to the rate of photosymthesis before water stress rather than to photosynthetic reserves accumulated before flowering [5, 11, 12]. This is in agreement with results obtained following 8 cycles of selection for drought tolerance where reduction in the anthesis-silking interval (ASI) observed was due to a greater accumulation of dry matter per spikelet. Therefore, the most efficient strategy for obtaining drought tolerant plants would be to select plants with either synchronized flowering or early female flowering. ASI is a quantitative trait and short ASI under water stress conditions is related to an increase in yield. Due to the complex nature of the trait and the difficulties in carrying out field experiments under water stress conditions, the determination of molecular markers associated with ASI and yield should provide an advantage in selection of drought tolerant materials within a breeding program.

2. MATERIALS AND METHODS

2.1. Plant materials

S6 lines derived by single seed descent from an S3 population H-353 which was formed from the cross Tuxpeño X Celaya.

2.2. Field conditions

Celaya, Guanajuato, Mexico,- Spring/Summer cycle 1994. The parental H-1 (long ASI) and H-2 (short ASI) lines and the segregating F2 population, were sown at INIFAP's field station in Celaya under six water stress regimes as indicated in Table I. The site at Celaya has clay soils and is 1,680 meters above sea level. In this season precipitation is zero and temperatures range from 15°C minimum to 32°C maximum. Plants were grown at a density of 75,000/hectare.

The arrangement of treatments was in split plots where the main plots were the water regimes and the sub-plots were the genetic materials. The different arrangements of treatments were distributed under a random block design with 4 replications. Evaluation of the dynamics of soil humidity was carried out using a neutron disperser for depths of 0-15, 15-30 and 30-60 cm pre-flowering and 0-15, 15-30, 30-60 and 60-90 cm post-flowering.

TABLE I. CORRELATION OF MARKERS AND TRAITS OF INTEREST USING POPULATION TAILS (TRAIT)

MARKER	ANTHESIS	SILKING	ASI	No. GRAINS	W100G	YIELD
UMC 4						
UMC 14		0.26*	0.34**	-0.34**	-0.29*	-0.28
A27	0.30**	0.40**	0.33**		-0.21*	
A36	0.44***	0.52***	0.33**	-0.30**		0.26*
A86	-0.22*	-0.27**		-0.56**	-0.46***	-0.50***
A87	-0.20*	-0.34***	-0.40***	0.27**	0.32**	0.38***
A140	-0.38***	-0.44***	-0.31**	0.22**	0.29**	0.25***
A152	-0.40***	-0.48***	-0.35**	0.46***	0.20*	0.42**
A155			-0.39**	0.51***	0.36***	0.44***
A158				0.41***	0.47***	0.40**
				0.24*	0.29**	0.26**

*,**, *** - significant at P=0.05, 0.01, and 0.001 levels respectively.
No. Grains = No. / ear, W100G = Weight of 100 kernels.

2.3. Southern blot analysis

Approximately 2.5 g of fresh tissue was used for DNA extraction by the method of Dellaporta et al. [13]. Following purification, DNA was digested with Hind III restriction enzyme, according to the manufacturers instructions, and fragments were separated on 0.8% agarose gels. Gels were blotted to Hybond N^+ nylon membrane (Amersham) by standard techniques and fixed. Digoxigen labeled probes were prepared either by random priming [14] or by standard PCR techniques using digoxigen-11-dUTP. Prehybridization was carried out for at least 30 minutes at 65°C, then hybridized at 65°C with gentle shaking overnight. Following hybridizaion the membranes were washed and exposed overnight to Dupont Cronex X-ray film.

2.4. AFLP analysis

AFLP analysis was carried out with the permission and using a protocol provided by Keygene, Holland [15, 16].

2.5. Statistical analysis

Statistical analysis involved ANOVA, GLM, correlation and regression analysis using SAS programs and Mapmaker QTL [17] for molecular marker correlations.

3. RESULTS AND DISCUSSION

Analysis of data from the field experiment showed that in general terms this experiment was successful; adequate stress conditions were applied during the period up to and including the flowering stage, whereas during the grain filling period, plants were unstressed. Although the average ASI was higher in most severely stressed environments, genotype rather than environment was shown to correlate with these differences as can be seen in Tables II and III.

In terms of yield, short ASI was correlated with higher yield. The F2 genotype showed the highest yield suggesting that certain components of the long ASI genotype contributed to the increase in yield observed in the F2 as compared to the short ASI genotype.

TABLE II. FIELD EVALUATION (MEAN ASI/GENOTYPE)

Population	N	Mean	SD
1 Long ASI	24	10.56	1.12
2 Short ASI	24	8.31	1.19
3 F_2	24	8.15	1.01
4 F_3	24	8.37	0.95
5 BC- P1	24	9.36	2.55
6 BC-P2	24	7.90	1.22

TABLE III. FIELD EVALUATION (MEAN ASI/ STRESS TREATMENT)

Level of stress	N	Mean	SD
1 Lowest	24	8.46	1.36
2	24	8.42	1.78
3	24	8.74	1.65
4	24	8.48	1.25
5	24	9.40	1.25
6 Highest	24	9.20	2.50

A total of 102 RFLP markers were tested for polymorphism between the two parental lines, of which 35 markers showed polymorphism. A total of 14 AFLP primer combinations have been analyzed revealing between 6 and 29 polymorphic bands per combination. In total, 261 polymorphic bands have been identified of which, to date, 72 have been analyzed in detail. AFLP's have been analyzed as dominant or, where possible, co-dominant markers as described by Van Eck et al. [18].

Two methods of analysis of marker and field data have been carried out. The first analysis involved comparing only the individuals with the most extreme phenotypes of the F2 population as reported by Koester et al. [19], in terms of flowering and yield data and correlating this data with marker genotypes. Ten plants from each extreme of four water treatment regimes were selected; Treatment 6 (T6) was left out since conditions were very severe and field data were thought to be unreliable and Treatment 1 (T1) was left out since virtually the same results were obtained as in T2. In total, 40 plants of each extreme were analyzed. The results obtained for these analyses are summarized in Table I. Only strong correlations with at least 3 traits have been included in the table although in total 37 markers with significant correlation to at least one trait were identified. As can be seen, negative values obtained for flowering traits correspond to positive values for yield and vice versa as would be expected.

The second method of analysis involves correlation of flowering and yield characteristics to marker genotypes in the T2 F2 population, using the specialized program Mapmaker QTL. The results of this analysis are summarized in Table IV. In this case only map regions correlating with at least two traits are shown, although a total of 17 regions correlating with at least one trait were identified. Again the tendency of correspondence between negative flowering values and positive yield values is reflected, although not perfectly.

A putative map showing the markers determined to be associated with flowering and yield traits, either by analysis of population 'tails' or by Mapmaker QTL, is presented in Fig. 1. This preliminary map shows one region where significant markers are common to both methods of analysis and in several cases both methods indicate significant markers on the same linkage group. The discrepancies in the results obtained by the different methods of analysis are probably due to the preliminary state of the map which was produced using only 43 markers and data from only the T2 F2 population. Unfortunately, four of the significant markers from the tails analysis have not yet been included and in general terms the map is very incomplete. It is hoped that as the map improves and becomes more robust, a stronger

correlation between significant markers identified by the different methods of analysis will be observed.

TABLE IV. CORRELATION OF MARKERS AND TRAITS IN WHOLE T2, F2 POPULATION (USING MAPMAKER QTL, LOD 2) (TRAIT)

MARKER	ANTHESIS	SILKING	ASI	No. GRAINS	YIELD
A24-A271	--	--		+	
A60-A101	--		+		+
A95-A158	--		--	+	+
A156-A95				+	+

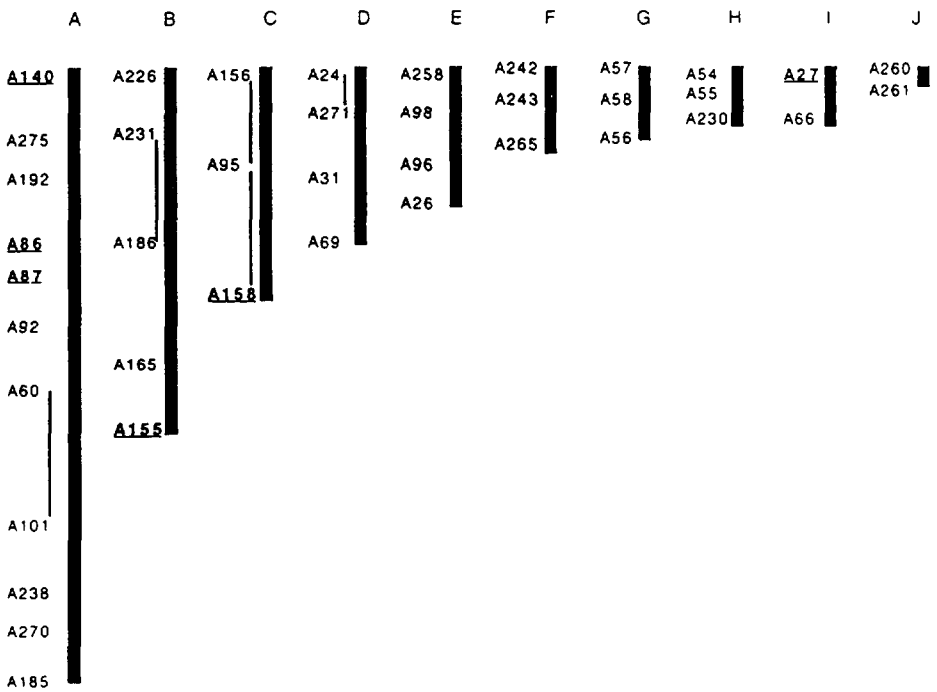


Fig. 1. Putative map showing markers related to flowering and yield traits. Numbers indicate markers, numbers underlined and in bold, indicate markers significant in 'tails' analysis. Vertical lines show regions where significant QTL's were found in Mapmaker analysis.

4. CONCLUSIONS/PERSPECTIVES

The project has in general terms been successful, the field experiment and a large proportion of the laboratory work have been completed. We plan to finish the molecular marker analysis of the T2 population and then proceed with analysis of the T5 population for which all molecular marker information is already available as raw data. Inclusion of the T5 population should help to improve the map and will allow a comparison of markers found to be significant under conditions of low (T2) and high (T5) stress. The final goal of the project is to use the marker information in an ongoing program for selection of drought tolerant materials.

REFERENCES

[1] FREGOSO, T.L.E., Efecto de la labranza y captación de lluvia sobre la estructura y algunas propiedades hidrofisicas del suelo e índices fisiotécnicos de maíz (*Zea mays* L.), Tesis de grado M.C., Colegio de Posgraduados, Chapingo, Mexico (1991) 233 pp.

[2] ORTIZ, S.C., Evaluación de la aptitud de las tierras de México para la producción de maíz, frijol y sorgo en condiciones de temporal, Tech. Bull., Soil Science Dept., Colegio de Posgraduados, Chapingo, México (1984).

[3] DENMEAD, O.T., SHAW, H.R., Effects of soil moisture stress at different stages of growth on the development and yield of corn, Agron. J. 52 (1960) 272-274.

[4] ECK, V.H., Effects of water deficits on yield, yield components, and water use efficiency of irrigated corn, Agronomy J. 78 (1986) 1035-1040.

[5] HERRERO, M.P., JOHNSON, R.R., Drought stress and its effects on maize reproductive systems, Crop Sci. 21 (1981) 105-110.

[6] BLUMP, A., Genotypic responses in sorghum to drought stress; I. Response to soil moisture stress, Crop Sci. 14 (1974) 361-364.

[7] BOYLE, M.G., BOYER, J.S., MORGAN, P.W., Stem infusion of liquid culture medium prevents reproductive failure of maize at low water potential, Crop Sci. 31 (1991) 1246-1252.

[8] EDMEADES, G.O., BOLAÑOS, J., HERNANDEZ, M., BELLO, S., Causes for silk delay in a lowland tropical maize population, Crop Sci. 33 (1993) 1029-1035.

[9] JURGENS, S.K., JOHNSON, R.R., BOYER, J.S., Dry matter production and translocation in maize subjected to drought during grain filling, Agron.J. 70 (1978) 678-682.

[10] SCHUSSLER, J.R., WESTGATE, M.E., Increasing assimilate reserves does not prevent kernel abortion at low water potential in maize, Crop Sci. 27 (1994) 639-648.

[11] BASSETTI, P., WESTGATE, M.E., Emergence, elongation and senescence of maize silks, Crop Sci. 33 (1993) 271-275.

[12] WESTGATE, M.E., BOYER, J.S., Reproduction at low silk and pollen water potentials in maize, Crop Sci. 26 (1986) 951-956.

[13] DELLAPORTA, S.L., WOOD, J., HICKS, J.B., A plant DNA minipreparation: Version II, Plant Mol. Biol. Reporter 1 (1983) 19-21.

[14] FEINBERG, A.P., VOGELSTEIN, B., A technique for radiolabelling DNA restriction fragment length polymorphisms to high specific activity, Annal. Biochem., 132 (1983) 6-13.

[15] LIN, J.J., KUO, J., AFLP™. A novel PCR-based assay for plant and bacterial DNA fingerprinting, Focus 17 (1995) 66-70.

[16] ZABEAU, M., Selective restriction fragment amplification, European Patent Application, Number 92402629.7, Europea Patent Office (1993).

[17] LANDER, E.S., BOTSTEIN, D., Mapping Mendelian factors underlying quantitative traits using RFLP linkage maps, Genetics 121 (1989) 185-199.

[18] VAN ECK, H.J., ROUPPE VAN DER VOORT, J., DRAAISTRA, J., VAN ZANDVORT, P., VAN ENCKEVOORT, E., SEGERS, B., PELEMAN, J., JACOBSEN, E., HELDER, J., BAKKER, J., The inheritance and chromosomal localization of AFLP markers in a non-inbred potato offspring, Mol. Breeding, 1 (1995) 397-410.

[19] KOESTER, R.P., SISCO, P.H., STUBER, C.W., Identification of quantitative trait loci controlling days to flowering and plant height in two near isogenic lines of maize, Crop Sci. 33 (1993) 1209-1216.

INCORPORATION OF CONVENTIONAL GENETIC MARKERS AND RAPD MARKERS INTO AN RFLP BASED MAP IN MAIZE

E.H. COE, Jr., M.D. McMULLEN, M. POLACCO
USDA-ARS, Plant Genetics Research Unit,
Columbia, Missouri, United States of America

G.L. DAVIS
USDA-ARS, Corn Host Plant Resistance Research Unit,
Mississippi State University, Mississippi, United States of America

S. CHAO
Institute of Botany,
Academia Sinica,
Nankang, Taipei, Taiwan

Abstract

Integration of classical genetic markers, in particular mutants, onto the maize Restriction Fragment Length Polymorphism (RFLP) map will provide the tools necessary to further our understanding of plant development and of complex traits. Initially integration was accomplished by visual alignment of common markers and sometimes involved the use of information from several different molecular maps to determine the relative placement of a single mutant. The maize core marker set was designed to provide a common set of markers which could be used for integration of map data. We have completed the mapping of 56 mutants on chromosome one relative to the core marker set. Phenotypes included whole plant, seedling, and kernel effects and represented a variety of biological processes. Since these mutants were previously located to chromosome arm, mapping required the use of only seven markers per mutant to define the correct bin location Two mistakes in marker order relative to the classical map were identified, as well as, six groups of mutants which require allelism testing Placement of mutants and cDNAs into bins using the core markers provides a necessary resource for identification of gene function in maize.

1. INTRODUCTION

Genetic and molecular maps in maize have been available for 61 and 11 years, respectively [1, 2]. Integration of the two will help us towards the goal of understanding complex traits. Establishment of a set of "core" markers which evenly divide the genome has provided the necessary bridge to accomplish integration of them [3, 4]. Through these markers any mutant mapped using publicly available clones can be placed on the maize bin map (http:\\www.teosinte.agron.missouri.edu). Individual bins comprise approximately 20 cM intervals along the chromosome. In addition to Restriction Fragment Length Polymorphism (RFLP) markers and mutants, Quantitative Trait Loci (QTLs) can also be assigned to bins. The most informative RFLP markers for studying complex traits are sequenced cDNAs (expressed sequence tags; ESTs) because of their potential to mark sites of known function. Randomly Amplified Polymorphic DNA (RAPD) markers, while more economical, present a less desirable alternative to cDNAs because they provide no potential information on function at the site they mark.

Given the myriad of mutants which have been identified in maize and the current technologies available for large scale sequencing, we are focusing our efforts in two areas: mapping of mutants relative to molecular markers and mapping of ESTs, with the goal of providing a resource of molecularly or functionally defined genes catalogued by location. Our specific objectives were to define a set of publicly available core markers for use in integrating mapping data and to use these markers to map maize mutants onto the bin map.

2. MATERIALS AND METHODS

2.1. Core markers

Potential core markers were chosen initially based on simple fragment pattern and even distribution at approximately 20 cM intervals on the chromosomes. Markers not among the previous core marker set identified by Gardiner *et al.* [3] were screened against inbreds A619, A632, B73, Mo17, CO159, and Tx303 using *Eco*RI, *Hin*dIII, *Eco*RV, *Bam*HI, *Dra*I, *Xba*I, *Bgl*II, and *Sst*I and the polymorphism rate was assessed. A marker was designated acceptable if it was polymorphic with a minimum of three of the eight enzymes with a majority of the inbred lines. A number of substitutions were made compared to the previous core marker set due to low levels of polymorphism or high fragment pattern complexity. Final choices were made based on even spacing, simple fragment pattern, high degree of polymorphism, and public availability. Markers meeting these criteria that had insert sizes less than 1000 base pairs were given preference to facilitate single-pass sequencing.

Core markers were prepared for sequencing using the alkaline lysis method. DNA quality was assessed using 0.8% agarose gels containing cut and uncut DNA. Quantity was determined using a spectrophotometer. Sequencing was performed either by dideoxy-termination reactions labeled with ^{35}S α-dCTP according to manufacturer recommendations for the T7 Sequenase Kit (US Biochem) or by PCR incorporation of fluorescently labelled bases followed by sample processing on the ABI sequencer. Results of the manual sequencing were entered into DNA to determine overlap. BLASTX searching was conducted against the nonredundant database of GenBank. All matches were evaluated using the MOTIFS program to determine whether homology occurred in a conserved protein domain.

2.2. Mutant mapping

Mutants used for this study were previously mapped to chromosome arm by genetic experiments involving other mutants or by cytogenetic tests. The mutants used represent whole plant, seedling and kernel defects and include a variety of biological processes. A list of mutants and phenotypes is provided in Table I. More detailed phenotypic descriptions are presented for the mutants which have been given gene designations.

One to four F_2 mapping populations were derived for each individual mutant where the nonmutant parent was one of the following inbreds, A619, A632, B73, or Mo17. Attempts were made to produce four segregating populations for each mutant to maximize the chance of obtaining polymorphisms for each of the core markers evaluated and to allow future evaluation of potential modifiers of trait expression.

Forty kernels per F_2 family were planted in the field. For mutants expressed in the seedling or adult plants, families were scored for presence of the mutant and individuals self-pollinated. Kernel phenotypes were scored on F_2 ears prior to planting in the field and again on F_3 ears. In families with seedling phenotypes, forty F_3 seed from each ear were evaluated in the sandbench to determine the genotype of each F_2 individual. In families with adult plant phenotypes, forty F_3 seed from each ear were evaluated in the field the following season to determine genotypes for each F_2 individual.

Tissue for RFLP analysis was collected from either field grown individuals or sandbench bulked F_3 progeny rows. 100 mg. of lyophilized tissue was extracted for each individual using

an ammonium acetate extraction technique. Extractions were divided into two aliquots and digested with *Eco*RI and *Hin*dIII. Southern analysis of *Eco*RI and *Hin*dIII digested DNA for each F_2 family was performed according to standard laboratory procedures [5]

TABLE I MUTANTS AND THEIR ASSOCIATED PHENOTYPES

Mutant[1]	Phenotype
ad*	adherent seedling
ad1	adherent 1--seedling leaves, tassel branches, and occasionally top leaves adhere
an1	anther ear--andromonoecious dwarf of intermediate stature
blh*	bleached seedling
Blh1	bleached 1--dominant plants have pale green midveins and base in the upper leaves of the plant
bnk*	brown kernel
br*	brachytic plant
cp*	collapsed kernel
ct2	compact plant 2--plant is semi-dwarfed with a club tassel
d*	dwarf
dcr*	defective crown
de*	defective kernel
dnt*	dent
et*	etched
hcf31	high chlorophyll fluorescence 31--yellow/green seedlings lacking chlorophyll a/b binding protein
l*	luteus
l16	luteus 16--yellow green leaves with patches that bleach to paler yellow
lls1	lethal leaf spot 1--chlorotic to necrotic lesions similar to those of *Helminthosporium carbonum* infected plants
nec*	necrotic seedling
pg*	pale green
pg15	pale green 15--light yellow green seedling with patches bleaching to white
rs2	rough sheath 2--short, zigzag plants with wart-like distorted leaves and sheaths
Smk*	small kernel
spc2	speckled 2--green seedlings containing light green speckles
spc*	speckled seedling
sr1	striate 1--leaves contain many white stripes
tlr*	tillered plant
ty*	tiny plant
v*	virescent leaf
Vg1	vestigial glume 1--glumes very small leaving cob and anthers exposed, upper leaves also have scant ligules
vp*	viviparous
vp5	viviparous 5--embryo fails to become dormant, white endosperm and seedling, encodes phytoene C-11,12 desaturase
vp8	viviparous 8--embryo doesn't become dormant, produces small, pointed-leaved seedlings
w*	white seedling
wl*	white luteus seedling
wlu5	white luteus 5--cream colored seedling
zb4	zebra crossbands 4--alternating bands of green, yellow-green, and white, more pronounced in cooler temperatures
zb7	zebra crossbands 7--alternating bands of green and pale green on glossy seedlings
zl1	zygotic lethal 1--homozygous recessive zygotes do not develop

[1] Mutant designations followed by an astericks have not yet been designated as distinct genes pending further information regarding map locations and allelism tests

Hybridizations were carried out using ^{32}P α-dCTP labelled probes in bulk lots of 40 membranes corresponding to a chromosome arm using each of the individual core markers for that arm. The hybridization mixture was reused the following day for a second set of 40 membranes. Prehybridization and hybridization protocols followed the standard laboratory procedures [5]. Following washing, the radioactive membranes were exposed to Kodak X-OMAT film in the presence of a Cronex-type intensifying screen at -80 C. Marker *bnl8.29* was substituted for *umc161* as *umc161* was not polymorphic in these materials.

Mapscores were aligned in core marker order in a spreadsheet and mutants were tentatively placed to an interval by minimizing the number of double-crossover events. Map locations were confirmed using Mapmaker for MacIntosh version 2.0. A second confirmation based on concurrent map locations of the same mutant mapped in different F_2 backgrounds was also used as a check.

3. RESULTS

3.1. Core markers

A set of ninety maize core markers were identified that best met the selection criteria. Table II lists the core markers, their bin number, and homology information. The average distance between core markers is 18 cM. Information regarding polymorphisms for the inbreds and enzymes tested is to be made available through the Maize Genome Database. Core marker sets are available through the probe request form of the database or by contacting the lab directly. The set consists of 75 genomic and 15 cDNA markers. Homology to genes of known function was identified by BLASTX searching of the nonredundant Genbank database for 20 of the 90 core markers with functionality of an additional 4 clones identified by targeted cloning experiments. Twenty-four percent of the genomic cores and 20% of the cDNA cores derived by non-targetted cloning had homology to genes with known function.

3.2. Mutant mapping

56 mutants were mapped to bins on maize chromosome 1 (Table III). Prior to this study, 44 of the mutants were known only to be located to chromosome arm, 21 on the short arm and 23 on the long arm. No mutants were mapped in bin 1.06 which is believed to contain the centromere based on translocation studies. The map order of *v5* relative to *srl* and *zb4* relative to *p1* was found to be reversed on the classical genetic map. Six groups of mutants with similar phenotype were identified based on bin assignment which are candidates for allelism testing. They are *zl1*, *et*-0634C*, *vp5*, and *cp*-1078A*; *ty**-0780A and *bnk*-1519C*; *wl*-1930* and *wl*-144*; *wlu5*, *w*-1890*, *wl*-47*, *wl*-56*, and *wl*-60*; *l*-129* and *l*-544*; and *spc*-370*, *spc2*, and *blh*-43*. One group, *ty*-0780A* and *bnk*-1519C*, was identified when it was noticed that some plants in the family segregating for brown kernel were also dwarf. Subsequent analysis revealed that these were the brown kernel individuals.

4. DISCUSSION

A fast, easy, cost-effective strategy for mapping a large number of mutants has been developed. Approximately 400 samples can be isolated by one individual in a day at a cost of one cent each. A maximum of seven probes are required to place a mutant into its correct bin. Three additional probes for a total of ten will place it in a five centiMorgan interval. By grouping the mutants into bins prior to testing for allelism, the number of crosses needed to determine allelic relationships is greatly reduced.

TABLE II. UNIVERSITY OF MISSOURI - COLUMBIA (UMC) MAIZE CORE MARKERS INCLUDING BIN ASSIGNMENTS AND HOMOLOGY INFORMATION

Marker name	Bin[1]	Homology information
p-tub1	1 01	β-tubulin
p-umc157	1.02	no match
p-umc76	1.03	no match
p-asg45	1 04	protein kinase, U26746, *Brassica napus*
p-csu3	1 05	no match
p-umc67	1 06	no match
p-asg62	1.07	no match
p-umc128	1.08	no match
p-csu164	1.09	no match
p-umc107	1.10	DNA-binding protein; transcription factor U39361 human
p-umc161	1.11	no match
p-bnl6.32	1.12	no match
p-bnl8 45	2.01	no match
p-umc53	2.02	no match
p-umc6	2.03	no match
p-umc34	2.04	no match
p-umc131	2.05	pistil-specific extensin Z14019 *Arabidopsis thaliana*
p-umc255	2.06	no match
p-umc5	2.07	no match
p-asg20	2.08	no match
p-umc49	2.09	no match
p-php20581	2.10	extensins Z46674 tomato
p-umc32	3.01	no match
p-csu32	3.02	no match
p-asg24	3.03	glutamyl-tRNA synthetase, Z91787 *Lupinus luteus*
p-asg48	3.04	no match
p-umc102	3.05	no match
p-bnl5.37	3.06	no match
p-bnl6 16	3.07	various different proteins
p-umc17	3.08	no match
p-umc63	3.09	no match
p-csu25	3.10	P450; U3907 rat
p-agr r115	4.01	not sequenced
p-php20725	4.02	no match
p-umc31	4.03	no match
p-npi386	4.04	various proteins containing polyproline runs
p-agr r37	4.05	no match
p-umc156	4.06	no match
p-umc66	4.07	plant transposon; X52387 potato
p-umc127	4.08	no match
p-umc52	4.09	extensin; X55681 tomato
p-php20608	4.10	no match
p-umc169	4.11	extensin; X55686 tomato
p-npi409	5.01	no match
p-umc90	5.02	no match
p-tub4	5.03	β-tubulin
p-bnl4.36	5.04	not sequenced
p-csu93	5.05	no match
p-umc126	5 06	no match
p-umc108	5.07	various different proteins
p-bnl5.24	5.08	no match
p-php10017	5.09	no match

TABLE II (cont)

Marker name	Bin[1]	Homology information
p-umc85	6 01	no match
p-umc59	6 02	no match
p-npi393	6 03	no match
p-umc65	6 04	no match
p-umc21	6 05	no match
p-umc38	6 06	no match
p-umc132	6 07	no match
p-asg7	6 08	no match
p-asg8	7 01	myb related proteins, X13294 human A-myb protein
p-asg34	7 02	methylmalonate-semialdehyde dehydrogenase M84911, *Pseudomonas aeruginosa*
p-asg49	7 03	no match
p-umc254	7 04	no match
p-umc245	7 05	no match
p-umc168	7 06	no match
p-npi220	8 01	no match
p-bnl9 11	8 02	leucyl-tRNA synthetase, M88581, *Bacillus subtilis*
p-umc124	8 03	choline kinase active site, U62317 human
p-bnl7 08	8 04	no match
p-bnl2 369	8 05	not sequenced
p-csu31	8 06	no match
p-npi268	8 07	no match
p-npi414	8 08	no match
p-agr r21	8 09	not sequenced
p-umc109	9 01	Constans protein, U62317, *Arabidopsis thaliana*
p-umc192	9 02	bronze 1
p-umc25	9 03	waxy 1
p-csu147	9 04	no match
p-umc95	9 05	no match
p-csu61	9 06	no match
p-asg12	9 07	no match
p-csu54	9 08	no match
p-php20075	10 01	gibberellin (and auxin) stimulated protein X63093, tomato
p-npi285	10 02	various calcium channel proteins, L29346 mouse
p-umc130	10 03	no match
p-umc64	10 04	no match
p-umc259	10 05	no match
p-umc44	10 06	no match
p-bnl7 49	10 07	homeobox domain, U34743 orchid

[1]Bins are defined as the interval between two adjacent core markers starting with the first (upper) core marker and continuing until just before the second (lower) core marker

TABLE III. BIN ASSIGNMENTS OF 56 MAIZE MUTANTS

Mutant	Bin assignement[1]
zl1	1 01
et-0634C*	1 01
vp5	1 01
Blh1	1 01
dcr-1176A*	1 01-1 02
cp-1078A*	1 01-1 02
nec-0495B*	1 01-1 02
pg15	1 02
sr1	1 02
Smk-845B*	1 02-1 03
vp-1136B*	1 02-1 04
ct2	1 03
hcf31	1 03
lls1	1 03
de-1345B*	1 03
pg-484B*	1 03
de-1081*	1 03-1 04
l16	1 04
rs-0780A*	1 04
zb4	1 04
bnk-1519C*	1 04
de-1390A*	1 04
wl-1930*	1 05
rs2	1 05
wl-144*	1 05
de 1057B*	1 05
wlu5	1 07
w-1890*	1 07
wl-47*	1 07
wl-56*	1 07
wl-60*	1 07
Vg1	1 07
an1	1 08
ad1	1 08
w-1802*	1 08-1 09
Mutant	Bin assignment[1]
cp-0948A*	1 08-1 09
*br**	1 08-1 09
de-1016A*	1 08-1 09
de-1420*	1 08-1 09
d 1352B*	1 09
l-129*	1 09
dnt-1185A*	1 09
l 544*	1 09
tlr-2245*	1 09-1 11
d-0998B*	1 10
spc-370*	1 10
v-55*	1 10
zb7	1 10
ad-582*	1 10
blh-43*	1 10
de-1061A*	1 10-1 11
spc2	1 10-1 11
pg-343*	1 10-1 11
v-245*	1 11
vp8	1 11

[1] Mutants listed with a range of bin assignments are the result of lack of polymorphism for the intervening core marker and could be assigned to an individual bin using additional markers

The maize RFLP and genetic maps can be aligned based on the framework established by the core markers. The current UMC (University of Missouri, Columbia) Maize RFLP map contains 982 loci, 49% of which have sequence information and 29% of which correspond to genes of known function [4]. Additional ESTs from the Brookhaven and INRA maps can be assigned to bins enhancing the number of loci with information about function [6, 7]. The current bin map maintained and updated in the Maize Genome Database contains 3516 binned loci including RFLPs, mutants, and cytological events. Combination of sequence and function information either as ESTs or phenotypic mutants provides the resource necessary for gene discovery in maize. Cataloguing the loci into bins enables identification of potential associations of functions with phenotypes including quantitative trait loci which warrant further investigation.

REFERENCES

[1] EMERSON, R.A., BEADLE, G.W., FRASER, A.C., A summary of linkage studies in maize, Cornell Univ. Agric. Exp. Stn. Memoir 180 (1935) 1-83.

[2] HELENTJARIS, R., SLOCUM, M., WRIGHT, S., SCHAEFER, A., NEINHUIS, J., Construction of genetic linkage maps in maize and tomato using restriction fragment length polymorphisms. Theor. Appl. Genet. 72 (1986) 761-769.

[3] GARDINER, J.M., COE, E.H., Jr., MELIA-HANCOCK, S., HOISINGTON, D.A., CHAO, S., Development of a core RFLP map in maize using an immortalized F2 population, Genetics 134 (1993) 917-930.

[4] YERK-DAVIS, G.L., McMULLEN, M.D., GRANT, D., MUSKET, T., BAYSDORFER, C., STAEBELL, M., XU, G., POLACCO, M., KOSTER, L., HOUCHINS, K., CHAO, S., COE, E.H., Jr., An improved maize map standard: ESTs and sequenced core markers in a 982-point map. (in preparation for submission to Genetics).

[5] RFLP Procedures Manual. Maize RFLP Laboratory. University of Missouri-Columbia, 44p.

[6] MATZ, E.C., BURR, F.A., BURR, B., Molecular maps based on TxCM and CoxTx recombinant inbred families, MNL 69 (1995) 257-267.

[7] CAUSSE, M., SANTONI, S., DAMERVAL, C., MAURICE, A., CHARCOSSET, A., DEATRICK, J., DEVIENNE, D., A composite map of expressed sequences in maize, Genome 39 (1996) 418-432.

TAGGING OF BLAST RESISTANCE GENE(S) TO DNA MARKERS AND MARKER-ASSISTED SELECTION (MAS) IN RICE IMPROVEMENT

J.Y. ZHUANG, J. LU, H.R. QIAN, H.X. LIN, K.L. ZHENG
Department of Biotechnology,
China National Rice Research Institute,
Hangzhou, China

XA9846997

Abstract

This paper reports progress made on the tagging of blast resistance gene(s) to DNA markers and on the initiation of marker-assisted selection (MAS) for blast resistance in rice improvement

A pair of near isogenic lines, K80R and K79S, were developed using a Chinese landrace Hong-jiao-zhan as the resistance donor. Ten putatively positive markers were identified by screening 177 mapped DNA markers Using the F_2 population of 143 plants and the derived F_3 lines, three Restriction Fragment Length Polymorphism (RFLP) markers (RG81, RG869 and RZ397) on chromosome 12 of rice were identified to be closely linked to the blast resistance gene *Pi-12(t)*. The genetic distance between *Pi-12(t)* and the closest marker RG869 was 5 1 cM. By employing the bulk segregant analysis (BSA) procedure, six of 199 arbitrary primers were found to produce positive Randomly Amplifiedl Polymorphic DNA (RAPD) bands Tight linkage between *Pi-12(t)* and three RAPD bands, each from a different primer, was confirmed after amplification of DNA of all F_2 individuals Two fragments were cloned and sequenced, and two sequence characterised amplified region (SCAR) markers were established.

In two other F_3 populations, Xian-feng 1/Tetep and Xian-feng 1/Hong-jiao-zhan, the blast resistance was found to be controlled by interactions of two or more genes One resistance gene was located in the vicinity of RG81 in both populations. Work to identify other gene(s) is currently under way.

Marker assisted selection for blast resistance was initiated Crosses were made between elite varieties and blast resistance donors to develop populations for DNA marker-assisted selection of blast resistance In addition, 48 varieties widely used in current rice breeding programs were provided by rice breeders DNA marker-based polymorphism among these varieties and resistance donors were analysed to produce a database for future MAS program.

1. INTRODUCTION

Rice blast, caused by *Pyricularia oryzae* Cav., is generally considered as the most important disease of rice because of its world-wide distribution and because of the severe losses in yield it may cause. Growing resistant varieties has been the most economical and effective way of controlling this disease. Unfortunately, blast resistance in rice varieties can be lost soon after large scale cultivation due to the development of new races or pathotypes of the pathogen. Breeding varieties with multiple resistance genes or a series of near isogenic lines (NILs) with different resistance genes (*i.e.* multi-lines) has been suggested as a solution to this problem [1, 2]. However, the reaction of different resistance genes to races of the pathogen may be similar, and the identification of blast resistance through inoculation with the fungus can be greatly limited by plant developmental stages and environmental factors. Conventional ways of host resistance identification would not always be helpful for rice breeders to determine whether different resistance genes have been integrated into a given line or a series of multi-lines.

On the other hand, indirect selection based on tightly linked genetic markers seems to be more promising in pyramiding the resistance genes and in the development of multi-lines. This relies on the exploitation of the tightly linked markers and the establishment of convenient and low cost detection procedures. The development of DNA restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) techniques has provided powerful tools for mapping genes of the interest [3, 4]. This paper reports our progress on the identification of DNA markers closely linked to a blast resistance gene, on the

studies of the interaction of different blast resistance genes, and on the initiation of DNA marker-assisted selection (MAS) for blast resistance.

2. MATERIALS AND METHODS

2.1. Tagging of a blast resistance gene to DNA markers

A pair of NILs, K80R (resistant to the rice blast) and K79S (susceptible) were developed [5]. The resistance donor used was a local Chinese *indica* cultivar Hong-jiao-zhan, which is well-known for its wide-spectrum and durable resistance to blast. The recurrent parent is an *indica* variety IR24.

An F_2 population of K80R/K79S consisting of 150 plants was grown at China National Rice Research Institute (CNRRI) in 1992. Blast resistance was evaluated by inoculation of the race ZB1 of *Pyricularia oryzae* Cav to each F_2 individual, and verified by inoculation to 12 plants of each F_3 line. DNAs of each F_2 individual as well as the four parental lines were extracted and subjected to RFLP analysis following methods described previously [6, 7].

Of 177 probes used, probe/enzyme combinations detecting polymorphisms between the donor and recurrent parents were used for the analysis of the NILs. Putatively positive results with a probe were examined further to see if the probe pattern co-segregated with blast resistance in the F_2 population. The computer program MAPMAKER [8] was used for linkage analysis. Distances between markers were presented in centimorgons (cM) derived using the Kosambi function [9].

To fine map the resistance gene with additional DNA markers, the bulk segregant analysis (BSA) procedure was employed. Equal quantities of DNAs from 10 homozygous resistant F_2 individuals were mixed to construct a resistant pool (R pool), and equal quantities of DNAs from 10 homozygous susceptible F_2 individuals were mixed to construct a susceptible pool (S pool). RAPD analysis of the pools was performed as described previously [5].

Primers generating polymorphic RAPD bands between the two pools were used to analyse their co-segregation with blast resistance in the F_2 population. RAPD fragments which co-segregated with blast resistance were cloned and sequenced. Two pairs of primers were synthesised for specific amplification.

2.2. Studies of gene interactions

Two F_3 populations were constructed, using a susceptible *indica* variety Xian-feng 1 as the common female parent, and blast resistance cultivars Tetep and Hong-jiao-zhan as the male parents, respectively. The three parental lines were inoculated with each of 20 blast races. The race ZC13 was selected to inoculate 161 F_3 lines of Xian-feng 1/Tetep and 175 F_3 lines of Xian-feng 1/Hong-jiao-zhan. Eighteen individuals of each line were inoculated. DNAs were extracted from bulked samples of 12 F_3 individuals of each line. RFLP analysis and specific amplifications were conducted by using DNA markers in the vicinity of mapped resistance genes on chromosome 12. BSA was then employed to identify RAPD markers linked to other resistance genes.

2.3. Initiation of MAS for blast resistance

Crosses were made between elite varieties and blast resistance donors in Hainan Province in the winter of 1995. The F_1 was grown in CNRRI in 1996 and backcrossed with elite varieties. A polymorphism survey was conducted using DNA markers linked to resistance genes.

Forty-eight varieties which were widely used in current rice breeding programs were provided by rice breeders. DNA marker-based polymorphism among the 48 varieties and resistance donors are being surveyed.

3. RESULTS AND DISCUSSION

3.1. Tagging of a blast resistance gene to DNA markers

Of the 177 RFLP probes tested, 75 were polymorphic between the donor and recurrent parents. They were then used to search for polymorphisms between the two NILs. When a probe produced an identical pattern for the donor parent and the resistant NIL, and produced an identical pattern for the recurrent parent and the susceptible NIL, this probe would likely be linked to a resistance gene(s). Ten probes of this class were found and termed putatively positive markers.

Most of the F_2 plants showed extreme responses to inoculation with conidial suspensions. One-hundred and ten plants were scored 0 or 1 (highly resistant) and 33 plants scored 7 or 9 (highly susceptible) to the race ZB1, fitting an expected ratio of 3:1 when the resistance was controlled by a single dominant gene (χ^2=0.33, P=0.50-0.75). Co-segregation of the blast resistance with putatively positive markers was then tested using the 143 plants.

Of the ten putatively positive markers, only three probes, RG81, RG869 and RZ 397, on chromosome 12 of rice, were found to be linked to the blast resistance gene.

The resistance genotypes of the F_2 individual were identified using the F_3 population, but of the 143 total, only 85 were verified. Map distances were estimated based on data from the 85 individuals. RG869 was most closely linked to the resistance gene, with a map distance of 5.1 cM. The gene for resistance to race ZB1 of *Pyricularia oryzae* Cav. was first tentatively named as *Pi-11(t)*, but later revised as *Pi-12(t)* [10].

Of the 199 10-mer arbitrary primers screened, 6 fragments, each generated by a different primer, were found to be polymorphic between the two pools (present in the R pool and absent in the S pool). After amplification of DNA from the two isolines and a subset of F_2 individuals, linkage between the resistance gene and three RAPD bands (1.3 Kb fragment produced by primer P622; 0.56 Kb fragment by primer P265, P265-560; and 0.35 Kb fragment by primer P286, P286-350) were confirmed. Complete co-segregation of the RAPD markers and blast resistance was detected against all the 143 F_2 individuals, indicating tight linkage of these three markers and the blast resistance gene.

The three fragments were cloned and used as probes to analyse RFLPs of the F_2 individuals. The 1.3-Kb and 560-bp fragments were found to contain repeat sequences. The 350-bp fragment was found to contain single copy sequence and detect a null allele in the susceptible individuals. The results of RFLP analysis using the clone of the 350-bp fragment are in agreement with those of RAPD analysis.

TABLE I. TWO SCAR MARKERS TIGHTLY LINKED TO *Pi-12(t)*

Marker	Sequence
P265-560	CAGCTGTTCAGTCGTTTG
	CAGCTGTTCATACAAGAAAT
P286-350	GCTCCGCATTAACGGGAAG
	AGCCGGCTCCGGAGGTGA

Clones of the 560-bp and 350-bp fragments were sequenced. Specific primers were synthesised for an establishment of (Sequence Characterised Amplification Region) SCAR markers (Table I). Specific amplifications of DNA from the two isolines and all the 143 F_2 individuals were made for P265-560. A 560-bp fragment was observed for all the resistant individuals but none of the susceptible ones, matching the result of RAPD analysis. For P286-350, polymorphsm between K80R and K79S was detected only after digestion of the PCR products with 4 cutters. The 350 bp fragment was observed for all the resistant individuals but none of the susceptible individuals.

3.2. Gene interactions

3.2.1. Gene interactions in the population of Xian-feng 1/Tetep

In both populations, three types of F_3 lines were observed in terms of their resistance segregation patterns, i.e. all individuals resistant to the race ZC13, segregation for resistance, and all individuals susceptible. They were referred as resistant lines, segregating lines and susceptible lines, respectively.

Of the total 161 F_3 lines of Xian-feng 1/Tetep, the number of resistant lines, segregated lines and susceptible lines were 68, 78 and 15, respectively. This fitted the expected ratio of 7:8:1 (P=0.275), when the resistance was controlled by duplicate interaction of two dominance genes (Table II). Of 1402 individuals in the 78 segregated F_3 lines, the numbers of resistant and susceptible plants were 1188 and 214, respectively. This was in good agreement with the expected ratio of 27:5 (P=0.710).

It was already known that a blast resistance gene *Pi-ta* in Tetep was closely linked to RFLP marker RZ397 on chromosome 12 [11]. In this study, RZ397 did not detect polymorphism between Xian-feng 1 and Tetep. Another linked marker RG81 was able to detect polymorphism between the two parents. Preliminary co-segregation analysis of RG81 and blast resistance indicated a gene for the resistance difference between Xian-feng 1 and Tetep was closely linked to RG81 (data not shown).

In order to detect the other resistance gene which was not linked to RG81, equal quantities of DNAs from 10 homozygous resistant F_2 individuals were mixed to construct a resistant pool (R pool), while those from 10 homozygous susceptible F_2 individuals were mixed to construct a susceptible pool (S pool). RAPD analysis of the pools was performed. Primers generating polymorphic RAPD bands between two pools were used to analyse their co-segregation with blast resistance. Thus far, three RAPD markers have been found to co-segregate with blast resistance, of which two were linked to RG81 and one was not linked to RG81. Mapping of the latter RAPD marker is underway using other mapping populations in our laboratory. After the marker is mapped, verification of the location of the two resistance genes will be conducted using F_6 or F_7 recombinant inbred lines of Xian-feng 1/Tetep.

TABLE II. GENETIC MODEL OF DUPLICATE INTERACTION OF TWO DOMINANT GENES FOR THE CONTROL OF BLAST RESISTANCE IN XIAN-FENG 1/ TETEP POPULATION

F_3 Phenotype	F_2 Genotype	Expected ratio
All plants were resistant	$R_1R_1R_2R_2$	1
	$R_1R_1r_2r_2$	1
	$r_1r_1R_2R_2$	1
	$R_1R_1R_2r_2$	2
	$R_1r_1R_2R_2$	2
Segregation	$R_1r_1R_2r_2$	4 (15R:1S) [a]
	$R_1r_1r_2r_2$	2 (3R:1S)
	$r_1r_1R_2r_2$	2 (3R:1S)
All plants were susceptible	$r_1r_1r_2r_2$	1
Total		16

[a]The numbers before ( ) refer to expected ratios for the number of F_3 lines, and those within ( ) refer to that for the number of individuals within each F_3 line, respectively. R= resistant; S= susceptible.

3.2.2. Gene interactions in the population of Xian-feng 1/Hong-jiao-zhan

Of the total 175 F_3 lines of Xian-feng 1/Hong-jiao-zhan, the number of resistant lines, segregating lines and susceptible lines were 31, 113, and 31, respectively. This ratio fitted none of single-gene or two-gene models, indicating the resistance difference between Xian-feng 1 and Hong-jiao-zhan may be controlled by three or more genes.

Supposing that the resistance is controlled by three genes while the joint effect of two homozygous susceptible genes resulted in susceptibility, the expected ratio of the numbers of resistant lines, segregating lines and susceptible lines would be 10:44:10 (Table 3). This ratio was well fitted by the observed data (P=0.491). Following the three gene controlling hypothesis, the expected ratio of the number of resistant and susceptible individuals in the 113 segregated lines was 135:41. This was also fitted by the observed data of 1545:488 (P=0.450), providing additional evidence for the hypothesis.

As described previously, a blast resistance gene *Pi-12(t)*, derived from Hong-jiao-zhan, was tightly linked to SCAR markers P265-560 and P286-350. By using P265-560, a 560-bp fragment was detected for Hong-jiao-zhan, and no fragment was detected for Xian-feng 1. After amplification of a subset of F_2 DNAs from bulked F_3 samples, the observed segregation of the 560-bp fragment was tested against the expectation (Table 4). The result (P=0.719) supported the hypothesis.

As the genetic control of the blast resistance in the population of Xian-feng 1/Hong-jiao-zhan seems to be rather complicated, identification of resistance genes other than *Pi-12(t)* will not be conducted until the two resistance genes in the population of Xian-feng 1/Tetep are mapped more precisely.

TABLE III. HYPOTHESIS OF THREE-GENE INTERACTION FOR THE BLAST RESISTANCE IN XIAN-FENG 1/HONG-JIAO-ZHAN POPULATION

F_3 Phenotype	Condition	F_2 Genotype			Expected ratio[a]
All plants resistant	Homozygosity for resistance alleles at two or all three loci	R_1R_1	R_2R_2	R_3R_3	1
		R_1R_1	R_2R_2	$--r_3$	3
		R_1R_1	$--r_2$	R_3R_3	3
		$--r_1$	R_2R_2	R_3R_3	3
All plants susceptible	Homozygosity for susceptible alleles at two or all three loci		r_1r_1	r_2r_2	r_3r_3 1
		r_1r_1	r_2r_2	r_3--	3
		r_1r_1	r_2--	r_3r_3	3
		R_1--	r_2r_2	r_3r_3	3
Segregation	All the others	R_1r_1	R_2r_2	R_3r_3	8 (27R:5S)[a]
		R_1r_1	R_2r_2	R_3R_3	4 (15R:1S)
		R_1r_1	R_2R_2	R_3r_3	4 (15R:1S)
		R_1R_1	R_2r_2	R_3r_3	4 (15R:1S)
		R_1r_1	R_2r_2	r_3r_3	4 (9R.7S)
		R_1r_1	r_2r_2	R_3r_3	4 (9R.7S)
		r_1r_1	R_2r_2	R_3r_3	4 (9R:7S)
		r_1r_1	R_2r_2	R_3R_3	4 (3R.1S)
		r_1r_1	R_2R_2	R_3r_3	4 (3R.1S)
		R_1R_1	r_2r_2	R_3r_3	4 (3R·1S)
Total					64

[a]The number before ( ) refer to expected ratios for the number of F_3 lines, and those within ( ) refer to that for the number of individuals within each F_3 line, respectively R= resistance; S= susceptible.

TABLE IV. THE NUMBER OF F_2 INDIVIDUAL WITH PRESENCE OR ABSENCE OF THE 560-BP FRAGMENT IN XIAN-FENG 1/HONG-JIAO-ZHAN

Resistance pattern	Observed number		Expected ratio	$P\chi^2$
	presence	absence	(presence : absence)	
Resistance	5	0	9 : 1	0.456
Segregation	20	7	32 : 12	0.875
Susceptible	5	10	3 7	0.778
Total	30	17		0.719

From the above results, it can be seen that the mode of gene interactions for blast resistance may vary greatly across populations. Using classical approaches, the mode of gene interactions can only be deduced statistically. When tightly linked markers become available, it should be possible to verify the genetic mode of action.

2.3. Initiation of DNA marker-assisted selection

Crosses were made between elite varieties and blast resistance donors in Hainan Province in the winter of 1995. Elite varieties used included an early *indica* variety Zhong 156 and several parental lines of commercial F_1 hybrids, and blast resistance donors used including Hong-jiao-zhan, C101A51 (*Pi-2*) and C101PKT (*Pi-4*). The F_1s was grown in CNRRI in 1996 and backcrossed with elite varieties. A polymorphism survey was conducted using DNA markers linked to resistance genes. In the following years, DNA marker-assisted selection for blast resistance will be employed.

Forty-eight varieties which were widely used in current rice breeding programs were provided by rice breeders. Polymorphism among the 48 varieties and resistance donors are being surveyed by using STS (Sequence Tagged Site) and RFLP markers. More than 40 STS markers have been used, and data are being collected. Based on five STSs linked to disease resistances (rice blast or bacterial leaf blight), pair-wise comparisons indicated that the polymorphism frequency was 13-52%. After digestion of the PCR products with 4 cutters, the frequency was 28-66%. When the data are all collected, they will be distributed to the rice breeders who provided the varieties. It is expected the database will be helpful to breeders for making appropriate decisions whenever they are interested in transferring resistance genes to their materials.

ACKNOWLEDGEMENTS

This research was supported by grants from the Rockefeller Foundation Rice Biotechnology Program, the International Atomic Energy Agency and the State Science and Technology Commission of China. The authors would like to thank Professors Shen Z. and H. Zuhua for providing rice seeds of NILs K80R and K79S, and other rice breeders in Zhejiang and Guangdong provinces for providing rice seeds for the establishment of the database, professors Yang C. and Chai R. for screening the blast resistance, and Shen B. for help during the early stages of this research. Thanks also due to Dr. Tanksley's laboratory at Cornell University, Ithaca, USA, for providing DNA clones. Mention should be made of the fact that part of the RFLP analysis was conducted in Dr. Kochert's laboratory at Georgia University, Atlanta, USA.

REFERENCES

[1] OU, S.H. Rice Disease, Commonwealth Agricultural Bureaux, Farham Royal (1985) 109-200.

[2] BROWING, J.A. and FREY, K.J. Multiline cultivars as a mean of disease control, Annu. Rev. Phytopathol. 7 (1969) 355-382.

[3] TANKSLEY, S. *et al.* RFLP mapping in plant breeding - new tools for an old science, Biotechnology 7 (1989) 257-264.

[4] WILLIAMS, G.K. *et al.* DNA polymorphisms amplified by arbitrary primers are useful as genetic marker, Nucleic Acids Res. 18 (1990) 22: 6531-6530.

[5] ZHENG, K.L. *et al.* Identification of DNA markers linked to a blast resistance gene in rice. Induced mutations and molecular techniques for crop improvement, Proceeding of an International Symposium on the Use of Induced Mutations and Molecular Techniques for Crop Improvement, Vienna, 19-23 June 1995, (1995) pp 253-261.

[6] ZHENG, K.L. *et al.* Restriction fragment length polymorphism in rice, Chin. J. Rice. Sci. 4 (1990) 4: 145-149.

[7] LIU, Y.J. and ZHENG, K.L. A simple method for extraction of DNA in rice, Chin. J. Rice Sci. 6 (1992) 1: 47-48.

[8] LANDER, E.S. *et al.* MAPMAKER: An interactive computer package constructing primary genetic linkage maps of experimental and natural populations, Genomics 1 (1987) 174-181.

[9] KOSAMBI, D.D. The estimation of map distances from recombination values. Ann. Eugen. 12 (1944) 172-175.

[10] ZHENG, K.L. *et al.* PCR-based marker-assisted selection in rice improvement. IRRI Discussion Paper Series No. 12. International Rice Research Institute, P.O. Box 933, Manila, the Philippines (1995).

[11] Yu, Z. *et al.* Tagging genes for blast resistance in rice via- linkage to RFLP markers. Theor. Appl. Genet. 81 (1991) 471-476.

RAPD TAGGING OF A SALT TOLERANCE GENE IN RICE

H. DING, G. ZHANG, Y. GUO, S. CHEN, S. CHEN
Plant Biotechnology Laboratory,
Institute of Genetics,
Academia Sinica,
Beijing, China

Abstract

Salinity, which is critical in determining the growth and development of plants, is a major problem affecting ever-increasing areas throughout the world. A salt tolerant rice mutant (M-20) was obtained from accession 77-170 (*Oryza sativa*) through EMS mutagenesis and selection *in vitro* [1, 4]. The use of 220 10-mer RAPD primers allowed the identification of a new molecular marker, whose genetic distance from a salt tolerance gene is about 16.4 cM.

1. INTRODUCTION

Salinity is a major problem affecting ever-increasing areas throughout the world. s. Research on plant osmoregulation through molecular biology is accompanied by crop breeding programmes including marker-assisted selection and genetic engineering. It is currently an area of active investigation seeking salt tolerance-relevant genes and their linked molecular marker(s). A salt tolerant rice mutant (M-20) was obtained from rice accession 77-170 (*Oryza sativa*) through EMS mutagenesis and selection *in vitro* [1, 4]. Inheritance analysis demonstrated that a major salt tolerance gene was present in these materials, and that it was associated with a single copy RFLP probe, RG4. The genetic distance between the trait and the marker was shown to be 7.0 +/- 2.9cM [2, 3, 4]. The use of 220 10-mer RAPD primers allowed the identification of a new molecular marker, whose genetic distance from a salt tolerance gene is about 16.4 cM.

2. MATERIALS AND METHODS

2.1. Plant materials

Anthers of rice 77-170 (*Oryza sativa*) were used to obtain calli on N_6 medium, which were then mutagenized with EMS. The selection of NaCl-tolerant mutants was carried out on N_6 medium containing 1% NaCl. After continuous selection, five mutant lines were obtained, the salt tolerance of which has been stably inherited over nine generations [1].

2.2. Evaluation of salt tolerance

The F_1 hybrids of 77-170 X M-20 were planted under normal conditions. Since it is possible to split rice into two parts in the seedling stage, one part of each F_2 plant was planted under normal conditions and the other part was planted in the saline pool containing 0.5% NaCl.

2.3. Rice DNA extraction

Three to five grams of rice leaf tissue were ground into a fine powder in liquid nitrogen, to which was added 16 ml of a 65°C preheated extraction solution (100 ml Tris-HCl, pH8.0; 50 mM EDTA; 500 mM NaCl; 1.25% SDS(w/v), add 0.38g Na_2SO_4 per 100 ml just before use), mixed well by inverting the tube and incubated at 65°C for 20 minutes. To this was added 5 ml of 5M KAc, followed by gentle mixing and incubation on ice for 20 minutes.

This was centrifuged and the supernatant transferred to a new tube to which an equal volume of chloroform: isoamyl (24:1) was added to remove protein and pigment. To this, 2/3 volume of isopropanol was added to the upper layer to precipitate DNA. RNase was added to dissolve the RNA. The DNA was resuspended in TE buffer (50 mM Tris-HCl, pH8.0; 10 mM EDTA).

2.4. Construction of DNA pools

Pure resistant and pure sensitive individuals of the F_2 generation were selected to construct two DNA pools, a salt tolerant pool (+) and a salt sensitive pool (-). The "+" pool was composed of 10 highly salt tolerant individuals A31, A34, A44, A57, A60, A64, A84, A89, A106 and A107, while the "-" pool contained 7 salt sensitive individuals A26, A38, A43, A46, A82, A88 and A93.

2.5. RAPD primers

All of the 10-mer RAPD primers were purchased from OPERON TECHNOLOGY INC.

2.6. RAPD analysis

The reaction mixture (25microl/tube) included template DNA (30ng), 1 microl. 2.5 mM dNTPs each, 2 microl. 10-mer RAPD primer (50 pmol), 2.5 microl. 10X reaction buffer (500 mM Tris-HCl, pH 8.3: 500 mM KCl; 15 mM $MgCl_2$; 0.01% Gelatin), 0.9 unit of Taq DNA polymerase (Institute for Genetics laboratory). The mixture was overlaid with 25 microl. of paraffin oil.

Amplification was performed on a Perkin Elmer DNA Thermal Cycler 480. After initial denaturation (five cycles for 1 min at 94°C, 1 min at 36°C, 2 min at 72°C) the reaction was then continued for another 40 cycles with the following procedure: 94°C for 20 s, 36°C for 1 min, and 72°C for 2 min. Finally, the procedure ended at 72°C for 10 min. Amplification products were electrophoresed in 1.5% agarose gel (containing 1microg./ml EB). The results were observed under UV light and recorded by photography.

3. RESULTS

Backcrosses of the 11th generation of salt tolerant mutant line and the original F_1 generation line were planted under normal conditions in the field. Individuals selected from the F_2 generation (100) were split into two parts; one planted under normal conditions and the other in a saline pool containing 0.5% NaCl. There was no obvious segregation among different individuals of the F_2 under normal conditions. In the saline pool, 72 and 78 individuals set seed, when M-20 and 77-170 were the respective maternal plants. The segregation ratio is about 3:1 (Table I). The same results were obtained from reciprocal crosses indicating that the mutation is controlled by nuclear genes and there exists a major gene enabling seed setting under saline conditions.

The two DNA pools were used as templates to perform RAPD amplifications. Three primers, OPS-12, OPS-04, and OPO-09, showed polymorphism between the "+" and the "-" pools. The base sequences of these primers are listed in Table II. Figure 1 shows some results of the RAPD screening.

TABLE I. NACL-TOLERANT SEGREGATIONS IN F_2 POPULATIONS OF M-20 X 77-170 AND GOODNESS OF FIT TO MONOHYBRID RATIO

Generation	Crosses	No. of seed-setting plants		No. of plants not setting seed		P(1 df)	No. of plants
		Saline	Normal	Saline	Normal		
R_{11}	M-20 x170	72	100	28	0	~0.50	100
	170xM-20	78	100	22	0	~0.50	100

TABLE II. SEQUENCES OF RAPD PRIMERS THAT DISPLAY DIFFERENTIAL PATTERNS

RAPD primers	Sequences
OPS-12	CTGGGTGAGT
OPS-04	CACCCCCTTG
OPS-09	TCCCACGCAA

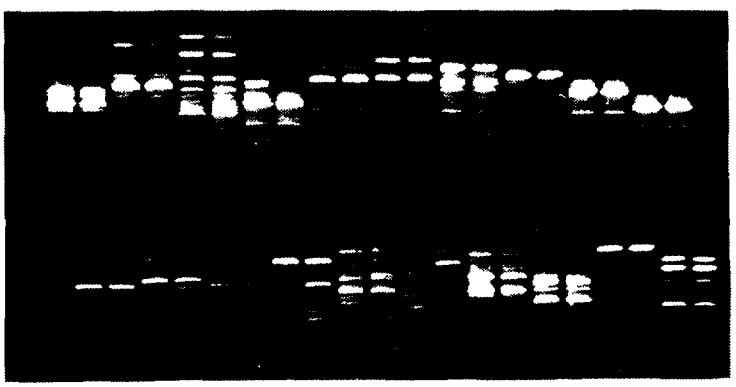


Fig. 1. Comparison of RAPD results from screening the "+" and "-" pools (the lanes alternate "+" and "-" pool; a different primer is used every two lanes).

Analysis of 80 individuals of the F_2 generation by use of the RAPD primers mentioned above. A 1 kb band was verified to be linked with the salt tolerance gene and the RFLP probe, RG4. Figure 2 shows this band and some of OPS-12 RAPD analysis in the F_2 generation.

The band shown in the figure is linked with RFLP probe RG4. Except for the two lanes noted, other lanes are all F2 individuals.

Fig. 2. The RAPD results of OPS-12 in the F_2 population.

The linkage between the RAPD marker, the RFLP probe RG4 and the locus relevant to salt tolerance was calculated with the program MAPMAKER. The result is shown in Figure 3. The genetic distance between the RAPD marker and RG4 is 5.0 cM, and between the RAPD marker and salt tolerance gene it is 16.4 cM.

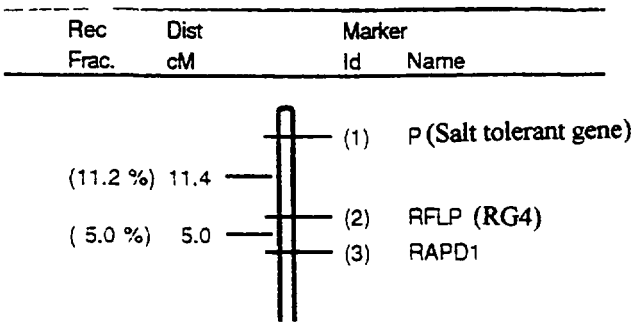


Fig. 3. The linkage between two probes and the locus relevant to salt tolerance.

3. DISCUSSION

Salt tolerance is a character controlled by multiple genes, and it has continuous phenotypic distribution. For this reason, it is regarded as a quantitative character. Despite this, there may exist a major gene responsible for a large part of the genetic variance for salt tolerance. In the F_2 generation of our materials, the ratio of salt tolerant to salt sensitive plants was about 3:1, where salt tolerance was evaluated on the ability to set seeds. This result indicated that salt tolerance is controlled by a major gene and modified by some other minor genes. Zhang et al. [3] tagged the major gene by a single copy probe, RG4, which is located on chromosome 7, at a genetic distance of 7.0 +/- 2.9 cM. We found a new marker near RG4, thus enhancing the possibility of mapping salt tolerance genes.

Although RAPD has some merits such as efficiency, simplicity and saving time, it still has a few shortcomings. Spurious bands often appear so that the amplification must be performed under specific conditions. The template DNA, polymerase concentration and the renaturation temperature should be kept in a suitable range. Moreover, we use individuals of pure character to construct the pools so as to diminish other factors irrelevant to salt tolerance.

We have to identify the phenotype of the F_2 by other criteria because of the nonexistence of an F_3 generation. It brings some uncertainty to our research and it is necessary for us to pay attention to DNA contamination and other sources of error. In the right part of Fig. 2, the pattern of the 6th lane is obviously different from others. It is suspected to be the result of DNA contamination.

REFERENCES

[1] CHEN, S.-L., Expression of M13 plant of NaCl-tolerant pollen plant in rice, In: Genetic Manipulation in Crops, Inst. of Genet. Chinese Acad. Sci., Beijing (1988) 85-86.

[2] CHEN, S., ZHU, L.-H., HONG, J., CHEN, S.-L., Molecular biological identification of a rice salt-tolerant line, Acta Bot. Sinica 33 (1991) 569-573.

[3] ZHANG, G.-Y., GUO, Y., CHEN, S.-L., CHEN, S.-Y., RFLP tagging of a salt tolerance gene in rice, Plant Sci. 110 (1995) 227-234.

[4] GUO, Y., CHEN S.-L., ZHANG, G.Y., CHEN. S., Salt-tolerance rice mutant lines controlled by a major effect gene were obtained by cell engineering technique, 24 (1997) 122- 126 [In Chinese, English Abst.].

RICE GENOME MAPPING AND ITS APPLICATION IN RICE GENETICS AND BREEDING

M.Y. EUN, Y.G. CHO, J.H. HAHN, U.H. YOON, B.Y. YI, T.Y. CHUNG
National Institute of Agricultural Science and Technology, RDA
Suwon, Republic of Korea

Abstract

An 'MG' recombinant inbred population which consists of 164 F_{13} lines has been developed from a cross between a Tongil type variety Milyang 23 and a Japonica type Gihobyeo by single seed descent A Restriction Fragment Length Polymorphism (RFLP) framework map using this population has been constructed Morphological markers, isozyme loci, microsatellites, Amplified Fragment Length Polymorphisms (AFLP), and new complementary DNA (cDNA) markers are being integrated in the framework map for a highly saturated comprehensive map. So far, 207 RFLPs, 89 microsatellites, 5 isozymes, 232 AFLPs, and 2 morphological markers have been mapped through international collaboration. The map contains 1,826 cM with an average interval size of 4 5 cM on the framework map and 3.4 cM overall (as of 29 October 1996)

The framework map is being used for analyzing quantitative trait loci (QTL) of agronomic characters and some physico-chemical properties relating to rice quality. The number of significant QTLs affecting each trait ranged from one to five, and 38 QTLs were detected for 17 traits The percentage of variance explained by each QTL ranged from 5 6 to 66.9%.

The isozyme marker, *Est1-2*, and two RFLP markers, *RG109* and *RG220*, were linked most tightly at a distance less than 1 cM with the semidwarf (*sd-1*) gene on chromosome 1. These markers could be used for precise *in vitro* selection of individuals carrying the semidwarf gene using single seeds or very young leaf tissue, before this character is fully expressed

Appropriate application of marker-assisted selection, using *Est1-2* and RFLP markers for the semidwarf character, in combination with other markers linked to genes of agronomic importance in rice, holds promise for improving the efficiency of breeding, and the high-resolution genetic and physical mapping near *sd-1*, aimed at ultimately cloning this valuable gene.

The Korea Rice Genome World Wide Web (WWW) Server was constructed to distribute the rice research and genomic information around world The Korea Rice Genome WWW Server and a mirror WWW site for Rice Genes are maintained at the National Institute of Agricultural Science and Technology, RDA (http //sun20.asti.re kr) and Department of Biological Science, Myongji University (http //bioserver myongji ac kr)

1. INTRODUCTION

Plant genomics has emerged as one of today's most important tools for studying plant biology. Genomics is the science of investigating genomes, both globally and locally, for the study of genome organization and evolution, physical mapping, and gene cloning using the tools of molecular biology. Historically, genomics received wide attention in the early 80's with the discovery that genetic maps could be made with molecular markers, such as restriction fragment length polymorphisms (RFLPs) [1]. Molecular markers on genetic maps serve as landmarks along chromosomes, and serve as entry points for gene cloning and physical mapping.

Rice, a staple food crop in Korea, is grown widely in every corner of the country. Rice breeding which is one of the most significant accomplishments in Korea became the focal point of the agricultural research and development. With the rapid population increase, there is still much current interest in breeding for consistent yields and resistance to diseases and insects, and environmental stresses.

In recent years, the National Institute of Agricultural Science and Technology (NIAST) has developed long-term research projects on rice genome mapping and its applications in genetics and practical breeding in the Korea Rice Genome Research Program (KRGRP). A comprehensive gene map has been constructed using a recombinant inbred population

developed at NIAST. Gene tagging experiments based on linkages between morphological characters and molecular markers have been carried out with intensive cooperation between scientists in the fields of biotechnology and breeding for the early utilization of these newly emerging high-technologies in practical breeding.

2. DEVELOPMENT OF A RECOMBINANT INBRED LINE AS A PERMANENT MAPPING POPULATION

The use of a recombinant inbred (RI) population as the basis for mapping provides an eternal mapping resource [2]. This means that obtaining DNA is not a limiting factor, and genetically pure seed can be reliably reproduced from each line. RI populations are especially advantageous for genetic analysis of quantitative traits because experiments can be replicated over years and environments using identical genotypes. If the parental combination selected for RI population development is of direct interest in a breeding program, the use of molecular marker-assisted breeding will be greatly facilitated.

An eternal RI population consisting of 164 F_{13} lines was developed from a cross between Milyang 23, (an Indica/Japonica derivative known as the Tongil type), and Gihobyeo (Japonica type) (hereafter the MG RI population) at NIAST. The parents were crossed in 1988 and F_2 seeds were selfed, with generations progressing via single seed descent (selected randomly) until the F6 generation. F7 seeds were planted in single rows in the field and the recombinant inbred lines were cultivated by row via single seed decent until the F_{13} generation (selected one major-plant type) (Table I).

TABLE I. DEVELOPMENT OF MILYANG 23/GIHOBYEO RECOMBINANT INBRED LINED FOR HIGHLY SATURATED MOLECULAR MAPPING IN RICE BY SINGLE SEED DESCENT

Milyang 23/Gihobyeo Recombinant Inbred Population for Highly Saturated Molecular Mapping

Generation : F_{13} seeds harvested ('96 Fall)
Method : Single Seed Descent
Population size : 164 lines
Characteristics :
- Excellent Population Structure (Abundant Seed and Easy to Propagate)
- Sufficient Population Size for Fine Mapping of Target Genes
- High Level of Polymorphism
- Immediate Availability of Pure Lines
- Many Agronomically Valuable Characteristics were Embodied by These Varieties (Especially for QTL mapping)
- Will be Available as **"International Reference Mapping Population"**

Milyang 23, the maternal parent, was selected from a cross between Suwon 232 and IR24 and released in 1976. Milyang 23 has many favorable genes and has been frequently used as a parent in rice breeding programs and a number of rice varieties have been developed from these progenies. Gihobyeo, the paternal parent, was developed and released in 1983 from a cross between Fuji 280 and BL 1. These two parents represent genetically divergent types, providing ample segregation of both molecular marker alleles and agronomically important genes and quantitative trait loci (QTL).

The MG RI population offers good resolution for the development of a highly saturated map of rice. The fact that individual RI genotypes are selfed and can be propagated indefinitely by seed makes this population especially useful for replicated experiments to localize and fine map genes and QTLs. The fact that both parental genotypes are agronomically acceptable makes it useful for near isogenic line development and marker-aided selection. The population and the associated molecular marker dataset are publicly available and it is therefore envisioned that it will be useful as an international mapping resource for rice.

3. CONSTRUCTION OF A MOLECULAR MAP

The construction of truly saturated maps requires the analysis of large numbers of DNA markers capable of sampling all regions of the genome. A combination of molecular markers capable of sampling all types of sequence configurations will provide the best genome coverage. RFLP markers generally represent single and low copy sequences, due to the nature of Southern analysis. Microsatellites or simple sequence repeats (SSR) are derived from repetitive DNA, but can be detected as individual loci because of the unique sequences flanking the microsatellite motifs [3]. Amplified fragment length polymorphism (AFLP) analysis complements other marker systems in that these markers may be derived from any portion of the genome that can be digested with restriction enzymes.

The recently developed AFLP technique complements other marker systems and efficiently detects abundant polymorphism. The principle of the AFLP method is basically quite simple. DNA is cut with restriction enzymes, and ds adapters are ligated to the end of the DNA-fragments. In this way, the sequence of the adjacent restriction site and the adapters serve as primer binding sites for subsequent amplification of the restriction fragments [4]. Selective nucleotides are added to the 3' ends of the PCR primers, which recognize only a subset of the restriction sites. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides are amplified. Substitution of the silver staining method in the AFLP technique gives rise to better resolution and convenience of handling over the use of radioisotopes [5]. This approach to molecular mapping promises great efficiency because of the ability to screen large numbers of DNA fragments in a single lane of a polyacrylamide gel, thus increasing the possibility of identifying polymorphisms and expediting the construction of high density linkage maps.

An AFLP map of rice has been constructed with the F_{11} recombinant inbred (RI) population. The mapping population was developed by a single seed descent method from an intercross between Milyang23 (M) (Tongil type) and Gihobyeo (G) (japonica type) and consists of 164 lines [17]. The polymorphism between parents was about 80 percent. A subset of the RFLP markers previously mapped by Causse *et al.* [6] and Kurata *et al.* [7], and microsatellite markers [8] were used to construct a framework map. We then integrated microsatellite and AFLP markers into the RFLP map, aiming to fill gaps and enhance the level of saturation [17]. With the five *EcoR*I+2 and *Mse*I+3 primers, 10 different primer combinations could be analyzed with the parents and 164 RI lines. Each primer combination generated a range of 73-134 bands visible on polyacrylamide gels, with a mean of 101 and an average of 27 polymorphic bands (Fig. 1), which are greater than those of AFLP map in barley [9, 10]. Of the 1 011 AFLP bands from 10 primer combinations, 269 (26.6%) were polymorphic in the MG RI population. The best two combinations are *E13/M59* and *E26/M47*, which showed over 30% polymorphism. So far, 232 AFLP markers have been integrated onto the RFLP map. The enzyme combination *EcoR*I/*Mse*I was good for small-genome crops such as rice (Fig. 1). It generated more bands and polymorphisms than the *Pst*I and *Mse*I

combination in DH population, which showed 945 AFLP bands from 20 primer combinations and 208 (21.8%) polymorphic bands [10]. The polymorphism rate with AFLP markers in the barley mapping population was 11.3% [9]. The polymorphism rate of AFLP markers is much smaller than RFLP markers, but they are extremely efficient because they allow the simultaneous analysis of a large number of bands per gel.

A total of 207 RFLP markers, 232 AFLP markers, 89 microsatellites and 5 isozyme loci and 2 morphological markers were integrated into the MG map. All marker types were well distributed throughout the 12 chromosomes. The integrated map covered 1,826 cM, and h ad an average interval size of 4.5 cM on the framework map, or 3.4 cM overall (as of Oct.29, 1996) (Fig. 2).

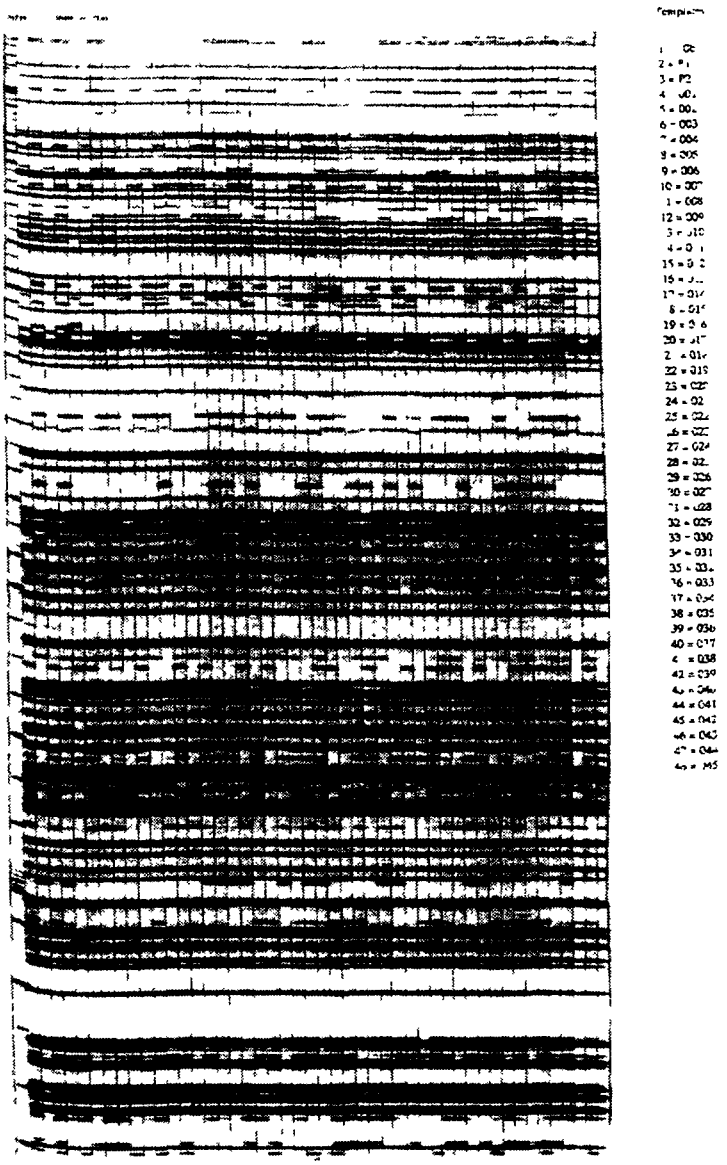


Fig. 1. Autoradiogram obtained with E13/M59 primer combination. In the left margin of the image the marker names and alignment bands are indicated. In the right margin the template identity is given. 10BL indicated the 10-base ladder; P1 and P2 indicate the parents, next are the original numbers from the MG RI population.

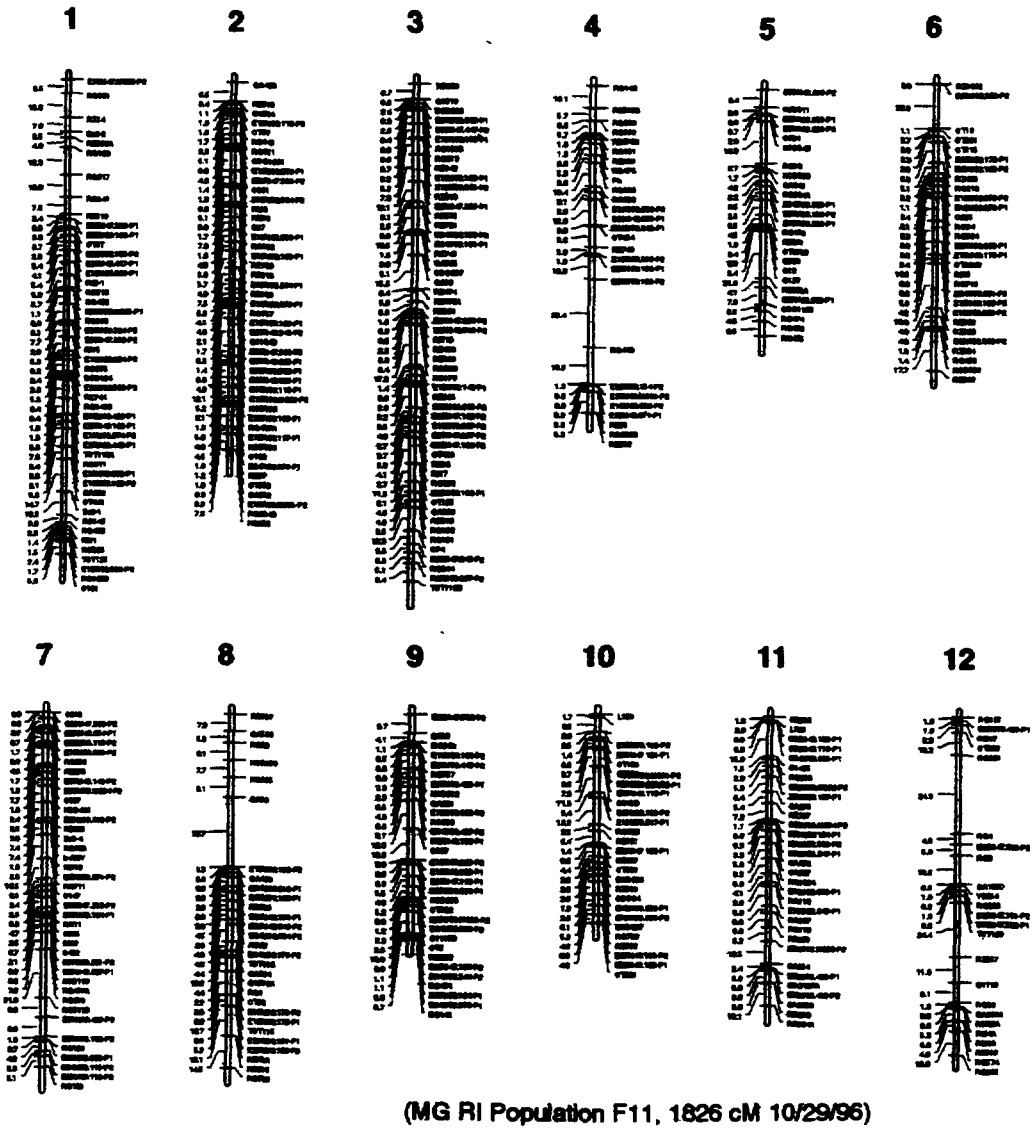


Fig. 2. The integrated map based on AFLP, RFLP, and microsatellite markers. Map distances (on the left) are given in cM (Kosambi function). AFLP markers are designated by E00M00.000 and highlighted by italics.

The existing MG map is a cornerstone of the Korean Rice Genome Research Program (KRGRP) and is being continuously refined through the addition of partially-sequenced cDNA markers derived from an immature seed complementary DNA (cDNA) library developed in Korea, and microsatellite markers developed at Cornell. The population is also being used for (QTL) analysis and as the basis for marker-assisted variety development.

4. QTL MAPPING OF AGRONOMIC CHARACTERS USING MILYANG 23/GIHOBYEO RI POPULATION

This study was carried out for the construction of a molecular map and QTL analysis of some agronomic traits. The 164 MG RILs of F_{11} derived from the cross between Milyang 23, Indica/Japonica hybrid type, and Gihobyeo, Japonica type, were evaluated for 21 quantitative traits, including physical properties of cooked rice, in 1995 at Iksan, in southwest Korea. Molecular map construction and QTL analysis was performed using MAPMAKER and

MAPMAKER-QTL programs. All traits examined fitted approximately a normal distribution and transgressive segregants were observed. The total genome size of the molecular map was 1,290.2 cM and 132 RFLP markers were located on twelve chromosomes, so the mean distance between markers was 9.8 cM. The number of significant QTL(s) (LOD2.0) affecting each trait ranged from one to five; a total of 38 QTLs were detected for 17 traits However, panicle length and hardness, cohesiveness and elasticity of cooked rice yielded no detectable QTL. The percentage of variance explained by each QTL ranged from 5.6% to 66.9%.

Four QTLs significantly affecting days to heading were detected and the percentage of phenotypic variance explained by each QTL ranged from 7.3 to 11.3%. Fitting the four QTLs simultaneously, 39.7% of the phenotypic variation could be accounted for. Only one QTL which significantly influenced culm length was identified, located on chromosome 1; it accounted for 59.0% of total phenotypic variation. Two QTLs were mapped for both the number of panicles per hill and the number of spikelets per panicle. The simultaneous fit of the two QTLs explained 17.1% and 26.4% of the total phenotypic variation, respectively. Three QTLs significantly affected 1,000-grain weight and they accounted for 7.4% of the total phenotypic variation. Only one QTL was mapped for both percent of ripened grains and brown/rough rice ratio and, each QTL accounted for 12.6% and 5.6% of the total phenotypic variation, respectively. Two significant QTLs, on chromosomes 8 and 9, showed association with grain yield. Cumulatively, the two QTLs explained 25.7% of the total phenotypic variation (Table II).

TABLE II. CHARACTERISTICS OF QTLS DETECTED FOR YIELD AND YIELD COMPONENTS IN MILYANG 23/GIHOBYEO RECOMBINANT INBRED ('MG' RI) POPULATION

Trait	QTL	Ch no	Markers bordering the QTL	Total length	QTL POS	Peak LOD	% Var *	Phenotypic effect**
No of panicles per hill	NPH 1	1	RG636-RG1028	8 7	6 0	2 70	8 1	1 06
	NPH 2	12	RG869-RZ816	36.7	34 0	2 76	9 0	1 16
No of spikelets per panicle	NSP 1	1	G1184A-RG140	39.0	30 0	4 08	19 6	-25 55
	NSP 2	3	RZ142-RZ319	16.2	14 0	2 19	6 8	-15.01
Percent of ripened grain	PRG 1	1	RG458-RG655	2 9	0 0	4 79	12 6	7 32
1,000 grain weight	GWT 1	1	RG317-RG462	20 3	20 0	2 12	8 5	-1 40
	GWT 2	2	RZ53-C601	12.3	4 0	4 18	12.2	1 68
	GWT 3	8	C825-KCD379	7.0	4 0	3 21	9 7	-1 50
Brown/ rough rice ratio	BR 1	4	RZ590-RG161	1.5	0 0	2 03	5 6	-0 74
Yield	YD 1	8	RG885-RG598	86 9	34 0	2 18	16 9	-62 65
	YD 2	9	RG662-RG451	14 5	10 0	2 75	8 8	-53 96

* Percentage of variance explained by each QTL.

** The signs, +(omitted) and -, preceding phenotypic effect, represent that the J alleles in homozygous state had higher phenotypic effects than the respective M alleles in homozygous state, and that the J alleles in homozygous state had lower phenotypic effects than the respective M alleles in homozygous state, respectively

Five QTLs, the highest number of QTLs among the investigated traits, affected grain length, and three QTLs affected grain width. When fitted simultaneously, the five QTLs in grain length explained 53.8% and three QTLs for grain width explained 38.8% of the total phenotypic variation. Two QTLs significantly affected grain thickness, and they explained 16.2% of the total phenotypic variation. Three significant QTLs were identified in both grain white core and white belly. They explained 35.1% and 91.6% of the total phenotypic variation, respectively. QTLs detected in grain white belly showed the highest percentage of variance explained among the evaluated traits. Three QTLs affected alkali digestion value, and they explained 32.4% of the total phenotypic variation when fitted simultaneously. Only one QTL was detected in adhesiveness, gumminess, and chewiness of cooked rice, and each QTL explained 14.6, 7.6 and 7.6% of the total phenotypic variation, respectively (Fig. 3, Table III).

TABLE III. NUMBER OF QTLS, CHROMOSOME NUMBER, TOTAL PERCENTAGE OF VARIANCE EXPLAINED BY QTLS (% VAR.), AND HERITABILITY (H^2) OF EACH TRAIT

Trait	No. of QTLs	Chromosome No.	% Var.	h^2
Days to heading (DOTH)	4	1, 3, 6, 7	39.7	-
Culm length (CL)	1	1	59.0	0.963
No. of panicles per hill (NPH)	2	1, 12	17.1	0.497
No. spikelets per panicle (NSP)	2	1, 3	26.4	0.914
% of ripened grain (PRG)	1	1	12.6	0.596
1,000 grain weight (GWT)	3	1, 2, 8	30.4	0.976
Brown/rough rice (BR)	1	4	5.6	0.314
Yield (YD)	2	8, 9	25.7	0.536
Grain length (GL)	5	1, 3, 5, 10, 12	53.8	0.920
Grain width (G)	3	2, 8, 12	38.8	0.879
Grain thickness (GT)	2	2, 10	16.2	0.382
White core (WC)	3	4, 6, 8	35.6	-
White belly (WB)	3	2, 7, 8	91.6	-
Alkali digestion value (ADV)	3	3, 7, 8	32.4	-
Adhesiveness (ADH)	1	1	14.6	0.935
Gumminess (GUM)	1	4	7.6	0.794
Chewiness (CHE)	1	4	7.6	0.703

QTLs for eight investigated traits were located on chromosome number 1. Other QTLs were found on all the other chromosomes in the complement (n = 12) but no significant QTL was found on chromosome number 11 (Table III). The relationships between heritability and percentage of variance explained by QTLs were very significant (Fig. 4).

5. LINKAGE OF SEMIDWARF GENE (*sd-1*) WITH AN ESTERASE LOCUS, Est I-2

The semidwarf gene (*sd-1*) in rice (*Oryza sativa* L.) is one of the most important single genes in the history of rice improvement. It was first identified in the Chinese variety Dee-geo-woo-gen (DGWG), and was first released in the Taiwanese variety Taichung Native 1 (TN-1) in 1956 [11]. This recessive allele causes reduced culm length and has been widely used to confer lodging resistance, high harvest index, responsiveness to nitrogen fertilizer, and favorable plant type in the breeding of high-yielding rice varieties [11].

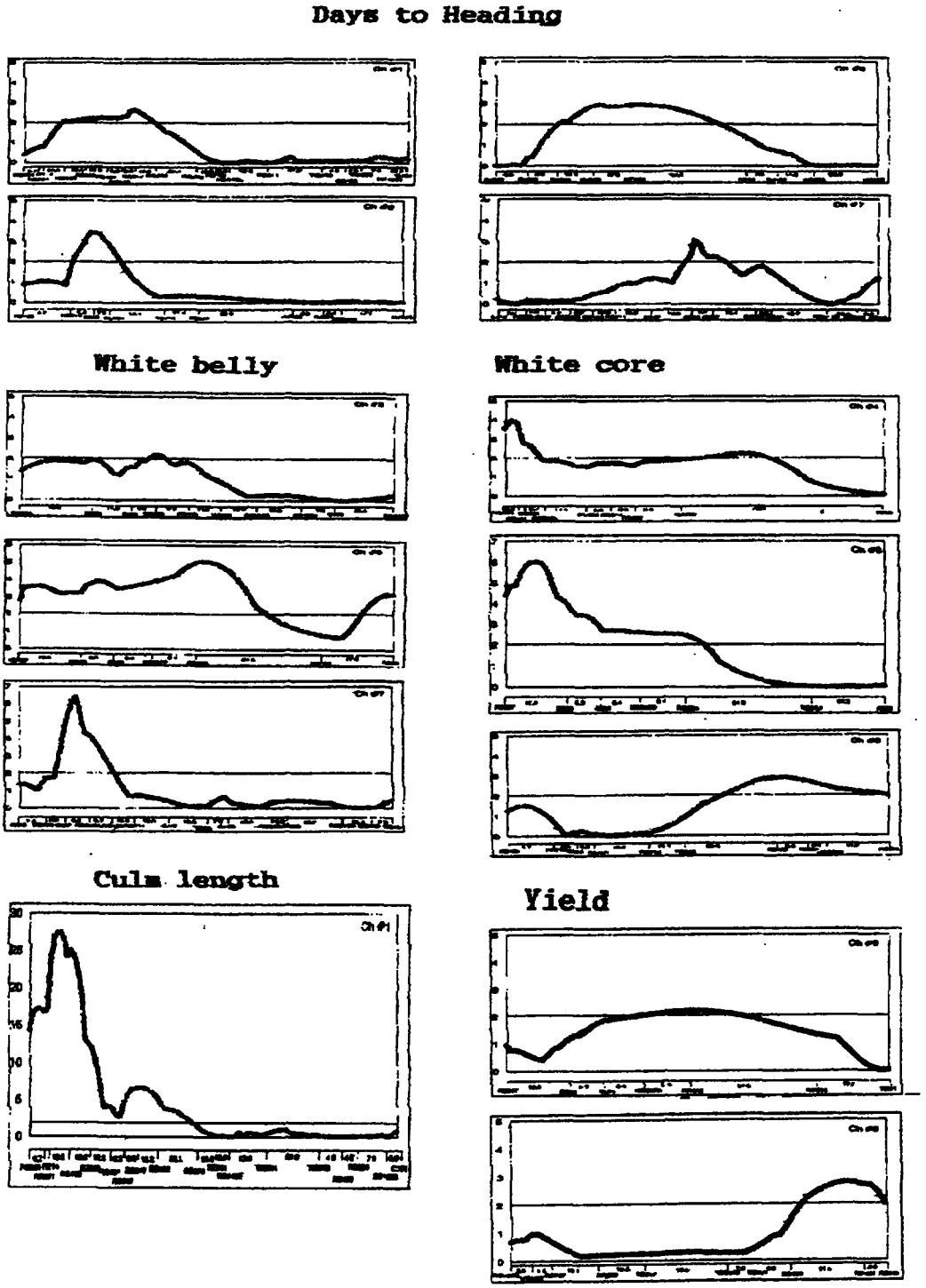


Fig. 3. QTL Likelihood plots indicating LOD scores and chromosome numbers for days to heading, white belly, white core, culm length, and yield. The left of each figure represents for LOD scores based on MAPMAKER-QTL and the top-right represents chromosome number. The RFLP linkage map used in the analysis is presented along the abscissa, in centiMorgan (cM) according to Kosambi (1944).

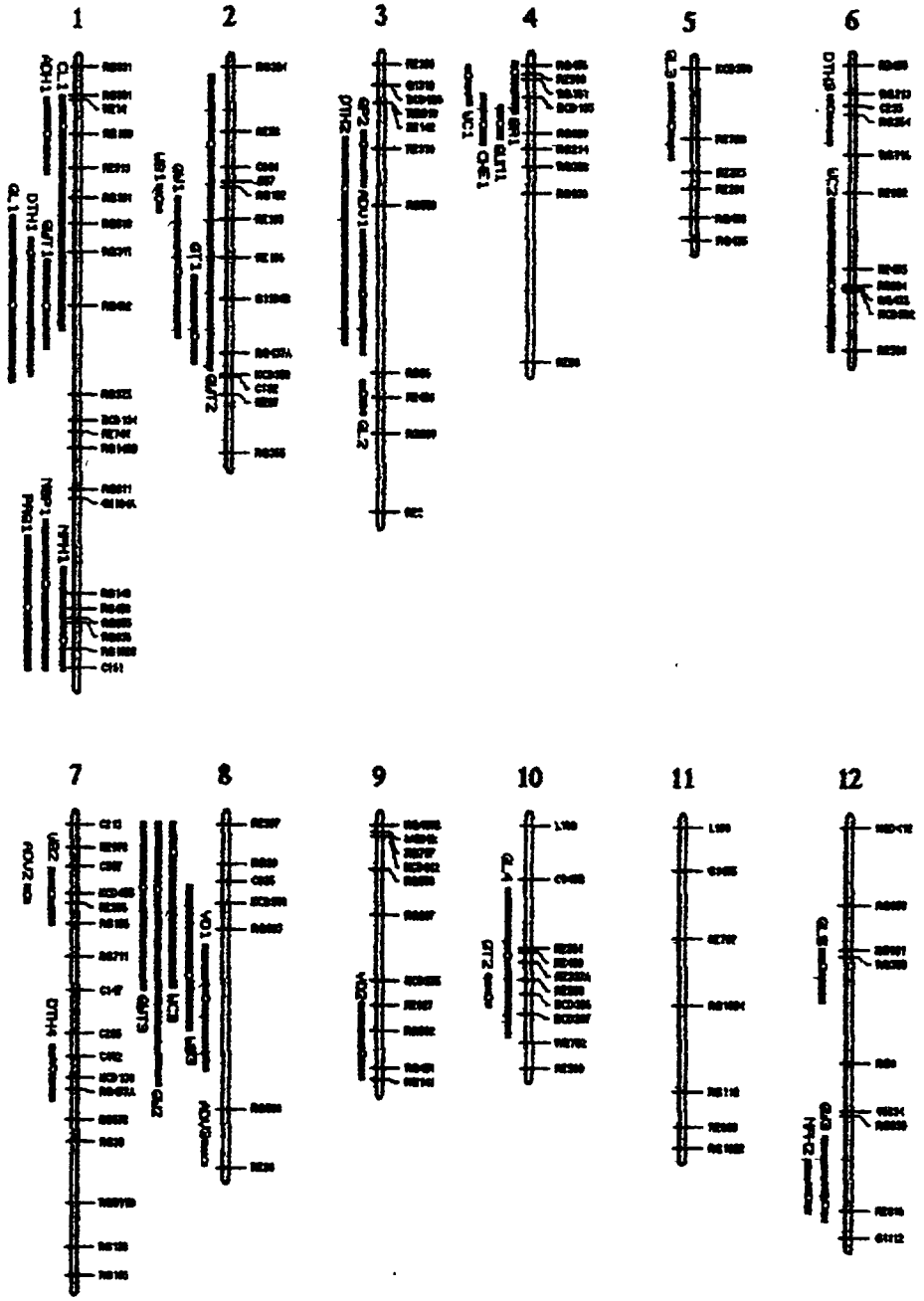


Fig. 4. Likelihood intervals for total QTLs mapped in F_{11} MG RILs. The position of QTL with LOD score above 2.0 is shown as the darkened bars. The names of the QTLs are given above or below the darkened bar and summarized in table 3. Open circles(d) on the darkened bars show the peak LOD scores that are the most probable position for the putative QTLs.

At least 60 dwarfing genes have been identified in rice. They are designated *d-1* to *d-60* [12]. Of these, *d-47*, or *sd-1*, has been most widely used in rice breeding. Most of the others have been used as phenotypic markers in genetic studies, but rarely used in plant breeding. From the classical linkage map, *sd-1* is known to be located on chromosome 1, and linked to *A* (anthocyanin activator), *Pp* (purple pericarp), *Pn* (purple node) and *Pau* (purple auricle) [13].

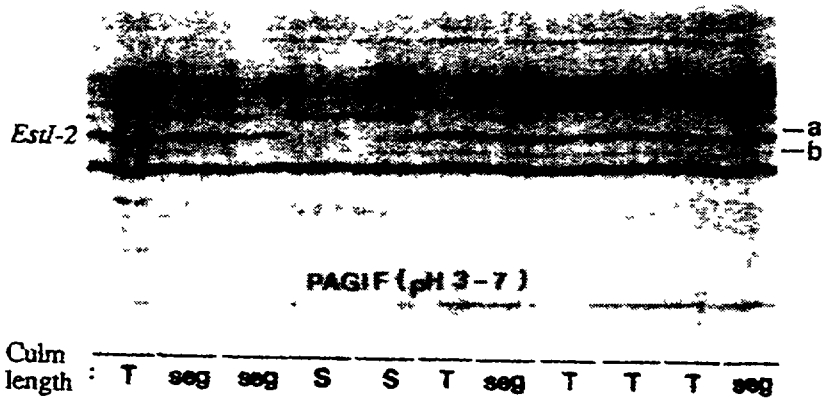


Fig. 5. Migration pattern of alleles at the EstI-2 locus in semidwarf and tall testers. a, semidwarf allele from Shiokari (sd-1), Taichung 65 (sd-1), and Milyang 23; b, tall allele from Shiokari and Taichung 65 (A, Pn, Pau). Segregation of EstI-2 alleles with culm lengths in F_3 families of Shiokari/Shiokari (sd-1); T, tall; S, short; seg, segregating for tall and short.

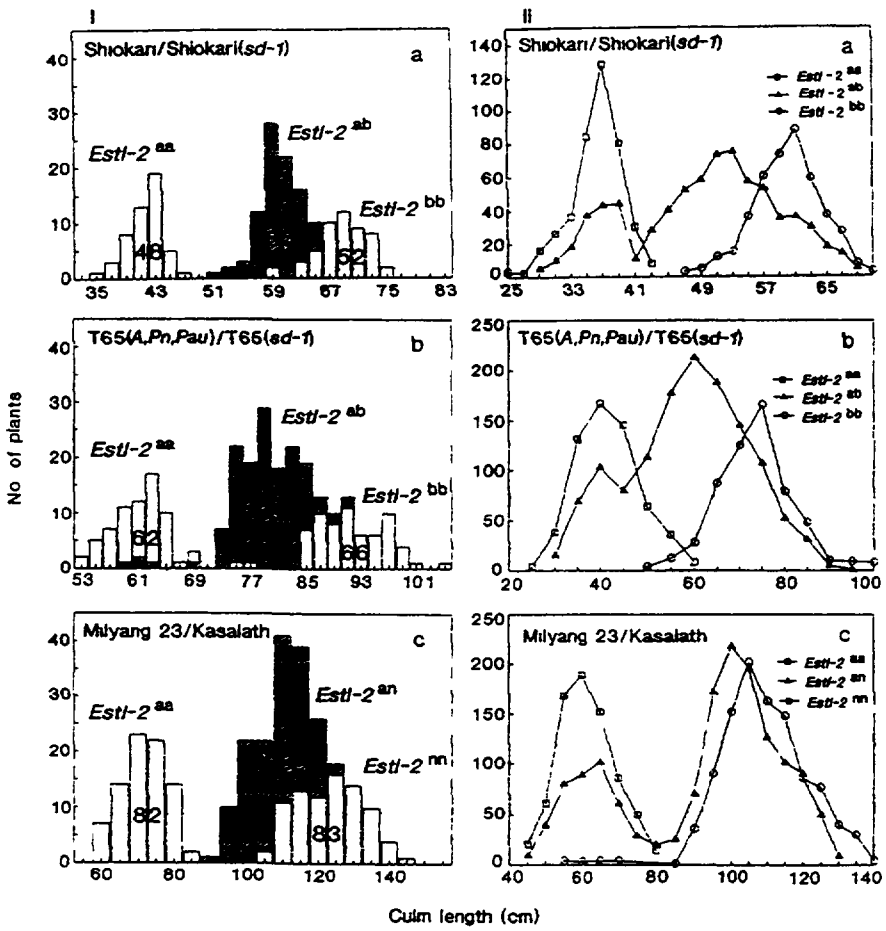


Fig. 6. The distributions of semidwarf and tall culm lengths and EstI-2 alleles in F_2 and F_3 families of (a) Shiokari/Shiokari (sd-1), (b) Taichung 65 (A, Pn, Pau)/Taichung 65 (sd-1), and (c) Milyang 23/Kasalath. I. Segregation of EstI-2 alleles in relation to culm length in F_2 populations. II. Segregation of EstI-2 alleles and culm lengths in F_3 families.

The linkage relationship between the semidwarf gene, *sd-1*, and the isozyme locus, *Estl-2*, was elucidated using segregating populations derived from crosses between several semidwarf testers and tall rice varieties. Bimodal distributions for culm length were observed in F_2 populations of three cross combinations, including Shiokari/Shiokari (*sd-1*), Taichung 65 (*A*, *Pn*, *Pau*)/Taichung 65 (*sd-1*), and Milyang 23/Kasalath. Taking the valley of the distribution curves as the dividing point, two height classes were apparent with a segregation ratio of 3 tall : 1 short, demonstrating this character to be under the control of a single recessive gene (Figs 5 and 6).

An inheritance study of esterase isozymes, based on isoelectric focusing (IEF), showed that the *Estl-2* locus had two active allozymes of monomeric structure and one null form, which were designated "*a*", "*b*", and "*n*", respectively [14]. Semidwarf testers such as Shiokari (*sd-1*), Taichung 65 (*sd-1*) and Milyang 23 have an active allozyme designated as *Estl-2*^{aa}, while the tall parents, Shiokari and Taichung 65 (*A*, *Pn*, *Pau*), have the active allozyme, *Estl-2*^{bb}, and Kasalath has a null form of the allozyme, *Estl-2*^{nn}. By dividing F_2 populations based on *Estl-2* allozyme patterns, culm length distributions exhibited trimodal curves. Most of the short plants had the homozygous *Estl-2*^{aa} pattern of the short parents, most of the tall plants had the homozygous pattern, *Estl-2*^{bb} or *Estl-2*^{nn}, and most of the intermediate plants had the heterozygous *Estl-2*^{ab} or *Estl-2*^{an} banding pattern. Linkage analysis indicated that *sd-1* and *Estl-2* were tightly linked (Fig. 6). These findings were also confirmed by segregation analyses in F_3 progenies. No recombinants were found among 171 F_3 families from the Shiokari/Shiokari(*sd-1*) combination, five recombinants were found among 267 F_3 families from Taichung 65 (*A*, *Pn*, *Pau*)/Taichung 65 (*sd-1*), and only two recombinants were found out of 237 F_3 families from Milyang 23/Kasalath. The recombination values were 0, 1.87 and 0.8%, respectively (Table IV).

TABLE IV. LINKAGE RELATIONSHIPS BETWEEN *ESTI-2* AND *SD-1* IN F_3 FAMILIES OF (A) SHIOKARI/SHIOKARI (*SD-1*), (B) TAICHUNG 65 (*A*, *PN*, *PA U*)/TAICHUNG 65 (*SD-1*) AND (C) MILYANG 23/KASALATH

Culm length	*Estl-2* allozymes in F_3			Total	LX^2	Recombination Value (%)
	aa	ab	b			
A: Shiokari/Shiokari (sd-1)						
TT	0	0	47	47		
Tt	0	80	0	80	351.0**	0
tt	44	0	0	44		
Total	44	80	47	171		
B: Taichung 65 (A, Pn, Pau) Taichung 65 (sd-1)						
TT	0	0	66	66		
Tt	1	133	1	135	504.5**	1.87
tt	63	3	0	66		
Total	6	136	67	267		
C: Milyang 23/Kasalath						
TT	0	0	67	67		
Tt	0	124	2	126	463 4**	0 8
tt	44	0	0	44		
Total	4	124	69	237		

6. MOLECULAR MAPPING OF SEMIDWARF GENE (*sd-1*)

To establish the location of the semidwarf gene, *sd-1*, the anthocyanin activator (*A*), purple node (*Pn*), purple auricle (*Pau*), and the isozyme locus, *EstI-2*, in relation to DNA markers on the molecular linkage map of rice, 20 RFLP markers, previously mapped to the central region of chromosome 1 [15], were mapped onto an F_2 population derived from the cross Taichung 65 (*A*, *Pn*, *Pau*)/Taichung 65 (*sd-1*). The *sd-1* and *EstI-2* were determined to be linked most tightly to RFLP markers *RG109* and *RG220*, which cosegregated with each other (Fig. 7). The distance between these RFLP markers and *sd-1* was estimated to be 0.8 cM, based on an observed recombination value of 0.8%. The order of genes and markers in this region of chromosome 1 was determined to be *sd-1*-(*EstI-2-RG220-RG109*)-*RG381-A-Pn-Pau* (Fig. 8).

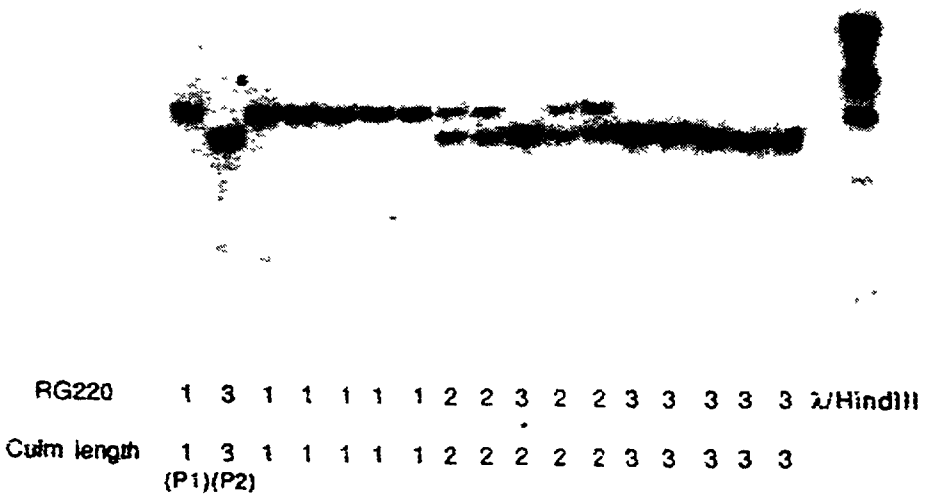


*Fig. 7. Cosegregation of the sd-1 gene and the RFLP marker RG220 in the F3 families derived from the cross Taichung 65 (A, Pn, Pau)/Taichung 65 (sd-1). XbaI-digested DNAs were blotted onto the filter. Genotypes: 1, homozygous for Taichung 65 (sd-1) (P1) allele; 2, heterozygous; 3, homozygous for Taichung 65 (A, Pn, Pau)(P2) allele; *, recombinant individual.*

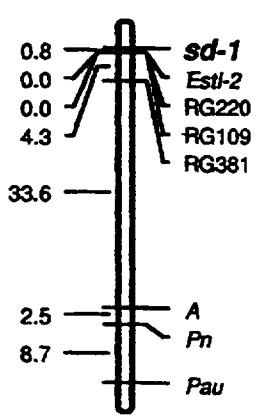


Fig. 8. Linkage map of rice chromosome 1 based on F_2 segregation of Taichung 65 (A, Pn, Pau)/Taichung 65 (sd-1). Kosambi cM to the left of the chromosome line; marker designations to the right of the chromosome line.

7. MARKER-ASSISTED SELECTION OF SEMIDWARF (*sd-1*) CHARACTER

Selection of a specific genotype from a segregating population is the main bottleneck in conventional breeding procedures. The utility of molecular markers in plant breeding is based on finding tight linkages between markers and genes of interest. Such linkages permit one to select indirectly for the presence of a desirable genotype by assaying for the molecular marker [16] and could significantly shorten the breeding cycle by their simplicity and reproductibility. In cases where a gene has been introduced into a wide array of germplasm from a single donor variety, markers identified in one population are likely to be useful in other populations that segregate for the gene.

The semidwarf phenotype cannot be clearly identified at the young seedling stage in rice. In addition, plant height is controlled by a variety of genes and is often normally distributed in breeding populations, making it difficult to unambiguously identify plants carrying the recessive *sd-1* gene. The efficiency of a breeding program would be increased if a reliable marker-assisted selection strategy could be used early in the life of the plants.

To test the efficacy of selection for *sd-1* based on the linked markers, *RG109* and *RG220*, and the isozyme locus, *EstI-2*, 203 50-day-old F_2 seedlings derived from the cross Milyang 23/Gihobyeo were analyzed for marker genotype. At this age, the semidwarf character could not be clearly detected based on phenotype. In addition, plant height was normally distributed in this population, making it difficult to unambiguously identify plants carrying *sd-1*. Thirteen seedlings homozygous for the *sd-1*-associated allele at *EstI-2*, *RG220* and *RG109*, and 13 seedlings homozygous for the *Sd-1*-associated allele at all three marker loci were selected for further genetic analysis.

At 20 days after heading, the culm lengths of these 26 plants were measured and the expected phenotype was confirmed in every case. These 26 plants were then selfed for four generations and F6 lines were evaluated to determine whether any recombination among the three molecular markers, or between these markers and the *sd-1* gene, could be detected. No recombinants were identified, confirming the tight linkage of these loci and the usefulness of marker assisted genotypic selection for this recessive semidwarf character before it is manifested phenotypically (Fig. 9).

Sequence Tagged Sites (STSs) are an alternative to cloned sequences as mapping markers. PCR allows the selective amplification of a region of DNA, providing that the sequence of a 20-30 nucleotide area flanking the area of interest is known. After end-sequencing the *RG109* insert at both ends, PCR primers were designed (Fig.10), and used to amplify the specific region (Fig. 11). The DNA product amplified by PCR had one band and it was cut into two bands by *Eco*R V in Milyang 23, but was not cut in Gihobyeo. This PCR pattern was the same as the RFLP pattern of RG 109 in segregating lines; it was cut into two bands in semidwarf progenies, one band in tall ones, and incompletely cut showing three bands in heterozygotes which segregated in the next generation. Thus it could be used as a CAPS (cleaved amplified polymorphic sequence) marker for efficient selection of *sd-1* genotypes *in vitro*.

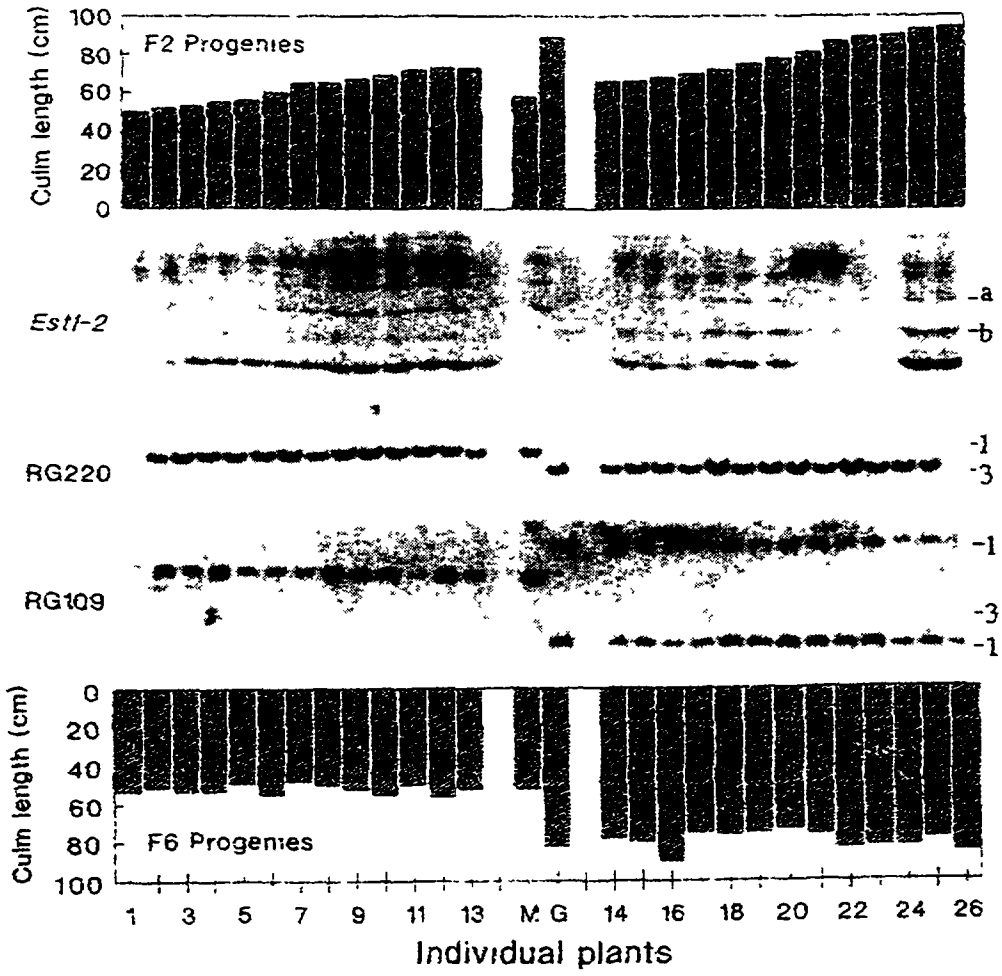


Fig. 9. Effect of in-vitro selection of plants at early stages, using molecular markers EstI-2, RG220, RG109, on culm length at late stages of the F_2 generation and that of progenies in the F_6 generation of Milyang 23/Gihobyeo. F_6 plants were grown under greenhouse condition during the winter ('91/'92).

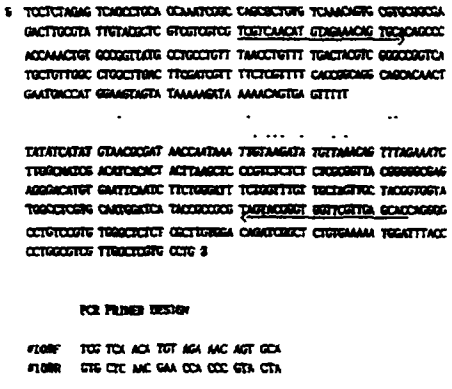


Fig. 10. Nucleotide sequence of RG 109 and locations of primers designed for the PCR amplificaiton of RG 109 region of chromosome 1.

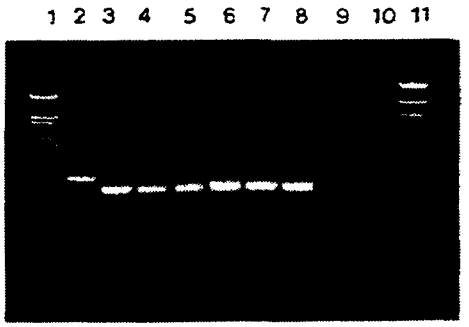


Fig. 11. PCR amplificaiton of RG 109 region with RG 109F and RG 109R primers.

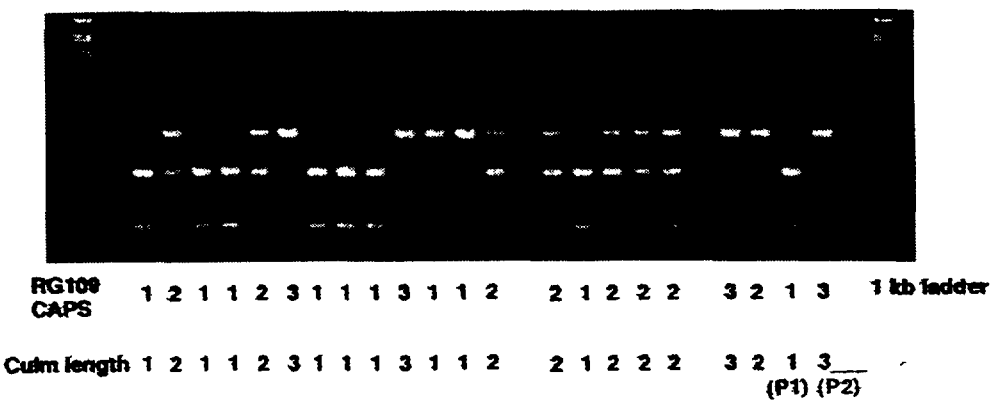


Fig. 12. CAPS analysis pattern after EcoR V digestion of DNAs amplified by PCR in F2 plants of Taichung 65 (A, Pn, Pau)/Taichung 65 (sd-1) and their parents.

In this study, we demonstrate that even when the expression of the target gene is modified by the presence of other, unidentified genes in the genetic background of a variety, tightly-linked markers can assist in accurately selecting for the target character. The markers, *EstI-2*, *RG109* and *RG220*, were useful in predicting stature at a young age before the effect of the semidwarf gene could be clearly detected phenotypically, and also provided an efficient way of selecting for the presence of *sd-1* in a population that showed continuous variation for plant height.

8. TOWARD MAP-BASED CLONING OF *sd-1* GENE

Map-based cloning is defined as the isolation of a gene based on its position on a genetic map and often includes four basic elements: target gene mapping, physical mapping, chromosome walking, and gene identification. Present studies are focused toward the isolation of semi-dwarf gene (*sd-1*) in rice as a model system for map-based gene cloning.

One recent advance that promises to greatly aid in the construction of contig maps is the development of new vectors for cloning large DNA fragments. They are yeast artificial chromosomes (YAC) and bacterial artificial chromosome (BAC) We have constructed rice YAC and BAC libraries for physical mapping and chromosome walking around the region of the *sd-1* gene; approximately 15,000 YACs with an average insert size of 150 kb (Fig. 13) and 5,000 BACs with an average insert size of 100 kb have been constructed.

The DNA probe *RG109*, the one most tightly linked to the *sd-1* gene, was used to screen the BAC libraries by colony hybridization and at least five overlapping BAC clones were isolated, ranging from 45 to 145 kb in insert size. To extend the BAC contigs toward the *sd-1* locus and to isolate suitable makers for the next step of the chromosome walk, the ends of BACs were isolated by using plasmid rescue and PCR methods (Fig. 14).

9. THE DEVELOPMENT OF THE KOREA RICE GENOME NETWORK AND RiceMac

The Korea Rice Genome WWW Server was constructed to distribute the rice research and economic information around the world. The Network provides EST sequences and their homology analysis results from rice immature seed cDNA which was performed by the Korea Rice Genome Research Group. The Network also supports the access to Rice Genes which is a rice specific Unix Database covering world wide rice research and genomic information. For the personal use of Rice Genes, RiceMac was developed.

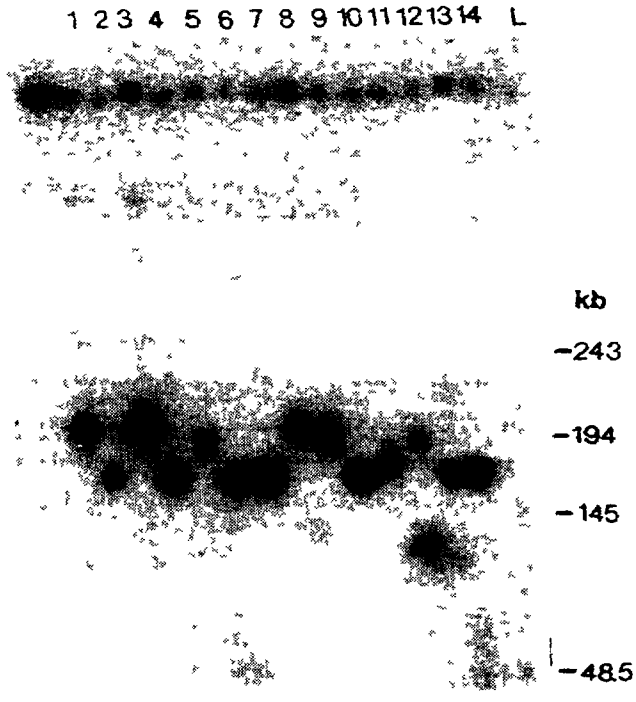


Fig. 13. Pulsed field gel separation of YACs and identification by Southern blot. The 1% agarose gel was run at 200V using ramped pulse times from 12.6 to 44.7 sec. for 24 hours in 0.5X TBE. The gel DNA was transferred onto nylon membrane. As probe, pBR322 labeled with ^{32}[P]dCTP was used.

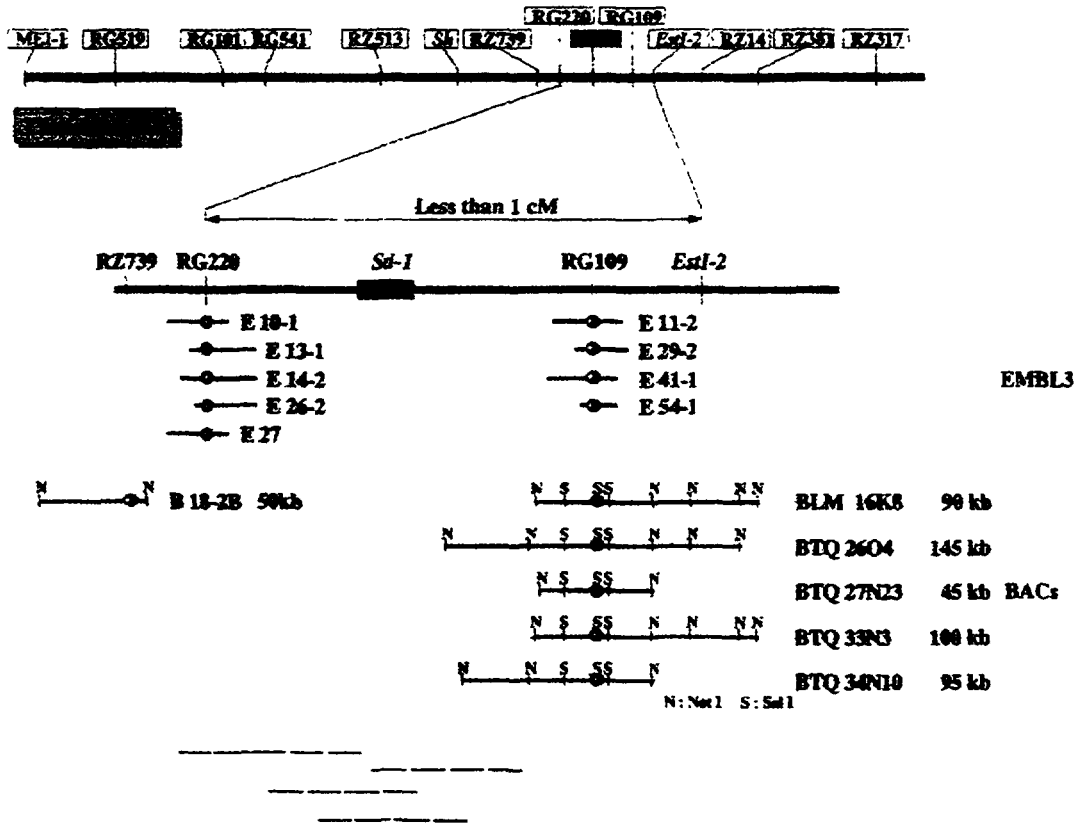


Fig. 14. Steps involved in map-based cloning of semidwarf (sd-1) gene of rice.

The first version, RiceMac 4.1.1, contains the 120 phenotypic images of marker mutants published by Dr. M. H. Hue along with the data of Rice genes 4.1.1. The RiceMac contains the rice research information such as authors, papers, abstracts, germplasm, mapping data, sequence, and probes information. The database also shows graphical presentation of molecular and morphological maps marked with probes according to their loci which are linked to their mapping data and blot images showing polymorphism. The database program can perform the DNA sequence analysis such as 3 frame translation, restriction analysis, codon usage analysis and splicing. The Korea Rice Genome WWW Server and the WWW mirror site of Rice Genes are maintained at the BioServer http://bioserver.myongji.ac.kr) of the Dept. of Biological Science, Myongji University and the Unix Server (http://sun20.asti.re.kr) of National Institute of Agricultural Science and Technology, RDA. The RiceMac (120 MB) is available as a compressed self-extracted file at the ftp archive (file://probe.nalusda.gov/pub/ricegenes) of Agricultural Genome Information Service(AGIS), National Agricultural Library (NAL), USDA. Also the CD-ROM version of RiceMac is available upon written request for those who do not have direct access to internet.

REFERENCES

[1] BOTSTEIN, D., WHITE, R.L., SKOLNCK, M., DAVIS, R.W., Construction of a genetic linkage map in man using restriction fragment length polymorphisms, Am. J. Hum. Genet. 32 (1980) 314-331.

[2] BURR, B., BURR, F.A., THOMPSON, K.H., ALBERTSEN, M.C., STUBER, C.W., Gene mapping with recombinant inbreds in maize, Genetics 118 (1988) 519-526.

[3] WEBER, J.L., MAY, P.E., Abundant class of human DNA polymorphism which can be typed using the polymerase chain reaction, Am. J. Hum. Genet. 44 (1989) 388-396.

[4] VOS, P., HOGERS, R., BLEEKER, M., REIJANS, M., VAN DE LEE, T., HORNES, M., FREITERS, A., POT, J., PELEMAN, J., KUIPER, M., ZABEAU, M., AFLP: a new concept for DNA fingerprinting. Nucleic Acids Res. 23 (1995) 4407-4414.

[5] CHO, Y.G., BLAIR, M.W., PANAUD, O., McCOUCH, S.R., Cloning and mapping of variety-specific rice genomic sequences:amplified fragment length polymorphisms (AFLP) from silver-stained polyacrylamide gels, Genome 39 (1996) 373-378.

[6] CAUSSE, M.A., FULTON, T.M., CHO, Y.G., AHN, S.N., CHUNWONGSE, J., WU, K., XIAO, J., YU, Z., RONALD, P.C., HARRINGTON, S.E., SECOND, G., McCOUCH, S.R., TANKSLEY, S.D., Saturated molecular map of the rice genome based on an interspecific backcross population, Genetics 138 (1994) 1251-1274.

[7] KURATA, N., NAGAMURA, Y., YAMAMOTO, K., A 300 kilobase interval genetic map of rice including 883 expressed sequences. Nature Genet. 8 (1994) 365-372.

[8] CHEN, X., TEMNYKH, S., XU, Y., CHO, Y.G., McCOUCH, S.R., (Development of a microsatellite map providing genome-wide coverage in rice (*Oryza sativa* L.), Theor. Appl. Genet. (1997) (in press).

[9] BECKER, J., VOS, P., KUIPER, M., SALAMINI, F., HEUN, M., Combined mapping of AFLP and RFLP markers in barley, Mol. Gen. Genet. 249 (1995) 65-73.

[10] MAHESWARAN, M., SUBUDHI, P.K., NANDI, S., XU, J.C., PARCO, A., YANG, D.C., HUANG, N., Polymorphism, distribution, and segregation of AFLP markers in a doubled haploid rice population, Theor. Appl. Genet. 94 (1996) 39-45.

[11] AQUINO, R.C., JENNINGS, P.R., Inheritance and significance of dwarfism in an indica rice variety, Crop Sci. 6 (1966) 551-554.

[12] KINOSHITA, T., Report of the committee on gene symbolization, nomenclature and linkage groups, Rice Genet. Newslet. 7 (1990) 16-50.

[13] SUH, H.S., HEU, M.H., The segregation mode of plant height in the cross of rice varieties. II. Linkage analysis of the semidwarfness of rice variety "Tongil", Korean J. Breed. 10 (1978) 1-6.

[14] EUN, M.Y., KIM, Y.K., CHO, Y.G., CHUNG, T.Y., Inheritance of esterase isozymes in rice by isoelectric focusing, Korean J. Breed. 22 (1990) 252-259.

[15] McCOUCH, S.R., KOCHERT, G., YU, Z.H., WANG, Z.Y., KHUSH, G.S., COFFMAN, W.R., TANKSLEY, S.D., Molecular mapping of rice chromosomes, Theor. Appl. Genet 76 (1988) 815-829.

[16] TANKSLEY, S.D., YOUNG, N.D., PATERSON, A.H., BONIERBALE, M.W., RFLP mapping in plant breeding: new tools for an old science. Biotech. 7 (1989) 257-264.

[17] CHO, Y.G., McCOUCH, S.R., KUIPER, M., POT, J., KANG, M.R., GROENEN, J.T.M., EUN, M.Y., Integration of AFLP and RFLP markers using recombinant inbred population of rice (*Oryza sativa* L.). Theor. Appl. Genet. (1997) (in press).

DETECTION OF GENETIC VARIABILITY IN BASMATI AND NON-BASMATI RICE VARIETIES AND THEIR RADIATION INDUCED MUTANTS THROUGH RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD)

S. FAROOQ, N. IQBAL, M. ARIF
Nuclear Institute for Agriculture and Biology (NIAB),
Faisalabad, Pakistan

Abstract

Random Amplified Polymorphic DNA (RAPDs) markers were utilized to detect polymorphism between pure lines and commercially available Basmati rice varieties to assess variation which may be helpful in quality control and varietal identification (Basmati-370 and derived radiation induced mutants), differentiation of mutants and parents, and identification of RAPD markers co-segregating with important agronomic traits including plant height, days to flower and grain quality. Basmati varieties were distinguished from non-Basmati varieties with the help of five diagnostic markers which will be useful for detecting mixing of non-Basmati and Basmati rices, currently a serious marketing problem. Different Basmati cultivars were identified with the help of diagnostic RAPD markers which can be used in quality control as well as for "fingerprinting" of cultivars Different radiation induced mutants were also successfully distinguished from the parents on the basis of variety specific and mutant specific markers which will be useful for varietal identification In addition to this, other markers were also identified which can differentiate mutants from each other and are being used for the fingerprinting of different mutants, particularly the dwarf mutants having similar appearance but different parentage For identification of RAPD markers co-segregating with plant height and days to flower, 50 F_2 plants and four F_3 families were studied from a reciprocal cross made between Kashmir Basmati (tall and early) and Basmati-198 (dwarf and late). Segregating bands were observed within these populations, and indicating the possible use of RAPD markers for tagging gene(s) of agronomic importance in rice

1. INTRODUCTION

The Nuclear Institute for Agriculture and Biology (NIAB) is undertaking research on the creation of genetic variability in rice, wheat, chickpea, cotton and mungbean through induced mutations, tissue culture and wide hybridization. The mutants of rice are being used as germplasm in crossbreeding programs aimed at improvement of plant type, early maturity and aroma [1]. Since most of these characters are polygenic and quantitatively inherited [2], selection of desirable plants through conventional breeding is difficult. Besides, most of these characters are highly influenced by the environment and often defy phenotypic identification due to the complex genotype x environment interactions that may affect the trait of interest. Under such circumstances, the true genetic potential of the plants might not be detected and it would be difficult to decide which plants should be selected and/or crossed to get recombinants of interest.

Utilization of molecular markers such as Random Amplified Polymorphic DNA (RAPD) [3] in breeding and selection programmes may circumvent some of these problems; RAPD markers have frequently been used in rice breeding programmes. For example, Fukuoka *et al.* [4] used RAPD markers to characterize different rice accessions. Munthali *et al.* [5] used RAPDs to fingerprint rice cultivars. Ronald *et al.*[6] used 895 random primers and 123 DNA markers to reference the gene that confers resistance to the pathogen *Xanthomonas oryzae*. Ashikawa *et al.* [7] determined a range of polymorphism in the rice genome by RAPD method. Yamazaki *et al.* [8] determined region-specific molecular marker using bulked segregant RAPD analysis. Antonio *et al.* [9] reported 1100 DNA markers consisting of genomic clones, complementary DNA (cDNA) clones and RAPD markers on the Restriction Fragment Length Polymorphism (RFLP) linkage map of rice. RAPD markers have also been used to characterize the rice blast fungus *Pyrcularia grisea* [10]. Yu and Nguyen [11] compared 9 upland and 4 lowland rice cultivars with the help of 42 random primers. They

obtained 260 PCR products, of which 80% were polymorphic. They concluded that RAPD analysis is a useful tool in determining genetic relationships among rice cultivars. Mohan *et al.* [12] used RFLP and RAPD markers to map the rice GM2 gene that confers resistance to biotype 1 of gall midge. Farooq *et al.* [13] used RAPD markers to characterize different wild and cultivated, salt tolerant and resistant rice species/varieties and identified genome specific, species specific and cultivar specific RAPD markers. Identification of species specific and cultivar specific RAPD markers in intergeneric F_1 hybrids of rice has also been made [14]; it was observed that the appearance of RAPD markers in interspecific F_1 hybrids is primer dependent. All these reports support the usefulness of RAPD markers in detecting genetic variability in rice. The goals of the current project were therefore, to utilize RAPD markers to detect polymorphism in pure lines, commercially available Basmati rice varieties (as a means to determine within variety variations which may be helpful in quality control and varietal identification), and their radiation induced mutants. A further goal was to identify RAPD markers co-segregating with plant height, days to flower and grain quality. Success in this programme would help to accelerate the identification, selection, characterization and utilization of mutants and identification of RAPD markers that can be used to accelerate breeding programmes.

2. MATERIALS AND METHODS

Traditional Basmati cultivars, their different radiation induced mutants and different non-Basmati rice varieties as described in Table I were collected from the rice breeding group of NIAB. Seedlings were raised in a rice nursery in the field and leaves were collected for DNA extraction. One month old nursery plants were transplanted to the field plots with an inter- and intra-row spacing of 20 cm. Agronomic practices were the same as reported by Cheema and Awan [15]. Leaf samples were again collected at the active tillering stage. Collected samples were dipped immediately in liquid nitrogen and stored at -85°C for further use.

Reciprocal crosses were made between Kashmir Basmati (tall and early) and Basmati-198 (dwarf and late) in the summer of 1993. F_1 seeds were selfed in 1994 and the segregating F_2 plants were planted in 1995, spaced as above. Selections were made from 500 individual plants on the basis of the plant type of Kashmir Basmati (10-15% reduction in plant height) and the plant type of Basmati-198 (flowers 3-4 weeks earlier). One F_2 individual, which showed 11-12% reduction in plant height, was selected from the cross between Kashmir Basmati and Basmati 198 and planted to create an F_3 population, 40 of which were selected at random on the basis of characters mentioned in Table II. Similarly, one individual, which showed a 6% increase in plant height and 5 week earlier flowering was selected from the reciprocal cross between Basmati 198 and Kashmir Basmati and planted to create an F_3 population, 40 of which were selected at random on the basis of characters mentioned in Table III.

Based on the same criteria, five individual plants from each cross direction were also selected for comparison in the F_3 (Tables II and III). Days to flowers were taken as the time, in days, from sowing to the appearance of the first panicle. Data with respect to plant height and heading date were recorded and leaf samples were collected for individual plants both in F_2 and F_3 generations.

Genomic DNAs from the leaves of all the test materials were extracted by using a CTAB method [16]. The concentration and quality of the DNAs, composition of reaction mixture, kind and source of primers and amplification conditions were similar to those used

earlier [17]. For the plants analyzed in groups, DNA extracted from individual plants were pooled and amplified using the same method. All reactions were repeated twice using fresh DNAs and data were scored from two good quality photographs.

TABLE I. DESCRIPTION OF RICE VARIETIES AND MUTANTS SELECTED FOR RAPD ANALYSES

Variety/mutant	Plt. height range (cm)	Days to flower range (days)	Significance
1. Kashmir Basmati	140-167	94-104	Fine grain, tall, highly aromatic
2. Basmati-370			
3. Basmati-Pak	115-130	114-120	Fine grain, tall and aromatic
4. Basmati-198			
5. DM-15-13-1(Bas.-Pak)	88-107	94-100	Fine grain, dwarf and aromatic
6. DM-179-1 (Bas. 370)	99-108	100-120	Fine grain, dwarf and aromatic
7. DM-107-4 (Bas. 370)			
8. DM-25 (Bas. 370)			
9. DM-2 (Bas. 370)			
10. IR-6	80-88	94-100	Coarse grain, dwarf and non-aromatic
11. IR-8			
12. Jhona-349	110-130	110-120 }	Coarse grain, tall and non aromatic
13. Pokkali	140-180	130-150 }	
14. CP-1	110-135	100-110 }	
15. EF-6 (Bas. Pak)	110-120	90-100 }	Fine grain, early and aromatic
16. EF-76-1 (Bas. 198)		}	
17. EF-15-1 (Bas. 198)		}	

( ) Names in parentheses indicate the source variety of the mutant line.

TABLE II. DESCRIPTION OF F_3 GROUPED AND INDIVIDUAL PLANTS SELECTED FROM A POPULATION OF THE CROSS KASHMIR BASMATI x BASMATI-198

Plt. No.	Plt. height (cm)	Days to flower	Height reduction (%)
1	107	95 (14)	23.57
2	97	96 (14)	30.71
3	140	96 (14)	normal
4	99	94 (14)	29.28
5	106	93 (13)	24.28
F3 population*	100-140**	90-100** (12-14)	-------
Group 1	107***	85- 89*** (12)	-------
Group 2	103***	97*** (13)	-------

* All plants in one cross, ** Ranges of all values, *** Mean values of 20 plants.
Numbers in parentheses indicate No. of weeks.

TABLE III. DESCRIPTION OF F_3 AND INDIVIDUAL PLANTS SELECTED POPULATION OF THE CROSS BASMATI-198 x KASHMIR BASMATI

Plt. No.	Plt. height (cm)	Days to flower	Induced earliness
1	103	98 (14)	1 week
2	94	97 (14)	1 week
3	100	87 (12)	3 weeks
4	110	99 (14)	1 week
5	105	91 (13)	2 weeks
F3 population*	90-110**	86-100 (12-14)	
Group 1	96***	90*** (13, 4 weeks early)	
Group 2	100***	97*** (14, 3 weeks early)	

* All plants in one cross, ** Ranges of all values, *** Mean values of 20 plants.
Numbers in parentheses indicate No. of weeks.

3. RESULTS

Three primers (S-13, S-19 and S-18) successfully identified Basmati from non-Basmati rice varieties by 5 RAPD fragments of which 2 each were specific for Jhona-349 and IR-6 and 1 fragment was specific for Basmati-Pak (Figs 1 and 2). Primer S-13 was used to amplify the mixture of Jhona-349 and Basmati-Pak which showed different relative intensities of fragments (Fig. 3). No fragments could be identified for the non-Basmati rice variety Pokkali, possibly indicating a lack of DNA. Four primers (S-13, R-15, R-17 and R-18) differentiated Basmati-Pak, Basmati 198, Kashmir Basmati and Basmati-370 from each other.

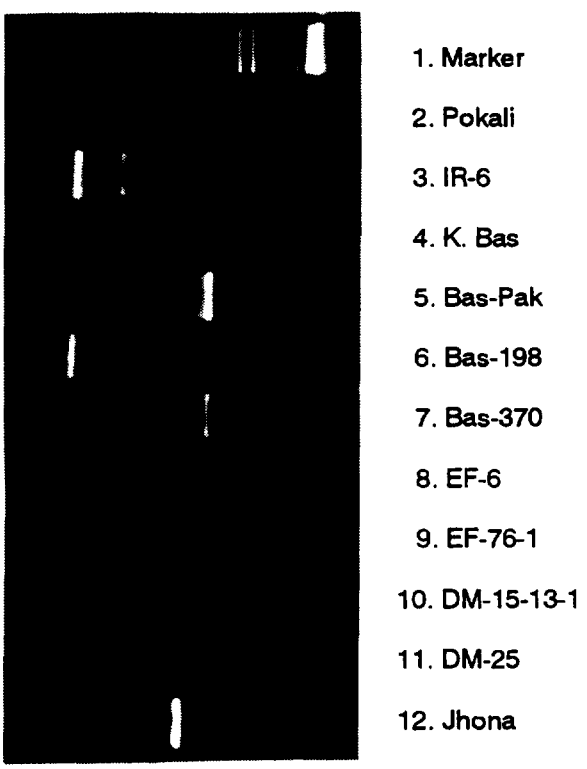


Fig. 1. Amplification profile produced by primer S-19 distinguishing non-Basmati varieties (lanes 3 and 12) and Basmati varieties (lanes3, 5, 6, 7 and 11).

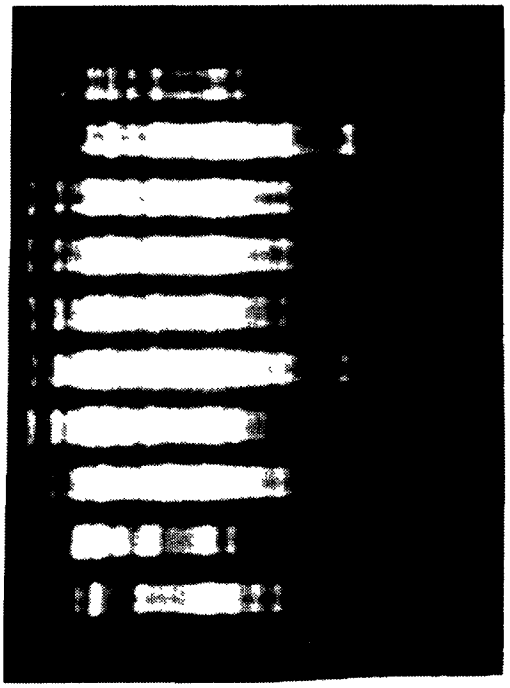


Fig. 2. Amplification profile produced by primer S-18 distinguishing non-Basmati varieties (lanes 2,11) from Basmati varieties (lanes3, 4, 5, 6).

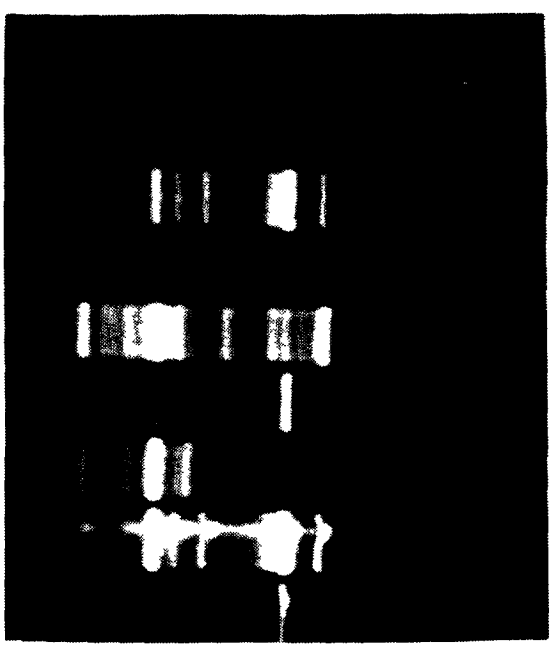


Fig. 3. Amplification profile produced by primer S-13 indicating reproducibility of major DNA fragments in Bas-Pak (lanes 3 and 8) and a different profile for a mixture of Bas-Pak (lane 6).

Comparisons of radiation-induced variants and Basmati parent varieties are shown in Figs 4 and 5. Three primers (R-15, R-17 and S-13) produced fragments which could be used to differentiate the mutants from their parents. DM-2, DM-25, DM-107-4 and Kashmir Basmati are mutants of Basmati-370 and showed band pattern differences. DM-15-13-1 and EF-6 are mutants of Basmati-Pak and showed band pattern differences. EF-76-1 is a mutant of Basmati-198 and showed band pattern differences.

Comparison of progeny and parents with primer R-08 showed segregating fragments (Fig. 6). Two plants showed 11-12% reduction in plant height (Fig. 6, lanes 4 and 5). Two plants showed 6-7% increase in plant height (Fig. 6, lanes 10 and 11). One plant showed 6% increase in plant height and 5 week earliness (Fig. 6, lane 8). The variation in phenotypic traits did not seem to correlate with band pattern differences.

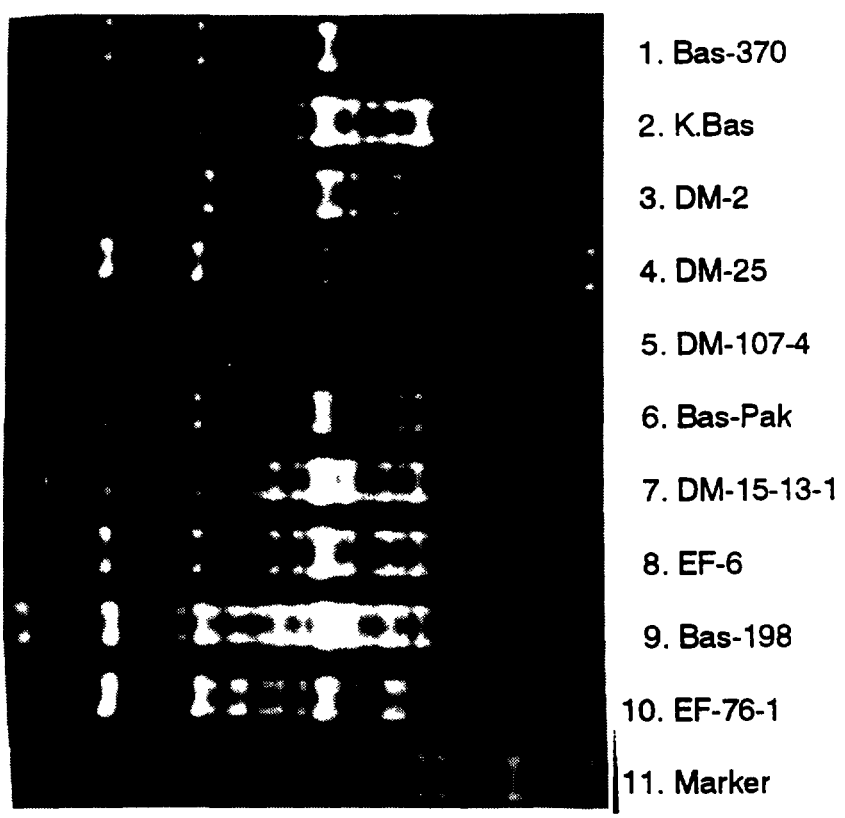


Fig. 4. Amplification profile produced by primer R-18 distinguishing Basmati varieties from each other (lanes1,2,6 and 9).

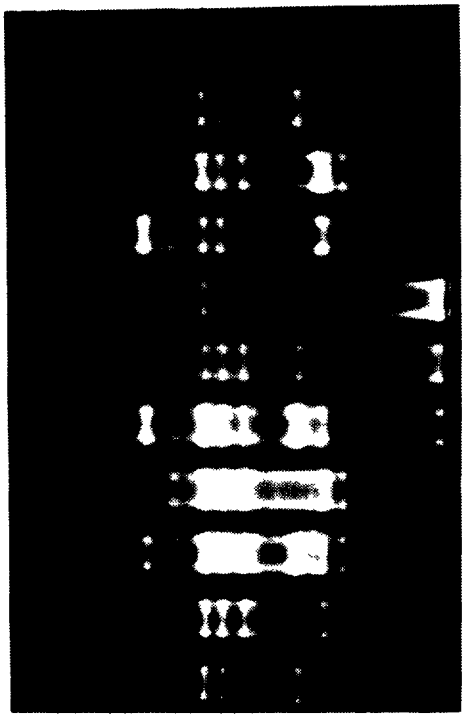


Fig. 5. *Amplification profile produced by primer S-13 distinguishing Basmati varieties (lanes 2,3,7 and 10) and radiation induced mutations (lanes 4,5,6,8,9 and 11) (see Table I).*

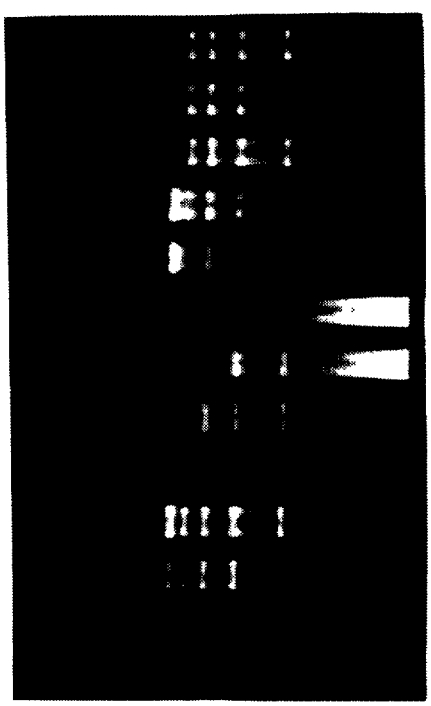


Fig. 6. *Amplification profile produced by primer R-08 comparing parents (lane 1 and 7) and selected progeny from reciprocal crosses of Kashmir Basmati and Basmati-198 (lanes 2-6 and 8-12).*

4. DISCUSSION

Molecular markers such as RFLP [18] and RAPDs [3] are capable of producing unique band patterns that can be used in rice breeding programs for different purposes [19]. Since RAPD markers are technically simple, quick to perform with small amount of DNA and do not require radioactive labelling [20] they are preferred over RFLP and are widely used, e.g. in lettuce [20], broccoli and cauliflower [21], *Arabidopsis thaliana* [22], maize [23], wheat [17] and apples [24]. In addition to this, RAPDs are also being used for detection of genetic polymorphism in cereals [25], gene introgression [26], identification of different fish species [27], estimation of the number of full sibling families of mosquitoes [28] identification of mutants [29, 30] and characterization of pathogens [31].

In the present project, we used RAPD markers for three main purposes: 1) identification of different Basmati cultivars, 2) comparison of mutants with their parents and 3) comparison of DNA fragments and trait segregation in Basmati rices.

Basmati rice cultivars, especially Basmati-370 which is considered the finest quality rice in the world, grows predominantly in Pakistan and about 40-45% of its production is being exported. Mixing low quality rice with Basmati rice affects the international consumer market which results in a loss of foreign exchange earnings. The problem could be avoided if strict quality control measures would be taken. Markers specific for Basmati cultivars can help identify any kind of mixing in the pure Basmati varieties and it has been shown in the present study that RAPD markers can be used for this purpose. Additional RAPD markers have also been identified which can discriminate between all the Basmati cultivars. These markers are reproducible and appear only in the specific Basmati variety and can serve as diagnostic markers for that particular variety and for its fingerprinting which is essentially required for varietal registration.

Mutation breeding is a supplementary approach for crop improvement and has played a productive role in sustainable agriculture. About 1790 different mutants have been listed in the database; many of them have been used for general cultivation and quite a large number have served as germplasm sources in breeding programmes[32]. Mutations represent changes at the DNA level and can be detected through the use of molecular markers. In the present study we have detected a number of band pattern differences which may be useful for distinguishing different mutants of Basmati varieties from their parents. Ultimately, markers specific for dwarf mutants would useful as probes for selection and/or induction of dwarfism in the rice breeding programmes, in addition to identification of dwarf mutants of similar appearance but different parentage.

RAPD markers have also been used for tagging disease resistance gene(s) in crops including stem rust [33] and crown rust resistant genes in oats [34], rust resistance genes in common bean [35] and vf gene for scab resistance in apple [36]. In the present study efforts have been made to identify RAPD markers co-segregating with plant height and days to maturity. Unfortunately, it was not possible to establish any correlations with the limited number of primers utilized here.

Plant height, days to maturity, aroma and other quality parameters are all quantitatively inherited traits [37, 38] and are governed by more than two sets of genes. The co-segregation of DNA fragments with a specific reduction in plant height, or early or delayed flowering may also co-segregate with other QTLs. The most important consideration in analysis and interpretation of QTL data is the threshold employed for inferring that a QTL is statistically

significant. Although, the present study was made on 50 F_2 plants and four F_3 families, the RAPD analyses presented are not based on the full set data but rather on plants selected on phenotypic extremes or which represent the best available estimates of the portion of the total phenotype and thus could be biased. The variance calculated on such data would be considered as overestimated compared to the calculations made on the full data set. The present study however, can serve as an index to compare the importance of different DNA fragments (markers) and their co-segregation with particular character(s). It could be inferred from the present study that tagging gene(s) with RAPD markers can produce more meaningful results if the analysis is made on the full data set, work which is currently in progress.

REFERENCES

[1] Twenty Years of NIAB. Fourth 5 years Report. NIAB, Faisalabad, Pakistan (1992) 1-9.

[2] AWAN, M.A., CHEEMA, A.A., TAHIR, G.R., The exploitation of induced genetic variability for the improvement of Basmati rice (*Oryza sativa* L). In: New Genetical Approaches for Crop Improvement. (Siddiqui, K.A., Faruqui, A.M. Eds., PIDC Printing Press (PVT) LTD. Karachi, (1982) 203-212.

[3] RAFALSKI, J.A., TIGNEY, S.V., WILLIAMS, J.G.K., RAPD markers: a new technology for genetic mapping and plant breeding. Ag. Biotech. News. Inf. 3 (1991) 646-648.

[4] FUKUOKA, S., HOSAKA, K., KAMIJIMA, O., Use of Random Amplified Polymorphic DNAs (RAPD) for identification of rice accessions. Jap. J. Genet. 67 (1992) 243-252.

[5] MUNTHALI, M., FORD-LLOYD, B.V., NEWBURY, H.J., The random amplification of polymorphic DNA for fingerprinting of plants. PCR Methods Appl. 1 (1992) 274-276.

[6] RONALD, P.C., ALBANO, B., TABIEN, R., ANENES, L., WU, K.S., McCOUCH, S., TANKSLEY, S.D., DNA markers in plant improvement. Genetic and physical analysis of the rice bacterial blight disease resistance locus XA21. Mol. Gen. Genet. 236 (1992) 113-120.

[7] ASHIKAWA, I., FUKUOKA, S., INOUE, T., MIYAO, A., MONNA, L., ZHONG, S., SASAKI, T., Polymorphism analysis of rice genome by PCR. Rice Genome 2 (1992) 6-7.

[8] YAMAZAKI, M., MIYAO, A., MONNA, L., ZHONG, H.S., TSUJI, T., TOUYAMA, T., SASAKI, T., Search RAPD markers for the brown plant hopper resistance gene Bph 1. Rice Genome 3 (1994) 3.

[9] ANTONIO, B.A., YAMANOTO, K., HARUSHIM, Y., SUE, N., SHOMURA, A., LIN, S.Y., INOUE, T., FUKUDA, A., SHIMANO, T., HAYASAKA, H., KUBOKI, Y., ZUNGSONTION, S., YANO, M., KURATA, N., NAGAMURA, N., More than one thousand markers mapped by RFLP. Rice Genome 2 (1993) 3-8.

[10] BERNARDO, M.A., NAQVI, N., LEUNG, H., ZEIGLER, R.S., NELSON, R.J., A rapid method for DNA fingerprinting of the rice blast fungus, *Pyricularia grisea*. Int. Rice Res. Notes 18 (1993) 8-50.

[11] YU, L.X., NGUYEN, H.T., Genetic variation detected with RAPD markers among upland and lowland rice cultivars (*Oryza sativa* L.). Theor. Appl. Genet. 87 (1994) 668-672.

[12] MOHAN, M., NAIR, S., BENTURE, J.S., RAO, U.P., BENNETT, J., RFLP and RAPD mapping of the rice GM2 gene that confers resistance to biotype 1 gall midge (*Oreseolia oryzae*). Theor. Appl. Genet. 87 (1994) 782-788.

[13] FAROOQ, S., SHAH, T.M., ARIF, M., IQBAL, N., Utilization of RAPD markers for the identification of cultivated and wild rice species. Pak. J. Bot. 27 (1995) 127-138.
[14] FAROOQ, S., SHAH, T.M., ARIF, M., IQBAL, N., Inheritance of RAPD markers in the F_1 interspecific hybrids of rice. Pak. J. Bot. 28 (1996) 51-59.
[15] CHEEMA, A.A., AWAN, M.A., Mode of reduction of internode length in a semidwarf mutant of *indica* rice cultivar, Basmati-370. Pak. J. Agric. Res. 3 (1982) 208-210.
[16] ROGERS, S.O., BENDICH, A.J., Extraction of DNA from plant tissues. Plant Mol. Bio. Manual. A6 (1988) 1-10.
[17] FAROOQ, S., SHAH, T.M., ASKARI, E., ZAIDI, A.A., ARIF, M., IQBAL, N., Identification of different wheat genotypes through polymorphism based on Random Amplified Polymorphic DNA (RAPDs). Pak. J. Bot. 26 (1994) 373-382.
[18] JAFFREYS, A.J., WILSON, V., THIEN, S.L., Individual specific "fingerprints" of human DNA. Nature 316 (1985) 76-79.
[19] McCOUCH, S.R., TANKSLEY, S.D., Development and use of Restriction Fragment Length Polymorphism in rice breeding and genetics. In: Rice Biotechnology, C.A.B. Int. Wallingford, Oxon, UK (1991) 109-133.
[20] MICHELMORE, R.W., PARAN, I., KESSELI, R.V., Identification of markers linked to disease resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA 88 (1991) 9828-9832.
[21] HU, J., QUIROS, C.F., Identification of broccoli and cauliflower cultivars with RAPD markers. Plant Cell Reports 10 (1991) 505-511.
[22] REITER, R.S., WILLIAMS, J.G.K., FELDMANN, K.A., RAFALSKI, J.A., TIGNEY, S.V., SCOLINKS, P.A., Global and local genome mapping in *Arabidopsis thaliana* by using inbred lines and Random Amplified Polymorphic DNA. Proc. Natl. Acad. Sci. USA, 89 (1992) 1477-1481.
[23] HEUN, M., HELENTJARIS, T., Inheritance of RAPD markers in F_1 hybrids of corn. Theor. Appl. Genet. 85 (1993) 961-968.
[24] LANDRY, B.S., LI, R.Q., CHEUNG, W.Y., GRANGER, R.L., Phylogeny analysis of 25 apple root stocks using RAPD markers and tactical gene tagging. Theor. Appl. Genet. 89 (1994) 830-847.
[25] WANG, G., CASTINGLIONE, S., ZHANG, J., FU, R.Z., MA, J.S., LI, W.B., SUN, Y.R., SALA, F., Hybrid rice (*Oryza sativa* L.) identification and parentage determination by RAPD fingerprinting. Plant Cell Reports. 14 (1994) 2-3.
[26] DURHAM, E.R., KORBAN, S.S., Evidence of gene introgression in apple using RAPD markers. Euphytica 79 (1994) 109-114.
[27] BARDAKCI, F., SKIBINSKI, D.O.F., Application of the RAPD technique in Tilapia fish: species and subspecies identification. Heredity 73 (1994) 117-123.
[28] APOSTOL, B.L., BLACK, W.C., MILLER, B.R., REITER, P., BEATY, B.J., Estimation of the number of full sibling families at an oviposition site using RAPD-PCR markers: Applications to the mosquito *Aedes aegypti*. Theor. Appl. Genet. 86 (1993) 991-1000.
[29] YANG, H.Y., SCHMIDT, H., Selection of a mutant from adventitious shoots formed in X-ray treated cherry leaves and differentiation of standard and mutant with RAPDs. Euphytica 7 (1994) 88-99.
[30] DENG, Z.N., GENTIL, A., NICOLOSI, E., DOMINA, F., VARDI, A., TRIBULATO, E., Identification of *in vivo* and *in vitro* lemon mutants by RAPD markers. J. Hort. Sci. 70 (1995) 117-126.
[31] CROWHURST, R.N., KING, F.Y., HAWTHORNE, B.T., SANDERSON, F.R., CHOIPHENG, Y., RAPD characterization of *Fusarium oxysporum* associated with wilt of angsans *(Pterocarpus indicus)* in Singapore. Mycological Res. 99 (1995) 14-18.

[32] MALUSZYNSKI, M., VAN ZANTEN, L., ASHRI, A., BRUNNER, H., AHLOOWALIA, B.S., ZAPATA, J.F., WECK, E., Mutation techniques in plant breeding. In: Induced Mutations and Molecular Techniques for Crop Improvement, IAEA, Vienna (1995) 489-504.

[33] PENNER, G.A., CHONG, J., LEVESQUE LEMAY, M., MOLNER, S.J., FEDAK, G., Identification of a RAPD marker linked to the oat stem rust gene Pg 3. Theor. Appl. Genet. 85 (1993) 902-905.

[34] PENNER, G.A., CHONG, J., WIGHT, C.P., MOLER, S.J., FEDAK, G., Identification of a RAPD marker for the crown rust resistance gene Pc 68 in oats. Genome 36 (1993) 818-820.

[35] HALEY, S.D., MIKLAS, P.N., STAVELY, J.R., BYRUM, J., KELLY, J.D., Identification of RAPD markers linked to a major rust resistance gene block in common bean. Theor. Appl. Genet. 86 (1993) 505-512.

[36] YANG, H.Y., KRUGER, J., Identification of RAPD marker linked to the vf gene for scab resistance in apples. Euphytica 77 (1994) 83-88.

[37] KOESTER, R.P., SISCO, P.O.H., STUBER, C.W., Identification of quantitative trait loci controlling days to flowering and plant height in near isogenic lines of maize. Crop Sci. 33 (1994) 1209-1216.

[38] PATERSON, A.H., DEVERNA, J.W., LANINI, B., TANKSLEY, S.D., Fine mapping of quantitative trait loci using selected overlapping recombinant chromosomes in an interspecific cross of tomato. Genetics 124 (1990) 735-742.

APPLICATION OF DNA RFLP PROCEDURES IN INTERSPECIFIC GENE TRANSFER: THE *Lr19* TRANSLOCATION OF WHEAT

R. PRINS
Agricultural Research Council (Small Grain Institute),
Department of Genetics,
University of Stellenbosch,
Stellenbosch, South Africa

G.F. MARAIS, A.S. MARAIS
Department of Genetics
University of Stellenbosch
Stellenbosch, South Africa

Z.A. PRETORIUS
Department of Plant Pathology,
University of the Orange Free State,
Bloemfontein, South Africa

B.J.H. JANSE
Department of Microbiology,
University of Stellenbosch,
Stellenbosch, South Africa

Abstract

Twenty-nine lines with deletions in the *Lr19* ('Indis') translocated chromosome segment were used to physically map *Thinopyrum* Restriction Fragment Length Polymorphism (RFLP) loci as well as the *Sr25* and *Sd1* loci. The relative distances between marker loci on the translocation were then calculated. The information was then used as an aid to characterize several recombined forms of the translocation. The data confirmed the reported homoeology between the *Lr19* segment and chromosome arm 7DL of wheat. Also, it seems that the *Lr19* translocation in 'Indis' is very similar to the *Lr19* segment in the T4 source and that the former may not derive from *Thinopyrum distichum*. Near-isogenic lines of the recombined segments were derived and used to study their expression of leaf rust resistance. It became evident that only one potentially useful recombinant was obtained in an earlier attempt to induce allosyndetic pairing between the *Lr19* translocation and 7DL of wheat.

1. INTRODUCTION

The *Lr19* translocation of wheat carries a leaf rust resistance gene which provides excellent leaf rust resistance world-wide. Unfortunately, in many countries *Lr19* cannot be used for breeding bread wheats as the translocated segment also carries a gene(s) that codes for yellow endosperm pigmentation [1-3]. Other known loci on the segment include: *Ep-D1* (endopeptidase), *Wsp-D1* (water-soluble protein), *Sr25* (stem rust resistance) and *Sd1* (segregation distortion) [4-6].

There are two sources of the *Lr19* translocation, the *Thinopyrum ponticum* derived *Lr19* or T4 [7] translocation and the 'Indis' translocation that was selected in the Dept. of Genetics, Stellenbosch in the backcross progeny of the 'Inia 66'(wheat)/*Thinopyrum distichum* cross in the early eighties. The origin of the 'Indis' translocation has, however, remained suspect as *Thinopyrum distichum*, as well as its amphiploid with common wheat are highly susceptible to local pathotypes of leaf and stem rust. The *Thinopyrum* segment (that has replaced a large portion of chromosome 7DL of wheat) does not pair with homoeologous wheat segments during meiosis, complicating attempts to recombine its genes or to study linkage relationships.

Following irradiation, Marais [5] derived 29 deletion mutants, each homozygous for a different deletion of the translocation. In an attempt to break the linkage between the leaf rust resistance and yellow endosperm loci, Marais [8] made use of pairing inhibitor *(ph1b, ph2b)* mutants in wheat ('CS') to induce allosyndetic pairing and crossovers between the *Lr19* segment and homoeologous areas of the wheat genome.

Four suspected recombinants giving white endosperm, three giving partially white endosperm, and one producing yellow endosperm but sometimes showing self-elimination, were recovered. The resistance in the four white selections was found to be associated with chromosomes other than 7D [9]. Knowledge of the relative positions of marker genes was required to characterize and select the most promising recombined forms. The deletion lines were used to determine the linear sequence of three marker genes on the translocated 7DL arm as: centromere-*Lr19-Wsp-D1-Y* [5]. They were used to characterize the suspected recombinants [8]. However, more markers were needed.

The aims of the present study were to: determine through deletion mapping the relative positions of further marker loci (especially DNA markers) on the 'Indis' translocation; use this knowledge to better characterize the suspected recombinants; study the expression of the leaf rust resistance of the original and apparently recombined forms; and determine whether the 'Indis' translocation could have been derived from *Thinopyrum distichum*.

2. MATERIALS AND METHODS

The deletion mutant lines were used to physically map three *Thinopyrum* RFLP loci, as well as the *Sr25* and *Sd1* loci. The eight suspected recombinants were also tested for the presence of the various marker loci. The original translocation and apparently recombined *Lr19* segments were incorporated through backcrossing into four common wheat backgrounds. These near-isogenic lines were compared for their resistance to five leaf rust pathotypes.

3. RESULTS AND DISCUSSION

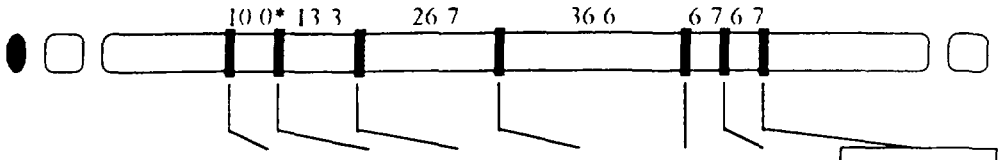
The deletion map results obtained by Marais [5] were integrated with the present data and are presented in Table I [10]. The mutants were ordered to reflect the sequence of marker genes studied. It can be assumed that irradiation induced random breaks on the translocation that resulted in these deletions. The frequencies with which the different mutants were obtained will, therefore, reflect the physical distances (arbitrary units) between loci. While the mutations were mostly caused by terminal deletions, at least two intercalary deletions may have occurred.

Three mutants had partially white endosperm which could suggest the presence of more than one *Y* locus or modified expression of a single locus. Similar phenotypes were recovered amongst the apparently recombined forms [8]. However, in view of the limited data, we chose to treat *Y* as being a single locus. Two mutants expressed stem rust resistance similar to that produced by *Sr25*, yet each has lost *Thinopyrum* chromatin on both sides of *Sr25*. It was assumed that the resistance does not derive from *Sr25* in these mutants.

Physical mapping of *Sd1* was problematic. Due to the complexity of the gametocidal interaction it was not possible to unequivocally distinguish genotypes showing complete, impaired or no gametocidal activity. The *Sd1* gene in translocation heterozygotes causes male and female gametes not carrying it to abort, and the severity of the effect depends on wheat responder genes in the genetic background [6]. Our results confirm the finding of Zhang &

Dvorák [11] that *Sd1* is situated between *Lr19* and the centromere. The absence of the *Xpsr105* and *Xpsr165* loci in three mutants, which have apparently lost all the marker loci, but still cause preferential transmission, normal transmission or self-elimination suggests that *Sd1* is situated proximal to these loci. The segregation distortion effects of 21 of the mutants were clearly different from that observed in crosses involving 'Indis' and 'Inia 66' and self-elimination occurred regularly. This would suggest that at least one further gametocidal gene may be involved. The gene probably has a distal location and was lost in the mutants described. It appears to enhance or to supplement the effect of *Sd1*.

TABLE I. DELETION MAP DATA



Mutant:	*Sd1*	*Xpsr 165*	*Xpsr 105*	*Xpsr 129*	*Lr19*	*Wsp-D1*	*Sr25*	*Y*
87M23-145	*a*	-	-	-	-	-	-	-
87M23-198	*a*	-	-	-	-	-	-	-
89M1-51	*b*	-	-	-	-	-	-	-
87M23-1	*b*	+	-	-	-	-	-	-
89M2-327	*a*	+	-	-	-	-	-	-
87M23-178	*a*	+	+	-	-	-	-	-
87M23-227	*c*	+	+	-	-	-	-	-
87M23-273	*a*	+	+	-	-	-	-	-
89M1-25	*a*	+	+	-	-	-	-	-
89M1-69	*d*	+	+	-	-	-	-	-
89M1-78	*a*	+	+	-	-	-	-	-
87M23-314	*a*	+	+	-	-	-	-	+*f,g*
87M23-3	*a*	+	+	+	-	-	-	-
87M23-27	*d*	+	+	+	-	-	-	-
87M23-108	*d*	+	+	+	-	-	-	-
87M23-115	*a*	+	+	+	-	-	-	-
87M23-118	*a*	+	+	+	-	-	-	-
87M23-128	*d*	+	+	+	-	-	-	-
89M2-416	*b*	+	-*e*	+	-	-	-	+*f,g*
87M23-219	*d*	+	+	+	-	-	+*f*	-
89M2-40	*c*	+	+	+	-	-	-	-
87M23-175	*c*	-*e*	+	+	-	-	-	-
89M2-39	*c*	+	+	+	-	-	-	-
87M23-103	*c*	+	+	+	+	-	+*f*	-
89M2-245	*d*	+	+	+	+	-	-	-
87M23-225	*d*	+	+	+	+	+	-	-
89M2-426	*d*	+	+	+	+	+	-	-
89M1-18	*b*	+	+	+	+	+	+	+*f,g*
87M23-266	*a*	+	+	+	+	+	+	+

*Percentage break points.

[a] Preferential/ normal transmission and self-elimination.

[b] Only self-elimination observed.

[c] Apparently normal segregation ratios, but low seed set.

[d] Only preferential transmission observed.

[a-d] Based on segregation of the mutated translocation in different full sib families.

[e] Intercalary deletion.

[f] Dubious classifications, see text (not considered in the calculation of physical distances).

[g] Intermediate phenotypes suggesting the possibility of more than one locus.

A total of 30 probable break points were recognized and used to estimate the physical distances between genes as indicated in this physical map. Most of the current maps for this segment simply show the markers as occurring within a linkage block. This is the first map depicting the linear sequence and estimated distances for the markers studied on this segment ($7el_1$). This also contradicts the results obtained by Kim et al. [12] that *Xpsr129* is situated distal to *Lr19*. The data confirmed the reported homoeology between the *Lr19* segment and chromosome arm 7DL of wheat.

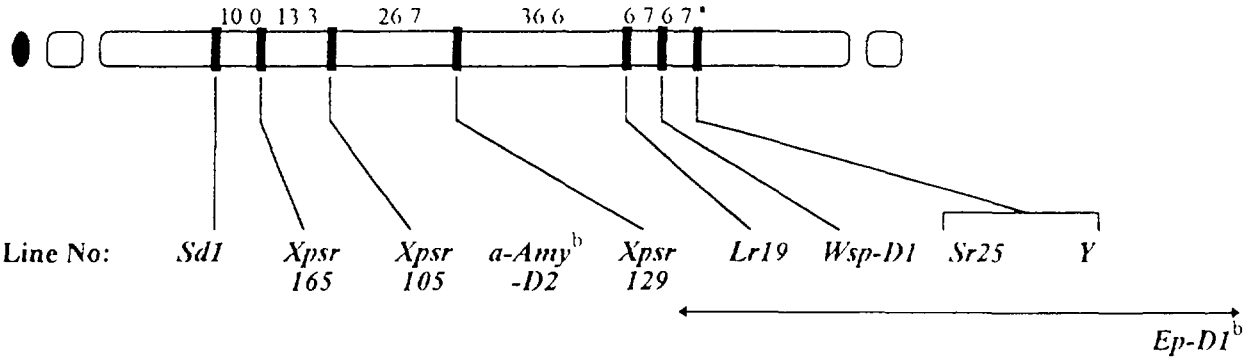
The recombinants were also characterized and rough physical maps of the apparently recombined *Lr19* segments were constructed as illustrated in Table II [13]. The selections were found to fall into two groups: those that retained large *Thinopyrum* segments, produce yellow or partially white endosperm and remained on chromosome 7D; and those that occur on different chromosomes and are not associated with yellow flour pigments. With respect to the first group, more distally located markers should be studied to determine if the distal parts of the three partially white selections have indeed been exchanged with wheat chromatin. The four promising white endosperm recombinants were initially selected on the basis of leaf rust resistance (one pathotype, UVPrt8) and absence of yellow endosperm pigmentation. Three of these selections (87M70-63, 88M22-157, 88M22-184) do not exhibit any of the *Thinopyrum* marker phenotypes except for having strong leaf rust resistance (unknown gene, *Lr?*) against one of five predominant pathotypes (UVPrt8). The latter selections have an altered resistance that could have resulted from the unintentional selection of a 'contaminating' resistance gene in the progeny following the induction of allosyndetic pairing. As will be discussed, the pedigree of 'Indis' is suspect and could have contributed such a gene.

The white endosperm recombinant 88M22-149 retained the *Thinopyrum* alleles of the *Xpsr129* and *Wsp-D1* loci, each of which can serve as a marker of its presence. It has also lost the *Xpsr129* locus on 7BL and regained the 7DL locus which would suggest that it was relocated to chromsome arm 7BL. Both the wheat (*Wsp-B1*) and *Thinopyrum*-derived (*Wsp-D1*) loci are expressed in this recombinant. This would imply that an unequal crossover event occurred which created a duplicated region. This recombinant has probably lost *Sd1* and has a very consistent tendency to self-eliminate. It is not clear from the results whether the strong self-elimination stems from the disruption of a complex of *Sd* genes or whether it is due to (or enhanced by) a disruptive chromosome duplication that occurred during recombination. It is the only true white endosperm recombinant that has retained the *Lr19* resistance and which provides complete resistance to the five pathotypes tested.

It seems likely that the 'Indis' translocation is simply a derivative of the T4 translocation. A comparison of the RFLPs obtained with a ditelosomic $7el_1$-*Thinopyrum ponticum* addition line (W743), 'Indis' and the T4 translocation, confirmed that the 'Indis' and T4 translocations and chromosome $7el_1$ of *T. ponticum* produce identical bands. Fragments corresponding to the polymorphisms in the translocation could not be detected in DNA extracts of two accessions of *Thinopyrum distichum* or its amphiploid with wheat.

The physical map of the *Lr19* segment enabled us to characterize the eight suspected recombinants. This led to the identification of only one true recombinant from the four originally suspected white recombinants. Should the *Lr19* resistance in this recombinant prove to be stable and not to be associated with any negative agronomic effects, it will be the only useful white endosperm recombinant that has been obtained following the use of the *ph* mutants. This illustrates that much effort is needed in tailoring the alien translocations for use in wheat breeding.

TABLE II. POLYMORPHISMS FOR MARKER LOCI IN SELECTIONS SUSPECTED TO HAVE RECOMBINED *LR19* TRANSLOCATED SEGMENTS (T: the *THINOPYRUM* allele; W: the common wheat allele: the alternative form which is a null condition or an allele that could not be positively identified, ?: unknown)



Translocation on chromosome arm 7DL: yellow and partially white endosperm										
87M70-348	?	T	T	-	T	T	T	T	T	-
88M22-42	?	W	W	W	T	T	T	T	T[c]	-
88M22-98	?	T	T	-	T	T	T	T	T[c]	-
88M22-103	?	T	T	-	T	T	T	T	T[c]	-
Unmodified	T	T	T	-	T	T	T	T	T	-
Resistance not associated with chromosome 7D: white endosperm										
87M70-63	-	W	W	W	W	*Lr?*[f]	W	-	-	W
88M22-157	-	W	W	W	W	*Lr?*[f]	W	-	-	W
88M22-184	-	W	W	W	W	*Lr?*[f]	W	-	-	W
88M22-149	-	W	W	W	T[d,e]	T	T[d]	-	-	W

[a] Physical deletion map constructed by Prins et al 1996a

[b] Mapped within this region by Chao et al 1989

[c] Partially white recombinants (see text)

[d] Retained the *Thinopyrum* locus and regained the wheat 7DL locus

[e] Lost the wheat 7BL locus

[f] Unknown resistance gene (*Lr?*)

ACKNOWLEDGEMENTS

We wish to thank the South African Wheat Board for financing the project. The cDNA clones were generously supplied by Dr. M.D. Gale, Institute of Plant Science Research, Cambridge Laboratory, Colney Lane, Norwich. UK. We also want to thank the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture for making the attendance of this meeting possible.

REFERENCES

[1] KNOTT, D.R., Mutation of a gene for yellow pigment linked to *Lr19* in wheat, Can. J. Genet. 22 (1980) 651-654.

[2] KNOTT, D.R., The effect of transfers of alien genes for leaf rust resistance on the agronomic and quality characteristics of wheat, Euphytica 441 (1989) 65-72.

[3] MARAIS, G.F., ROUX, H.S., PRETORIUS, Z.A., PIENAAR, R. de V., Resistance to leaf rust of wheat derived from *Thinopyrum distichum* (Thunb.) Löve. Proc. 7th Int. Wheat Genet. Symp., Cambridge, 1988 (Miller, T.E., Koebner R.M.D., Eds) IPSR, Cambridge (1988) 369-849.

[4] MARAIS, G.F., MARAIS, A.S., The assignment of a *Thinopyrum distichum* (Thunb.) Löve-derived translocation to the long arm of wheat chromosome 7D using endopeptidase polymorphisms, Theor. Appl. Genet. 79 (1990) 182-186.

[5] MARAIS, G.F., Gamma irradiation induced deletions in an alien chromosome segment of the wheat 'Indis' and their use in gene mapping, Genome 35 (1992) 225-229.

[6] MARAIS, G.F., Genetic control of a response to the segregation distortion allele, *Sd-1d*, in the common wheat line 'Indis', Euphytica, 60 (1992) 89-95.

[7] SHARMA, D., KNOTT, D.R., The transfer of leaf rust resistance from *Agropyron* to *Triticum* by irradiation, Can. J. Genet. Cytol. 8 (1966) 137-143.

[8] MARAIS, G.F., The modification of a common wheat-*Thinopyrum distichum* translocated chromosome with a locus homoeoallelic to *Lr19*, Theor. Appl. Genet. 85 (1992) 73-78.

[9] MARAIS, G.F., Characterization of modified forms of a *Lr19* translocation. Proc. 8th Int. Wheat Genet. Symp., Beijing, 1993, Li, Z.S., Xin, Z.Y. Eds., China Agric. Scientech Press, Beijing (1993) 937-941.

[10] PRINS, R., MARAIS, G.F., JANSE, B.J.H., PRETORIUS, Z.A., MARAIS, A.S., A physical map of the *Thinopyrum*-derived *Lr19* translocation, Genome 39 (1996) 1013-1019.

[11] ZHANG, H.B., DVORÁK, J., Characterization and distribution of an interspersed repeated nucleotide sequence from *Lophopyrum elongatum* and mapping of a segregation distortion factor with it, Genome 33 (1990) 927-936.

[12] KIM, N.-S., ARMSTRONG, K., KNOTT, D.R., Molecular detection of *Lophopyrum* chromatin in wheat-*Lophopyrum* recombinants and their use in the physical mapping of chromosome 7D, Theor. Appl. Genet. 85 (1993) 561-567.

[13] PRINS, R., MARAIS, G.F., PRETORIUS, Z.A., JANSE, B.J.H, MARAIS, A.S., A study of modified forms of the *Lr19* translocation of common wheat. Theor. Appl. Genet. (1997) (in press).

[14] CHAO, S., SHARP, P.J., WORLAND, A.J., WARHAM, E.J., KOEBNER, R.M.D., GALE, M.D., RFLP-based genetic maps of wheat homoeologous group-7 chromosomes. Theor. Appl. Genet. 78 (1989) 495-504.

MICROSATELLITES IN WHEAT AND THEIR APPLICATIONS

P. STEPHENSON, G.J. BRYAN*, J. KIRBY, M.D. GALE
John Innes Centre,
Norwich Research Park,
Norwich, United Kingdom

Abstract

The development of large panels of simply analyzable genetic markers for diversity studies and tagging agronomically important genes in hexaploid bread wheat is an important goal in applied cereal genetic research. We have isolated and sequenced over two-hundred clones containing microsatellites from the wheat genome, and have tested 150 primer pairs for genetic polymorphism using a panel of ten wheat varieties, including the parents of our main mapping cross. A total of 125 loci were detected by 82 primer pairs, of which 105 loci from 63 primer pairs can be unequivocally allocated to one of the wheat chromosomes. A relatively low frequency of the loci detected are from the D-genome (24%). Generally, the microsatellites show high levels of genetic polymorphism and an average 3.5 alleles per locus with an average polymorphism information content (PIC) value of 0.5. The observed levels of polymorphism are positively correlated with the length of the microsatellite repeats. A high proportion, approximately one half, of primer pairs designed to detect simple sequence repeat (SSR) variation in wheat do not generate the expected amplification products and, more significantly, often generate unresolvable Polymerase Chain Reaction (PCR) products. In general our results agree closely with those obtained from other recent studies using microsatellites in plants.

1. INTRODUCTION

In recent years the use of genetic mapping techniques based upon Polymerase Chain Reaction (PCR) methodology using simple sequence repeat (SSR) markers has begun to supersede the use of Restriction Fragment Length Polymorphisms (RFLP). A recent mouse map contains over 4000 microsatellite markers [1]. However, microsatellite markers have been developed less rapidly in plants. Plant chromosomes carry microsatellites at relatively low frequencies when compared to mammals [2]. The relatively slow adoption of microsatellite technology is due to the high development cost of SSR markers. Nevertheless, microsatellites are ubiquitous and have the potential to provide extremely polymorphic, codominant marker systems in plants [3, 4, 5]. Previous studies suggest that microsatellites occur at high enough frequencies to allow their isolation in relatively large numbers from plant genomes.

Hexaploid bread wheat shows relatively low levels of RFLP, most likely a result of its narrow genetic base [6]. The formation of hexaploid wheat, in all likelihood, traces back to a single fortuitous hybrid only 10 000 years ago [7]. This lack of marker polymorphism in hexaploid wheat necessitates the use of very wide crosses for efficient map generation. RFLPs also show a considerable degree of clustering on the genetic map, and it is possible that other types of marker may show different patterns of genetic distribution across cereal genomes. There is an unquestionable need for more highly polymorphic genetic marker systems than RFLPs can provide, particularly in crosses and breeding schemes using adapted varieties. Simple sequence repeats have previously been shown to be useful as genetic markers in wheat [8, 9]. In order to develop markers for genetic mapping and diversity studies in hexaploid wheat and related species using microsatellites, we have isolated different microsatellite repeats from the wheat genome by hybridization screens of small-insert genomic libraries. We have isolated a substantial number of SSRs and have screened them for genetic polymorphism using a panel of wheat varieties. Where possible, these markers have been assigned to particular wheat chromosomes using aneuploid analysis. There are some problems

*Current address: Scottish Crop Research Institute, Invergowrie, Dundee, United Kingdom.

with the development of SSRs in wheat, associated with the extremely large genome size and high repetitive DNA content typical of cereal species.

2. MATERIALS AND METHODS

2.1. Genetic stocks/wheat varieties

The following varieties were used for the evaluation of polymorphism and were chosen to represent material used in current wheat breeding programmes, together with the parents of the main mapping cross used to construct the RFLP map in this laboratory: "Cappelle-Desprez", "Brigadier", "Herzog", "Hereward", "Soissons", "Timgalen", "Hope", "RL4137", and "Chinese Spring" and a synthetic hexaploid (IPSR 1190903) hereafter referred to as "Synthetic" [10, 11]. The aneuploid stocks used for the determination of chromosomal origins of PCR products were the 21 nullisomic-tetrasomic lines of "Chinese Spring" [12].

2.2. Library construction/microsatellite isolation

Small insert libraries of the wheat variety "Chinese Spring" were constructed as follows: DNA (1-10 µg) was digested with *Sau*3AI. DNA fragments of the desired size range (ie. 300-500 bp) were size-selected and purified from agarose gels using Prepagene (Biorad) or DEAE paper (Schleicher and Schuell). Purified DNA fragments were ligated into the *Bam*HI site of M13mp18. Ligations were transformed into JM101/JM105 by electroporation (Biorad Gene Pulser). M13 plaques were screened with γ-^{32}P end-labelled oligonucleotide probes.

Positive plaques were suspended in 1ml of L-broth and rescreened using serial dilutions from the original plaque suspension. Three dilutions from each clone (10^{-7}, 10^{-8}, 10^{-9}) were plated in ordered arrays onto seeded bacterial lawns in 24 cm square petri dishes, at a density of 70 clones per petri dish, and then rescreened with the original probe oligonucletide. Single positive plaques were isolated and phage DNA isolated by standard small-scale procedures.

2.3. PCR screening of positive clones

In order to minimise the amount of unnecessary sequencing, a PCR screen was used to check each putative positive SSR-bearing clone for the presence and position of a microsatellite. PCR screens were performed using three primer pairs: reaction #1 - M13 Forward + M13 Reverse; reaction #2 - M13 Forward + SSR primer A; Reaction #3 - SSR primer B + M13 Reverse. SSR primers A and B are the microsatellite probe repeat sequence and its complementary sequence respectively. 1µl of phage suspension was amplified in 50µl reaction containing 10mM Tris-HCl (pH8.3), 1.5mM $MgCl_2$, 50mM KCl, 100µM dNTPs, 2µM each primer, 0.1 unit *Taq* polymerase. Cycling conditions were as follow: five cycles of 94°C for 1 min., 45°C for 1 min., 72°C for 1 min., followed by 25 cycles of 94°C for 1min., 60°C for 1 min., and 72°C for 2 min. PCR was completed by a final incubation at 72°C for 5 min. For those clones selected by simultaneous hybridization with two different oligonucleotide probes, it was necessary to perform the PCR screen using five reactions per clone (i.e. as above, but with two pairs of reactions using different microsatellite repeat primers). This procedure effectively determined whether the clone contained a microsatellite, the position of the microsatellite within the clone, and in some cases, the type of microsatellite. Following Senior *et al.* [13], an arbitrary 8 base pair tail (5'-TAGCCTAG-3') was added to the 5-prime terminus of the simple sequence repeat oligonucleotide PCR primer.

2.4. Sequencing of microsatellite containing clones

Phage DNA was isolated by standard small-scale procedures and DNA sequencing was performed with the AutoRead Sequencing kit (Pharmacia) analyzed with an Automated Laser Fluorescence (ALF) DNA sequencer (Pharmacia)

2.5. PCR amplification and product analysis

DNA was extracted from fresh or freeze-dried plant tissue as described by Sharp *et al.* [14]. PCR primers were designed from the microsatellite flanking sequences either by inspection or by analysis using the 'oligo' primer analysis software (National Biosciences Inc.). Primers were chosen to have annealing temperatures in the range 58-65°C. DNA (100 ng) was PCR amplified in 30 µl reactions containing: 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 10 mM KCl, 200nM of each primer, 200 µM of each dNTP, and 2.5 units of *Taq* DNA polymerase (Stoffel fragment, Perkin-Elmer). Cycling conditions were as follows: an initial denaturation step of 4 min at 94°C, followed by 30 cycles of 30 sec at 95°C, 1 min at 61°C, and 1 min at 73°C. After cycling the reactions were incubated for 5 min at 73°C. PCR products were analyzed on standard denaturing sequencing gels (6% polyacrylamide (19:1 Acrylamide:Bis), 8M urea). Products were visualised by silver staining (Promega Inc.), or by the use of autoradiography where γ-^{33}P-labelled oligos were used as primers.

2.6. Estimation of polymorphism information content

We have estimated the polymorphism information content (PIC), assuming homozygosity of wheat varieties, following Anderson *et al.* [15]:

$$PIC_i = 1 - \sum_{j=1}^{n} p_{ij}^2$$

where p_{ij} is the frequency of the *j*th pattern for marker *i* and the sum is made over *n* patterns.

2.7. Chromosome assignment of SSRs

PCR amplifications were performed with DNA from a set of 21 nullisomic-tetrasomic lines of "Chinese Spring". The microsatellite loci were assigned to chromosomes corresponding to the nullisomic-tetrasomic template for which either no PCR product was obtained, or one of the PCR products was missing, provided that the templates for all of the other twenty lines generated the relevant amplification product.

3. RESULTS

3.1. Cloning data and frequency of microsatellites in libraries

The M13 libraries were probed with six different microsatellite sequences: $(CA)_{16}$, $(GA)_{16}$, $(CAA)_{10}$, $(GAA)_{10}$, $(ACG)_{10}$, $(CAG)_{10}$. Approximately 2000 positive plaques were picked from the primary screens of approximately 700,000 M13 clones. Of these, 1500 positive clones were re-screened and, wherever possible, single plaques isolated. From these hybridizations, it was possible to plaque purify approximately 700 M13 clones. These plaques were then screened by PCR to check the presence, nature, and position of the microsatellites within the clones. The addition of an 8bp tail to the microsatellite repeat primer appears to help anchor the SSR PCR primer and to restrict slippage of the SSR primer along the microsatellite array, resulting in more discrete PCR products. Only positive clones with a satisfactory location of the SSR were selected for DNA sequencing.

3.2. Sequencing data

Of 277 M13 clones sequenced, 222 were found to contain a microsatellite in the sequenced portion of the clone. Use of the PCR screen obviated the need for sequencing a further 430 clones. Flanking primers from 150 sequences were synthesized. The majority of microsatellite sequences contain either a $(CA)_n$ or a $(GA)_n$ repeat (~80%), which indicates that, as might be expected, the frequency of these types of dinucleotide repeats is significantly higher than the selected trinucleotide repeats in the wheat genome. Approximately 15% of clones contained more than one type of microsatellite repeat and usually, the different repeat types are adjacent, or very nearly so, in the clones.

3.3. PCR results and levels of genetic polymorphism

Primers from 82 of the 150 clones amplify a PCR product of the predicted size from the variety Chinese Spring, the source of the M13 clones. Primers from 63 of these clones amplify products which can be assigned to a chromosome. The remaining 68 primers either produce no detectable PCR products, incorrectly sized PCR products or a larger number of bands which cannot be genetically analyzed with ease. Several clones contain more than one type of microsatellite repeat (Table I).

TABLE I. FREQUENCY OF MICROSATELLITE MOTIFS

Repeat type	Number found	Frequency	Sub-totals	GA	CA
GA	58	38.66%		38.66%	
CA	40	26.66%	65.33%		26.66%
CA/GA	7	4.66%		4.66%	4.66%
CA/GT	2	1.33%			1.33%
CA/CT	7	4.66%		4.66%	4.66%
CA/TA	4	2.66%			2.66%
GA/TA	3	2.00%	15.33%	2.00%	
CAA	13	8.66%			
GAA	7	4.66%			
Other 3bp	9	6.00%	19.33%		
Total	150			49.98%	39.97%

3.4. Localization of microsatellites to chromosomes

The distribution of chromosomal locations of microsatellite loci is shown in Table II. For several of the SSR primer pairs, it has been possible to identify the chromosomal origin of more than one PCR product. These loci are spread across the three wheat genomes as follows: A genome: 38 loci (36%), B genome: 42 loci (40%), D genome: 25 loci (24%).

In only four cases does one of the additional loci appear to originate from a homoeologous chromosome but these are not polymorphic and so homeology cannot be proven. In 11 cases more than one PCR product appear to originate from the same chromosome, suggesting the possibility of duplicated chromosome specific sequences

containing microsatellites. Only two of these multiple products have been mapped and they map to the same locus. In 27 cases, in which the primers amplified discrete product, the PCR products are not absent in any of the NT lines, suggesting amplification from a duplicated sequence at two or more chromosomal locations in the wheat genome, in all cases these loci are not polymorphic.

TABLE II. DISTRIBUTION OF MICROSATELLITE LOCI BY GENOMES

	A	B	D	Total	%
Chromosome 1	4	1	3	8	7.6%
Chromosome 2	7	5	3	15	14.4%
Chromosome 3	5	11	1	17	16.2%
Chromosome 4	7	8	6	21	20%
Chromosome 5	2	7	3	12	11.4%
Chromosome 6	6	3	1	10	9.5%
Chromosome 7	7	7	8	22	21%
Total	38	42	25	105	
%	36%	40%	24%		
PIC all loci	0.4	0.4	0.38		
PIC polymorphic loci	0.49	0.53	0.43		

4. DISCUSSION

4.1. Identification of useful microsatellite loci

In this report we describe the isolation of a substantial number of microsatellites from the wheat genome. During our SSR isolation procedure, over 50% of putative positive clones were discarded during the secondary screening/plaque-purification process. Approximately 60% of the plaque-purified clones were rejected following the PCR screen, obviating the need for the sequencing of over 400 clones. Of 277 clones sequenced, 127 were deemed unsuitable for primer synthesis. Therefore, as a proportion of the number of positive clones from the first screen, only 10% of the isolated SSRs were selected for primer synthesis. Owing to our stringent selection criteria, we have been able to make PCR primers against a high proportion of the sequenced clones.

Given our experience of large attrition rates of positive clones, it may be advisable in future, to opt for efficient SSR enrichment strategies to reduce expenditure of time and resources in clone purification [16, 17]. Future SSR isolation programmes should combine an efficient enrichment strategy, with one that avoids the cloning of highly repetitive flanking sequences from the wheat genome. The use of methylation-sensitive restriction enzymes, such

as *Pst*I and *Hpa*II, during the enrichment and cloning processes could possibly be used to address this problem [18].

4.2. SSR polymorphism

Based on a set of ten varieties, our microsatellites show an average PIC value of 0.51. However, given the large difference in microsatellite length, the average PIC values are very similar, suggesting that, beyond a critical length, the level of polymorphism exhibited by SSRs does not increase dramatically. The mean number of alleles seen in this study of ten genotypes is 3.6. We have tested a few of our SSRs on larger sets of more diverse material and larger numbers of alleles have been detected. The level of polymorphism seen in this study is considerably higher than that shown by RFLPs, although we do not have an estimate based on the same set of varieties used in this study. The data of Chao *et al.* [6], when subjected to a PIC analysis, gives an average figure of 0.06.

No effect on levels of polymorphism due to the different types of microsatellite can be seen from our data. The PIC values of polymorphic (CA)n repeats average 0.5, (GA)n repeats average 0.55, chimeric repeats average 0.65 and the 3 base pair repeats average 0.37.

4.3. Additional products generated by microsatellite primers

The SSR primers generally detect additional loci. The additional loci show lower levels of polymorphism (average PIC of 0.36, 2.4 alleles per locus). Sometimes the observed size difference between PCR products from different loci exceeds the length of the microsatellite in Chinese Spring, which implies that the microsatellite may be absent or that the flanking sequence has been deleted or rearranged. We feel that it is important to analyze the DNA sequences of these additional products, in order to understand the basis of multilocus microsatellite amplification in hexaploid wheat. A common problem when using a set of unrelated material for the analysis of microsatellites, is the unequivocal identification of alleles for the same locus for primers detecting multiple loci, especially where the products from the different loci are of very similar size.

In this study, a number of the 150 synthesised primer pairs gave rise to a larger number of PCR products than can be resolved or analyzed with ease. In several cases these products were observed as smears or stuttered ladder patterns on polyacrylamide gels. Obviously, with the high levels of repetitive sequences present in the wheat genome, it is likely that a relatively large number of microsatellites will be within DNA sequences that are themselves repetitive sequences. During the isolation of SSRs, we have isolated clones which appear to contain short repetitive motifs in the flanking DNA. It is important to establish the proportion of wheat microsatellites which are embedded in repetitive flanking DNA and to see if such microsatellites can be rendered useful by judicious primer selection. However again, the use of methylation sensitive restriction digests for the initial library construction may reduce the proportion of non-useful amplification products.

4.4. Primer transportability

We have seen very little evidence of homoeologous amplification in this analysis. It is not yet known if SSRs isolated from wheat represent homoeologous sequences, and whether the failure of primers to amplify across the three wheat genomes is due to polymorphism in their flanking DNA. There are important questions concerning the transportability of PCR primers raised against genomic sequences, across the three wheat genomes and beyond, into

other grass genomes. The data of Röder *et al.* [9] suggest that these types of PCR marker may not transfer well to other species, in the way that RFLPs have been shown to do very effectively. Our results also show a low level of transportability across the three wheat genomes and to other cereal genomes.

TABLE III. TRANSPORTABILITY OF MICROSATELLITES

	Number transported	Number polymorphic
Wheat	44 tested	26 ********
Diploid	34 (77%)	
Rye	24 (55%)	3 (1 dominant)
Barley	18 (40%)	3 (1 dominant)
Maize	7 (16%)	2 (0 dominant)
Rice	5 (11%)	1 (0 dominant)

These results contrast greatly with results obtained using PCR primers raised against cDNAs, which exhibit very high levels of transportability across cereal genomes [19]. This also contrasts with data from mammalian microsatellite studies, whereby primers raised against one species, often work on others [20]. This question of marker transfer between species is assuming an ever increasing level of significance in genetic studies in grasses and other organisms, given the current level of investment in comparative genetic mapping. The development of these expensive PCR-based markers with the ability to transcend species barriers in grasses is likely to be an important area of marker development in the coming years.

REFERENCES

[1] DIETRICH, W.F., MILLER, J.C., STEEN, R.G., MERCHANT, M., DAMRON, D., NAHF, R., GROSS, A., JOYCE, D.C., WESSEL, M., DREDGE, R.D., MARQUIS, A., STEIN, L.D., GOODMAN, N., PAGE, D.C., LANDER, E.S., A genetic-map of the mouse with 4,006 simple sequence length polymorphisms, Nature Genet. 7 (1994) 220-245.

[2] LAGERCRANTZ, U., ELLENGREN, H., ANDERSSON, L., The abundance of various polymorphic microsatellite motifs differs between plants and vertebrates, Nucleic Acids Res. 21 (1993) 1111-1115.

[3] AKKAYA, M.S., BHAGWAT, A.A., CREGAN, P.B., Length polymorphisms of simple sequence repeat DNA in soybean, Genetics 132 (1992) 1131-1139.

[4] MORGANTE, M., OLIVIERI, A.M., PCR-amplified microsatellites as markers in plant genetics, Plant J. 3 (1993) 175-182.

[5] WU, K-S., TANKSLEY, S.D., Abundance, polymorphisms and genetic mapping of microsatellites in rice, Mol. Gen. Genet. 241 (1993) 225-235.

[6] CHAO, S., SHARP, P.J., WORLAND, A.J., WARHAM, E.J., KOEBNER, R.M.D., GALE, M.D., RFLP-based genetic maps of wheat homoelogous group 7 chromosomes, Theor. Appl. Genet. 78 (1989) 495-504.

[7] HILLMAN, G.C., The plant remains from Tell Abu Hureyra: a preliminary report, Proc. Prehistoric Soc. 41 (1975) 306-322.

[8] DEVOS, K.M., BRYAN, G.J., COLLINS, A.J., STEPHENSON, P., GALE, M.D., Application of two microsatellite sequences in wheat storage proteins as molecular markers, Theor. Appl. Genet. 90 (1995) 247-252.

[9] RÖDER, M.S., PLASHKE, J., KÖNIG, S.U., BÖRNER, A., SORRELLS, M.E., TANKSLEY, S.D., GANAL, M.W., Abundance, variability and chromosomal location of microsatellites in wheat, Mol. Gen. Genet. 246 (1995) 327-333 .

[10] MCFADDEN, E.S., SEARS, E.R., The origin of *Triticum spelta* and its freethreshing hexaploid relatives, J. Hered. 37 (1946) 81-89.

[11] SEARS, E.R., A synthetic hexaploid wheat with fragile rachis, Wheat Inf. Serv. 41-42 (1976) 31-32.

[12] SEARS, E.R., The aneuploids of common wheat, Missouri Agric. Exp. Stn. Res. Bull. 572 (1954) 1-59.

[13] SENIOR, M.L., HEUN, M., Mapping maize microsatellites and polymerase chain reaction confirmation of the targeted repeats using a CT primer, Genome 36 (1993) 884-889.

[14] SHARP, P.J., KREIS, M., SHEWRY, P., GALE, M.D., Location of beta-amylase sequence in wheat and it's relatives, Theor. Appl. Genet. 75 (1988) 289-290.

[15] ANDERSON, J.A., TANKSLEY, S.D., SORRELLS, M.E., Optimizing parental selection for genetic linkage maps, Genome 36 (1993) 181-186.

[16] KARAGYOSOV, L., KALCHEVE, I.D., CHAPMAN, V.M., Construction of random small-insert genomic libraries enriched for simple sequence repeats, Nucleic Acids Res. 21 (1993) 3911-3912.

[17] KANDPAL, R.P., KANDPAL, G., WEISSMAN, S.M., Construction of libraries enriched for sequence repeats and jumping clones, and hybridization selection for region specific markers, Proc. Natl. Acad. Sci. USA 91 (1994) 88-92.

[18] CHEUNG, W.Y., MOORE, G., MONEY, T.A., GALE, M.D., *Hpa*II library indicates 'methylation-free islands' in wheat and barley, Theor. Appl. Genet. 84 (1992) 739-746.

[19] BRYAN, G.J., STEPHENSON, P., COLLINS, A., SMITH, J.B., GALE, M.D., Isolation and characterisation of microsatellites from hexaploid bread wheat, Theor. Appl. Genet. 94 (1997) 557-563.

[20] SCHLOETTERER, C., AMOS, W., TAUTZ, D., Conservation of polymorphic simple sequence loci in Cetacean species, Nature 354 (1991) 63-65.

ADVANCES IN SORGHUM GENETIC MAPPING WITH IMPLICATIONS FOR SORGHUM IMPROVEMENT

XA9847003

M. LEE
Department of Agronomy,
Iowa State University,
Ames, Iowa, United States of America

Abstract

DNA markers and genetic maps have become important tools for direct investigations of several facets of crop improvement and will provide vital links between plant breeding and basic plant biology The markers and maps will become more important for increased crop production because plant genetics will be required to extend or replace extant management practices such as chemical fertilizers, pesticides, and irrigation [1] Despite the importance of the sorghum crop, comprehensive genetic characterization has been limited Therefore, the primary goal of this research program was to develop basic genetic tools to facilitate research in the genetics and breeding of sorghum The first phase of this project consisted of constructing a genetic map based on restriction fragment length polymorphisms (RFLPs) The ISU sorghum map was created through linkage analysis of 78 F2 plants of an intraspecific cross between inbred CK60 and accession PI229828 [2] Subsequent mapping efforts in several labs have enriched the sorghum map to the point where it now contains over 1,500 loci defined by RFLPs and many others defined by mutant phenotypes and QTLs The ISU map consists of 201 loci distributed among 10 linkage groups covering 1299 cM Comparison of sorghum and maize RFLP maps on the basis of common sets of DNA probes revealed a high degree of conservation as reflected by homology, copy number, and colinearity Examples of conserved and rearranged locus orders were observed

The same sorghum population was used to map genetic factors (mutants and QTLs) for several traits including vegetative and reproductive morphology, maturity, insect, and disease resistance Four QTLs for plant height, an important character for sorghum adaptation in temperate latitudes for grain production, were identified in a sample of 152 F2 plants [3] whereas 6 QTLs were detected among their F3 progeny These observations and assessments of other traits at 4 QTLs common to F2 plants and their F3 progeny indicate some of these regions correspond to loci (*dw*) previously identified on the basis of alleles with highly qualitative effects Four of the six sorghum plant height QTLs seem to be orthologous to plant height QTLs in maize Other possible instances of orthologous QTLs included regions for maturity and tillering These observations suggest that the conservation of the maize and sorghum genomes encompasses sequence homology, colinearity, and function

The genetic information and technology developed on the basis of DNA markers could be used in several facets of breeding, genetics, and other basic biological investigations In addition, DNA markers have been used to survey large collections of elite sorghum germ plasm to determine the degree of genetic relationships and genetic diversity [4] RFLP data seem to portray genetic relationships more accurately than the methods based exclusively on the coancestry coefficient This information provides the basis for more accurate perceptions of genetic relationships and diversity. Recently, PCR-based markers (microsatellites, simple sequence repeats, SSRs) have become available for sorghum Thirty-two SSR loci have been mapped throughout the sorghum genome More SSR loci will be developed so that a rapid, reliable, and non-radioactive genetic marker system will be available for sorghum research in the near future

1. INTRODUCTION

Knowledge of a crop's genetic architecture will become more important for increased crop production because plant genetics will be required to extend or replace extant management practices such as chemical fertilizers, pesticides, and irrigation Such knowledge will include more detailed descriptions of genome organization, the crop's gene pools, and genes and pathways controlling important phenotypes. In many instances, DNA markers and genetic maps will be important tools for direct investigations of these areas and will provide vital links between plant breeding and basic plant biology [1].

Many of the limitations of plant breeding methods have been rooted in the status of the technical infrastructure for conducting genetic analyses. Breeders and geneticists of all crops have lacked an informative and integrated genetic context to aid interpretation and

conciliation of perspectives provided by seemingly different approaches to genetic improvement. The result has been a situation resembling the Tower of Babel with breeders, geneticists, cytogeneticists, taxonomists, molecular biologists, plant pathologists, and other factions contributing to the confusion. A key component of the infrastructure and context of future plant breeding programs will be genetic maps. The maps, when fully integrated, will have several roles: 1) provide a focal point and hub for data derived from the perspectives of myriad disciplines for each crop; 2) constitute a vital two-way avenue connecting plant breeding and basic plant biology; 3) contribute essential information for positional cloning of genes; 4) facilitate a considerable and directed expansion of a crop's gene pool through comparative mapping of related and unrelated taxa; 5) accelerate identification and incorporation of useful genes into cultivars; and 6) contribute important clues toward understanding the biological basis of complex traits and phenomena important to crop improvement. The significance of these and other roles and their implementation will vary with the repertoire of genetic technologies available to the crop, breeding methods and goals, and the nature of the crop's nuclear genome. However, the foundation provided by the maps have had, and will have a positive impact on the genetic improvement of crop species in many instances.

2. DEVELOPMENT OF A GENETIC MAP FOR SORGHUM AND COMPARATIVE MAPPING WITH MAIZE

In terms of cultivated area, sorghum (*Sorghum bicolor* L. Moench) is the world's fifth leading cereal [5]. Despite the importance of the sorghum crop, comprehensive genetic characterization has been limited. Therefore, the primary goal of this research program was to develop basic genetic tools to facilitate research in the genetics and breeding of sorghum. The specific objectives were to 1) develop a complete genetic linkage map for sorghum based on DNA markers, 2) identify the genetic locations of genes of interest to sorghum breeding programs, 3) integrate the sorghum genetic map(s) with maps of allied grass species, and 4) assess the genetic diversity and relationships of sorghum germ plasm.

In sorghum, several maps have been developed for various objectives by different research groups. These maps are being compared and integrated on the basis of probes exchanged among various laboratories (G. Hart, 1996 personal communication). The RFLP linkage map produced at ISU (Iowa State University) was created through linkage analysis of 78 F2 plants of an intraspecific cross between inbred CK60 and accession PI229828 [2]. These parents were selected to produce the mapping population for several reasons; 1) adequate DNA polymorphism, 2) parental difference for resistance to an important insect pest, the aphid, *Schizapus graminum* (greenbug, race E), 3) parental difference for resistance to the fungal pathogen, *Peronsclerospora sorghi*, the causal agent of downy mildew, 4) parental divergence for numerous morphological and developmental traits (e.g. plant height, flowering, and tillering), 5) no known chromosomal polymorphisms and 6) presumably, rates and patterns of recombination more representative of other intraspecific crosses. Selection of this population and use of maize DNA probes to detect RFLPs permitted rather efficient collection of extensive genetic information for sorghum and the means of relating the information to that of maize and other grasses.

The map consists of 201 loci distributed among 10 linkage groups covering 1299 cM. Presumably the number of linkage groups correspond to the basic chromosome complement of sorghum (n=10). The RFLP loci were detected through hybridizations with probes of maize genomic (52), maize cDNA (124), and sorghum genomic (10) clones. Most probes detected a single RFLP locus (172) but there was evidence of genomic duplication as many probes

(76) detected more than one band with a strong signal. However, in this population the additional bands were usually monomorphic. Segregation data at 95% of the loci fit expected ratios for an F2 generation of a cross between two homozygous parents. Loci with deviant ratios were located predominantly to one region of linkage group B. All features of this initial map have been verified in a second and larger sample from this F2 population used for mapping quantitative trait loci (QTL [3]).

Recently, a collaboration among my lab, the University of Milan, Italy (E. Pe and G. Taramino) and the USDA/ARS (S. Kresovich, Griffin, GA, USA) has identified genetic loci using simple sequence repeats (SSRs or microsatellites). Genomic DNA clones or their sequences containing di-, tri- or tetra-nucleotide repeats were selected from either Genbank sequences or specially constructed libraries. Flanking sequences were then used to construct primers of 25-30 base pairs. PCR amplification of sorghum genomic DNA with these primers typically amplifies one predominant DNA fragment that is easily observed on agarose (4% Metaphor) gels stained with ethidium bromide. The first report of this collaboration identified seven SSR loci in the CK60/PI229828 mapping population [6]. Mapping with SSR primers developed by the USDA/ARS (S. Kresovich) will bring the total number of loci to 32 [7]. Additional efforts to develop primers for detecting SSRs in sorghum have been recently initiated (G. Hart, 1996, personal communication). Based on rather limited assessments and efforts, the utility of the primers has been limited to sorghum. In sorghum, however, SSRs do detect more DNA polymorphisms than RFLPs (M. Lee, unpublished). Therefore, this DNA marker system offers several advantages for breeding and genetics programs.

The maize DNA clones used to construct maize RFLP maps, and subsequent mapping of new maize cDNA clones, permitted comparative linkage analysis. Maize and sorghum are both diploid (2n=2x=20) but the maize nuclear genome is 3-4 times larger than the sorghum genome. Comparison of sorghum and maize RFLP maps revealed a high degree of conservation as reflected by homology, copy number, and colinearity. Over 60% of the maize clones, genomic and cDNA, produce strong hybridizations signals with sorghum. Many of the loci linked in maize (45 of 55 tested) were also linked in this sorghum population. Examples of conserved and rearranged locus orders were observed.

3. MAPPING MUTANTS AND QTL'S IN SORGHUM AND MAIZE

The same sorghum population was used to map genetic factors (mutants and QTLs) for several traits including vegetative and reproductive morphology, maturity, insect, and disease resistance. This presentation will emphasize analysis of genetic factors affecting plant height, an important character for sorghum adaptation in temperate latitudes for grain production. Evaluations of the traits were conducted with 152 F2 plants [3] and their F3 progeny (Ahnert, D.A., Pereira, M.G. and Lee, M. unpublished). Analysis of the F2 plants detected 4 unlinked QTLs for plant height accounting for 63% of the variation. The QTLs were located to linkage groups A, B, E, and H. Positive, additive genetic effects were estimated at 15-32 cm and alleles for increased stature were derived from the tall parent, PI229828. Tallness was dominant or overdominant at 3 QTLs whereas short stature was dominant at the fourth on linkage group H. Epistasis was evident for one pair of QTLs on linkage groups A and E. Analysis of F3 progeny verified all features of the QTLs detected in F2 plants and detected 2 additional QTLs for plant height in two other linkage groups, D and F.

Analysis of other traits identified several QTLs linked and unlinked to the plant height QTLs. Two QTLs for maturity (number of heat units to flowering) were identified as being

linked to two plant height QTLs. Regions initially identified as plant height QTLs were associated with several other traits (from 1 to 10). Only the QTL on linkage group E was specific for plant height. Collectively, these data and assessments of other traits at the 4 QTLs common to the F2 plants and F3 progeny indicate some of these regions correspond to loci (*dw*) previously identified on the basis of alleles with highly qualitative effects. These observations and linkage relationships will be assessed with sets of near isogenic lines for each to the 4 *dw* loci.

4. COMPARATIVE QTL MAPPING

On the basis of integrated RFLP maps, the positions and effects of sorghum and maize plant height QTLs were compared. Four of the six sorghum plant height QTLs seem to be orthologous to plant height QTLs in maize. The putative orthologous regions are (sorghum linkage group and maize chromosome) as follows: A & long arm of chromosome 1, D & chromosome 5, E & long arm of chromosome 6, and H and chromosome 9. The regions of the maize plant height QTLs also contain genetic loci defined by mutants with qualitative effects on stature such as *brl* and *anl* on chromosome 1, *nal* and *tdl* on chromosome 5, *pyl* on chromosome 6, and *d3* on chromosome 9. The effects of some of these maize mutants strongly resemble those of the sorghum plant height QTLs and *dw* loci. At least 3 of those maize loci, *anl*, *brl*, and *d3* have been tagged with transposons or cloned by various laboratories. These sequences could be used to isolate the related gene from sorghum and further assess the degree and nature of conservation between these two genomes.

Comparative QTL analysis identified evidence for several other orthologous regions. For example, a region of linkage group A (*isu033-isu123*) was strongly associated with tillering and production of lateral branches. The LOD values were 2.8 and 8.7 in F2 plants and F3 families. As indicated by comparative mapping with RFLP loci and QTLs for plant height, this region of the sorghum genome is most closely related to the long arm of chromosome 1 of maize. This region of the maize genome is the site of a genetic locus, *tbl*. The mutant phenotype at that locus is characterized by the production of many tillers and lateral branches in a manner strongly resembling the tillering QTL in sorghum. Other possible instances of orthologous QTLs included regions for maturity. These observations suggest that the conservation of the maize and sorghum genomes encompasses sequence homology, colinearity, and function despite their divergence millions of years ago and subsequent evolution in different hemispheres with contrasting ecogeographical conditions.

5. SORGHUM BREEDING AND GENETICS WITH DNA MARKERS

The genetic information and technology developed on the basis of DNA markers could be used in several facets of breeding, genetics, and other basic biological investigations of sorghum. For example, sorghum breeders in temperate regions routinely transfer short stature and photoperiod insensitivity from adapted temperate inbred lines into exotic (tropical) germ plasm through several generations of backcrossing. The goal of the backcross breeding is to maximize the recovery of the tropical germplasm with a growth habit adapted to temperate latitudes and mechanized harvesting. Once the traits necessary for temperate adaptation have been adequately recovered, the converted tropical germ plasm may be evaluated in temperate regions for traits. Similar programs are conducted for many crops (e.g. rice, maize, and wheat), traits (e.g. resistance to diseases and insects, grain quality, male sterility), and for diversifying and enhancing gene pools (e.g. adapting temperate germplasm to tropical environments and vice versa).

The U.S. Sorghum Conversion Program [8] has used a backcrossing scheme to introduce genes for short stature and photoperiod insensitivity from temperate, adapted parent to the tropical, unadapted parent. According to that scheme, adapted segregants are selected on the basis of stature and ability to flower at the temperate latitude. Self-pollinated seed from such segregants is selected and used to backcross to the recurrent, tropical parent in a tropical environment. That backcross generation is self-pollinated in the tropical location and selfed seed is sown in the temperate location to identify segregants of appropriate stature and flowering response. Typically, 4 or more cycles of backcrossing, selfing, and selection are used to convert a tropical parent.

In this instance, DNA markers could be used to eliminate generations of backcrossing and to ensure that the tropical germplasm was indeed transferred. For example, DNA fingerprints of adapted segregants could be determined and used as a selection criteria prior to backcrossing. This should be especially effective for expediting recovery of chromosome regions from the tropical parent unlinked to the genes needed for adaptation to the temperate latitudes. For example, Pereira and Lee [3] identified short-statured, adapted F2 plants with RFLP genotypes closely resembling (60-75% similarity over 111 loci) the tall or short parent. By selecting the short plants with the greatest degree of resemblance to the tall parent, the backcross conversion could be completed in fewer generations. In addition, DNA marker loci could be used to minimize linkage drag from the adapted, donor parent in the vicinity of the genes affecting adaptation. Through such a scheme, the conservation and conversion of the tropical germ plasm would be optimized. Also, breeders would have the greatest opportunity to identify new and valuable genes from exotic germ plasm.

6. ASSESSING GENETIC DIVERSITY IN SORGHUM BREEDING PROGRAMS

One consequence of modern agricultural practices, which generally emphasizes maximum productivity with acceptable quality and uniformity, has been a reduction of genetic diversity of the primary gene pool under cultivation with similar fates for the secondary and tertiary gene pools of most major crops. That trend may be exacerbated in crops such as sorghum in which F1 hybrid seed is produced using cytoplasmic male sterility. Even though the extent of the reduction may be largely unquantifiable, it is generally assumed that valuable and irreplaceable genes have been lost or ignored, plant genetic resources have been shrinking at accelerated rates, and crop-based agriculture has become more vulnerable to the vagaries of climate and associated biotic and abiotic stresses. Undoubtedly there is considerable merit, validity, and controversy associated with each point. Facts and anecdotes aside, the consequences of a narrow genetic base of major crops have been experienced sporadically throughout recent history often with significant human and economic costs. Therefore, an awareness of genetic diversity and management of crop genetic resources has been an important component of plant improvement programs.

The prospects of utilizing DNA marker technology for managing germ plasm collections has been the subject of a recent and comprehensive review [9]. How do plant breeders assess genetic diversity and relationships among elite germ plasm? Many of the methods used by germ-plasm managers have been used by plant breeders (e.g. morphology and ecogeographical data). In addition, plant breeders often have access to pedigree information, performance records (e.g. combining ability, progeny evaluation, selection and breeding history), and inferences gleaned from various mating designs. The strength of some of the methods is that they are often based on direct assessments of what the breeder needs to know about the germ plasm. Such methods will be extremely difficult to improve. However, some methods and concepts have relied on weak genetic foundations, if any. For

some plant breeding practices, that may constitute a weakness which reduces their efficiency. At least some of these deficiencies may be satisfied in part by DNA markers.

One of the most pervasive measures of genetic relationships in elite crop germ plasm has been Malecot's coefficient of coancestry (f), which provides an estimate of the degree of genetic similarity between two individuals [10]. This measure estimates the probability that two randomly drawn, homologous genes (alleles) from each of two individuals are identical by descent. The measure has been based on Mendelian inheritance and probability and has been calculated under several assumptions: 1) absence of selection, mutation, migration, and drift; 2) regular diploid meiosis; and 3) no relationship ($f = 0$) for individuals without verified common ancestors [10]. Several common features of plant breeding programs have represented departures from these assumptions: 1) intense selection, 2) drift due to small sample sizes, 3) irregular nondiploid meiosis for some crops, and 4) unknown or incorrect pedigree records. Nevertheless, this method of estimating the degree of similarity will create information each generation and has been used by crop breeding programs.

DNA markers have been used to assess large collections of elite sorghum germ plasm to determine the degree of genetic relationships and genetic diversity [4]. A set of 58 R lines, mostly from Kafir, and 47 B lines, mostly from Zera-zera or Feterita were surveyed with 104 DNA probes. The RFLP data clearly identify two different gene groups of inbred lines (pollen vs. seed parents of F1 hybrids; R and B lines, respectively) and document the high degree of genetic similarity among members of certain gene pools (e.g. B lines). On average, 3.6 RFLP-band patterns per probe were observed for R lines whereas only 3 were detected for B lines. Estimates of genetic similarity based on RFLP fingerprints were 0.67 and 0.76 for R and B lines. Cluster analysis of RFLP data further divided R lines into 2 distinct groups representing derivatives from Feterita and Zera-zera.

Similar assessments may be made without DNA markers but the methods require extensive pedigree records and generations of breeding. This requires generations of careful record keeping and is based on several assumptions mentioned previously. However, there was only a modest positive correlation between estimates of genetic similarity based on RFLP data and those based on the coancestry coefficient ($r=0.46$ and 0.43 for related sets of R and B lines). In this instance, information derived from DNA markers is substituting for the considerable time and records needed for traditional analysis of pedigrees and lineages. Also, RFLP data seem to portray genetic relationships more accurately than the methods based exclusively on the coancestry coefficient.

7. SUMMARY AND CONCLUSIONS

The state of sorghum genetics has changed dramatically within the last five years. In 1990, the sorghum genetic map consisted of nine linkage groups with each group containing 2-9 loci. Most of the 260 identified genes had not been mapped. Less than six years later, there are several genetic maps of sorghum each with ten well defined linkage groups based on RFLPs and PCR-based maps are under development. Exchange of DNA clones and mapping information has facilitated map development to the point where the genetic map contains at least 1,500 RFLP loci. Mapping resources have also improved with the development of several populations of recombinant inbred lines (RILs) suitable for mapping factors controlling resistance to biotic and abiotic stress and other important attributes. The RILs are usually available to any research group in the world. Thus, a powerful and practical means of collecting and compiling genetic information for sorghum has been established. The RFLP, QTL and genetic (mutant) maps of sorghum are now being integrated with those of

other grasses such as maize and rice. These connections should create many opportunities to transfer information, technology and materials among research groups working with these species. In many regards, in the last five years, there has been more advancement in the basic infrastructure needed for sorghum improvement than there has been in the previous fifty years.

REFERENCES

[1] LEE, M., DNA Markers and plant breeding programs. Adv. Agron. 55 (1995) 265-343.

[2] PEREIRA, M.G., LEE, M., BRAMEL-COX, P., WOODMAN, W., DOEBLEY, J., WHITKUS, R., Construction of an RFLP map in sorghum and comparative mapping in maize. Genome 37 (1994) 236-243.

[3] PEREIRA, M.G., LEE, M., Identification of genomic regions affecting plant height in sorghum and maize, Theor. Appl. Genet. 90 (1995) 380-388.

[4] AHNERT, D., AUSTIN, D., LEE, M., LIVINI, C., OPENSHAW, S.J., SMITH, J.S. C., PORTER, K., DALTON, G., Genetic diversity among elite sorghum inbred lines assessed with DNA markers and pedigree information. Crop Sci. 36 (1996) 1385-1392.

[5] DOGGET, H., Sorghum, Longmans, Green & Co, London (1988).

[6] TARAMINO, G., TARCHINI, R., FERRARIO, S., LEE, M., PE, E., Characterization and mapping of simple sequence repeats (SSRs) in Sorghum bicolor. Theor. Appl. Genet. (1997).

[7] BROWN, S.M., HOPKINS, M.S., MITCHELL, S.E., SENIOR, M.L., WANG, T.Y., DUNCAN, D.D., GONZALEZ-CANDELAS, F., KRESOVICH, S., Multiple methods for the identification of polymorphic simple sequence repeats (SSRs) in sorghum (*Sorghum bicolor* L. Moench), Theor.Appl.Genet. 93 (1996) 190-198.

[8] DUNCAN, R., BRAMEL-COX, P., MILLER, F.R., Contributions of introduced sorghum germplasm to hybrid development in the USA. In: Use of Plant Introductions in Cultivar Development-Part 1. (H.L Shands, L.E. Weisner, Eds.), Special Publ. No. 17, Crop Sci. Soc. Am., Madison, WI, USA (1991) 69-101.

[9] BRETTING, P.K., WIDRLECHNER, M.P., Genetic markers and plant genetic resource management, Plant Breeding Revs. 13 (1995) 11-86.

[10] MELCHINGER, A.E., Use of RFLP markers for analysis of genetic relationships among breeding materials and prediction of hybrid performance, (Proc. Int. Crop Sci. Symp. Ames, IA, USA, 1992), (BUXTON, D.R., SHIBLES, R., FORSBERG, R.A., BLAD, B.L., ASAY, K.H., PAULSEN, G.M., WILSON, R.F., Eds), Crop Sci. Soc. Am., Madison, WI, USA (1993) 621-628.

COMPARATIVE GENOME ANALYSIS AND RESISTANCE GENE MAPPING IN GRAIN LEGUMES

N.D. YOUNG
Department of Plant Pathology,
University of Minnesota,
St. Paul, Minnesota, United States of America

Abstract

Using DNA markers and genome organization, several important disease resistance genes have been analyzed in mungbean (*Vigna radiata*), cowpea (*Vigna unguiculata*), common bean (*Phaseolus vulgaris*), and soybean (*Glycine max*) In the process, medium-density linkage maps consisting of restriction fragment length polymorphism (RFLP) markers were constructed for both mungbean and cowpea Comparisons between these maps, as well as the maps of soybean and common bean, indicate that there is significant conservation of DNA marker order, though the conserved blocks in soybean are much shorter than in the others DNA mapping results also indicate that a gene for seed weight may be conserved between mungbean and cowpea Using the linkage maps, genes that control bruchid (genus *Callosobruchus*) and powdery mildew (*Erysiphe polygoni*) resistance in mungbean, aphid resistance in cowpea (*Aphis craccivora*), and cyst nematode (*Heterodera glycines*) resistance in soybean have all been mapped and characterized. For some of these traits resistance was found to be oligogenic and DNA mapping uncovered multiple genes involved in the phenotype

1. INTRODUCTION

DNA marker technology has profoundly transformed plant genetics and breeding. Using DNA markers, highly saturated linkage maps of important crop species can be quickly constructed and used to characterize genome organization, locate genes of interest, and carry out marker-assisted selection for traits of economic importance. Two areas that have been especially fruitful are comparisons between the genomes of related, but sexually incompatible taxa, and the mapping of genes that underlie disease resistance phenotypes. Significantly, DNA markers can be used to identify and characterize genes involved in both monogenic and polygenic traits. This has been especially useful in the analysis of quantitative disease resistance characters in plants.

2. RESULTS AND DISCUSSION

2.1. Construction of DNA marker maps for mungbean and cowpea

A genetic linkage map of mungbean consisting of 172 markers including 151 random genomic DNA and 20 cDNA RFLP loci and one pest (*Callosobruchus*) resistance locus was constructed [1]. All but six markers were assigned to 11 coherent linkage groups, plus three small groups with four markers or less. The linked loci covered 1570 centiMorgans (cM) with an average distance of 9 cM. A majority of the mapped loci (121 out of 171) corresponded to single- and low-copy sequences as previously defined. Markers detecting more than one locus, exhibiting aberrant ratios and dominant/null phenotypes were detected as well in the current map. Although the number of linkage groups in the present map does not coincide yet with the 11 known chromosomes of mungbean, the overall appearance of the 11 coherent linkage groups in the map corresponds closely to the karyotype description reported previously.

A genetic linkage map for cowpea was constructed using 58 plants from an F2 population derived from a cross between an elite cowpea line (IT2246-2) and a non-cultivated wild relative (TVn 1963) [2]. These two lines share the same primary gene pool, although the

F1 hybrid between them showed partial pollen fertility. Between these two genotypes, restriction fragment length polymorphisms (RFLPs) were detected by about 22% of the genomic clones from various sources. Seed coat texture and pod shattering were also scored on the F2 plants. Eighty three loci (comprising 79 genomic, 5 RAPDs, 4 cDNA and 1 morphological trait) were distributed on 10 linkage groups which span 680 cM.

2.2. Comparative genome analysis between mungbean and cowpea

Genome relationships between mungbean and cowpea based on the linkage arrangement of RFLP markers were investigated [3]. A common set of probes derived from cowpea, common bean (*P. vulgaris*), mungbean, and soybean (*G. max*) *Pst* I genomic libraries were used to construct the genetic linkage maps. In both species, a single F2 population from a cross between an improved cultivar and a putative wild progenitor species was used to follow the segregation of the RFLP markers. Approximately 90% of the probes hybridized to both mungbean and cowpea DNA, indicating a high degree of similarity in the nucleotide sequences among these species. A higher level of polymorphism was detected in the mungbean population compared to the cowpea population. Loci exhibiting duplications, null phenotypes and distorted segregation ratios were detected in both populations. The mungbean and cowpea genomes were compared based on the copy number and linkage arrangement of 53 markers mapped in common between the two species. Results indicate that nucleotide sequences are conserved, but variation in copy number were detected and some rearrangements in linkage order appeared to have occurred since the divergence of the two species. Entire linkage groups were not conserved but several large linkage blocks were maintained in both genomes.

2.3. Comparative genome mapping among grain legumes

A set of 219 DNA clones derived from mungbean, cowpea, common bean, and soybean was used to generate comparative linkage maps among mungbean, common bean, and soybean [4]. The maps allowed an assessment of linkage conservation and colinearity among the three genomes. Mungbean and common bean, both of the subtribe Phaseolinae, exhibited a high degree of linkage conservation and preservation of marker order. Most linkage groups of mungbean consisted of only one or two linkage blocks from common bean (and vice versa). The situation was significantly different with soybean, a member of the subtribe, Glycininae. Mungbean and common bean linkage groups were generally mosaics of short soybean linkage blocks, each only a few centiMorgans in length. These results suggest that it would be fruitful to join maps of mungbean and common bean, while knowledge of conserved genomic blocks would be useful in increasing marker density in specific genomic regions for all three genera. These comparative maps may also contribute to enhanced understanding of legume evolution.

2.4. Orthologous seed weight genes in cowpea and mungbean

Using genetic maps of mungbean and cowpea, we located major quantitative trait loci (QTL) for seed weight in both species [5]. Two unlinked genomic regions in cowpea contained QTLs for seed weight accounting for 52.7% of the variation for seed weight. In mungbean there were four unlinked genomic regions accounting for 49.7% of the variation. In both cowpea and mungbean the genomic region with the greatest effect on seed weight spanned the same RFLP markers in the same linkage order. This suggests that the QTL in this

genomic region have remained conserved through evolution. This inference is supported by the observation that a significant interaction (*i.e.* epistasis) was detected between the QTL in the conserved region and the same unlinked RFLP marker locus in both species.

2.5. Bruchid resistance in mungbean

Bruchids (genus *Callosobruchus*) are among the most destructive insect pests of mungbeans and other members of the genus,*Vigna*. Genetic resistance to bruchids was previously identified in a wild mungbean relative, TC1966. To analyze the underlying genetics, accelerate breeding, and provide a basis for map-based cloning of this gene, we mapped the TC1966 bruchid resistance gene using restriction fragment length polymorphism (RFLP) markers [6]. Fifty-eight F2 progeny from a cross between TC1966 and a susceptible mungbean cultivar were analyzed with 153 RFLP markers. Resistance mapped to a single locus on linkage group VIII, approximately 3.6 centiMorgans from the nearest RFLP marker. Because the genome of mungbean is relatively small (estimated to be between 470 and 560 million base pairs), this RFLP marker may be suitable as a starting point for chromosome walking.

2.6. Powdery mildew resistance in mungbean

We used RFLPs to map genes in mungbean that confer oligogenic resistance to the powdery mildew fungus, *Erysiphe polygoni* [7]. DNA genotypes for 172 RFLPs spanning 1570 centiMorgans of the mungbean genome were assayed in a population of 58 F2 plants. This population was derived from a cross between a moderately powdery mildew resistant (VC3890A) and a susceptible (TC1966) mungbean parent. F3 lines derived from these F2 plants were then assayed in the field for powdery mildew response and the results were compared to the RFLP genotype data, thereby identifying loci associated with powdery mildew response. Three genomic regions were found to have an effect on powdery mildew response, together explaining 58% of the total variation. At 65 days after planting, two genomic regions on linkage groups 3 and 7 were significantly associated with powdery mildew resistance. In both cases, the allele from VC3890A was associated with increased resistance. At 85 days, a third genomic region on linkage group 8 was also associated with powdery mildew response. In this case, the allele from the susceptible parent (TC1966) was the one associated with higher levels of powdery mildew resistance.

2.7. Aphid resistance in cowpea

A cross between an aphid (*Aphis craccivora*) resistant, cultivated cowpea, IT84S-2246-4, and an aphid susceptible wild cowpea, NI 963, was screened for both aphid phenotype and RFLP marker segregation [8]. One RFLP marker, bg4D9b, was found to be tightly linked to the aphid resistance gene (*Rac1*) and several flanking markers in the same linkage group (linkage group 1) were also identified. The close association of *Rac1* and RFLP bg4D9b presents a potential for positional cloning of this insect resistance gene.

2.8. Cyst nematode resistance in soybean

Several sources of soybean cyst nematode (*Heterodera glycines*; SCN) resistance have been identified in soybean. This study was conducted to identify quantitative trait loci that control disease response in three commonly used sources of SCN resistance [9]. Using genetic markers, we analyzed three segregating soybean F2 populations ['Evans' X 'Peking', Evans X Plant Introduction (PI) 90763, and Evans X PI 88788] and compared the results with those

of a previous study involving PI 209332 [10, 11]. In each case, multiple races of the nematode were tested. To uncover putative resistance loci, F2 DNA marker genotypes at between 63 and 99 loci in each population were contrasted with cyst indices averaged from 12 F2:3 progeny individuals. Four independent partial SCN resistance loci were uncovered at $P < 0.0002$ (probability per locus). One of these loci, located at the top of linkage group 'G' near RFLP locus C006V, was significant at $P < 0.0001$ in all populations and races tested. Other significant loci included one near RFLP A378H at the opposite end of linkage group 'G' from C006V, another on linkage group 'J' near marker B032V-1, and a fourth on linkage group 'N' near marker A280Hae-1. Comparisons between different SCN races indicated that some of the putative resistance loci behave in a race-specific manner. These results may serve as a resource for SCN researchers and soybean breeders by summarizing a wide range of genetic data on the soybean-SCN interaction.

ACKNOWLEDGEMENTS

The author gratefully acknowledges collaboration in the research described in this paper with: D. Menancio-Hautea, University of the Philippines, Los Baños, Philippines, and C. Fatokun, International Institute of Tropical Agriculture, Lagos, Nigeria (Sections 2.1, 2.2 and 2.4); S. Boutin, University of Minnesota, St. Paul, MN, USA, R. Shoemaker, USDA-ARS, Iowa State University, Ames, IA, USA, and E. Vallejos, University of Florida, Gainesville, FL, USA (Section 2.3); Doo-Hwan Kim, Asian Vegetable Research and Development Center, Shanhua, Taiwan (Section 2.5); D. Danesh, University of Minnesota, St. Paul, MN, USA (Section 2.6); G. Myers, Louisiana State University, Baton Rouge, LA, USA, and C. Fatokun, International Institute of Tropical Agriculture, Lagos, Nigeria (Section 2.7); V. Concibido, University of Minnesota, St. Paul, MN, USA, and J. Orf, University of Minnesota, St. Paul, MN, USA (Section 2.8).

This research was also assisted by technical support of: R. Denny, L. Kumar, D. Lange, T. Olson, N. Talekar, Z.-H. Yu, and S. Shanmugasundarum. The research was supported by grants from the International Atomic Energy Agency, the Rockefeller Foundation, the Minnesota Soybean Research and Promotion Council and the Minnesota Agricultural Experiment Station.

REFERENCES

[1] MENANCIO-HAUTEA, D., KUMAR, L., DANESH, D., YOUNG, N., RFLP linkage map for mungbean (*Vigna radiata* L. Wilczek), in: Genetic Maps, 1992. S.J. O'Brien, ed. Cold Spring Harbor Press, Cold Spring Harbor, NY (1993) pp. 6.259-6.260.

[2] FATOKUN, C., DANESH, D., YOUNG, N., RFLP linkage map for cowpea (*Vigna unguiculata* L. Walp.), in: Genetic Maps, 1992. S.J. O'Brien, ed. Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. (1993) 6.256-6.258.

[3] MENANCIO-HAUTEA, D., FATOKUN, C., KUMAR, L., DANESH, D., YOUNG, N., Comparative genome analysis of mungbean (*Vigna radiata* L. Wilczek) and cowpea (*V. unguiculata* L. Walpers) using RFLP mapping data, Theor. Appl. Genet. 86 (1993) 797-810.

[4] BOUTIN, S., YOUNG, N., OLSON, T., YU, Z.-H., SHOEMAKER, R., VALLEJOS, C., Genome conservation among three legume genera detected with DNA markers, Genome 38 (1995) 928-937.

[5] FATOKUN, C., MENANCIO-HAUTEA, D., DANESH, D., YOUNG, N., Evidence for orthologous seed weight genes in cowpea and mungbean based on RFLPs, Genetics 132 (1992) 841-846.

[6] YOUNG, N., KUMAR, L., MENANCIO-HAUTEA, D., DANESH, D., TALEKAR, N., SHANMUGASUNDARUM, S., KIM, D., RFLP mapping of a major bruchid resistance gene in mungbean (*Vigna radiata*, L. Wilczek), Theor. Appl. Genet. 84 (1992) 839-844.

[7] YOUNG, N., DANESH, D., MENANCIO-HAUTEA, D., KUMAR, L., Mapping oligogenic resistance to powdery mildew in mungbean with RFLPs., Theor. Appl. Genet. 87 (1993) 243-249.

[8] MYERS, G., FATOKUN, C., YOUNG, N., RFLP mapping of an aphid resistance gene in cowpea (*Vigna unguiculata* L. Walp.), Euphytica 91 (1996) 181-187.

[9] CONCIBIDO, V.C., LANGE, D.A., DENNY, R.L., ORF, J.H., YOUNG, N.D., Genome mapping of soybean cyst nematode resistance loci in 'Peking', PI 90763, and PI 88788 using DNA markers. Crop Sci. 37 (1997) 258-264.

[10] CONCIBIDO, V.C., DENNY, R.L., LANGE, D.A., ORF, J.H., YOUNG, N.D., RFLP mapping and marker-assisted selection of soybean cyst nematode resistance in PI 209332, Crop Sci. 36 (1996) 1643-1650.

[11] CONCIBIDO, V., YOUNG, N., LANGE, D., DENNY, R., DANESH, D., ORF, J., Targeted comparative genome analysis and qualitative mapping of a major partial resistance gene to soybean cyst nematode, Theor. Appl. Genet. 93 (1996) 234-241.

LIST OF PARTICIPANTS

Atanassov, A.	Institute of Genetic Engineering, 2232 Kostinbrod 2, Bulgaria
Chen, Sh.	Institute of Genetics, Academia Sinica, Datum Road, Beijing 100101, China
Davis, G.	USDA-ARS, Corn Host Plant Resistance Research Unit, Mississippi State University, MS, USA
Eun, M.Y.	National Institute of Agricultural Science and Technology, RDA, Suwon, 441-707, Republic of Korea
Farooq, S.	Nuclear Institute for Agriculture and Biology (NIAB) Jhang Road, Faisalabad, Pakistan
Lee, M.	Department of Agronomy, Iowa State University, Ames, IA, USA
Prins, R.	Agricultural Research Council (Small Grain Institute), P/a Department of Genetics, University of Stellenbosch, 7600 Stellenbosch, South Africa
Simpson, J.	Centro de Investigaciones y Estudios Avanzados (CINVESTAV), Irapuato, Mexico
Stephenson, P.	John Innes Centre, Norwich Research Park, Norwich, NR4 7UH, United Kingdom
Weck, E. *(Scientific Secretary)*	Plant Breeding and Genetics Section, Joint FAO/IAEA Division, International Atomic Energy Agency, P.O. Box 100, A-1400 Vienna, Austria
Young, N.D.	Department of Plant Pathology, University of Minnesota, St. Paul, MN 55108, USA
Zhuang, J.Y.	Department of Biotechnology, China National Rice Research Institute, Hangzhou, 310006, China