IAEA-TECDOC-1047

Use of novel DNA fingerprinting techniques for the detection and characterization of genetic variation in vegetatively propagated crops

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 Mumbai, India, 24–28 February 1997

INTERNATIONAL ATOMIC ENERGY AGENCY IAEA

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

USE OF NOVEL DNA FINGERPRINTING TECHNIQUES FOR
THE DETECTION AND CHARACTERIZATION OF GENETIC VARIATION IN
VEGETATIVELY PROPAGATED CROPS

IAEA, VIENNA, 1998
IAEA-TECDOC-1047
ISSN 1011-4289

Printed by the IAEA in Austria
October 1998

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.

FOREWORD

Vegetatively propagated crops, such as banana and plantain, sweet potato, yam, sugarcane and cassava, represent important sources of food in the developing countries. Although some of these crops may produce seeds, they must for practical purposes be propagated vegetatively. As normal plant breeding strategies based on genetic hybridization are of limited value or not applicable to such crops, it is necessary to assess the genetic diversity already existing in these crops and to design breeding strategies accordingly. If the existing genetic variation is shown to be too narrow for breeding purposes, one promising possibility for the introduction of genetic variability is the use of mutations induced by radiation or chemical mutagens.

In 1992, at the outset of this CRP, DNA fingerprinting strategies offered a unique opportunity for the measurement of genetic variability, especially in vegetatively-propagated species. These DNA fingerprinting methods, in conjunction with other molecular marker methods, allow: the characterisation of genetic diversity induced by radiation or chemical mutagenesis, or in vitro culture-derived variants; the evaluation of genetic diversity among cultivars and wild species in germ plasm banks to allow the identification of suitable breeding strategies (mutation breeding, conventional plant breeding); and fingerprinting various lines or mutants to exclude misuse.

This CRP focused on: the detection of genetic diversity induced by mutagenic treatment or in vitro culture; the development of crop-specific markers; and increasing co-operation between molecular biologists in advanced laboratories and plant breeders and molecular biologists in the developing countries.

The success of this CRP is evidenced by the introduction and application of new molecular methods by laboratories in developing countries, especially for the analysis of local crop genetic diversity. These exciting preliminary results show the potential for applications in crop improvement but much work remains to be done. Many of the vegetatively propagated species are "orphan crops", under-investigated on the international level. The development of new uses of transgenesis for the development of edible vaccines should not be overlooked. The challenge that remains is in the application of these new tools for practical end-user oriented improvements in vegetatively propagated crops.

The present publication summarizes the third and final Research Co-ordination Meeting on the Use of Novel DNA Fingerprinting Techniques for the Detection and Characterization of Genetic Variation in Vegetatively Propagated Crops. 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.

Results and conclusions from other IAEA sponsored programmes dealing with mutation techniques and molecular characterization of genetic variation were summarized recently in IAEA-TECDOC-1010, Application of DNA Based Marker Mutations for Improvement of Cereals and Other Sexually Reproduced Crop Plants, IAEA, Vienna (1998).

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

1. INTRODUCTION

Since mankind has shifted from life as nomad hunters and gatherers to that of sedentary farmers, good husbandry has implied selecting appropriate robust and productive individuals for plant and animal stocks. From this stage onwards, breeding was empirical and based on morphological aspects and performance of the individuals chosen to carry the population into the next generation. By these "naked eye polymorphisms" (NEP) the farmers selected the most vigorous plants giving high yield or those having other desirable features. The systematic application of empirical knowledge became a selective pressure. The results of this selective pressure are manifested in the wide array of present day cultivated crops, vegetatively or sexually propagated.

Vegetatively propagated crops contribute significantly to the world food supply. Because of their predominantly vegetative mode of multiplication, additional challenges exist for their genetic analysis and improvement. However, it must be noted that most vegetatively propagated crops can be reproduced sexually, which, although problematic at times, permits their analysis in segregating populations. During the five year duration of this co-ordinated research project (CRP), experience has been gained in building productive collaborations between molecular geneticists and breeders whereby a common language, based on biotechnology, has been elaborated. This now allows effective communication between breeders and molecular geneticists to develop strategies to resolve problems through collaboration. As a spin-off, these scientists have developed capacities to exchange and transfer knowledge and know-how. The monologues of "Biodiversity Centers" and "Biotechnology Centers" have merged into a profitable dialogue.

An intensive research effort has led to rapid breeding progress in the development of sexually propagated crops during the past decade. As a consequence, the genomes of many of these crops are better known now, molecular marker techniques have been developed, screened, and optimized for the best results, whole sets of allele-specific markers are available, and reasonably saturated genetic maps have been established. In comparison with these achievements, the vegetatively propagated crops as a group are almost totally under-researched.

They represent orphan crops in several respects. Most of them are grown in the tropical or sub-tropical regions of the world where infrastructure and research capacities are insufficient. Therefore, even tissue culture has not advanced as yet to the level that is standard for most sexually propagated crops, not to speak of induced mutation breeding or the application of molecular techniques. Most of the vegetatively propagated crops are minor on a global scale, some are the crops of small scale farmers and the poor (e.g. yam). Therefore, there is little long term research on the majority of these crops, with banana and potato as exceptions. Efficient breeding strategies have not been developed, or breeding is relatively difficult when compared with sexually propagated crops.

As already described in the conclusions of the fourth FAO/IAEA Research Co-ordination Meeting (RCM) (Vienna, November 1996) on The Application of DNA Based Marker Mutations for Improvement of Cereals and Other Sexually Reproduced Crop Species (IAEA-TECDOC-1010), the pressure on modern breeders caused by the urgent demand for more productive and better adapted, disease resistant and high yielding varieties of food crops,

intensifies the need for their continuous improvement. The essence of genetic improvement is the optimization of gene interactions, which is based on utilizing the available genetic diversity. The latter can be obtained from natural populations, sexual crosses (via recombination, segregation and selection), spontaneous mutations (genic and chromosomal), induced mutations (physical and chemical mutagens), and insertional mutagenesis (transposable elements, T-DNA, retroposons).

DNA markers are integrally connected with the success of molecular breeding. Moreover, markers, preferably seedling or juvenile markers, are needed by breeders to identify new gene sources in the available biodiversity, to select parents in order to increase heterosis, to decrease the number of backcross generations for gene introgression breeding programmes, for marker assisted selection (MAS) and, ultimately, for gene isolation and transfer via map-based or deletion-based cloning. These have already been extensively described in IAEA-TECDOC-1010.

Polymerase chain reaction (PCR) technology has boosted the scientific output and yield of this worldwide co-operative research. Based on the PCR technology, neither a complex laboratory infrastructure nor highly trained and skilled staff are required to effectively genotype entire breeding populations with large numbers of individuals, as proven by marker assisted cattle breeding. PCR analysis is very simple, robust and reliable; a minimal quantity of total genomic DNA, even only partially degraded, suffices as a template for multiplex amplifications. Germplasm assessment and genotyping for identification purposes (breeder's rights, variant authentication, conformity test) can be achieved with polymorphic loci (genetic fingerprinting). The homogeneity of in vitro mass propagated material can easily be monitored using recurrent checks during the whole process. PCR assays may be readily automated with no further human interference between sampling in situ (in the fields) and analysis "in silico" (on the computer screen), which will allow the transfer of molecular markers to the "grass roots".

2. ACHIEVEMENTS

Various molecular marker technologies have been used to measure genetic diversities of vegetatively propagated crops as shown in the individual papers presented in this RCM. The building of this research network has permitted the transfer of technology and the enrichment of knowledge and plant genetic resources available to individual projects where technologies of DNA profiling [restriction fragment length polymorphism (RFLP); randomly amplified polymorphic DNA (RAPD); simple sequence repeats (SSR); amplified fragment length polymorphism (AFLP); DNA amplification fingerprinting (DAF); and randomly amplified microsatellite polymorphism (RAMPO)] have been evaluated. Cost, convenience, reliability and information content have been recognized as key criteria for selecting an appropriate profiling technology.

The association of markers and morphological traits led to the generation of maps in several species which allow the exploitation of alternative life cycles of vegetatively propagated plants. For example, in banana one important strategy is the identification of the diploid accessions most closely related to modern cultivars for improvement of desirable traits in these diploids by MAS, thus recreating polyploid, sterile and parthenocarpic cultivars. Another example is yam, *Dioscorea* spp., where mapping of the genome of *Dioscorea tokoro*, a dioecious diploid species closely related to the economically more important yam crops, allowed the tagging of important genes, fostering isolation of these genes for eventual

transformation of yam for genetic improvement. In this fashion, where linkage analysis (detection of linkage disequilibrium) is possible, there are no technological barriers to extend these strategies to vegetatively propagated crops of interest, and to genetically engineer agronomically important genes.

2.1. Specific achievements of this CRP

The participants in this CRP have investigated various vegetatively propagated species with different molecular marker systems. The details of their progress are presented below.

Burg, Austria (7637/CF) – The identification and cloning of minisatellite type repetitive elements of the oak genome supplies a new marker system for the analysis of the genetic diversity of oak resources.

Leal, Cuba (8137/RB) – Molecular evidence of somaclonal variation has been found in sugarcane. There is possibly a role for several genes involved in the osmotolerance response (ATPase, delta-pyrroline-5-carboxylase reductase, osmotin and heat shock proteins).

Lagoda, France (7422/CF) – A common basis was established in which breeders communicated their problems to molecular geneticists. Bridging the communication gap clarified the need for a molecular breeding programme on banana and plantain requiring the input of a mapping and a genetic diversity analysis programme. The development of additional markers was a prerequisite for applications outside the laboratory, especially PCR detection kits. This has been achieved and will be completed in the future by developing sequence tagged microsatellite sites (STMS) markers and sequence characterized amplified regions (SCARs) from AFLP.

Sangwan, France (7480/CF) – In cassava, induced genetic variations have been characterized *in vitro*, Using PCR-based markers (RAPD and microsatellites). The RAPD analyses were reproducible and showed distinct polymorphic bands.

Kahl, Germany (7227/CF) – For yam, an intense application of various DNA profiling techniques and sequence comparisons of chloroplast tRNA gene regions revealed genetic diversity in various *Dioscorea* species, various accessions of economically important yams and parents for crosses, e.g. *D. tokoro*. The same techniques were employed to clarify taxonomic problems in *D. bulbifera*, phylogenetic relationships between *D. cayensis* and *D. rotundata* (Guinea yam), and between these and their putative progenitors. For the first time, a defined cross of selected parents of *D. tokoro* permitted profiling progeny. For greater exploitation of the RAPD polymorphisms, a new hybridization-based method, RAMPO, was designed that expands the information content of a single gel several-fold.

Parida, India (8491/RB) – Molecular markers were used for genetic characterization, species identification, establishing phylogenetic and species relationships in the Indian mangrove species. Markers have also been used to select priority areas for conservation and consolidation of genotypes with the capacity for tolerance to salinity.

Rao, India (7856/RB) – Tissue cultures have been established from thirteen banana genotypes belonging to different genomic groups including wild diploids. *In vitro* multiple shoot cultures have been irradiated and in the early stages of field planting, regenerants have exhibited chlorophyll variations, earliness, changes in plant height and plant type and further

observations of these will follow. Molecular studies for finger printing have been initiated with different banana accessions and variants isolated from irradiation experiments.

Lavi, Israel (7423/CF) – Several kinds of DNA markers (including minisatellites, AFLP and SSRs) were applied to both mango and avocado. Achievements include: identification of individuals, study of genetic relationships, identification of linkage between some DNA markers and genes coding for agriculturally important traits in avocado, and the generation of a preliminary genetic map in avocado.

Low, Malaysia (7229/RB) – Somaclonal variations and changes in DNA methylation were demonstrated for the first time in rubber trees, *Hevea brasiliensis*, by various DNA fingerprinting techniques. Gamma irradiation resulted in changes in DNA profiles, but these changes were complicated by, and indistinguishable from, somaclonal variations.

Mignouna, Nigeria (7228/CF) – Genetic variation among cassava varieties was analysed with RAPD markers. The molecular taxonomy of cultivated and wild yams was established with RAPD and microsatellite markers.

Terauchi, Japan (7228/CF) – High levels of genetic diversity have been found in natural populations of wild yam suggesting the importance of maintaining a small number of large populations to protect genetic diversity.

Iqbal, Pakistan (7231/RB) – Local varieties of sugarcane and banana were compared with RAPDs to assess their genetic diversity.

Mansvelt, Republic of South Africa (7638/RB) – RAPD markers were used to generate fingerprints for differentiation of deciduous fruit cultivars. Gene transfer technology was developed for deciduous fruit trees using *Agrobacterium* as a vector.

Gresshoff, USA (7232/CF) – Arbitrary primer technology using DAF was optimized using mini-hairpin primers, secondary amplification of DAF products (ASAP), 7M urea-10% PAGE, a 55°C annealing temperature, and maintenance of high primer (3 μM) and low template (1-2 ng/ 20 μL reaction volume) concentrations. Molecular markers were valuable for anchoring yeast artificial chromosome (YAC) and bacterial artificial chromosome (BAC) clones on molecular linkage maps facilitating the next step of map-based cloning of genes induced by mutagenesis.

3. RECOMMENDATIONS

Based on the experience from this CRP and current achievements in plant science, it is recommended that there be a future focus on the application of molecular tools in solving important problems related to vegetatively propagated crops in developing countries. In sharp contrast to our ignorance about basic features of vegetatively propagated crops, most of them are nevertheless important staple foods, especially in developing countries. Moreover, the centers of genetic diversity of most of these crops are located in the developing world. Based on molecular techniques in the field of DNA markers and induced mutations, the common language which was established among all participants during this CRP and the highly valuable plant material existing in the developing countries, the following recommendations were made:

3.1. General recommendations

- Research on vegetatively propagated crops should be continued, or even increased, in the future.
- The loose network established in the present CRP has been effective and valuable, and should be maintained and strengthened either through organizational (other granting agencies, national programmes, institutional sources) and/or other means (e-mail, germ plasm exchange, collaborative projects, exchange of personnel).
- Establishment of collaborative research groups centered on an individual crop should be encouraged. These teams should carry out collaborative and co-ordinated research in order to address practical problems specific to each crop and thus contribute to its improvement. Molecular tools for increasing the efficiency of crop improvement should be applied in crops which are agriculturally important to developing countries and which do not benefit from the investment of major scientific effort. Simultaneously, the application of these recommendations will also increase the efficiency of technology transfer to developing countries.
- Molecular markers have proven their potential for identifying genetic variability in vegetatively propagated crops. Use of this successful technology and its transfer should be continued.
- The simplification and optimization of techniques should be a major aim for the future. Obvious examples include the substantial reduction of recommended reaction volume for AFLP and the use of microsatellite capture or magnetic beads rather than colony hybridization for STMS primer generation. These improvements should prepare the transfer of the technologies to laboratories in developing countries.
- The multiplicity of DNA marker technologies should be maintained, and expanded if required by a specific problem, as each technology [RAPD; RFLP; AFLP; DAF; sequence tagged microsatellite sites (STMS), RAMPO] has already provided useful results in a range of crops. Despite differences in cost, reliability, and need for prior sequence information, no single technique provides clear-cut experimental advantages.

3.2. Recommendations for crop selection and techniques for mapping

This CRP has prepared the ground for advanced research by developing and applying techniques for the characterization of genetic diversity, taxonomic and phylogenetic relationship, cultivar identification, and in exceptional cases, the preparation of preliminary core maps, in banana and *Dioscorea tokoro*, Tokoro yam. The techniques and markers can be used directly to continue research towards mapping and characterization of mutations and agronomically interesting traits in vegetatively propagated crops.

3.2.1. Crop selection criteria for future programmes

The following criteria should be used in selecting appropriate vegetatively propagated crops to be included in future collaborative programmes:

- It must be a food crop.
- It should be a crop of developing countries.

- It should be under-researched in terms of induced mutations and/or molecular techniques.

3.2.2. Suggested list of crops

These crop selection criteria are satisfied so far for the following: banana/plantain, yam, grapes, mango, pineapple, sago palm, strawberry, sugarcane, and sweet potato. Other crops may also be found which satisfy these criteria.

3.2.3. Plant material

Improvement of several agronomic traits is of particular relevance: disease and stress tolerance, quality and high yield. For agronomic improvement of such traits, access to segregating populations is a pre-requisite. Such populations are already available for several crops, including banana, yam, sugarcane, mango and strawberry. For other species, however, such populations are either unavailable or at early stages of development. This demands the development of such segregating populations.

3.2.4. Molecular tools

The use of STMS primers is extremely simple and reproducible, in contrast to their expensive, time consuming, and comparably inefficient and laborious generation. Therefore, we propose a strong collaboration between advanced laboratories and institutions in the developing countries; exchange of scientists, collaborative projects such as the generation of STMS primers in advanced laboratories, and use of STMS markers in developing laboratories. Due to the high multiplexing capacity, the AFLP technique allows the generation of many more polymorphic, yet dominant, markers in an experiment than any other technique. The following recommendations can be made.

- The establishment of core maps for vegetatively propagated crops is strongly recommended. This should include both co-dominant and dominant marker systems, that are based on PCR, the STMS and AFLP techniques.
- STMS mapping should aim at establishing a core map (or even a landmark map) and AFLP can then generate markers to saturate the core map and/or to bridge gaps present in established maps.
- The core map should contain about 100 STMSs or about 300 - 400 AFLPs for most plant genomes. Again, cooperative projects are encouraged.
- Other marker types, including RFLP and RAPD, may still be exploited for this type of research.

ISOLATION AND CHARACTERIZATION OF REPEAT ELEMENTS OF THE OAK GENOME AND THEIR APPLICATION IN POPULATION ANALYSIS

S. FLUCH, K. BURG
Austrian Research Centre,
Seibersdorf, Austria

Abstract

Four minisatellite sequence elements have been identified and isolated from the genome of the oak species *Quercus petraea* and *Quercus robur*. Minisatellites 1 and 2 are putative members of repeat families, while minisatellites 3 and 4 show repeat length variation among individuals of test populations. A 590 base pair (bp) long element has also been identified which reveals individual-specific autoradiographic patterns when used as probe in Southern hybridisations of genomic oak DNA.

1. INTRODUCTION

Forest ecosystems are valuable natural resources providing manifold economic, environmental and social functions. As threats to their genetic diversity endanger their stability and adaptability there is an urgent need to study genetic diversity of tree populations. Population genetic studies should provide answers to questions that are fundamental to understand forest ecosystems and to design programs for the conservation of genetic resources. Regardless of specific goals or applications, empirical population genetics requires genetic variation (polymorphism), thus providing "genetic markers". Population geneticists have traditionally employed unusual phenotypes, proteins (mainly isoenzymes), or secondary compounds as genetic markers. DNA markers in forest genetics have received only limited attention, as compared to their extensive use in other organisms. Population genetic studies usually involve the analysis of large numbers of individuals at many gene loci. Therefore, rapid and economic genetic screening methods are needed.

Several types of variable DNA loci in an organism can be detected with various tools, including: restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), microsatellites, DNA-fingerprinting and minisatellites. In this study we were interested in identifying minisatellite type repeat elements of the oak genome. By locating loci of this sort, a multi-allelic system can be built up which is useful both in population studies and in genetic mapping.

In our strategy we intended to isolate repetitive DNA sequence elements of the oak genome (*Quercus robur* and *Quercus petraea*) in order to search for minisatellite/DNA-fingerprint sequences among them.

2. MATERIALS AND METHODS

Leaf samples were collected from Austrian and Hungarian oak populations and the DNA isolated as described [1]. RAPD amplification of total oak DNA by Operon primers was made according to the manufacturers recommendations. Molecular biological techniques were essentially done as described by Sambrook *et al.*[2]. The computer analysis of the DNA sequence was made using the PC/Gene program of IntelliGenetics, PCR primer sequences were generated by the Oligo5 software of the National Biosciences Inc. and the gels were analysed by RFLPScan of Scanalytics.

3. RESULTS

3.1. Identification and isolation of minisatellite elements

We identified oak nuclear genome repetitive elements in two oak species, *Quercus robur* and *Q. petraea*, in two different ways: further analysis of RAPD fragments and screening small insert oak DNA libraries.

3.1.1. Identification of repetitive elements from RAPD fragments

We applied the suggestion that PCR fragments generated by the RAPD method may have a preference to be generated on repetitive DNA by nesting the primers in an appropriate spacing and orientation for yielding PCR fragment(s). This idea was also successful in isolating microsatellite repeats of Daphnia [3]. Therefore RAPD fragments generated on isolated total oak DNA were screened for yielding multiband hybridisation patterns by applying them as probes in Southern hybridisations on total oak DNA. Two out of three fragments tested yielded the expected hybridisation pattern, one showing uniform multibanding, while the other resulted in an individual specific hybridisation pattern containing more than 30 autoradiographic bands ranging from 0.5 to 6 kb on *Hae*III digested oak DNA. This latter fragment (OP-D7-2.3) was analysed in detail. It was possible to identify the part of the fragment responsible for the individual-specific banding pattern (fingerprint element), however, the sequence analysis of the fragment revealed the presence of three other repetitive elements (Minisatellite 1, 2 and 3)(Fig. 1).

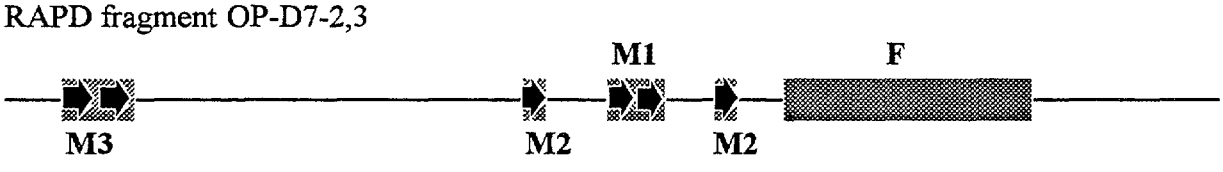


FIG. 1. Locations of the minisatellite elements 1,2 and 3 (M1, M2 and M3) and the fingerprint sequence (F) on the 2.3 kb long RAPD fragment generated by the D7 primer of Operon Tech. The arrows indicate the orientation of the repeat units.

3.1.1.1. Fingerprint element

An approximately 590 bp long part of the OP-D7-2.3 fragment has been identified which still yields the individual specific hybridisation pattern on Southern blots of *Hae*III digested total oak DNA, resulting in 20-25 hybridisation bands (Fig.2). Using either the first or second half of this sequence as a probe in Southern hybridisations, the generated autoradiographic pattern remained identical (not shown) suggesting that this sequence may represent a long repeat element of the genome, since within this 590 bp we could not identify any repeated sequence elements.

3.1.1.2. Minisatellite 1

The minisatellite 1 sequence shows a tandem repeat motif of 112 bp, with 50 and 62 bp repeat units in it. Both of the repeats contain a 13 bp long sequence of 100% similarity containing an *Mnl*I restriction site. Southern hybridising *Mnl*I digested oak DNA with the repeat motif as a probe yields a ladder type autoradiographic pattern with an estimated repeat length of approximately 100 bp, suggesting that the sequence may represent a repeat family

(Fig. 3 A). In some individuals additional bands appeared on the autoradiogram indicating length variability of the repeat unit (Fig 3 A, sample 3) PCR amplification of the region by flanking primers shows a low level of length polymorphism, 10% of the individuals also contain a PCR fragment approximately 60 bp shorter, possibly indicating the presence of a single repeat element in that particular fragment

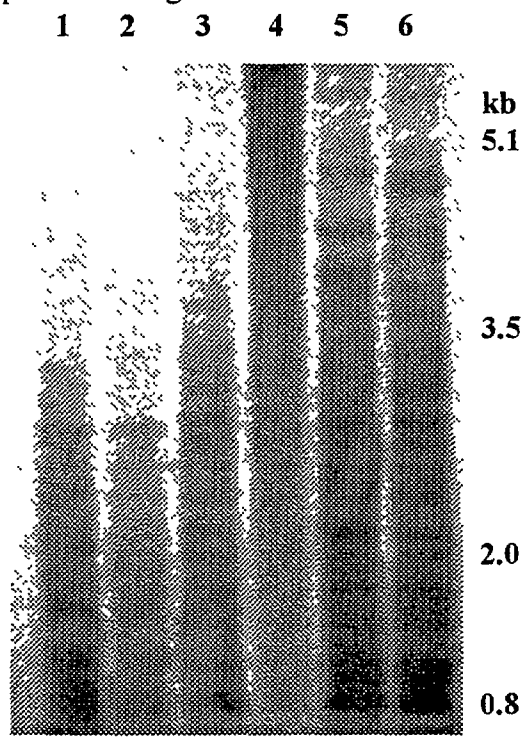


FIG 2. Autoradiographic pattern generated by the 590 bp long fingerprint element on HaeIII digested total oak DNA of six individuals.

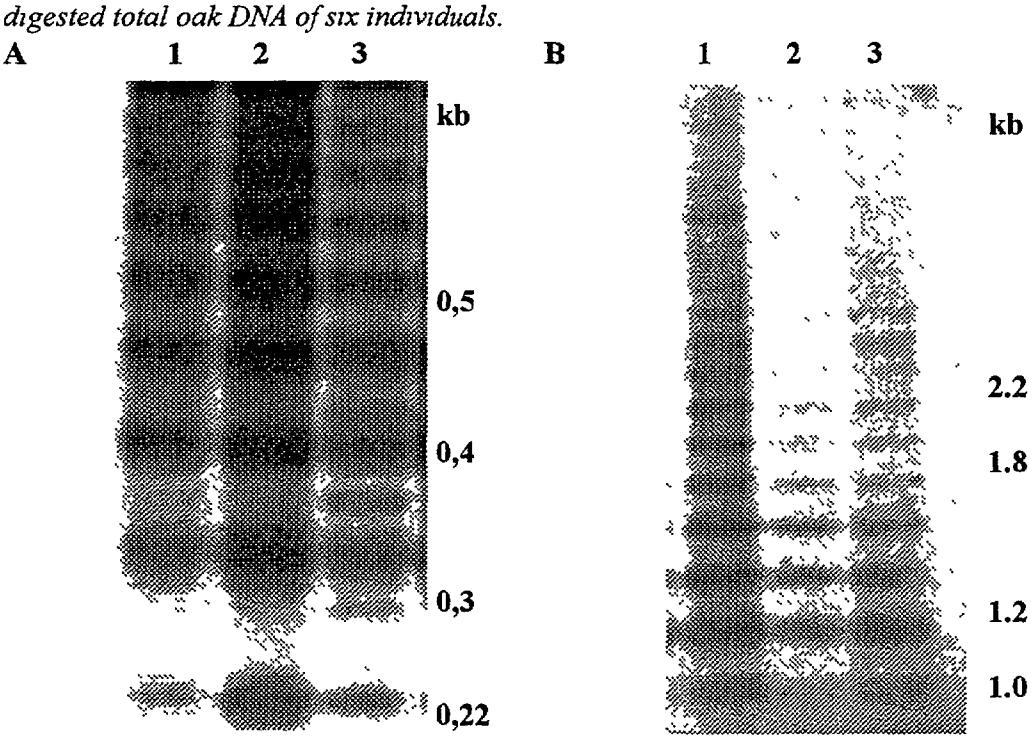


FIG. 3. Autoradiographic profiles generated by the minisatellite sequences 1 (A) and 2 (B) used as hybridisation probes. A) Oak total DNA was digested by MnlI restriction endonuclease and the fragments separated on a 1% agarose gel. B) Oak total DNA digested by HaeIII and separated on 1% agarose.

3.1.1.3. Minisatellite 2

Single representatives of this repeat can be found approximately 300 bp apart flanking the minisatellite1. The repeated sequence is about 84 bp long with an identity of 79% while the putative "core" is approximately 43 bp long with 92% similarity. In this sequence we could not detect any common enzyme site(s), however, on *Hae*III digested genomic blots a similar ladder type hybridisation pattern was observed as with the minisatellite1 with a repeat length of approximately 150 bp (Fig. 4).

3.1.1.4. Minisatellite 3

The sequence shows a tandem repeat motif of 74 bp with a nearly perfect identity (97%) of the 37 bp long individual repeats. PCR amplification of the region by flanking primers has so far yielded four different fragments. The length of the generated fragments differ always with the approximate size of the individual repeat unit. Analysis of 64 individuals revealed that the allele containing two repeat elements is predominant (80%), while alleles with a single element share 12%, with three elements 5% and with possibly seven repeat elements 3% respectively.

3.1.2. Identification of repetitive elements from small fragment DNA libraries

By screening oak small fragment DNA libraries for the presence of microsatellite sequences we could identify a 1 kb long clone in which a microsatellite, as well as a minisatellite sequence, could be found close together. The microsatellite region (pOAG17) contains eleven units of an AG repeat which is separated from the minisatellite region by approximately 100 bp. The minisatellite region (Minisatellite 4) contains three tandemly arranged 65, 79 and 38 bp long repeats. PCR amplification of this region showed moderate variability, yielding seven different length variant alleles (Table 1).

The region was amplified with PCR primers flanking the combined microsatellite-minisatellite region.

The PCR primers used for the amplification of the minisatellite sequence were located outside of the microsatellite-minisatellite combined region, because primers nested between the two regions in order to amplify exclusively the minisatellite locus in several individuals yielded no PCR product, suggesting the possible instability of the genome at that location.

TABLE I. ALLELE DISTRIBUTION OF THE MINISATELLITE 4 SEQUENCE IN A TEST POPULATION OF 39 INDIVIDUALS

Allele size (in bp)	Number of alleles	Fraction of total alleles %
620	1	0.013
470	8	0.103
450	2	0.026
392	60	0.769
335	3	0.038
320	1	0.013
140	3	0.038

4. DISCUSSION

Using RAPD fragments as well as a fragment containing a microsatellite element as a source we could identify four different minisatellite DNA sequence elements of the oak species *Quercus robur* and *Quercus petraea*. The size of these repeat elements ranges from 37 bp to 70 bp as far as the putative core sequences are concerned. When used as probes in Southern hybridisations, two of the minisatellites (1 and 2) yield a ladder type autoradiographic pattern, suggesting that they may represent a repeat family.

Furthermore, a 590 bp long sequence element has also been identified which if used as a hybridisation probe yields an individual-specific autoradiographic pattern.

Both the RAPD and the DNA library fragments contain more than one repeat element in relative close proximity. The RAPD fragment contains three different minisatellite (1, 2 and 3) and the fingerprint elements within a 2.3 kb region, while the library fragment contains a microsatellite sequence near the minisatellite sequence (Minisatellite 4).

Minisatellite regions 3 and 4 show a low level of length variability when PCR amplified by flanking primers.

ACKNOWLEDGEMENTS

This work was supported by the research grant P10800-MOB of the FWF (Austrian Research Foundation).

REFERENCES

[1] BURG, K., ZECHMEISTER-MACHART, M., GLÖSSL, J., SCHMIDT, J. Oak chloroplast-DNA polymorphisms detected by restriction fragment length polymorphism (RFLP) Annales des Sciences Forestieres **50** (1993) Suppl. 1, 66s-69s.

[2] SAMBROOK, J., FRITSCH, E.F., MANIATIS, T. Molecular Cloning: A Laboratory Manual, Second edition. Cold Spring Harbor Laboratory Press (1989).

[3] ENDER, A., SCHWENK, K., STADLER, T., STREIT, B., SCHIERWATER, B. "RAPD identification of microsatellites in Daphnia", Molecular Ecology **5** (1996) 437-441.

USE OF ARBITRARY DNA PRIMERS, POLYACRYLAMIDE GEL ELECTROPHORESIS AND SILVER STAINING FOR IDENTITY TESTING, GENE DISCOVERY AND ANALYSIS OF GENE EXPRESSION

P. GRESSHOFF
Plant Molecular Genetics and Center for Legume Research,
Institute of Agriculture,
University of Tennessee,
Tennessee, USA

Abstract

To understand chemically-induced genomic differences in soybean mutants differing in their ability to enter the nitrogen-fixing symbiosis involving *Bradyrhizobium japonicum*, molecular techniques were developed to aid the map-based, or positional, cloning [1]. DNA marker technology involving single arbitrary primers was used to enrich regional RFLP linkage data. Molecular techniques, including two-dimensional pulse field gel electrophoresis, were developed to ascertain the first physical mapping in soybean, leading to the conclusion that in the region of marker pA-36 on linkage group H, 1 cM equals about 500 cM [2]. High molecular weight DNA was isolated and cloned into yeast or bacterial artificial chromosomes (YACs/ BACs). YACs were used to analyze soybean genome structure, revealing that over half of the genome contains repetitive DNA. Genetic and molecular tools are now available to facilitate the isolation of plant genes directly involved in symbiosis. The further characterization of these genes, along with the determination of the mechanisms that lead to the mutation, will be of value to other plants and induced mutation research.

1. INTRODUCTION

The precise recognition of molecular differences between organisms of different traits is thought to permit the eventual understanding of underlying structure-function relationships. This has become a major paradigm of the various "genome projects", but may require additional information about protein folding and function.

To understand the complexity of eukaryotic genomes, molecular genetic analysis is coupled with classical genetics. Molecular marker technology has permitted the construction of recombination as well as physical maps. The eventual goal is to have available the entire genome sequence for several major plants and animals, permitting functional comparisons and determination of fundamental rules governing gene expression, genome plasticity, and evolution.

To isolate genes defined by mutant phenotypes has become a challenge. In some organisms such as the crucifer *Arabidopsis thaliana* [3] or the cereal crop *Zea mays*, insertional mutagenesis has facilitated this process [4]. In most plants, this is not feasible because of low transformation frequencies. Accordingly, other approaches of gene discovery needed to be developed [5]. In some cases, complementation in *Escherichia coli* or yeast mutants is an alternative; this requires the functional sharing of biochemical steps, as frequently found in metabolic pathways. However, the approach is not available, when the analysis of a developmental pathway such as nodulation or flowering is required. In those cases, gene discovery can be achieved through a strategy called positional or map-based cloning [6]. The essence of the approach is to utilize the co-segregation of a molecular marker and the causative

gene for a known phenotype (as defined by mutation) to isolate the relevant genomic region [7].

Map-based cloning for gene discovery involves (i) detection of genetic differences [8], (ii) determination of the mode of inheritance, (iii) association of a molecular marker with the segregating phenotype [9], (iv) determination of marker order in the relevant genomic region, (v) isolation of anchored high molecular weight clones carrying either one or two of the closely flanking molecular markers in either yeast or bacterial artificial chromosomes (YACs and BACs; [10,11,12]), (vi) detection of candidate gene sequences on the isolated YAC/BAC, and finally (vii) verification of the candidate gene sequences through functional complementation using gene transfer technology [13].

2. RESULTS

2.1. Generation of genetic diversity

Genetics is based on the comparison of heritable differences. At times these exist in a population, especially when it is in its natural state. However, many crop plants have been selected for a long time and genetic bottlenecks have restricted variation. Likewise, natural relatives are too distinct to be valuable in a breeding programme, or carry alterations that are based on multigenic traits, which are difficult to dissect genetically.

In such cases, as with soybean (*Glycine max* L. Merrill) and genetic variation for nodulation and nitrogen fixation, induced mutagenesis is a way to generate genetic variation. Both physical and chemical mutagenesis of soybean were used to produce nodulation mutants. Irradiation by gamma-rays was found to be less effective than ethyl methanesulphonate (EMS) mutation [8,14]. Using EMS with soybean cultivar Bragg, several symbiotic mutants were isolated [1,13,15]. All are single Mendelian recessives. Three major classes were isolated: non-nodulation (equivalent to 'resistance to *Bradyrhizobium*'), supernodulation (absence of autoregulation of nodulation leading to nitrate-tolerant nodulation; nts) and absence of nitrate reductase (for review see [1]). For these mutants there is no information concerning the nature of the mutation. Reversions have not been observed. Non-nodulation and supernodulation mutants nod49, nod139 and nts382 are non-allelic and segregate independently [16]. We are attempting to clone each locus by positional cloning.

2.2. Detection of molecular diversity: use of molecular markers

As a first step in this direction, RFLPs were useful but limited and costly. However, they were mostly co-dominant and robust. The 1990s brought amplification technology either driven by specific or arbitrary primers. The techniques include randomly amplified polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR), DNA amplification fingerprinting (DAF), amplified fragment length polymorphism (AFLP), selective amplification of microsatellite polymorphic loci (SAMPL), cleaved amplified polymorphic sites (CAPS), randomly amplified microsatellite polymorphism (RAMPO) and simple sequence repeats (SSR).

We developed and optimized the DAF technology, based on single short arbitrary primers (see [17]; US patent No. 5,413,909). The procedure was improved through the use of minihairpin primers that gave rich amplification profiles for a wide range of species (turfgrass, soybean, pea, several forest trees and others). Such primers contain a mini-hairpin of 4 nucleotides, flanked by a double and opposed G:C stem [18]. The 3' end of the hairpin is extended through the use of three selective nucleotides [19,20]. Each mini-hairpin is thus

available as a 64 primer set. We have found that primers with different hairpin sequences, but identical 3 nucleotide extensions on their 3' end give different amplification markers [21,22]. The amplification products are routineLY separated on polyacrylamide gels (thin, 10%, denaturing with 7M urea) and stained with silver (as commercialized through Promega Inc. in their Silver Sequence kit, and Pharmacia in their Plus One kit). All kits are based on a patented procedure (see [19]; US patent Nos. 5,567,585 and 5,492,810).

Molecular approaches were used to determine further details about genome structure in soybean. For example, we discovered a 92 base pair (bp) satellite DNA that exists in about 100,000 copies clustered in four centromeric regions [5]. Likewise, we used soybean YACs to amplify their insert DNA with degenerate PCR primers and fluorescent label to detect soybean metaphase chromosomes with different distributions of repeated DNA sequences [10].

2.3. DAF improvements

It was recognized that different thermocyclers, characterized by different ramping regimes, give different amplification profiles. However, when we programmed a MJ Research thermocycler to have the same ramping regime as we used in an Ericomp Twinblock thermocycler, the same profile was obtained.

The ability of DAF to detect molecular differences was increased through mini-hairpin primers [19] and ASAP, in which DAF products from a first reaction were reamplified with arbitrary primer or primers representing a potential microsatellite sequence [20].

We found that increasing the annealing temperature to 55°C gave strong amplification profiles (30 s. at 96°C, 60 s. at 55°C, then 72°C for 30 s., for 35 cycle (Ghassemi and Gresshoff, in preparation). We use Stoffel enzyme (Perkin-Elmer) at 4 units per 20 µL (or 2 units per 10 µL to save enzyme); primer concentration is usually 3 µM. Primer quality was determined by running 20% polyacrylamide gel electrophoresis (PAGE) gels and silver staining. Separation of normal DAF products is by 10% PAGE. Such gels are backed by GelBond from FMC and are air-dried to permit permanent storage. DNA bands can be excised with the polyacrylamide and silver, still producing good reamplification [23]. Reamplification may lead to multiple products, some smaller, some larger than expected. We clone and then select a large number of transformants and select the clone with the expected amplification product. At times, multiple products may be cloned, leading to a paradox relating to proper fragment assignment. We regularly then test the clone for hybridization to a DAF-blot as well as a genomic blot. Frequently, a DAF polymorphism is no longer polymorphic on a DAF-blot, suggesting that the difference stems from quantitative differences. It is worthwhile to clone the "null" area as a control allowing, if necessary, the detection of comigrating bands and their amplification products. The critical feature of a DAF marker is to be able to detect the PCR difference on a DAF gel by Southern hybridization [24].

High concentration PAGE gels have the problem of drying out. We have available an "anti-cracking solution", combining glycerol, ethanol and water. We have alternative methods for semi-automated gel separation. most attractive is capillary electrophoresis [25]. This still requires gel separation but it is at high resolution and semi-automatic. We used the Phast-System and find it operationally difficult [26].

2.4. Some applications of DAF technology

2.4.1. Turfgrass

We are actively involved in commercial turfgrass DNA analysis [25,27]. This involves mainly bermudagrass, that is propagated as triploid clones. Several clones are used for different applications; genetic diversity occurs for unknown reasons. We have used DAF to sort out these differences, and concluded for one genotype (Tifway 419) that contamination is the major cause of genetic instability. DAF data were accepted in a court decision of Boca Del Mar Country Club vs. Aetna Insurance (December 1996).

2.4.2. Soybean

We have used DAF in two applications. The first involved a blind comparison of ten soybean genotypes to establish the relatedness [28]. We matched the pedigree as well as the dendrogram established by 53 RFLP clones. This is due to the greater multiplex power of DAF.

DAF was used in bulked segregant analysis (BSA) for markers close to the supernodulation (*nts-1*) gene [29]. A window of homozygosity was created using the RFLP haplotype of the region. A linked marker was found and converted into a sequence characterized amplified region (SCAR). BSA was used with DAF to look for additional markers next to *enod2*, which maps close to the "*I*" locus on linkage group 1 (Ghassemi and Gresshoff, in preparation). Homozygotes for *enod2* were selected and pooled to give a localized richness of markers. The *enod2* gene was identified as *enod2b*, because the 5' region contained a microsatellite that was mapped using specific primers to the same position as the RFLP. Other nodulin genes failed to map in the same region, suggesting that in soybean, genes involved in a common developmental process are not clustered.

2.4.3. Pea

As in soybean, BSA was coupled with DAF and found two markers close to the *sym31* locus governing non-nitrogen fixation in pea. One marker was placed about 3.7 cM, the other 16 cM, from *sym31* (Men and Gresshoff, unpublished data).

2.4.4. Aphids

In collaboration with Dr. Chuck Niblett (Florida) and Farshid Ghassemi (University of Tennessee), we used DAF to analyze aphids that spread a major citrus virus from South America. Individual aphids were profiled. The population analysis suggested that of 3 Brazilian populations (which are distinct), one became mobile and infected Costa Rica, then Cuba, and now southern Florida. Major crop damage is expected as there is no known resistance.

2.4.5. Forest trees

As part of a forensic test, we profiled dried leaf DNA from tulip poplar, white oak and beech. DNA profiles were compared to fresh leaf samples taken in the vicinity of the putative crime scene.

2.4.6. YAC and BACs

We have constructed BACs and YACs for two legumes, and have demonstrated their utility. For example, three putative YACs and two BACs homologous to pUTG-132a, closely linked to the supernodulation gene of soybean (0.7±0.5 cM) were obtained. We are planning to construct a contig in this chromosomal region and will use endclones as well as DAF markers generated from the YAC/BAC candidates [24]. YACs were also used together with degenerate primer PCR to obtain high intensity FISH images allowing the determination of chimerism in YACs as well as painting chromosomes with either anchored or random YACs. YACs with high hybridization strength value (HSV) to total soybean DNA were found to contain repeated DNA, while low HSV YACs gave single target spots [10].

2.4.7. *Lotus japonicus*

We are developing this plant with short life span and high transformation and regeneration potential as a model legume [21,30]. YACs and BACs have been constructed [12]; transformation is possible in four to six months using two *Agrobacterium* strategies; reporter genes have been expressed as well as some developmentally sensitive cDNA and promoter regions. We have established the classical and molecular genetics of the plant [21], and constructed a skeletal map based on DAF markers and NEP (naked eye polymorphisms = morphological traits). DAF markers were predominantly dominant, but were detected at high frequencies between genotypes 'Gifu' and 'Funakura', the parents for the F_2 and F_6 RIL population (available for sharing). Some markers were inherited in an apparently uniparental fashion, confirming data of Prabhu and Gresshoff [31]. Alternatively, the marker may be repeated, giving skewed segregation.

3. DISCUSSION

Although we see different marker systems available, we feel that DAF fulfills many requirements for DNA profiling. The increased annealing temperature has even further increased the robustness of the technique. While AFLPs are deemed very useful, we are concerned about clustering of markers when using *Eco*RI and *Mse*I. Perhaps the use of *Pst*I or *Hin*dIII as the rare cutter can correct this problem. In a detailed study using 2 genotypes and replicate restriction /amplification, we found that AFLP still produced an average of 9.1 % irreproducible bands. AFLP (a term actually first coined by my laboratory, but used by KeyGene Inc for an alternative procedure; see [17]) may be convenient for large scale DNA identity testing, but may be limited for map-based cloning and marker-assisted selection.

Recently we utilized our experience with DAF to look at differential display products from soybean [32]. We propose the use of high annealing temperature as well, along with high primer concentration (3 μM).

The use of arbitrary primer technology has enriched our tool kit to investigate the nature of genomes. More is to come with the advent of oligonucleotide arrays produced lithographically on silicon wafers (genosensors). Perhaps large scale automation with low cost through-put will then be possible for application in agricultural and medical screening.

REFERENCES

[1] GRESSHOFF, P.M. Molecular genetic analysis of nodulation genes in soybean, Plant Breeding Reviews **11** (1993) 275-318.

[2] FUNKE, R.P., KOLCHINSKY, A. and GRESSHOFF, P.M. Physical mapping of a region in the soybean (*Glycine max*) genome containing duplicated sequences, Plant Mol. Biol. **22** (1993) 437-446.

[3] FELDMANN, A.K. T-DNA insertion mutagenesis in *Arabidopsis*: mutational spectrum, Plant J. **1** (1991) 71-82.

[4] BAKER, B., SCHELL, J., LÖRZ, H. and FEDEROFF, N. Transposition of the maize controlling element "Activator" in tobacco, Proc. Nat. Acad. Sci. USA **83** (1986) 4844-4848.

[5] KOLCHINSKY, A.M. and GRESSHOFF, P.M. A major satellite DNA of soybean is 92 base pairs tandem repeat, Theor. Applied Genetics **90** (1995) 621-626.

[6] WICKING, C. and WILLIAMSON, B. From linked marker to gene, Trends in Genetics 7 (1991) 288-290.

[7] BENT, A.F., KUNKEL, B.N., DAHLBECK, D. BROWN, K.L., SCHMIDT, R., GIRAUDAT, J., LEUNG, J., and STASKAWICZ, B.J. *RPS2* of *Arabidopsis thaliana*: A leucine-rich repeat class of plant disease resistance genes, Science **265** (1994) 1856-1860.

[8] CARROLL, B.J., MCNEIL, D.L. and GRESSHOFF, P.M. Mutagenesis of soybean (*Glycine max* (L.) Merr.) and the isolation of non-nodulating mutants, Plant Sci. **47** (1986) 109-114.

[9] LANDAU-ELLIS, D., ANGERMÜLLER, S.A., SHOEMAKER, R.C., and GRESSHOFF, P.M. The genetic locus controlling supernodulation co-segregates tightly with a cloned molecular marker, Mol. Gen. Genetics **228** (1991) 221-226.

[10] ZHU, T., SHI, L., FUNKE, R.P., GRESSHOFF, P.M. and KEIM, P. Characterization and application of soybean YACs to molecular cytogenetics, Mol. Gen. Genetics **252** (1996) 483-488.

[11] WOO, S.S., RASTOGI, V.K., ZHANG, H.-B., PATERSON, A.H., SCHERTZ, K.F., and WING, R.A. Isolation of megabase-size DNA from sorghum and applications for physical mapping and bacterial and yeast artificial chromosome library construction, Plant Molecular Biology Reporter **13** (1995) 82-94.

[12] PILLAI, S., FUNKE, R.P., and GRESSHOFF, P.M. Yeast and bacterial artificial chromosomes (YAC and BAC) clones of the model legume *Lotus japonicus*, Symbiosis **21** (1996) 149-164.

[13] GRESSHOFF, P.M. "The interface between RFLP techniques, DNA amplification and plant breeding", New Diagnostics in Crop Sciences, (SKERRITT, J.H. and APPELS, R. Eds.). CAB International, England (1995) 101-125.

[14] CARROLL, B.J. and GRESSHOFF, P.M. Isolation and initial characterisation of soybean mutants with an altered constitutive nitrate reductase mutants NR328 and NR345 of soybean (*Glycine max*), Plant Physiol. **81** (1986) 572-576.

[15] CARROLL, B.J., MCNEIL, D.L. and GRESSHOFF, P.M. Isolation and properties of soybean mutants which nodulate in the presence of high nitrate concentrations, Proc. Nat. Acad. Sci. USA **82** (1985) 4162-4166.

[16] MATHEWS, A., CARROLL, B.J. and GRESSHOFF, P.M. The genetic interaction between non-nodulation and supernodulation in soybean: an example of developmental epistasis, Theor. Appl. Genet. **79** (1990) 125-130.

[17] CAETANO-ANOLLÉS, G., BASSAM, B.J. and GRESSHOFF, P.M. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers, Bio Technology **9** (1991) 553-557.

[18] CAETANO-ANOLLÉS, G. and GRESSHOFF, P.M. Staining nucleic acids with silver: an alternative to radioisotopic and fluorescent labeling, Promega Notes **45** (1994a) 13-18.

[19] CAETANO-ANOLLÉS, G. and GRESSHOFF, P. M. DNA amplification fingerprinting using arbitrary mini-hairpin oligonucleotide primers, Bio Technology **12** (1994b) 619-623.

[20] CAETANO-ANOLLÉS, G. and GRESSHOFF, P.M. Generation of sequence signatures from DNA amplification fingerprints with mini-hairpin and microsatellite primers, Bio Techniques **20** (1996) 1044-1056.

[21] JIANG, Q. and GRESSHOFF, P.M. Classical and molecular genetics of the model legume *Lotus japonicus*, Mol. Plant-Microbe Interact. **10** (1997) 59-68.

[22] YAZDI-SAMADI, B., QIAN, D., GHASSEMI, F., ALLEN, F., and GRESSHOFF, P.M. Soybean cyst nematode resistance: genetics, biology and molecular biology, Physiology and Molecular Biology of Plants **2** (1996) 25-48.

[23] WEAVER, K., CAETANO-ANOLLÉS, G., GRESSHOFF, P.M. and CALLAHAN, L.M. Isolation and cloning of DNA amplification products from silver stained polyacrylamide gels, Bio Techniques **16** (1994) 226-227.

[24] KOLCHINSKY, A.M., FUNKE, R.P. and GRESSHOFF, P.M. DAF-amplified fragments can be used as markers for DNA from pulse field gels, Bio Techniques **14** (1993) 400-403.

[25] CAETANO-ANOLLÉS, G., CALLAHAN, L.M, WILLIAMS, P.E., WEAVER, K. and GRESSHOFF, P.M. DNA amplification fingerprinting analysis of bermudagrass (*Cynodon*): genetic relationships between species and interspecific crosses, Theor. Appl. Genetics **91** (1995) 228-235.

[26] BAUM, T.J., GRESSHOFF, P.M., LEWIS, S.A. and DEAN, R.A. Characterization and phylogenetic analysis of four root-knot nematode species using DNA amplification fingerprinting and automated polyacrylamide gel electrophoresis, Mol. Plant-Microbe Interact. 7 (1994) 39-47.

[27] CAETANO-ANOLLÉS, G., CALLAHAN, L.M. and GRESSHOFF, P.M. Inferring the origin of bermudagrass (*Cynodon*) off-types by DNA amplification fingerprinting in phyto-forensic applications, Crop Science **37** (1997) 81-87.

[28] PRABHU, R.R., JESSEN, H., WEBB, D. LUK, S., SMITH, S. and GRESSHOFF, P.M. Genetic relatedness among soybean (*Glycine max* L.) lines revealed by DNA Amplification fingerprinting, RFLP and pedigree data, Crop Science **37** (1997) 1590-5.

[29] KOLCHINSKY, A.M., LANDAU-ELLIS, D. and GRESSHOFF, P.M. Map order and linkage distances of molecular markers close to the supernodulation (*nts-1*) locus of soybean, Mol. Gen. Genet. **254** (1997) 29-36.

[30] STILLER, J., MARTIRANI, L., TUPPALE, S., CHIAN, R.-J., CHIURAZZI, M. and GRESSHOFF, P.M. High frequency transformation and regeneration of transgenic plants in the model legume *Lotus japonicus*, J. exp. Bot. **48** (1997) 1357-1365.

[31] PRABHU, R.R. and GRESSHOFF, P.M. Inheritance of polymorphic markers generated by short single oligonucleotides using DNA amplification fingerprinting in soybean, Plant Mol. Biol. **26** (1994) 105-116.

[32] GRESSHOFF, P.M., SENOO, K., PADILLA, J., LANDAU-ELLIS, D., FILATOV, A., KOLCHINSKY, A.M. and CAETANO-ANOLLÉS, G. "Molecular and genetic insights into shoot control of nodulation in soybean", Biological Nitrogen Fixation for Ecology and Sustainable Agriculture, (LEGOCKI, A., Ed.) Springer Verlag, (1997) 271-274.

COMBINED AMPLIFICATION AND HYBRIDIZATION TECHNIQUES FOR GENOME SCANNING IN VEGETATIVELY PROPAGATED CROPS

G. KAHL, J. RAMSER, R. TERAUCHI*
Biocentre, University of Frankfurt,
Frankfurt am Main, Germany

C. LOPEZ-PERALTA
IRGP, Colegio de Postgraduados,
Montecillo, Edo. de Mexico,
Texcoco, Mexico

H.N. ASEMOTA
Biotechnology Centre,
University of the West Indies,
Mona, Kingston, Jamaica

K. WEISING
School of Biological Sciences,
University of Auckland,
Auckland, New Zealand

Abstract

A combination of PCR- and hybridization-based genome scanning techniques and sequence comparisons between non-coding chloroplast DNA flanking tRNA genes has been employed to screen *Dioscorea* species for intra- and interspecific genetic diversity. This methodology detected extensive polymorphisms within *Dioscorea bulbifera* L., and revealed taxonomic and phylogenetic relationships among cultivated Guinea yams varieties and their potential wild progenitors. Finally, screening of yam germplasm grown in Jamaica permitted reliable discrimination between all major cultivars. Genome scanning by microsatellite-primed PCR (MP-PCR) and random amplified polymorphic DNA (RAPD) analysis in combination with the novel random amplified microsatellite polymorphisms (RAMPO) hybridization technique has shown high potential for the genetic analysis of yams, and holds promise for other vegetatively propagated orphan crops.

1. INTRODUCTION

The design of a series of molecular techniques and the development of molecular markers during the past decade catalyzed plant genome research dramatically. Even large genomes can now be scanned rapidly and reproducibly without any sequence information or cloning steps, and this process generates genomic or DNA fingerprints of varying complexity. One of the main goals in genomic fingerprinting was to reduce the enormous complexity of the original genome into simple patterns, that would allow visualization of sequence differences between two or more genomes. Such differences originate from mutations, such as transitions, transversions, inversions, insertions, deletions, or generally small- or large-scale DNA rearrangements, that occurred in one, but not the other genome, and may be detected by either amplification or hybridization techniques. DNA amplification exploits the polymerase chain reaction (PCR), a technique that allows multiplication of a target sequence exponentially, with the help of short, flanking oligodeoxynucleotide primers. DNA hybridization in turn is based on

* Present address: Kyoto University, Kyoto, Japan.

the sequence-specific Watson-Crick interactions between two single-stranded DNAs, the target and the probe. In each case, a "fingerprint" is produced, a multi-band pattern detected by either ethidium bromide staining of the electrophoretically separated amplification products or autoradiography of hybrids between a radioactively labelled probe and its target sequence.

Both technologies have been applied extensively to diverse problems in plant biology, such as the reliable identification of individuals, clones, breeding lines, cultivars, hybrids, parents and progenies, the estimation of intra- and interspecific relatedness and diversity, the analysis of phylogenetic relationships, the demonstration of gene flow in populations, the genetic and physical mapping of agronomically important traits, their marker-assisted selection, and the isolation of candidate genes by map-based cloning (for a review, see Winter and Kahl [1]). The repertoire of fingerprinting techniques also extends into screening of expressed genes (RNA fingerprinting, differential display [2,3,4]).

The amplification fingerprint techniques fall into two broad categories. While random amplified polymorphic DNA (RAPD) analysis [5], arbitrary-primed PCR (AP-PCR) [6], DNA amplification fingerprinting (DAF) [7] and related methods employ primers of arbitrary sequence to amplify anonymous regions of the template DNA, another class of techniques targets repetitive DNA: microsatellite-primed PCR, (MP-PCR) [8]; anchored microsatellite-primed PCR, (AMP-PCR) [9]. Both classes of techniques as well as various modifications and combinations thereof, such as random amplified microsatellite polymorphisms (RAMPO) [10], are now globally used, primarily because they are relatively cheap, do not require any prior DNA sequence information, are easy to handle, and fast. They meet, however, with several problems, one of them being the sensitivity to amplification parameters. For example, RAPD patterns can usually be reproduced by one and the same experimentor only, and are therefore not robust. Also, the homology of comigrating amplification bands is obscure, unless tested by hybridization. In spite of these and other drawbacks, the amplification fingerprint techniques found wide acceptance as opposed to hybridization-based fingerprinting ("DNA fingerprinting"). The latter method is a derivative of RFLP analysis [13]: completely restricted genomic DNA is electrophoresed in agarose, the restriction fragments either transferred to a membrane or, alternatively, the gel dried, and the blot or dried gel hybridized to (non)radioactively labelled, mini- or microsatellite-complementary probes. The hybrid molecules between probe and target are detected by chemiluminescence or autoradiography This so-called oligonucleotide fingerprinting technique, though experimentally demanding, time-consuming and expensive, is extremely reliable, reproducible and robust, and allows detection of polymorphisms in an otherwise monotonous genetic background [11,12]. Oligonucleotide or microsatellite fingerprinting was successfully applied to various problems in plant taxonomy, phylogeny, breeding and protection of breeder's rights (e.g., identification of cultivars or clones [13]).

Recently, we developed a technique that combines amplification and hybridization procedures for genome scanning. This novel strategy was derived from the idea that any RAPD or MP-PCR reaction would generate thousands of different amplification products of varying abundance. Only a few (i.e. between 1 and 20) such products can be visualized by ethidium bromide staining, whereas most of them remain below detection level. However, these low-abundance amplicons, invisible after ethidium bromide staining of agarose gels, can be detected if they carry microsatellites (stretches of mono-, di-, tri- or tetranucleotide tandem repeats such as $[A]_n$, $[TA]_n$, $[AAT]_n$, or $[GATA]_n$). And since microsatellites are ubiquitous components of all eukaryotic genomes, detection of these second-level DNA markers is possible with (non) radioactively labelled microsatellite motifs, hybridization and lumino- or

autoradiography. We have named this novel technique RAMPO (Random Amplified Microsatellite Polymorphism Detection [14]).

The potential of a combined arbitrary primer technology and microsatellite hybridization detection approach is here exemplified with the mainly (sub) tropical genus *Dioscorea*, of which the edible species ("yams") possess substantial cultural, social and economic importance in West Africa, the Caribbean, the Indian Subcontinent, Southeast Asia and Polynesia, and are typically vegetatively propagated crops.

2. MATERIALS, METHODS AND PROBLEMS

2.1. Materials

The plant material belongs to three classes. First, 23 accessions of aerial yam (*Dioscorea bulbifera*), originating from different geographical regions were probed for intraspecific diversity and relatedness (Table I). Second, 21 accessions of cultivated Guinea yam (*D. rotundata* and *D. cayenensis*) were analyzed together with 21 accessions belonging to seven of their potential progenitor species in order to re-examine Guinea yam taxonomy and phylogeny (Table II). Third, eleven cultivars belonging to five different yam species grown in Jamaica were used to establish a cultivar identification system for Jamaican yam germplasm (Table III).

TABLE I. *DIOSCOREA BULBIFERA* COLLECTION

Accession	Variety	Origin
DB1	*anthropophagorum* (cultivated)	Antananarivo; Madagascar
DB3	*anthropophagorum* (cultivated)	Lushoto; Tanzania
DB9303	*anthropophagorum* (cultivated)	Majan I; Ethiopia
DB9304	*alveolata* (wild)	Majan II; Ethiopia
DB9307	*anthropophagorum* (cultivated)	Majan III; Ethiopia
DB5	*suavior* (cultivated)	Townsville (Qld.); Australia
DB6	*elongata* (wild)	Nambayufa; Papua New Guinea
DB7	*sativa* (cultivated)	Tonga Island
DB8	*sativa* (cultivated)	Oahu Island; Hawaii
DB9	*sativa* (wild)	Hawaii Island; Hawaii
DB10	*heterophylla* (wild)	Sandimen; Taiwan
DB12	*suavior* (cultivated)	Wushe; Taiwan
DB13	*vera* (wild)	Lanyu Island; Taiwan
DB16	*heterophylla* (wild)	Cheng Mai; Thailand
DB18	*heterophylla* (wild)	Cheng Sen; Thailand
DB235	*vera* (wild)	Aki, Oita; Japan
DB247	*vera* (wild)	Tosa Ichino, Kochi; Japan
DB267	*vera* (wild)	Nakahanda, Oita; Japan

Accession	Variety	Origin
DBNC1	unknown (wild)	Mamie; New Caledonia
Bot I	unknown	Bot. Garden Karlsruhe; Germany
Bot II	unknown	Bot. Garden Darmstadt; Germany
Bot III	unknown	Bot. Garden Giessen; Germany
D. burkilliana	(wild)	Oni Gambari; Nigeria

TABLE II. GUINEA YAMS COMPLEX AND WILD RELATIVES

Species	Code	Origin
D. rotundata	rot1	Delta Nigeria
D. rotundata	rot 2	Delta Nigeria
D. rotundata	rot 3	Yoruba Land, Nigeria
D. rotundata	rot 4	IITA, Nigeria
D. rotundata	rot 5	Nibo; Nigeria
D. rotundata	rot 6	Mbiri; Nigeria
D. rotundata	rot 7	Nigeria
D. rotundata	rot 8	Togo
D. rotundata	rot 9	Nigeria
D. rotundata	rot 10	Nigeria
D. rotundata	rot 11	Manchester, Jamaica
D. rotundata	rot 12	St. Ann, Jamaica
D. rot./D. cay.	rot/cay 1	Manchester, Jamaica
D. rot./D. cay.	rot/cay 2	Nigeria
D. cayenensis	cay 1	Nigeria
D. cayenensis	cay 2	Ghana
D. cayenensis	cay 3	Ivory Coast
D. cayenensis	cay 4	Ivory Coast
D. cayenensis	cay 5	Manchester, Jamaica
D. cayenensis	cay 6	Manchester, Jamaica
D. cayenensis	cay 7	St. Elizabeth, Jamaica
D. abyssinica	aby 1	Bode Sadu, Nigeria
D. abyssinica	aby 2	Mokwa, Nigeria
D. abyssinica	aby 3	Mokwa, Nigeria
D. praehensilis	prae 1	IITA forest, Nigeria
D. praehensilis	prae 2	Ore, Nigeria

Species	Code	Origin
D. praehensilis	prae 3	Omo junction, Nigeria
D. liebrechtsiana	lieb 1	Omo, Nigeria
D. liebrechtsiana	lieb 2	Omo, Nigeria
D. liebrechtsiana	lieb 3	Oni Gambari, Nigeria
D. smilacifolia	smil 1	IITA forest, Nigeria
D. smilacifolia	smil 2	Onne, Nigeria
D. smilacifolia	smil 3	Omo, Nigeria
D. minutiflora	min 1	Ifon, Nigeria
D. minutiflora	min 2	Omo, Nigeria
D. minutiflora	min 3	Ore, Nigeria
D. togoensis	togo 1	IITA forest, Nigeria
D. togoensis	togo 2	Bode Sadu, Nigeria
D. togoensis	togo 3	IITA forest, Nigeria
D. burkilliana	burk 1	IITA forest, Nigeria
D. burkilliana	burk 2	Oni Gambari, Nigeria
D. burkilliana	burk 3	Oni Gambari, Nigeria
D. bulbifera	bulb 1	Oita, Japan
D. alata	alat	St. Elizabeth, Jamaica
D. opposita	oppo	Kyoto, Japan
D. trifida	trif	St. Catherine, Jamaica
D. sansibarensis	sansi	Berlin, Germany
Tamus communis	tam	Berlin, Germany

2.2. Methods

Total genomic DNA was isolated from fresh or lyophilized leaves using either a modified Dellaporta technique specifically adapted to yam leaves or tubers [15,16] or a modified CTAB procedure with subsequent purification of the DNA by CsCl buoyant density gradient centrifugation [17]. Both DNAs are of sufficient purity for short-term RAPD or MP-PCR experiments. However, for longer periods of storage or long-term experiments with necessary repetitions, only CsCl-purified DNA proved to be stable and gave consistent and reproducible results [18]. Therefore this additional purification is strongly recommended and was included in the experiments with both aerial and Guinea yam. RAPD analysis was performed essentially as described [18], MP-PCR followed the procedure of [8], and RAMPOs were detected by published techniques [14,19]. Unequivocal positions of RAPD and MP-PCR bands were transformed into binary character matrices, and pairwise distances calculated with simple matching, as well as the Jaccard coefficient and the similarity index according to Nei and Li

[20]. Additive trees based on distance matrices were generated by the neighbor-joining method [21]. Alternatively, binary character matrices were analyzed by the split decomposition approach [22].

TABLE III. JAMAICAN YAM SPECIES AND CULTIVARS

Species	Cultivar	Sampling location
D. alata	St. Vincent	A, B, C, H, L, M
	Sweet Yam	E, G, H, M
	White Yam	E, B
D. cayenensis	Roundleaf	E, A, B, C, D, F, H, I
	Blackwiss	E, B, D, H, I, K, M
D.cayenensis/ rotundata	Tau Yam	E, F
D. rotundata	Lucea Yam	E, I, M
	Negro Yam	E, I, F, G, K, M, L
	Mozella	E, F
D. trifida	Yampie Yam	E, C, D
D. esculenta	Chinese Yam	E, D, K
Tamus communis		Botanical Garden, Frankfurt; Germany

Abbreviations: Local name,District; A. Stony Hill, St. Andrew; B. Mt. Moreland,St. Catherine; C. Sygoville, St. Catherine; D. Kentts, Clarendon; E. Mandeville, Manchester; F. Brown Town, St. Ann; G. Clarement, St. Ann; H. Faiths Pen, St. Ann; I. Rosehall, St. Elizabeth; K. Dalton, St. Elizabeth; L. Caysham, St. Elizabeth; and M. Cesnock, Hanover.

The amplification and sequencing of three non-coding regions encompassing chloroplast tRNA genes $trnT_{UGU}$, $trnL_{UAA}$ and $trnF_{GAA}$ as well as evaluation of the sequence data were described [23,40].

2.3. Problems

2.3.1. *Dioscorea bulbifera*

The monocotyledonous Dioscoreaceae comprise about 600 different species with mainly tropical and subtropical habitats. A subset of these species, collectively referred to as "yam" are grown as economically important tuber crops, especially in Africa ("yam belt"), the Caribbean basin, Asia and Polynesia. One of the cultivated species, *D. bulbifera* L. (aerial yam) is somewhat exceptional, since it develops edible aerial bulbils from meristems at the base of the petioles, is the sole *Dioscorea* species present both in Africa and Asia in wild forms, and possesses an extremely high intraspecific morphological polymorphism (e.g. plasticity of bulbil shape and flesh colour). This extreme morphological variation led to several controversial taxonomical concepts. Either Asian and African forms were regarded as distinct and different species (Asian *D. bulbifera*, African *D. anthropophagorum*; both with a series of varieties [24]), or classified as only one species with one African variety, and nine different Asian varieties [25]. More recently, the African form was also subdivided into several varieties [26].

Finally, on the basis of chloroplast RFLP data two primary clusters were prevalent, an African cluster (three accessions) and an Australasian cluster with twelve accessions [27].

2.3.2. Guinea yam complex

The culturally and economically most important tuber crop of the "yam belt" in West Africa is referred to as "Guinea yam". It consists of two forms, distinguishable by the colour of the tuber flesh: the "yellow yam" (*D. cayenensis*), and the "white yam" (*D. rotundata*). The archetypes of both forms are distinct, but many intermediates and about 500 - 2500 cultivars present an almost insurmountable task for unequivocal classification. Moreover, an almost exclusive vegetative propagation over many hundreds of years has rendered many cultivars sterile, so that no crossing experiments can be performed. Even in fertile cultivars, crossing by hand pollination is demanding, because the flowers are tiny and the pollen is sticky. Since the normal fertilization is insect-borne, cross- contamination with pollen from other species is a problem [28] All these obstacles make a traditional approach to solving the species problem difficult, if not impossible. Consequently, different views exist as to the taxonomy of Guinea yams. Both forms were either thought to represent one single, yet highly evolved species [29], or two clearly separable species. [30], or two well-separated clusters with some intermediate forms [31,32].

Also, the phylogeny of Guinea yam is obscure. Earlier investigations implied more than ten different species of the *Enantiophyllum* section as progenitors [24]. Ecological considerations led to the view that *D. rotundata* evolved from hybridization events between the rainforest species, *D. cayenensis*, and the savannah species, *D. praehensilis* [33]. Chemotaxonomical data also suggested a polyphyletic origin of the complex [34]. Finally, nuclear and chloroplast RFLP analyses detected a close relationship between *D. rotundata* on one hand, and *D. abyssinica, D. liebrechtsiana* and *D. praehensilis* on the other. *D. cayenensis* cultivars shared the same chloroplast genotype with these potential progenitors, but also a nuclear rDNA marker with *D. burkilliana, D. minutiflora* and *D. togoensis*. These data were interpreted to indicate that *D. cayenensis* was a hybrid and *D. rotundata* a true species [35].

2.3.3. Cultivar identification

Yam cultivars are generally distinguished by morphological criteria, which, however, are dependent on environmental parameters and therefore quite variable and not reliable. Moreover, unequivocal cultivar identification is hindered by linguistic barriers. For example, most cultivated forms of yams have never been named conclusively. Therefore, each location has its own repertoire of names for cultivars, and even cultivars from different species are often called by the same name. We chose the Jamaican yams with eleven local varieties from the five cultivated species *D. rotundata, D. trifida, D. esculenta, D. alata* and *D. cayenensis* as a model system to identify yam cultivars by DNA profiling.

3. RESULTS AND DISCUSSION

The results of this research have both technical and scientific dimensions.

3.1. The techniques

Since RAPD analysis is based on an enzymatic reaction driven by primers of arbitrary sequence, small changes in the reaction conditions might change the results. Moreover, yam

DNA is probably associated with firmly bound proteins which may interfere with restriction or amplification [36]. Therefore it is an absolute prerequisite for reliable and reproducible RAPD-, MP-PCR- or RAMPO-experiments to use highly purified DNA and to optimize the reaction conditions for each technique. All the DNAs used for intraspecific studies of aerial and Guinea yams were separated from RNAs and proteins by CsCl gradient centrifugation. This procedure yielded template DNAs that allowed accurate quantification (by spectrophotometry) and reliable template concentrations in all the experiments, clear-cut banding patterns with all the techniques, and extreme reproducibility. Also, the large numbers of detectable RAPD bands in our experiments can be attributed to the high quality of the template.

The RAMPO technique expands the information content of a single RAPD- or MP-PCR gel severalfold, since the previously bound radioactive probe can be washed off the blot before it is rehybridized with the next probe. The probe repertoire is enormous, and $(A)_{16}$, $(GA)_8$, $(CA)_8$, $(GAA)_5$, $(CAA)_5$, $(CCTA)_4$, $(GTGA)_4$ and the telomeric $(CCCTAAA)_3$ all detected RAMPOs, whether a RAPD- or MP-PCR gel was used [19]. The RAMPO patterns are highly reproducible, and usually less complex than the underlying RAPD- or MP-PCR gels. We regard RAMPOs as second-level DNA markers. Pattern complexity can be tailored by primer/probe combinations, and seems to be higher in RAMPOs based on MP-PCR as compared to RAPD experiments. The level of detected polymorphisms is similar to that obtained with RAPD or MP-PCR alone. We consider RAMPO bands to be more reliable than RAPD or MP-PCR amplicons, because not only the amplification fragment size, but also the hybridization signal intensity (indicative of the presence and copy number of the microsatellite) can be scored simultaneously, and the problem of false interpretation of homology is therefore greatly reduced.

3.2. Intraspecific variability (*D. bulbifera*)

The extent of genetic diversity and the genetic relationships between different *D. bulbifera* accessions belonging to different varieties were successfully probed with RAPD and RAMPO techniques [14,18]. The ten arbitrary primers revealed 375 different band positions among the 23 *D. bulbifera* accessions used, and only eight of them were monomorphic between all *D. bulbifera* DNAs. This considerable intraspecific variability exceeds that detected in tomato [37] or groundnut [38]. As should be expected from the exclusive vegetative propagation of cultivars, within-cultivar variability in *D. bulbifera* was nil or negligible. With few exceptions, the accessions grouped together according to their varietal affiliation.

Using different algorithms, the data nevertheless led to robust phenograms with a clear separation of the African and Asian-Polynesian accessions (see also [27] and [39]). This result lends credence to Burkill's hypothesis that both populations were separated from each other about 10 million years ago, when southwestern Asia began to desertify. The Asian and Polynesian accessions also group separately, but the distinction is less clearcut, suggesting a more recent separation of both groups (Fig.1). RAMPO banding corroborates the hypothesis of divergent evolution of the African from the Asian-Polynesian *D. bulbifera*, since several $(GA)_8$ RAMPO bands are only present in the African accessions [14].

3.3. The taxonomy and phylogeny of the Guinea yams complex

RAPD, MP-PCR and RAMPO analyses as well as sequence comparisons of non-coding chloroplast DNA were employed to solve two major problems surrounding the Guinea yams

complex: first, are *D. rotundata* and *D. cayenensis* to be considered as two distinct species, and second, what are the progenitors of cultivated Guinea yams?

Our RAPD and MP-PCR, but also RAMPO, data strongly support the two species concept [30], because phenograms and split graphs clearly separated the *D. rotundata* and *D. cayenensis* accessions, and - equally important - RAMPO bands were detected that were diagnostic for either *D. cayenensis* or *D. rotundata* [23], though the different cultivars originated from different locations (here Africa and Jamaica). Two morphological intermediates grouped together with *D. rotundata* (Fig.2). No intraspecific sequence variation was found in the non-coding chloroplast DNA regions investigated, which could be expected in view of the relative stability of the chloroplast genome. In yams, this marker type appears to be useful at the species or genus levels only, but not informative intraspecifically [23]. The only problem we are facing is the relatively small number of accessions that could be involved (21 accessions of Guinea yam and 21 accessions belonging to seven putative progenitor species). Two morphological intermediates grouped together with *D. rotundata*.

Taken together, our data support the hypothesis that *D. cayenensis* and *D. rotundata* belong to two different taxa.

Now, which wild species represent the actual progenitors of cultivated Guinea yams? It was already known from chloroplast RFLP analyses, that both forms of cultivated Guinea yam shared the same chloroplast type as *D. abyssinica, D. liebrechtsiana* and *D. praehensilis* [35]. Our RAPD-, MP-PCR and chloroplast sequence data altogether support this presumptive progenitor-successor relationship. Also, the occurrence of shared RAMPO bands points to *D. rotundata, D. liebrechtsiana* and *D. praehensilis* as one related group [23].

On the other hand, *D. burkilliana, D. minutiflora, D. smilacifolia* and *D. togoensis* each possess a different chloroplast DNA type, but share a common nuclear rDNA fragment with *D. cayenensis* [35]. These characteristics were interpreted as an indication for the hybrid nature of *D. cayenensis*, incorporating features of both precursor groups. The tentative female parent would then either be *D. rotundata, D. abyssinica, D. liebrechtsiana* or *D. praehensilis*, the male parent a member of the other progenitor group [35]. At least some of our RAMPO data support the hybrid nature of *D. cayenensis*; this species shared common RAMPO fragments with *D. minutiflora, D. burkilliana*, and also *D. smilacifolia*.

Our data are consistent with the possibility that *D. cayenensis* is an asymmetric hybrid, whose female donor would have been *D. rotundata, D. praehensilis, D. abyssinica* or *D. liebrechtsiana*, donating the mass of nuclear DNA and the chloroplast genome. The smaller part of the nuclear DNA would then have been contributed by either *D. burkilliana, D. minutiflora* or *D. smilacifolia*. And it is this part that can be detected by a subset of RAPD and RAMPO fragments. The *D. cayenensis* cultivars, as a group, being genetically very similar, would then originate from one and the same hybridization event [23].

3.4. Cultivar identification of Jamaican yams

The yam species grown in Jamaica are representative for the whole Caribbean basin. Historically, these yams have been introduced more recently via the slave trade route from West Africa, and belong to five species: *Dioscorea alata, D. cayenensis, D. esculenta, D. rotundata*, and *D. trifida*. All these species in turn split into many cultivars, of which those from *D. alata* and *D. cayenensis* contribute the bulk of Jamaican (or Caribbean) yam

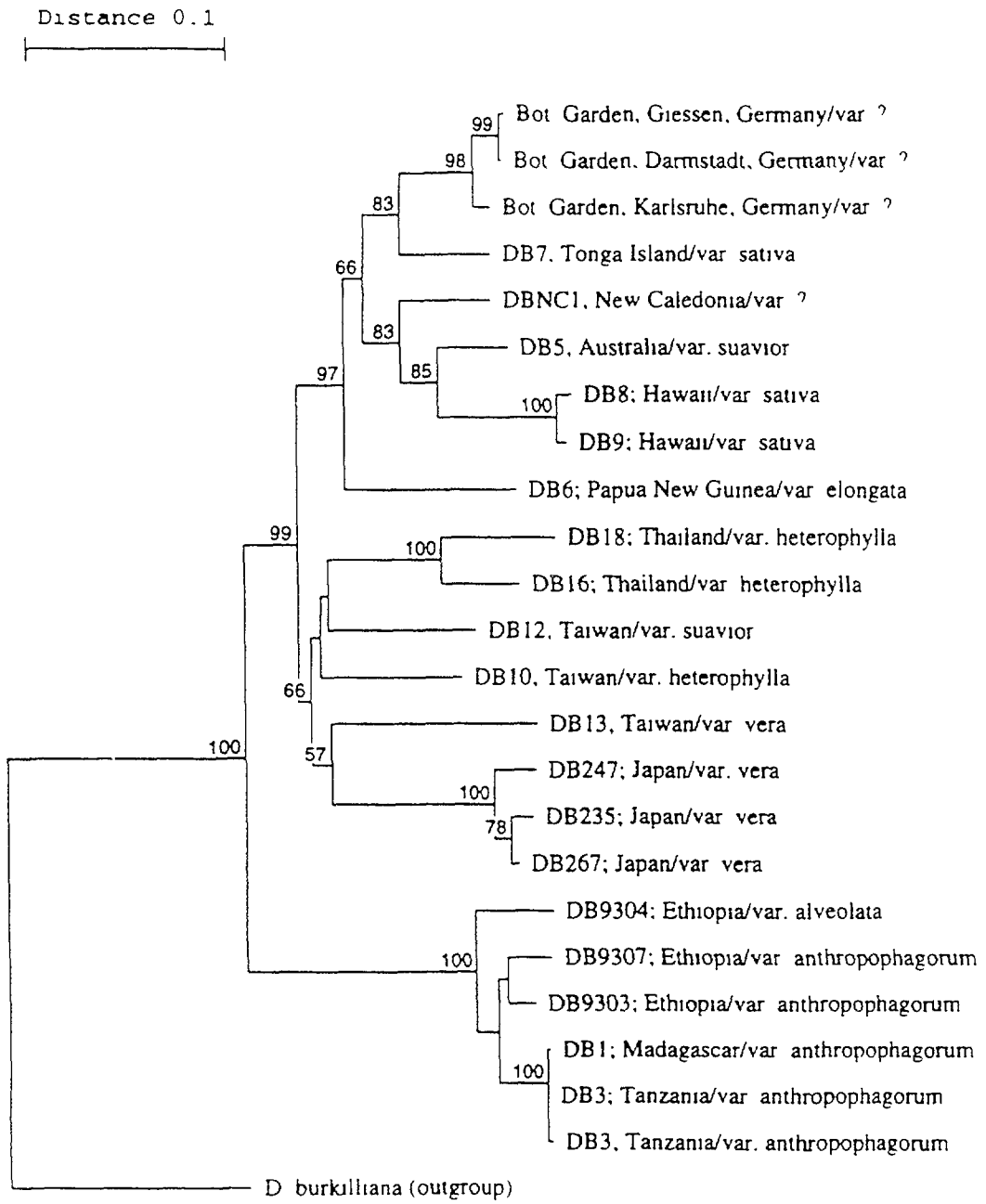


FIG 1 Neighbour-joining tree of D bulbifera accessions based on RAPD analysis Presence/absence of bands at 375 positions revealed by 10 different Operon primers was transformed into a distance matrix using the simple matching coefficient of similarity and analyzed by the neighbor-joining option of the NTSYS-pc software package Dioscorea burkilliana was included as an outgroup to root the dendrogram Numbers at the nodes represent bootstrap values The relative genetic distance is indicated by the horizontal bar.

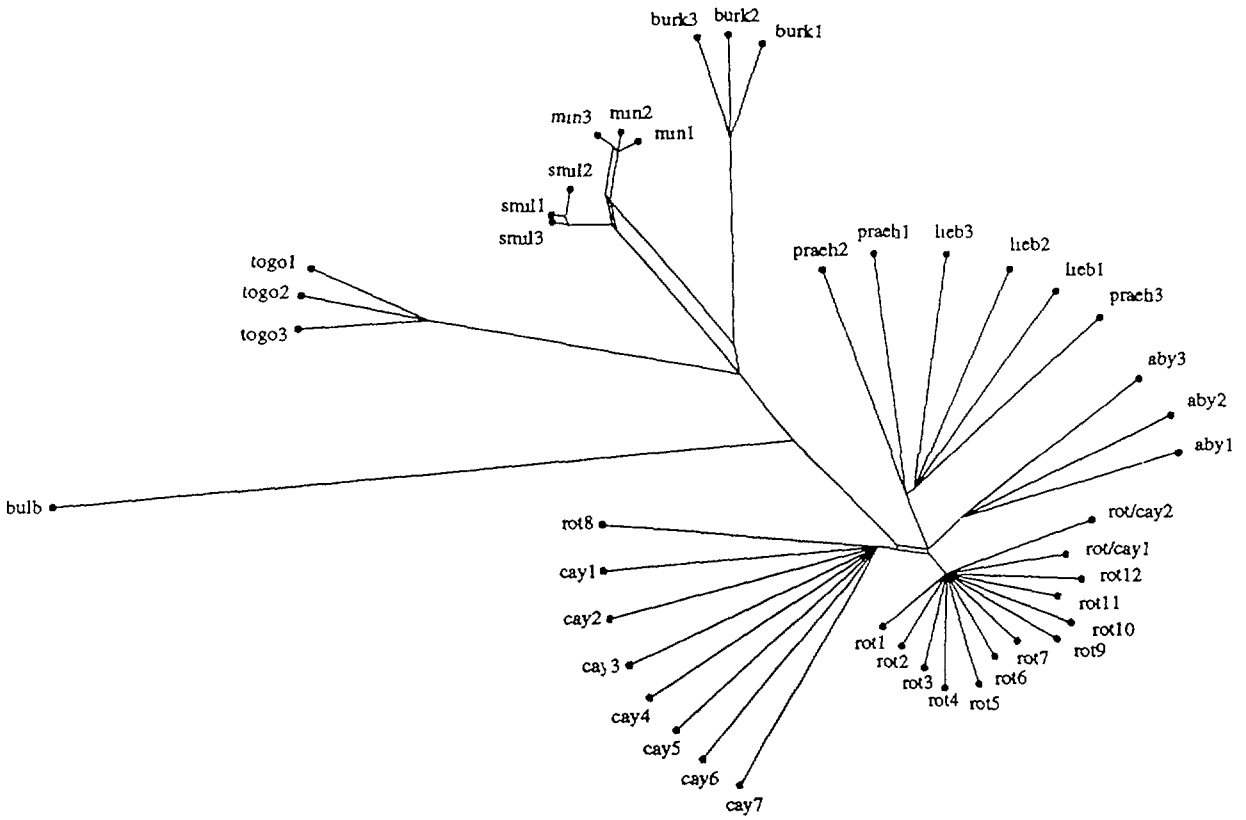


FIG 2 Split decomposition analysis of the relatedness of D rotundata, D cayenensis and their putative wild ancestor species, based on 246 RAPD and MP-PCR characters

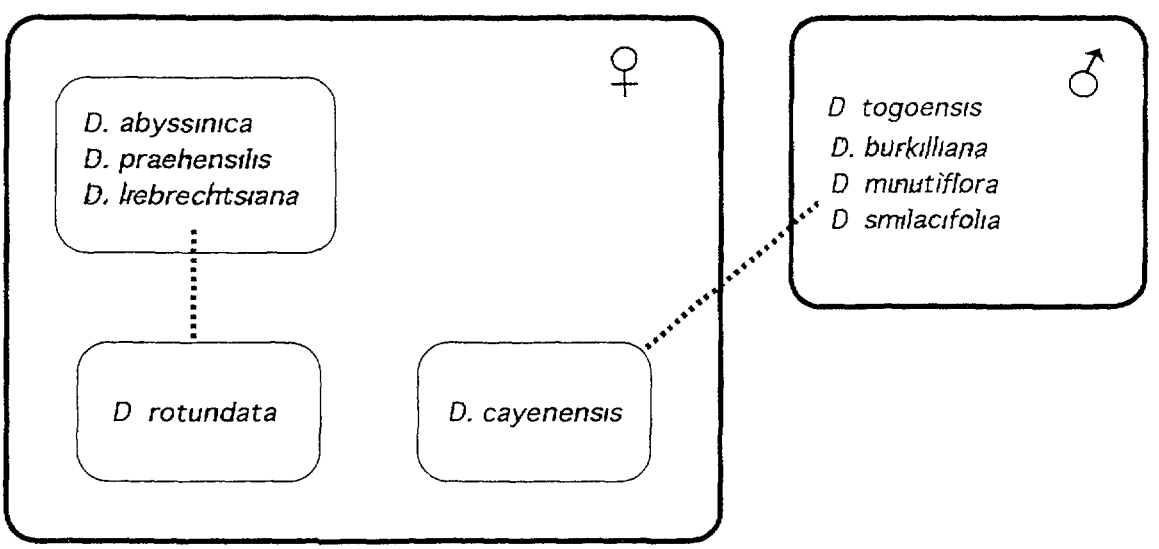


FIG 3 Hypothetical origin of Guinea Yam (D rotundata/D cayenensis) In this scheme D cayenensis represents an asymmetric hybrid whose presumptive female parent is either D rotundata D praehensilis D abyssinica or D liebrechtsiana contributing the chloroplast genome and most of the nuclear DNA Each of D burkilliana D minutiflora or D smilacifolia could be the male parent origin of a minor part of nuclear DNA (that can be traced by a subset of RAPD and RAMPO markers)

production. Over the past decade, increasing exports to the US added to the economic value of this crop. In spite of their importance as a food and cash crop, yam cultivars have not yet been unequivocally classified.

A sound classification can be achieved with RAPDs. After careful adjustment of reaction conditions, clear-cut banding patterns were produced from nine of fifteen arbitrary primers. These RAPD patterns were highly reproducible and somatically stable (i.e., identical patterns were obtained with either leaves, shoots or roots). RAPDs readily discriminated between the different *Dioscorea* species, and cultivars as well. Cultivar identification is most desirable, both for protection of breeders rights and improvement programs, and can be achieved unambiguously with RAPDs [41]. RAPD fingerprints are therefore reliable and mitotically stable tools to typify yam cultivars. As expected, intracultivar variability was negligible, though different plants of the same cultivar were taken from different growing areas in Jamaica [41]. This lack of intracultivar variability also holds for other vegetatively propagated crops [42,43, 44,45], though exceptions of this rule are known [46].

The combined amplification and hybridization techniques are powerful tools for solving even delicate problems of the taxonomy and phylogeny of vegetatively propagated crops, and to protect breeder's rights by unequivocally profiling different cultivars of a taxon, exemplified here with the genus *Dioscorea* (yam).

ACKNOWLEDGEMENTS

Research of the authors was supported by IAEA (Research Contract 7227/CF), DFG (grants Ka 332/14-1, 14-2 and 14-3), the Alexander von Humboldt-Foundation (R.Terauchi), CONACYT Mexico (C. López-Peralta), EDF (H.N. Asemota), FAZIT-Foundation (K.Weising) and the "Freunde und Förderer der Universität" Frankfurt am Main. We appreciate the editorial assistance of Mrs. Sieglinde Kost.

REFERENCES

[1] WINTER, P. and KAHL, G. Molecular marker technologies for plant improvement, World J. Microbiol. Biotechnol. **11** (1995) 438-448.

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

[3] WELSH, J., CHADA, K., DALAL, S.S., CHENG, R., RALPH, D. and MCCLELLAND, M. Arbitrarily primed PCR fingerprinting of RNA, Nucleic Acids Res. **20** (1992) 4965-4970.

[4] LIANG, P. and PARDEE, A.B. Recent advances in differential display, Current Opinion in Immunology 7 (1995) 274-280.

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

[6] WELSH, J. and MCCLELLAND, M. Fingerprinting genomes using PCR with arbitrary primers, Nucleic Acids Res. **18** (1990) 7213-7218.

[7] CAETANO-ANOLLÉS, G., BASSAM, B.J. and GRESSHOFF, P.M. DNA amplification fingerprinting: a strategy for genome analysis, Plant Mol. Biol. Rep. **9** (1991) 294-307.

[8] WEISING, K., ATKINSON, R.G. and GARDNER, R.C. Genomic fingerprinting by microsatellite-primed PCR: a critical evaluation, PCR Meth. Applications **4** (1995) 249-255.

[9] ZIETKIEWICZ, E., RAFALSKI, A. and LABUDA, D. Genome fingerprinting by simple sequence repeat (SSR)- anchored polymerase chain reaction amplification, Genomics **20** (1994) 176-183.

[10] WU, K.S., JONES, R., DANNEBERGER, L. and SCOLNIK, P.A. Detection of microsatellite polymorphisms without cloning, Nucleic Acids Res. **22** (1994) 3257-3258.

[11] WEISING, K. and KAHL, G. "Hybridization-based microsatellite fingerprinting of plants and fungi", DNA markers: protocols, applications and overviews (CAETANO-ANOLLÉS, G., GRESSHOFF, P.M., Eds.), Wiley & Sons, N.Y (1997).

[12] WEISING, K., KAEMMER, D., WEIGAND, F., EPPLEN, J.T. and KAHL, G. Oligonucleotide fingerprinting reveals probe-dependent levels of informativeness in chickpea (*Cicer arietinum* L.), Genome **35** (1992) 436-442.

[13] WEISING, K., WINTER, P., HÜTTEL, B. AND KAHL, G. Microsatellite markers for molecular breeding. J. Crop Prod. 1 (1998) 113 - 143.

[14] RICHARDSON, T., CATO, S., RAMSER, J., KAHL, G. and WEISING, K. Hybridization of microsatellites to RAPD: a new source of polymorphic markers, Nucleic Acids Res. **23** (1995) 3798-3799.

[15] DELLAPORTA, S.L., WOOD, J. and HICKS, J.B. A plant DNA minipreparation: version II, Plant Mol. Biol. Rep. **1** (1983) 19-21.

[16] ASEMOTA, H.N. A fast, simple and efficient miniscale method for the preparation of DNA from tissues of yam (*Dioscorea spp.*), Plant Mol. Biol. Rep. **13** (1995) 214-218.

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

[18] RAMSER, J., LÓPEZ-PERALTA, C., WETZEL, R., WEISING, K. and KAHL, G. Genomic variation and relationships in aerial yam (*Dioscorea bulbifera* L.) detected by random amplified polymorphic DNA, Genome **39** (1996) 17-25.

[19] RAMSER, J., WEISING, K., CHIKALEKE, V. and KAHL, G. RAMPO: Increased Informativeness of RAPD Analysis by Detection of Microsatellite Motifs, BioTechniques **23** (1997) 285-290.

[20] NEI, M. and LI, W.H. Mathematical model for studying genetic variation in terms of restriction endonucleases, Proc. Natl. Acad. Sci. USA **76** (1979) 5269-5273.

[21] SAITOU, N. and NEI, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees, Mol. Biol. Evol. **4** (1987) 406-425.

[22] BANDELT, H. J. and DRESS, A.W.M. Split decomposition: a new and useful approach to phylogenetic analysis of distance data, Mol. Phylogenet. Evol. **1** (1992) 242-252.

[23] RAMSER, J., WEISING, K., LÓPEZ-PERALTA, C., TERHALLE, W., TERAUCHI, R. and KAHL, G. Molecular marker-based taxonomy and phylogeny of Guinea Yam (*Dioscorea rotundata- D. cayenensis*). Genome (1997)(submitted).

[24] CHEVALIER, A. Contribution à l'étude de quelques espèces africaines du genre *Dioscorea*, Bull. Mus. Natl. Hist. Nat. Sér. 2, **8** (1936) 520-525.

[25] PRAIN, D. and BURKILL, I.H. An account of the genus *Dioscorea* in the East. Part I: The species which twine to the left, Ann. Roy. Bot. Gard. Calcutta **14** (1936) 1-20.

[26] MIÈGE, J. "De quelques caractères discriminatoires entre les taxons intraspécifiques de *D. bulbifera* L.", Yams (Ignames). (MIÈGE, J., LYONGA, S.N. Eds., Oxford University Press, Oxford (1982).

[27] TERAUCHI, R., TERACHI, T. and TSUNEWAKI, K. Intraspecific variation of chloroplast DNA in *Dioscorea bulbifera* L., Theor. Appl. Genet. **81** (1991) 461-470.

[28] ZOUNDJIHEKPON, J., HAMON, S., TIO-TOURÉ, B. and HAMON, P. First controlled progenies checked by isozymic markers in cultivated yams (*Dioscorea cayenensis/rotundata*), Theor. Appl. Genet. **88** (1994) 1011-1016.

[29] MARTIN, F.W. and RHODES, A.M. The relationship of *Dioscorea cayenensis* and *D. rotundata*, Trop. Agric. (Trinidad) **55** (1978) 193-206.
[30] ONYILAGHA, J.C. and LOWE, J. Studies on the relationship of *Dioscorea cayenensis* and *Dioscorea rotundata* cultivars, Euphytica **35** (1985) 733-739.
[31] HAMON, P. and TOURÉ, B. Characterization of traditional yam varieties belonging to the *Dioscorea cayenensis-rotundata* complex by their isozymic patterns, Euphytica **46** (1990) 101-107.
[32] HAMON, P. and TOURÉ, B. The classification of the cultivated yams (*Dioscorea cayenensis-rotundata* complex) of West Africa, Euphytica **47** (1990) 179-187.
[33] AYENSU, E.S. and COURSEY, D.G. Guinea yams. The botany, ethnobotany, use and possible future of yams in West Africa, Econ. Bot. **26** (1972) 301-318.
[34] MIÈGE, J. "Etude chimiotaxonomique de dix cultivars de Cote d'Ivoire relevant du complexe *Dioscorea cayenensis-rotundata*", Yams (Ignames) (MIÈGE, J., LYONGA, S.N., Eds.), Oxford University Press, Oxford (1982).
[35] TERAUCHI, R., CHIKALEKE, V.A., THOTTAPILLY, G. and HAHN, S.K. Origin and phylogeny of Guinea yams as revealed by RFLP analysis of chloroplast DNA and nuclear DNA, Theoret. Appl. Genet. **83** (1992) 743-751.
[36] SCHÄFER, W., GÖRZ, A., and KAHL, G. T-DNA integration and expression in a monocot crop plant after induction of *Agrobacterium*, Nature **327** (1987) 529-532.
[37] WILLIAMS, C.E. and ST. CLAIR, D.A. Phenetic relationships and levels of variability detected by restriction fragment length polymorphism and random amplified polymorphic DNA analysis of cultivated and wild accessions of *Lycopersicon esculentum*, Genome **36** (1993) 619-630.
[38] HALWARD, T.M., STALKER, H.T., LARUE, E.A. and KOCHERT, G. Genetic variation detectable with molecular markers among unadapted germplasm resources of cultivated peanut and related species, Plant Mol. Biol. **18** (1992) 315-325.
[39] BURKILL, I.H. The organography and evolution of *Dioscoreaceae*, the family of yams, J. Linn. Soc. Lond. Bot. **56** (1960) 319-412.
[40] TABERLET, P., GIELLY, L., PANTOU, G. and BOUVET, J. Universal primers for amplification of three noncoding regions of chloroplast DNA, Plant Mol. Biol. **17** (1991) 1105-1109.
[41] ASEMOTA, H.N., RAMSER, J., LOPEZ-PERALTA, C., WEISING, K. and KAHL, G. Genetic variation and cultivar identification of Jamaican yam germplasm by random amplified polymorphic DNA analysis, Euphytica **92** (1996) 341-351.
[42] KAEMMER, D., AFZA, R., WEISING, K., KAHL, G. and NOVAK, F.J. Oligonucleotide and amplification fingerprinting of wild species and cultivars of banana (*Musa* spp.), BioTechnology **10** (1992) 1030-1035.
[43] CASTIGLIONE, S., WANG, G., DAMIANI, G., BANDI, S., BISOFFI, S. and SALA, F. RAPD fingerprints for identification and taxonomic studies of elite poplar (*Populus* spec.) clones, Theor. Appl. Genet. **87** (1993) 54-59.
[44] HOEPFNER, A.S., NYBOM, H., CARLSON, U. and FRANZEN, R. DNA fingerprinting useful for monitoring cell line idendity in micropropagated raspberries, Acta Agric. Scand. Sect. B., Soil Plant Sci. **43** (1993) 53-57.
[45] WOLFF, K., ZIETKIEWICZ, E. and HOFSTRA, H. Identification of chrysanthemum cultivars and stability of DNA fingerprint patterns, Theor. Appl. Genet. **91** (1995) 439-447.
[46] NOVY, R.G., KOBAK, C., GOFFREDA, J. and VORSA, N. RAPDs identify varietal misclassification and regional divergence in cranberry (*Vaccinium macrocarpon* (Ait.), Pursh. Theoret. Appl. Genet. **88** (1994) 1004-1010.

RESULTS OF A *MUSA* MAPPING PROJECT

P.J.L. LAGODA, J.-L. NOYER, F.-C. BAURENS, L. GRIVET,
L.-M. RABOIN, C. AMSON, G. AUBERT, L. COSTET, C. LANAUD
CIRAD-GERDAT BIOTROP Laboratory, AGETROP,
Montpellier, France

D. DAMBIER, J. GANRY,
H. TEZENAS-DU-MONTCEL, J.-P. HORRY,
CIRAD-FLHOR, Montpellier, France

F. BAKRY, P. OLLITRAULT, F. CARREEL
CIRAD-FLHOR, Guadeloupe, France

E. AUBOIRON, E. FOURE, C. JENNY, K. TOMEKPE
CRBP, Nyombe, Cameroon

A. GRAPIN
CATIE, Turrialba, Costa Rica

A.S. FAURE
Généthon, Paris, France

F. RESCHŒUR
GenomeExpress SA, Grenoble, France

D. GONZALEZ-DE-LEON
CIMMYT, Mexico-City, Mexico

Abstract

A completed map, based on two selfed progenies from two banana cultivars (M53 and SFB5) is presented (roughly 1200 cM). More than three hundred markers are linked in 11 linkage groups representing the genome (2n=22) of *Musa acuminata*. Roughly one third of the markers are co-dominant restriction fragment polymorphisms (RFLPs; one hundred) or microsatellites (thirty). Two thirds of the markers are dominant amplified fragment length polymorphisms (AFLPs; 10% could be considered to be co-dominant). The mean linkage distance is 3 cM, but marker density still should be increased on a couple of linkage groups. Particularities for a mapping job in banana are discussed. Due to *Musa acuminata* sub-species specific translocations, up to 36% of all the markers tested show important segregation distortions. The need for a cooperative mapping initiative based on a proposed "frame-map" harbouring evenly spaced co-dominant "anchor" markers is proposed. CIRAD has published 45 sequence tagged microsatellite sites (STMS) in the EMBL database which are accessible at: "http://www.ebi.ac.uk/" using the keywords LAGODA and MICROSATELLITE (EMBL accessions X87258 to X87265, X90740 to X90750 and Z85950 to Z85977).

1. INTRODUCTION

Banana and plantain belong to the *Musaceae* family and are among the tallest monocotyledons. They are crops of primary economic importance for many tropical countries, either as a local staple food or for exportation. Unfortunately, they can be considered "orphan crops" because they are seldom included in international genome analysis initiatives. They are,

however, an example of higher eukaryotes (reviewed in [1]) whose cytogenetic structure is made more complex through chromosomal repatterning due to considerable chromosome structural differentiation within the *Musa* species (reviewed in [2]). Natural reproductive barriers exist, therefore, between *acuminata* wild type diploid subspecies, an obvious component of subspecies divergence, and genetic diversity of the species as a whole. This fact is of paramount importance to breeders, seriously limiting options in improvement strategies. Diploid cultivars are essentially inter-(sub)species hybrids, heterozygous for one or several translocations (reviewed in [2]). The tendency towards male-sterile material and genic female sterility further complicates the use of these materials as female parents. Sterility (both genic female (male?) and caused by structural hybridity), parthenocarpy (i.e. autonomous stimulus to pulp growths) and subsequent formation of triploids (resulting from the fusion of reduced and non-reduced gametes following nuclear restitution) are the milestones of domestication which created the present vegetatively propagated, sexually sterile cultivars. Molecular knowledge of the complex genome structure of banana and plantain is thus mandatory to better understand *Musa* genetics. In the late 1980s, CIRAD started to use molecular markers on banana to study the genetic diversity of the *Musaceae* and to investigate marker assisted breeding ([3] and [4] and references therein) for resistance to important diseases such as Black Leaf Streak Disease (BLSD). The present report will focus on mapping the banana genome.

2. MATERIALS AND METHODS

2.1. Plant material (mapping populations)

Eighty-nine individuals have been used to build the "M53" map. The parental clone, M53, is a synthetic hybrid:

(*Musa acuminata malaccensis* 'Kedah' type x *Musa acuminata banksii* 'Samoa' type)
x
(AAc 'Paka' type x *Musa acuminata banksii* 'Samoa' type)
|
"M53"

M53 originally stems from the genetic improvement program headed by Dr. K. Shepherd in Jamaica. The progeny was produced at CIRAD, French West Indies (Guadeloupe). The progeny of the selfed M53 shows segregation for the resistance to BLSD (Bunchy Leaf Disease). More than 750 other progenies of the same cross are being evaluated in the field for BLSD resistance at the CRBP in Cameroon.

Our second mapping population was a selfed progeny of clone SFB5. The parental clone, SFB5, is a hybrid between SF265(AAcv) and *Musa acuminata* ssp. *banksii* type 'Banksii' (AAs). Ninety-two individuals have been used to build the "SFB5" map [5].

2.2. Detection of molecular markers and map construction

Amplified fragment length polymorphisms (AFLP) were performed using six primer pairs from the kit of GIBCO-Life Technology, with the protocol provided by the supplier. Variable number of di-nucleotide repeats (VNDR) were obtained and analysed as previously described [6, 7]. Restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD) and isozyme analyses were performed as previously described [4,8]. Maps were constructed using the JoinMap 1.4 software [9]. A critical LOD value of 4 was used to establish linkage groups.

TABLE I. FERTILITY AND ESTIMATED NUMBER OF TRANSLOCATIONS IN M53 AND SFB5

				Observed poly-valents per metaphase (maximum number of translocations) I = mono-, II = di-, III = tri-, IV = tetra- valents				
Individual	Viable pollen total number	Fertility %	Metaphase spreads number	(0) 9 II	(1) 9 II, 1 III, & 1 I	9 II & 1 IV	(2) 8 II & 1 VI	7 II, 1 IV, 1 III & 1 I	Translocations number
M53	510 (545)	93	14	12	1	1	0	1	2
SFB5	908 (969)	94	15	10	2	2	1	0	2

(modified from [4])

3. RESULTS

Pollen fertility and metaphase spreads of the parental accessions of the two mapping populations, M53 and SFB5, have been previously described (Table I, [4]). Pollen fertility, evaluated according to the method described by Alexander [10], was rather high (93%), whereas up to one third of the analysed metaphase spreads showed chromosomal structures compatible with 2 "heterozygous" translocations for both accessions.

A composite map was constructed based on common markers in the two mapping populations. Mapping of the "SFB5" population has been previously reported [5]. This map is based on RFLPs (61 probes, eight of which gave duplicated loci), RAPDs (thirteen loci) and isozymes (4 loci, MDH, POXc, EST, PGM). We recently added 16 microsatellite sites. This map now comprises 106 markers, 89 of which are mapped on 15 linkage groups. Four markers appeared as two independent, tightly linked pairs of loci, thirteen loci remained unlinked at a LOD (Log of the Odds) value of 4.

The available polymorphism in the "M53" population permitted the mapping of 61 RFLP probes, five of which revealed duplicated loci, and 29 microsatellite loci. Thus a "frame-map" of 95 co-dominant "locus-specific anchor markers" was produced. The use of RAPD and AFLP technologies allowed for a rapid addition of eleven and 204 dominant markers, respectively. Seventeen pairs of AFLP markers (34 fragments) appeared to be alternative alleles of seventeen loci. Every candidate AFLP-pair was revealed by the same set of primers, they had close positions on the analysing gel and no 'double absences' were observed for the same individual. They were tentatively treated as seventeen co-dominant markers. The resulting genetic map comprises 294 markers. Two hundred and seventy six markers were mapped on eleven linkage groups. Six markers appeared as three independent tightly linked pairs of loci and nine markers remained unlinked at a LOD value of 4. The total size of this map was 1177 cM. Segregation distortions were calculated for all the markers. Twenty AFLP markers with a significant c^2 at the 0.001 level were excluded.

The "M53" and "SFB5" maps had 97 locus specific markers in common (81 RFLP and sixteen microsatellites). Co-linearity was observed between linkage groups of the two maps. This allows for the construction of a composite map (1227 cM) at a LOD score of 4.75. This composite map links 373 isozyme, microsatellite, RFLP, RAPD and AFLP markers in 11 linkage groups. One tightly linked AFLP pair (provided by "M53") doesn't associate with any linkage group and two STMS, four AFLP from "M53" as well as one RFLP and two isozyme markers from "SFB5" stay unlinked at the chosen LOD score. Mean linkage distance is 3 cM. Up to 36 % of the tested markers showed important segregation distortions. Most of these markers associate in a very large linkage group at LOD £ 3.0, but are dissociated in different linkage groups at LOD ^{3} 4.0. Of special interest are three RFLP markers pMaCIR1001, pMaCIR1111 and pMaCIR257. These RFLP markers are amongst the markers showing the most distorted segregation. They are also the only RFLP markers permutating linkage when comparing both maps (Fig. 1). More studies are needed to test whether this is indicative of a translocation breakpoint.

4. DISCUSSION

4.1. Mapping and translocations

The most important problem for mapping the banana genome seems to be the variability of chromosome structure. Translocations are a common phenomenon in diploid bananas [2]. Banana chromosomes are tiny, which explains the paucity of cytogenetic data (reviewed in [4]). These data are not explicit enough to identify whether the translocation(s) involved: an unbalanced translocation, a segment from one chromosome is transferred on top of another, nonhomologous, chromosome; the insertion of a segment from one chromosome into the chromosomal arm of another non-homologous chromosome; or the exchange of chromosomal ends between two non-homologous chromosomes.

According to Burnham [11] the reciprocal translocation (exchange) is the most common process observed in somatic cells or at meiosis. Translocations may occur spontaneously due to the imbrication of bivalents, entangled chromosomes, homozygosity of special genes (e.g. *sticky, st1* in corn), growth from aged seeds or be induced by exogenous stimuli like chemical treatments or irradiation. At meiosis the homologous regions of the hybrid chromosomes produced by translocation will associate. Meiosis will proceed normally if both members of the hybrid chromatid couple experienced the same structural modification. The translocation is then called homozygous. In structural hybrids, when one member of the associated chromosomes suffered structural modification, the translocation is then called heterozygous. At prophase I, the association of four chromosomes (two pairs), sharing partial homologies, produces a complex chromatid configuration (tetravalent, visible as a structure resembling a cross). How this complex configuration behaves at meiosis depends on the frequency of the chiasmata and on their relative location in relation to the centromere position and orientation. If two heterozygous translocations co-exist in the same cell, two tetravalents may be observed if both events don't share a common chromosome or one hexavalent, if one chromosome is implicated in both structural modifications (Table I, Fig. 2).

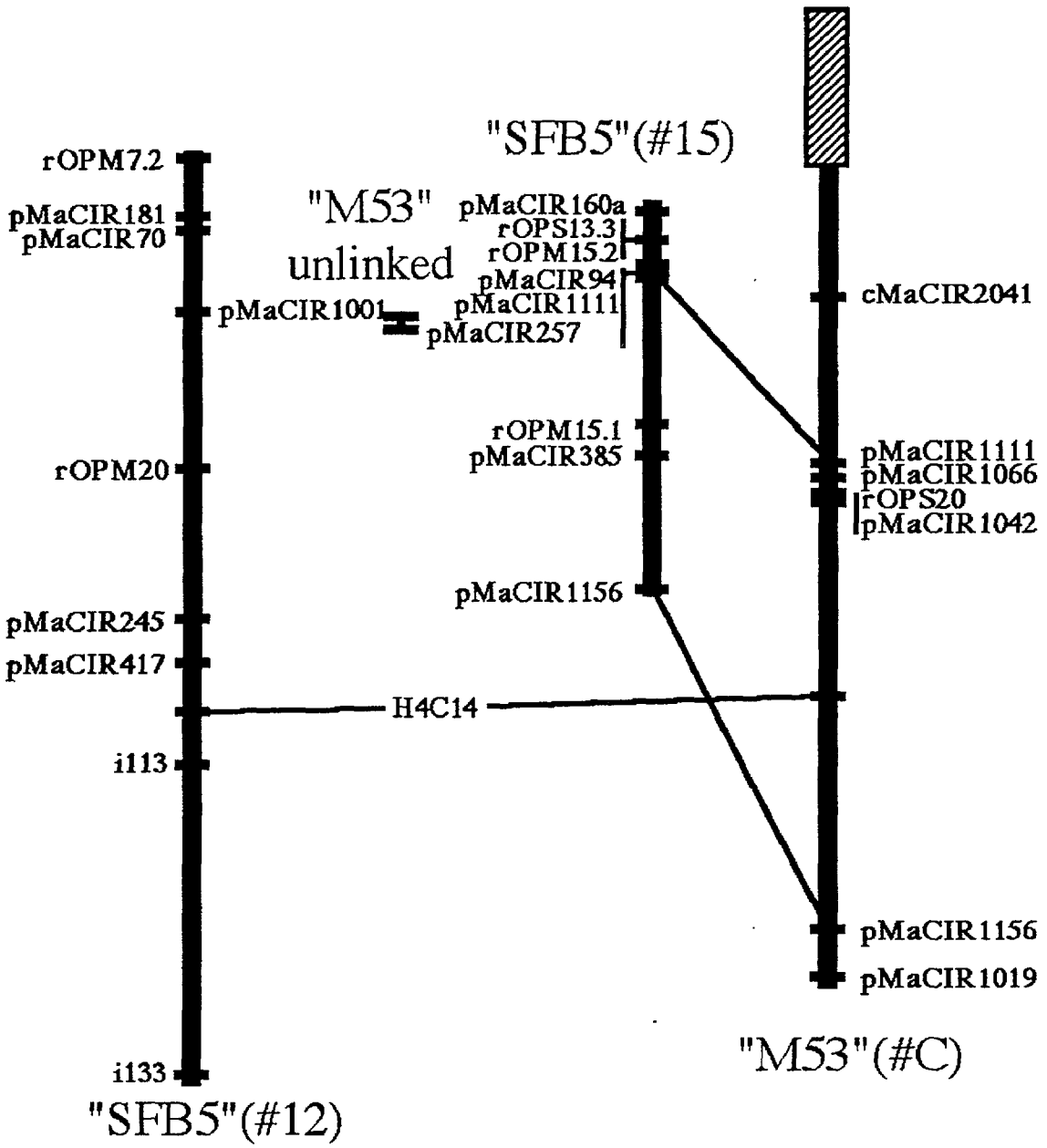


FIG. 1. Detail from the comparison of the "SFB5" and "M53" maps. The candidate translocation breakpoint (to be investigated further) is indicated as: "M53" unlinked. The probe type is indicated by the probe name: pMaCIRxxxx, RFLP (genomic); cMaCIRxxxx, RFLP(cDNA); rOPXxx.x, RAPD; ixxx, STMS; and H4C14, RFLP (histone).

There are at least three important genetic consequences of structural hybridity. First, nonhomologous chromosomes involved in a reciprocal translocation will associate during meiotic prophase I to form the polyvalents described above, thus creating inter-chromosomal linkage. These complex structures may be considered as a single recombination unit or linkage group. This might explain why at a LOD score between 3 and 4 both our maps include nine linkage groups (basic chromosome number n = 11), one being a very long "mega-linkage group" harbouring significantly more markers than the eight other linkage groups. Thus, genes usually segregating independently, because they are normally situated on different chromosomes in one parent, will show variable degrees of linkage in the progenies of structural hybrids. Second, the recombination rate is reduced near translocation breakpoints due to steric

hindrance of the heterochromatin structure [11] (Fig. 2) and genetic distances between the proximal loci will appear shortened. Third, at meiosis, the disjunction of the multiple chromosome associations at metaphase will result in mostly unbalanced distributions of interchanged chromosomal segments. Some gametes will be unviable and their gene marker) combinations will not be represented in the progeny due to the differential gametic viability.

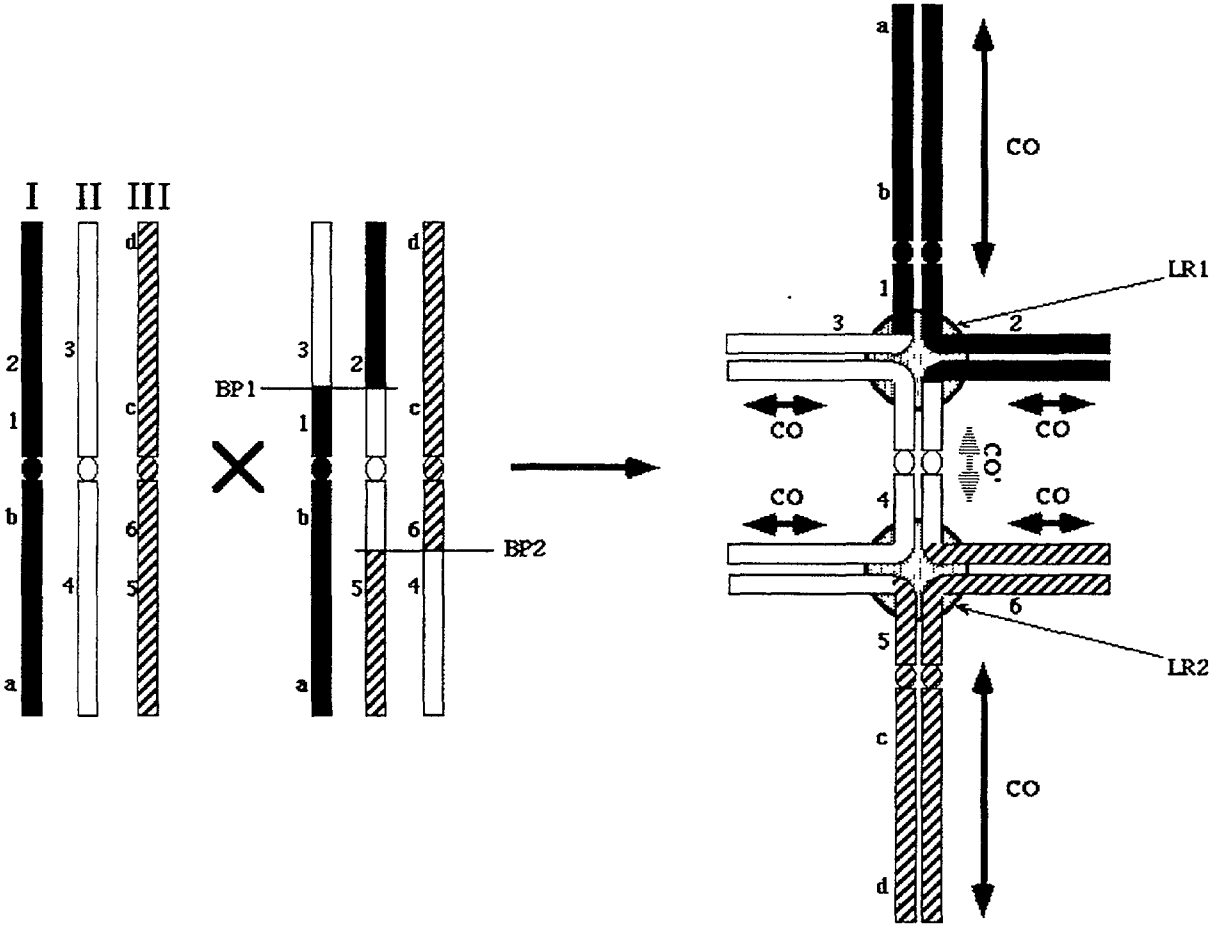


FIG. 2. Diagrammatic representation of meiotic pairing in a hypothetical structural heterozygote for two reciprocal translocations involving 3 pairs of chromosomes (modified from [1].The complements of the parental accessions differ by two reciprocal interchanges involving 3 pairs of chromosomes (I, II, III). BP1, BP2 = translocation breakpoints. CO = segments of putatively unaffected crossing over potential. LR1, LR2 = zones with reduced recombination rate. Loci linked in both parents: {1, b, a} and {d, c, s}. CO' = region of variable crossing over potential. Loci linked in parent 1 (I, II, III) but not in parent 2: 1&2, 3&4 and 5&6. Loci linked in parent 2 but not in parent 1: 1&3, 2&5 and 4&6. Linkage groups {a - b} and {c - d} are the most likely to be unaffected by translocations. "Hybrid" linkage groups {1, 2, 3} and {4, 5, 6} will appear independant only if the crossing over rate is high at CO' between BP1 and BP2. Loci 1, 2 and 3 (idem for loci 4, 5 and 6) may appear "linked" more or less tightly depending on crossing over rate between each of them and the proximal breakpoint. Crossing over may be sterically hindered depending on position relative to the proximal LR and the centromere.

This phenomenon will be observed as a distortion of the expected marker segregation on rearranged chromosomes. This might explain the severe distortion of some of our mapping markers and the fact that 36% of the tested markers show varying levels of distortion. The majority of the markers showing some degree of distortion are found in the "mega-linkage group" described above. At a LOD score ^{3} 5.0, both maps break up into 14 ("M53") and 15

("SF5") linkage groups (these remain stable up to a LOD score of 10). Depending on the relative position of translocation breakpoints and centromeres (Fig. 2), the crossing over frequency and the percentage of most homozygous gametes at the translocation breakpoints, the "mega-linkage group" would produce up to 15 fragments behaving as autonomous linkage units at increasing LOD scores (Fig. 2, computer simulations).

In fact, the mechanism responsible for the resulting segregation has not yet been completely understood. Chromosomes having suffered translocation(s) and normal chromosomes will migrate to opposite poles; all resulting gametes will thus inherit a complete set of genes and gametic viability and fertility will be high. Non-homologous, translocated, alleles will migrate towards the same pole. The resulting gametes will inherit one normal and one translocated chromosome, thus their gene sets will harbour duplications of genes and suffer loss of genes, and differential gametic viability and fertility will depend on the relative importance of the duplicated and/or lost genome segments.

In conclusion, both the probability of finding unwanted linkages and the strength of these linkages may increase. As a corollary, the precision of QTL localization will be adversely affected.

4.2. Molecular markers

Comparing both maps from different sub-species hybrids, it has been noted, as expected, that collinearity of co-dominant markers (RFLP, Microsatellites) is highly conserved. The efficiencies of the different marker systems, RFLP and microsatellites, in a comparative mapping approach are roughly equivalent [7].

The usefulness of microsatellite derived markers is emphasised by the utilization of relatively simple laboratory techniques (polymerase chain reaction(PCR), non radioactive detection, potential for automated analysis) compared to RFLP markers [6,7]. Detecting microsatellite loci is expensive, but using already established markers is cost effective. Nevertheless, development of microsatellite markers is currently limited in plants, in general, by the number of available published sequence data for most species. This is particularly the case for tropical crops such as banana, but an increasing number of research groups are working on the molecular characterisation of these and there is no doubt that more DNA sequences will be available in the near future.

RAPD and AFLP techniques have in common the ability to rapidly produce a great number of signals which can be scored. These data can be introduced into evaluation algorithms in order to be analysed statistically. These analyses are the bases of identification systems, genetic diversity estimations and mapping. These markers are, however, "anonymous", random markers, meaning that the fragments produced in these assays must be scored as dominant (presence *vs.* absence) markers. To identify the specific loci, and to identify the homologous alleles of the specific loci, one must invest more effort into sub-cloning and eventually sequencing these fragments, producing cleaved amplified polymorphic sites (CAPS) and sequence characterized amplified regions (SCARs). This is the main reason these marker systems are good identification tools for clonal identification or to assist breeding of near-isogenic lines (NILs). In a mapping approach they may be used to rapidly saturate a frame map based on co-dominant markers, knowing that the information deficiency of dominant markers, as compared to co-dominant markers in a F2 type mapping approach, decreases confidence in QTL map positions and marker order.

A complete comprehensive map of *Musa* spp. will be attainable through the comparison of several maps based on parents with different karyotypes. This can not be accomplished by a single research group. Thus, we are selecting a subset of our co-dominant markers, spaced 10-20 cM apart, to construct a "frame map" which could be easily saturated using AFLP, as an example. Eventually this "anchor marker set" is bound to be distributed to interested researchers in a cooperative research effort. Forty-five STMS from CIRAD are published in the EMBL database and accessible via "http://www.ebi.ac.uk/" using keywords LAGODA and MICROSATELLITE (EMBL accessions X87258 to X87265, X90740 to X90750 and Z85950 to Z85977).

The different maps eventually produced could be compared through the relative localisation of the same anchor markers. The comparative data resulting from these efforts should be used to map translocation breakpoints in order to produce a *Musa* consensus or core map.

5. CONCLUSION

An almost complete map harbouring eleven linkage groups, ideally corresponding to the basic number of chromosome (n=11) has been produced. The total length is about 1200 cM. Significant levels of segregation distortion were observed, particularly for AFLP markers. Those distortions could be related to the two translocations observed on meiotic preparations of M53 and SFB5 [4].

No structural differences could be detected between the 'SFB5' and 'M53' maps. This allowed the straightforward construction of a frame-map of the *Musa* genome. It now comprises 119 loci with 94 RFLP, 24 microsatellites and one isozyme. The density of co-dominant markers will have to be improved for a couple of linkage groups.

The development of PCR-based markers is a key objective of our mapping program. AFLP technology also appears to be a fast and powerful tool for saturating the "frame-map". A mean of 33 markers were revealed for each primer pair and about 10% could be considered to be co-dominant. It is probable that a combination of SCARs from AFLP and microsatellites will be used in the future to transfer genotyping "to the field".

The difficulties with breeding improved triploid cultivars are now easy to understand. Classical strategies involve crossing a diploid accession (e.g., wild type), usually a fertile one showing some desirable trait, onto a good triploid recipient cultivar having good female restitution. Other strategies include breeding improved diploids, both cultivated and wild type accessions, in order to reconstruct triploid cultivars by crossing the improved diploid onto a tetraploid, artificially induced or not. Insufficient knowledge about the genomic origins and composition of diploid and polyploid cultivars complicates the choice of the best diploid materials for desirable introgressions. The overall effects of structural hybridity, as shown, will interfere with the breeders efforts at recombining and transferring desirable traits from wild and cultivated diploids, sometimes forcing them, unwittingly, to attempt bridging crosses to circumvent these reproductive barriers or to avoid apparent linkage of undesirable traits. On the other hand, translocations can also be used to the breeder's advantage for the transfer, or localization, of particular gene loci [1].

As far as breeding *Musaceae* is concerned, molecular markers have proven to be invaluable tools. Having these tools handy, breeders, the real work is still to be done!

REFERENCES

[1] GONZALEZ-DE-LEON, D. and FAURE, S. "Genetic Mapping of the Banana Diploid Genome: toward an integrated approach to the study of the *Musa* genome and the use of molecular marker technologies in *Musa* breeding", Proceedings of the Workshop on "Biotechnology Applications for Banana and Plantain Improvement", San José, Costa Rica, 27-31 January 1992 (edited by INIBAP, Montpellier, France) (1993) 29-51.

[2] SIMMONDS, N.W. The Evolution of the Bananas, Longmans, London, UK (1962).

[3] CARREEL, F. Etude de la diversité génétique des bananiers (genre Musa) à l'aide de marqueurs RFLP, Ph.D. Thesis, INA, Paris-Grignon, France (1994).

[4] FAURE, S. Contribution à la cartographie génétique du génome des bananiers diploides (*Musa acuminata*) à l'aide de marqueurs moléculaires, Ph.D. Thesis, Ecole Nationale Supérieure Agronomique de Rennes, N°9310, SérieC, N° de Série 23. Rennes, France (1993).

[5] FAURE, S., NOYER, J.L., HORRY, J.P., BAKRY, F., LANAUD, C., and GONZALEZ DE LEON, D. A molecular marker-based linkage map of diploid bananas *(Musa acuminata)*, Theoretical and Applied Genetics **87** (1993) 517-526.

[6] LAGODA, P.J.L., NOYER, J.L., DAMBIER, D., BAURENS, F.C., and LANAUD, C. "Abundance and distribution of simple sequence repeats in the *Musaceae* family: microsatellite markers to map the banana genome", The Use of Induced Mutations and Molecular Techniques for Crop Improvement (Proc. Symp. Vienna, 1995) IAEA, Vienna, Austria, 287-295.

[7] KAEMMER, D., FISCHER, D., JARRET, R.L., BAURENS, F.C., GRAPIN, A., DAMBIER, D., NOYER, J.-L., LANAUD, C., KAHL, G., and LAGODA, P.J.L. Molecular Breeding in the Genus *Musa*: a strong case for STMS marker technology, Euphytica **96** (1997) 49-63.

[8] HORRY, J.P. Chimiotaxonomie et organisation génétique dans le genre *Musa*. Université Paris XI, Centre d'Orsay, France (1989) 95pp.

[9] STAM, P. Construction of integrated genetic linkage maps by means of a new computer package: Joinmap, Plant Journal **3** (5) (1993) 739-744.

[10] ALEXANDER, M.P. Differential staining of aborted and nonaborted pollen, Stain Technol. **44** (1969) 117-122.

[11] BURNHAM, C.R. Chromosomal interchanges in plants, Bot. Rev. **22** (1956) 419-552.

GENERATION AND APPLICATION OF SSR MARKERS IN AVOCADO

D. SHARON, U. LAVI
Institute of Horticulture, ARO Volcani Center,
Bet-Dagan, Israel

P.B. CREGAN
United States Department of Agriculture, Agricultural Research Service,
Beltsville, Maryland, United States of America

J. HILLEL
Faculty of Agriculture, Hebrew University of Jerusalem,
Rehovot, Israel

Abstract

Simple Sequence Repeat (SSR) DNA markers were generated and applied to avocado. An SSR marker is based on a pair of primers which are synthesized on the basis of DNA sequences flanking a microsatellite. These markers are PCR based, quite polymorphic and abundant in several species. These are the markers of choice in the human genome. The number of SSR markers in the avocado genome was calculated to be about 45,000, with the A/T microsatellite being the most frequent (1 in 40 kb). SSR markers are quite expensive to generate due to the required multi-step procedure; Screening a genomic library, about 66% of the positive clones turned out after sequencing to be SSR containing clones. In only about 55% of these, was it possible to synthesize primers and, of this group, only about 50% of the markers were useful for typing a specific family. Typing of five avocado cultivars using 59 SSR markers results in one to eight alleles per locus, mean heterozygosity ranging between 0.51 and 0.66 and gene diversity ranging between 0.42 and 0.66. The SSR markers were used to estimate the genetic relationships between various *Persea* species. The number of alleles in these species ranged between five and twelve with heterozygosity levels between 0.11- 0.78 and gene diversity between 0.69-0.89. A preliminary genetic map, based on these SSR markers together with some DNA fingerprints (DFP) and randomly amplified polymorphic DNA (RAPD) markers, was drawn. The map consists of 12 linkage group having two to five markers each. Linkage analysis with several quantitative trait loci (QTLs) was performed by genetic typing and phenotypic assessment of the progeny of a controlled cross. The result of the interval mapping suggests that the gene(s) coding for the existence of fibers in the flesh, are probably linked to linkage group 3.

1. INTRODUCTION

Avocado (*Persea americana* Mill.) is a sub-tropical fruit-tree, with 24 chromosomes (2n=24) and haploid genome size of 8.83 x 10^8 bp. The classical avocado breeding programs are inefficient mainly because of a long juvenile phase, large tree size and limited genetic knowledge. These obstacles may be alleviated by applying marker assisted selection (MAS).

In the last few years, four types of genetic markers have been used in avocado. Degani *et al.* [1] used isozyme markers mainly to study the level of selfing and out-crossing in 'Fuerte'. Furneir *et al.* [2] used two restriction fragment polymorphism loci (RFLP) loci to study the relationships between Persea species and between avocado cultivars. DNA fingerprints (DFP), using multilocus minisatellite and microsatellite probes were applied for identification of cultivars and races [3] and for detection of linkage between a gene affecting fruit skin color and a specific DFP band [4].

Simple sequence repeat (SSR) markers have been shown to be highly polymorphic in human [5], animals [6] and plants. Preliminary studies of simple sequence repeat markers in fruit-trees were carried out: two SSR markers were isolated from a cross between lemon and orange and found to be polymorphic between citrus and related species [7]. Thomas and Scott [8] isolated five microsatellite loci from grapevine and found that the heterozygosity level of these loci ranged between 69% and 88%. Lavi *et al.* [9] generated 30 dinucleotide microsatellite markers and demonstrated the high level of polymorphism of two of them.

In this study, we present the generation of avocado-specific SSR and the application of these markers for genetic analyses as well as construction of the first integrated genetic linkage map of avocado.

2. MATERIALS AND METHODS

2.1. Plant material

Trees were grown at the Akko Experiment Station and the Agricultural Research Organization, Bet Dagan, Israel. Leaves were taken from 50 offspring of the cross 'Pinkerton'x'Ettinger' and from the following *Persea* species: *P. brobonia, P. cinerascens, P. floccosa, P. gigantea, P. indica, P. longipes, P. nubigena, P. schiedeana, P. schiedeana 05, P. schiedeana 06, P. steyermarkii Allen.*

2.2. DNA isolation

DNA was isolated from very young leaves using the CTAB (cetyltrimethyl ammonium bromide) method [10], following a few modifications [3].

2.3. DNA fingerprinting

Ten micrograms of DNA were digested with *Hinf*I restriction endonuclease. Electrophoresis and hybridization are detailed elsewhere [4].

2.4. Randomly amplified polymorphic DNA (RAPD) markers

Reaction mixes for RAPD markers contained 30 ng of genomic DNA, 1.5 mM Mg^{2+}, 0.2 mM of primer, 250 mM of each nucleotide, 1X Taq buffer (containing 20 mM $(NH_4)SO_4$, 75 mM Tris-HCl, pH 9.0, 0.1% Tween) and 0.5 unit of Taq DNA polymerase in a total volume of 25ml. The reaction mixes were overlaid with 40ml of mineral oil. Reactions were denatured at 94°C for 3 min before cycling which consisted of a 1 min denaturation at 94°C, 1 min annealing at 36°C, and 1 min extension at 72°C for 44 cycles. Cycles were followed by an extension step for 5 min at 72°C. Amplification products were analyzed by electrophoresis in 1.5% agarose gels and detected by staining with ethidium bromide. RAPD primers used are from set #7 (numbered 601-700) of the University of British Columbia.

2.5. Genomic library

Genomic DNA of 'Pinkerton' was cut with the restriction enzyme *Sau*3AI. DNA fragments were size selected on agarose gels and fragments (400-600bp) were cloned in pBluescript II KS^+ and transformed into *E. coli* Inv-a strain. Transformants were grown in LB media with 50mg/ml Ampicillin. White colonies were transferred into microtiter plates and

stamped onto Hybond N+. Colonies were grown, lysed, denatured and neutralized. Membranes were hybridized to end-labeled g-^{32}p oligonucleotide probes. Probes were: $(A)_{30}$, $(CT)_{10}$, $(GT)_{10}$, $(CAC)_6$, $(TCC)_6$, $(TCT)_6$, $(GATA)_4$, $(GACA)_4$ and $(GGAT)_4$.

2.6. SSR definition

Sequences of positive clones were searched for any type of repeated sequences. A repeat was identified as an SSR if the number of repeats (n)>14 for mononucleotide repeat, (n)>6 for di-nucleotide repeats, (n)>3 for tri-nucleotide repeats and (n)>2 for tetra-nucleotide (or more) repeats.

2.7. Selection and synthesis of primers

SSR-containing clones were analyzed using the Primer Detective Software or PRIMER 0.5. Primers having melting temperatures of at least 60°C and which would amplify products with predicted lengths of 75-210bp were synthesized.

2.8. Genotyping

PCR reaction mixes for SSR markers contained 30 ng of genomic DNA, 1.5 mM Mg^{2+}, 0.15 mM of 3' and 5' end primers, 100 mM of each nucleotide, 200mg/ml BSA, 0.1 ml of 3,000 Ci/mmol a-^{32}P dCTP, 1X Taq buffer (containing 50 mM Tris-HCl, pH 9, 0.1% Triton X-100), and one unit of *Taq* DNA polymerase in a total volume of 10ml. The reaction mixes were overlaid with 20ml of mineral oil. Reactions were denatured at 94°C for 30 s before cycling which consisted of a 15 s denaturation at 95°C, 25 s annealing at 45°C or 50°C (depending on the primers), and 25 s extension at 68°C for 32 cycles. This was followed by an extension step for 2 min at 68°C. Ten ml of loading buffer (95% formamide; 0.02 M EDTA, pH 8; 1% bromophenol blue; 1% xylene cyanol; and 10 mM NaOH) were added and 3ml of the reaction were loaded on a DNA sequencing gel containing 6% polyacrylamide, 8 M urea and 1xTBE and electrophoresed at 50 Watt for three to four hours. Gels were dried and exposed over-night to an Fuji X-ray film. The length of the SSR alleles was determined using a sequence ladder of M13 as size marker.

2.9. Heterozygosity analysis

Gene Diversity (GD) was calculate according to [11]: $GD = 1-\Sigma pi^2$ where pi is the frequency of the i th pattern. Heterozygosity level was calculated as the proportion of heterozygote genotypes from all tested genotypes.

2.10. Evolutionary analysis

Phylogenetic trees was performed using the maximum parsimony method with the 'PAUP' software.

2.11. Map construction

For map construction, two-point analyses of all possible combinations were performed using MAPMAKER/EXP 3.0 (12) and JoinMap (13). Linkage was determined when the LOD score was >3.0. Linkage analysis of loci that showed significant deviation from Mendelian

expectation was tested using the c^2 test in addition to the LOD score calculation. The final map was drawn using MAPMAKER and was verified using several mapping programs.

2.12. Detection of association between DNA markers and loci controlling fruit traits

One way analysis of variance was performed between offspring genotypes (of each marker locus) and each of the nine fruit traits using the JMP computer program. The Contrast-Analysis was used in order to search for possible allelic interaction affecting the content of fibers in the flesh. The interval mapping method was performed between the 12 avocado linkage groups and each of the nine fruit traits. The paternal or maternal interval mapping analysis of the 12 linkage groups was made using MAPMAKER/QTL software [12]. The combined interval mapping analysis between linkage group 3 and the content of fibers in the flesh was made using MapQTL software [14].

3. RESULTS

3.1. Abundance of microsatellites in the avocado genome

An avocado genomic library containing about 10,000 clones with a mean insert size of 500 bp was screened with various SSR repeats (Table I). This library represents about 0.57% of the avocado genome ($5x10^6$bp out of 883Mbp). The frequency assessment of SSRs, based on positive clones, suggests that $(A/T)_n$ and $(TC/AG)_n$ sequences are the two most frequent microsatellites: 1 in 40kb and 1 in 68kb respectively. A total of 236 positive clones were identified suggesting about 45,000 microsatellite sequences in the avocado genome (Table I).

3.2. Generation of SSRs

Two hundred and twenty five positive clones were sequenced (from one side), of which 172 gave reliable sequence data (Table II). Of these 172 clones: 12 (7%) did not contain any type of repeat; the sequences of 47 clones (27%) contained short repeats; and 113 clones (66%) contained an SSR. Suitable primers could not be selected in 51 (45%) of the SSR-containing clones (Table III) due to location of the repeat near the cloning site (41 clones) or low melting temperature (10 clones), leaving 62 (55%) SSR primer pairs.

TABLE I. FREQUENCY OF NINE REPEAT TYPES IN THE AVOCADO GENOMIC LIBRARY

Repeat type	No. of screened clones	No. of positive clones (%)	Predicted ratio of SSRs per kb	Predicted No. of SSRs in the genome
(A/T)n	10,000	125(1.25)	1: 40	~22,000
(TC/AG)n	10,000	74 (0.74)	1: 68	~13,000
(GT/CA)n	10,000	27 (0.27)	1: 185	~ 4,800
(CAC/GTG)n	5,000	3 (0.06)	1: 833	~ 1,050
(TCC/AGG)n	5,000	2 (0.04)	1:1250	~ 700
(TCT/AGA)n	5,000	2 (0.04)	1:1250	~ 700
(GATA/CTAT)n	3,000	0 (0)	<1:1500	< 590
(GACA/CTGT)n	3,000	0 (0)	<1:1500	< 590
(GGAT/CCTA)n	3,000	3 (0.1)	1: 500	~ 1750
Total	**10,000**	**236(2.36)**		**~45,200**

TABLE II. FROM POSITIVE CLONES TO SSR-CONTAINING CLONES

	TC/AG	GT/CA	Others	Compound	Percent
No. of positive clones	66	21	9	10	
Good sequence	51	15	9	8	100%
No repeat	2	2	5	1	7%
Short repeat	10	5	1	2	27%
SSR-containing clones	39	8	3	5	66%

TABLE III. FROM SSR-CONTAINING SEQUENCES TO PRIMERS

	A/T	TC/AG	GT/CA	Others	Compound	Percent
SSR-containing sequences	51	33	6	9	14	100%
SSR near the cloning site	15	14	2	4	6	36%
Primers not found	6	2	2	0	0	9%
Primers were found	**30**	**17**	**2**	**5**	**8**	**55%**

The 62 SSR markers which were developed in this study, combined with 29 from a previous avocado library [9], and one from the 5' region of the cellulase gene (GenBank data) were used to analyse the avocado genome.

3.3. Polymorphism and heterozygosity levels in SSR loci

Fifty nine avocado SSR loci were used to analyse the polymorphism and heterozygosity level of the avocado genome. The dinucleotide repeats $(AG)_n$ are the most polymorphic SSR loci (of those examined) in avocado and thus presented the highest mean number of alleles per locus and highest Gene Diversity value (Table IV). The mean heterozygosity in the five cultivars was estimated to be 62%, and varied between 58% and 70%. 'Horshim' was the cultivar with the highest level of heterozygosity (Table V). In summary, the analysis of 59 loci in five cultivars indicated an average of 3.4 alleles per locus and a mean heterozygosity of 62%.

TABLE IV. IDENTIFICATION OF SSR MARKERS IN FIVE AVOCADO CULTIVARS

Gene Diversity	Average No. of alleles (range)	No. of markers	Repeat element
0.66	4.1 (1-8)	29	$(AG)_n$
0.42	3.0 (1-6)	19	$(A)_n$
0.56	2.5 (1-5)	7	Compound
0.55	2.6 (1-4)	4	Others
0.56	**3.4**	**59**	**Sum**

TABLE V. HETEROZYGOSITY LEVEL (HL) OF FIVE AVOCADO CULTIVARS

Simple repeat	'Ettinger'	'Pinkerton'	'Bacon'	'Horshim'	'XX102'	Mean
AG	68	68	67	82	63	70
A	65	79	67	60	65	67
Mix	30	30	43	60	33	39
Others	40	20	20	60	50	38
Total	59	62	58	70	58	62

3.4. Evolution of the *Persea* genus

Nine *Persea* species were chosen to detect genetic distances between *Persea* species using ten SSR markers. Fig. 1 represents the phylogenetic tree constructed by PAUP software. The three avocado races are located on close branches although the Mexican race seems to be closest to the other *Persea* species which can be divided into four main groups.

3.5. Polymorphism within family

Each parent of the mapping population ('Pinkerton' and 'Ettinger') was screened with each of the 92 primer pairs. Five SSRs gave no PCR product (Table VI), 14 gave non-reliable or multiple products, 25 gave non-informative patterns (non-polymorphic, or both parents homozygous for different alleles). Forty eight of 92 analysed SSRs were used for typing 50 offspring of the mapping population. Two primer pairs amplified products from two different loci each and thus a total of 50 loci were scored.

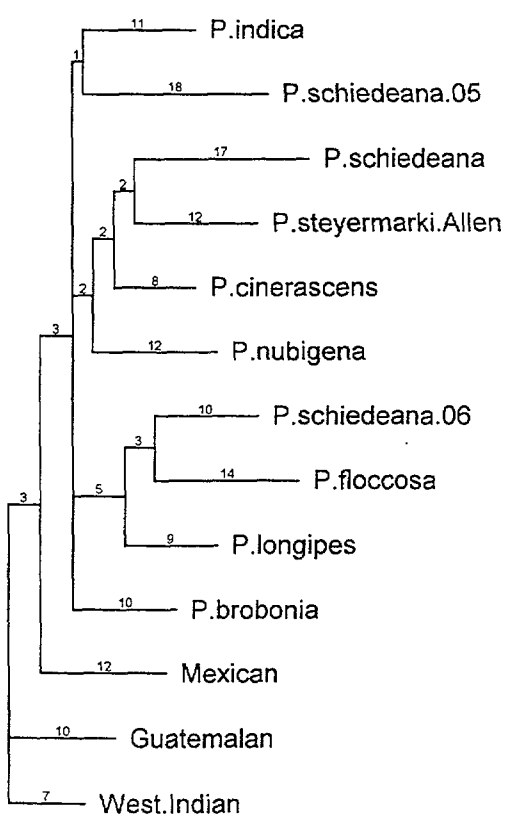


FIG. 1. Evolution tree of Persea genus.

TABLE VI. TYPING THE PROGENY OF THE CROSS 'PINKERTON' X 'ETTINGER'

	TC/AG	GT/CA	Others	Compound	Total	Percent
Currently available	36	3	5	19	92	100%
No PCR product	3	0	1	1	5	5%
Non-reliable product	4	0	0	3	14	15%
Non-informative	7	2	1	6	25	27%
Typed	22	1	3	9	48	52%

3.6. Avocado linkage map

The genotypes of the two parents ('Pinkerton' and 'Ettinger') and 50 offspring were determined using 50 SSRs, 17 RAPDs and 23 DFP bands [4]. All 90 loci were used for two point linkage analysis using the following programs: CRI-MAP, JoinMap, LINKAGE, LINKEM and MAPMAKER. The two-point analysis of LINKAGE and CRI-MAP programs resulted in identical LOD scores and *theta* values and the two-point analysis of LINKEM and MAPMAKER resulted in identical LOD scores and *theta* values. The last two programs gave higher values of LOD score (about 10% higher compared to the results obtained by LINKAGE and CRI-MAP). The two point analysis of JoinMap and MAPMAKER resulted in similar LOD (Log of the Odds) score values (except for markers showing segregation distortion).

The paternal, maternal and combined maps are presented in Fig. 2. The combined map consists of 12 linkage groups and 34 markers. The number of markers per linkage group varies between two to five and the length of the 12 linkage groups varies from 14.7cM to 68.7cM. A specific region on linkage group 3 varies significantly between the paternal and maternal maps. The maternal distance between locus AVA13 and locus AVA04 is almost twice the parental distance (14.7cM versus 7.6cM). Although, in other linkage groups only minor differences can be detected between paternal and maternal maps, the maternal distances are higher than the paternal ones (21% difference on the average). Analysis of marker distribution on the linkage groups did not reveal any deviation from the Poisson distribution, indicating no significant deviation from randomness. The total map length is 352.6cM.

3.7. Association between avocado fruit traits and DNA markers

Sixty offspring of the cross 'Pinkerton' X 'Ettinger' were analysed to detect associations between 90 DNA markers (50 SSRs, 23 DFP bands and 17 RAPDs) and loci controlling nine avocado fruit traits. Each marker was analysed against each of the nine fruit traits. To avoid a false positive result, association was declared only if P was equal or less than 0.01. The positive associations are presented in Table VII. For seven out of the nine traits, positive associations were found for at least one marker.

The location of loci controlling fruit traits was determined by the interval mapping procedure while each trait was analysed against each linkage group and the results are shown in Table VIII. In three cases, high LOD score levels (>2.0) were identified. A locus controlling skin gloss was found to be in association with linkage group 6, which is composed of two

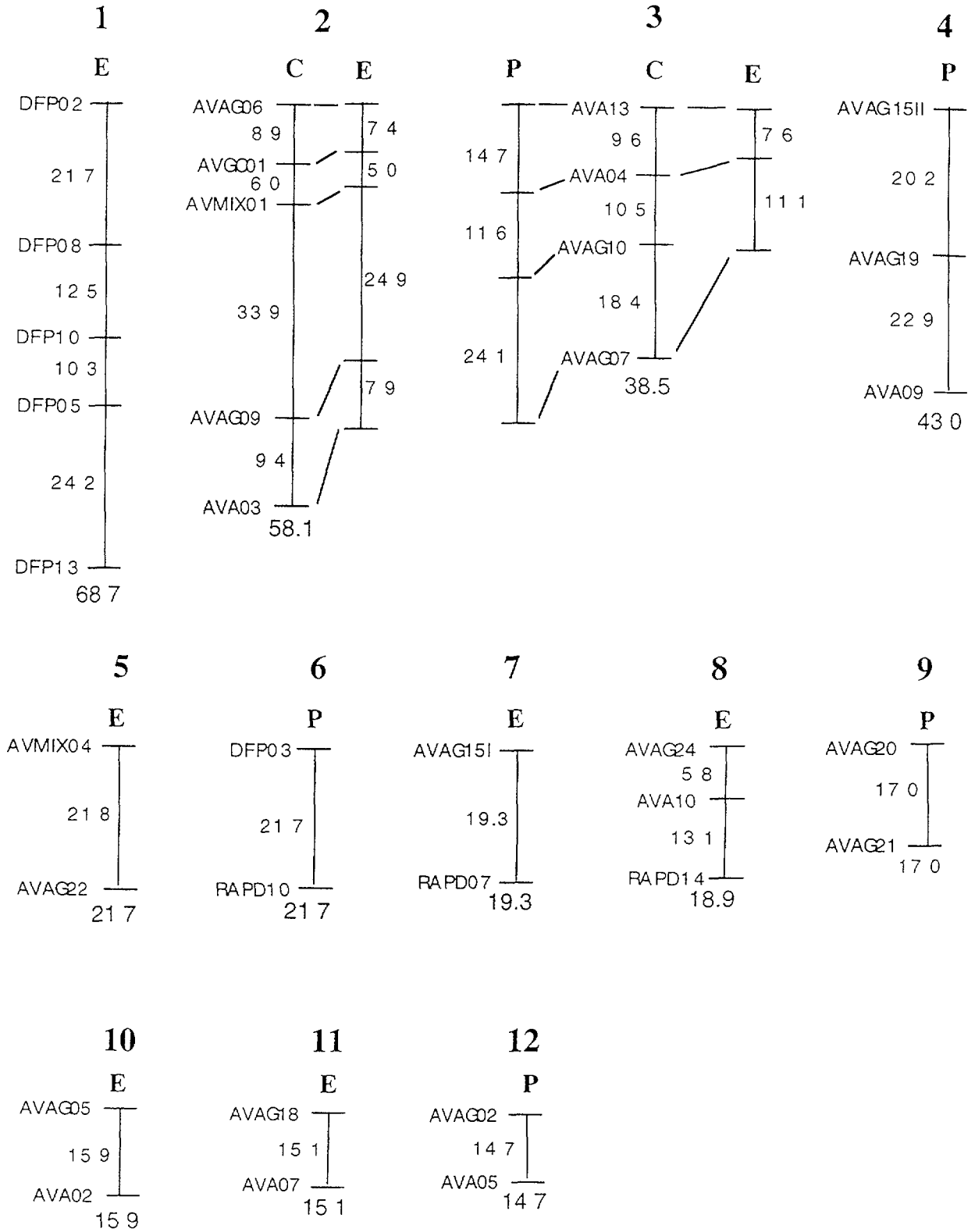


FIG 2 Avocado genetic linkage groups E- 'Ettinger' map, P- 'Pinkerton' map, C- Combined map Distances between markers are in CentiMorgan

TABLE VII. RESULTS OF ONE-WAY ANALYSIS OF VARIANCE OF AVOCADO FRUIT TRAITS

Trait	Marker	Marker's location	Polymorphism source	P value
Skin gloss	AVAG11	Un-mapped	'Ettinger'	0.0014
	AVAG13	Un-mapped	'Ettinger'	0.01
	AVA08	Un-mapped	'Ettinger'	0.0014
	DFP03	Group 6	'Pinkerton'	0.005
	RAPD10	Group 6	'Pinkerton'	0.004
Skin surface	AVAG15II	Group 4	'Pinkerton'	0.004
	AVAG23	Un-mapped	'Pinkerton'	0.009
	AVA07	Group 11	'Ettinger'	0.01
Skin thickness	AVAG14	Un-mapped	'Ettinger'	0.009
Seed size	AVAG24	Group 8	combined	0.0006
	AVAG12	Un-mapped	'Pinkerton'	0.007
Skin peeling	AVAG01	Un-mapped	'Ettinger'	0.006
	AVAC01	Un-mapped	'Ettinger'	0.003
	AVMIX06	Un-mapped	'Ettinger'	0.005
Fibers in the flesh	AVAG07	Group 3	combined	0.007
	AVA04	Group 3	combined	0.00001
	AVA13	Group 3	combined	0.01
Flesh taste	AVMIX04	Group 5	'Ettinger'	0.01
	DFP05	Group 1	'Ettinger'	0.008

markers, each having two alleles in 'Pinkerton'. The effect of this association on the progeny skin surface explains 25% of the trait variance. A locus controlling skin surface was found in association with linkage group 9, which is composed of two SSR markers each having two alleles in 'Pinkerton'. The effect of the locus on the skin surface progeny average explains 38% of the trait variance.

3.8. A locus(i) controlling fibers in the flesh

A locus controlling fibers in the flesh was found to be located on linkage group 3. This linkage group is composed of four SSR markers, and locus(i) controlling fibers in the flesh was

TABLE VIII. RESULTS OF THE INTERVAL MAPPING ANALYSIS TO DETECT LINKAGE BETWEEN LOCI CONTROLLING FRUIT TRAITS AND MARKER LOCI

Trait	Linkage group	LOD score value	Detected map	Effect (in SD values)	% variance
Skin gloss	6	2.9	'Pinkerton'	1.17	25
Skin surface	4	1.7	'Pinkerton'	-0.78	16
	9	2.6	'Pinkerton'	-1.20	38
	11	1.7	'Ettinger'	-0.81	17
Fibers in the flesh	3	5.4	Combined	0.62	37.5
Flesh taste	5	1.5	'Ettinger'	-0.81	16

located close to AVA04, having a LOD score value of 5.4. The locus effect on the progeny average explains 37.5% of the trait variance.

Fig. 3a presents the parental phases of the marker alleles on linkage group 3 and the parental haplotypes marked as P1 and P2 for 'Pinkerton' and E1 and E2 for 'Ettinger'. The offspring are thus divided to various haplotype combinations where the four parental groups are the majority. Fig. 3b presents contrast analysis between the alleles of the markers located on the haplotype combination E1+P1 and the alleles of the other haplotype combinations. The association between the locus(i) controlling fibers in the flesh and the alleles of each marker on E1+P1 haplotype is significant (except for the marker AVAG10). This result indicates that a locus controlling fibers in the flesh is located within this linkage group close to the marker AVA04 and having an extremely high significance level ($P=4.4x10^{-8}$). Furthermore, this locus controls high level of fibers in the flesh only when specific allele combinations are present (interaction between alleles). Namely, only when P1 alleles are present in the genome in combination with E1 alleles is the level of fibers in the flesh significantly high. The association between the locus(i) controlling fibers in the flesh and the marker AVAG10 is not significant because only 'Pinkerton' is a heterozygote for this marker and thus using this marker one can not distinguish between offspring having E1 haplotype and offspring having E2 haplotypes.

4. DISCUSSION

4.1. Generation and polymorphism of SSR in avocado

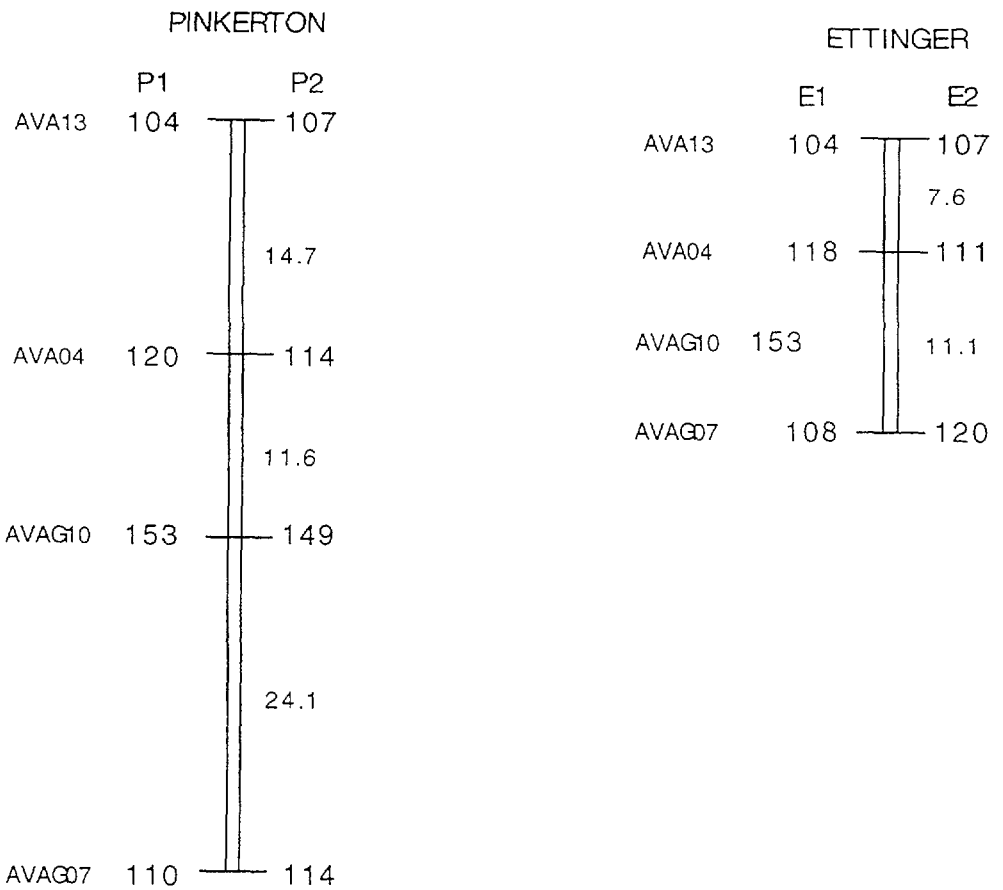
While in the human genome [5], $(GT/CA)_n$ repeats are the most frequent, the $(TC/AG)_n$ and $(AT/TA)_n$ repeats seem to be the most frequent in plants genomes, while in avocado we found that the two most frequent repeats are $(A/T)_n$ and $(TC/AG)_n$. The high number of predicted microsatellites in the avocado genome (~45,200, Table I) indicates that an SSR based map with high resolution can be obtained.

RFLP and isozyme markers show low heterozygosity levels in avocado compared to the results of this study. This is due to the high level of polymorphism in VNTR loci compared with RFLP and isozymes. For breeding purposes, a high degree of heterozygosity among cultivars can be an advantage, providing a high degree of variation among seedlings that might be exploited by selection. On the other hand, this situation limits the ability of a breeder to predict offspring performance.

4.2. Mapping the avocado genome

Of the 90 SSR, RAPD and DFP markers that were analyzed, 34 were mapped in linkage groups and 56 markers remain un-linked. The proportion of un-linked loci (62%) is higher than in other maps. In soybean, 40 SSRs were integrated into a known map using 60 F_2 plants (15). When only the 40 SSRs were analyzed, 18 of them (45%) remained un-linked. Poisson distribution analysis, indicated that the SSR markers are randomly dispersed in the avocado genome. Marker distribution analyses in other species has shown similar results (15). Linkage was found between a RAPD marker and a DFP band (linkage group 6) but not between SSR markers and DFP bands. The lack of linkage between SSR and DFP markers could be explained based on the finding that DFP loci are not randomly distributed but rather clustered in centromeric and telomeric regions. The differences in recombination frequencies between the paternal and maternal genomes are in agreement with [16]. They detected differences in

a



b

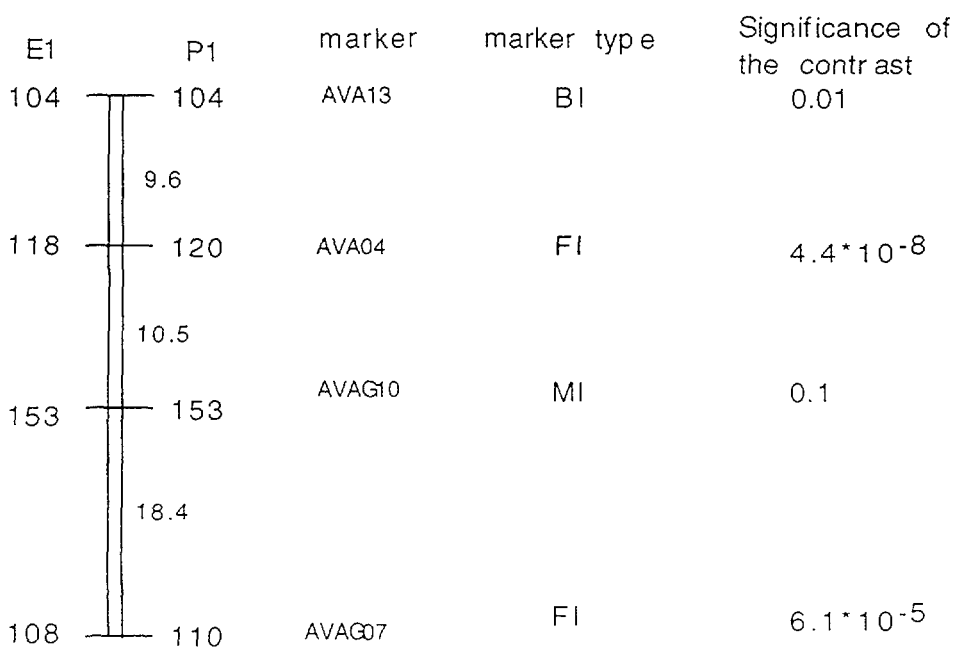


FIG.3. a. Parental haplotypes of linkage group 3.b. Contrast analysis between SSR markers on linkage group 3 and fibers in the flesh.

recombination frequency in tomato using the same cultivar as either the female or the male parent.

None of the computer mapping programs available to date can efficiently map the genome of an out-crossing species using polymorphic markers. The mapping approach which we used to map the avocado genome consists of transforming the avocado F1 mapping data to BC1 data which meet the MAPMAKER requirements. The result of this analysis was verified using other computer programs and found to be reliable. The loblolly pine genome, which is another out-crossing species, was mapped using the JoinMap computer [17]. This computer program is capable of analysing this data set, except for markers with segregation which deviates from Mendelian expectations.

4.3. Avocado fruit traits linked to DNA markers

The detection of association between genetic markers and QTLs is complicated in families resulting from a cross between heterozygote parents [18]. The main problems includes type I error in which a QTL is falsely detected as linked to a marker, and type II error in which no linkage is detected even though such linkage does exist [19]. Type I error is caused, in most of the cases, by using a high number of loci and a high number of traits and a type II error results from the demand of only high LOD score and P values. Thus QTLs which are far from the marker or have a small effect on the trait would not be detected. In the present study, 90 markers and nine traits (total of 810 pairwise comparisons), were detected. A significant value of 6.2×10^{-5} should be used to avoid type I error. This value should be corrected due to the existence of 12 linkage group having 34 linked markers (612 pairwise comparisons) resulting in a significant value of 8.2×10^{-5}. However, this high P value can cause a type II error and thus association between loci controlling fruit traits and DNA markers having a P value of P=0.01 or less were presented. The only association in which this P value was less than 8.2×10^{-5} is the one between linkage group 3 and the locus controlling fibers in the flesh on which we focused for further studies.

To verify the results, we used two statistical approaches: one-way analysis of variance and interval mapping. In most cases when association was found in the one-way analysis of variance it was also detected by the interval mapping except for two cases: the association between the marker AVAG24 and locus controlling seed size, and between the DFP marker DFP05 and locus controlling flesh taste. In these two cases there were only two alleles of the markers in the family, and the LOD score values of the interval mapping analysis were between 0.5 and 1. In one case, skin surface on linkage group 9, an association was detected by the interval mapping but could not be detected in the one-way analysis of variance. Although, a significant P value ($P<0.05$) could be detected, we did not consider this value as an indication for association.

It is noteworthy that no association was detected between fruit weight and any of the marker loci using both statistical approaches (P values of 0.02 and 0.03 were detected with two markers). On the other hand, there was a significant difference in the mean fruit weight between the offspring and the parents. This negative result can be explained by the finding that the trait is highly affected by the environment and heritability is quite low (20). Mhameed *et al.* [4] reported that no linkage was found between DFP bands and fruit weight even for ($0.01<P<0.05$) in this family. This result is compatible with the results obtained in this work and marked the difficulty in detecting markers which are linked to a locus controlling fruit weight.

In the case of the existence of fibers in the flesh, the offspring average is slightly higher than the parents performance, suggesting that a dominant component is involved in the determination of the trait. The Contrast Analysis suggested that three out of the four genotype groups of the marker AVA04, showed a low level of fibers in the flesh while the fourth group, having haplotypes E1 and P1, showed a high level of fibers in the flesh. This result can be explained by a negative dominant factor which is located on the haplotypes E2 and P2 and thus the fibers level of the offspring having one of those haplotypes is low compared with the offspring that lack these haplotypes.

The results presented in this study emphasize the importance of using highly polymorphic markers especially for map construction and detection of linkage with QTLs. The SSR markers which were used in this study, allow better detection of association with QTL compared with RFLP, RAPD or DFP markers.

REFERENCES

[1] DEGANI, C., GOLDRING, A., ADATO, I., EL-BATSRI, R., and GAZIT, S. Pollen parent effect on outcrossing rate, yield, and fruit characteristics of 'Fuerte' avocado, HortScience **25** (1990) 471-473.

[2] FURNIER, G.H., CUMMINGS, M.P., and CLEGG, M.T. Evolution of the avocados as revealed by DNA restriction site variation, J. Hered. **81** (1990)183-188.

[3] LAVI, U., HILLEL, J., VAINSTEIN, A., LAHAV, E., and SHARON, D. Application of DNA fingerprints for identification and analysis of avocado, J. Amer. Soc. Hort. Sci. **116** (1991) 1078-1081.

[4] MHAMEED, S., HILLEL, J., LAHAV, E., SHARON, D., and LAVI, U. Genetic association between DNA fingerprint fragments and loci controlling agriculturally important traits in avocado (*Persea americana* Mill.), Euphytica **81** (1995) 81-87.

[5] HUDSON, T.J., STEIN, L.D., GERETY, S.S., MA, J., CASTLE, A. B., SILVA, J., SLONIM, D.K., BAPTISTA, R., KRUGLYAK, L., XU, S.H., HU, X., COLBERT, A.M.E., ROSENBERG, C., REEVE-DALY, M.P., ROZEN, S., HUI, L., WU, X., VESTERGAARD, C., WILSON, K.M., BAE, J.S., MARITA, S., GANIATSAS, S., EVANS, C.A., DEANGELIS, M.M., INGALLS, K.A., NAHF, R.W., HORTON, L.T., ANDERSON, M.O., COLLYMORE, A.J., YE, W., KOUYOUMJIAN, V., ZEMSTEVA, I.S., TAM, J., DEVINE, R., COURTNEY, D.F., RENAUD, M.T., NGUYEN, H., O'CONNOR, T.J., FIZAMES, C., FAURE, S., GYAPAY, G., DIB, C., MORISSETTE, J., ORLIN, J.B., BIRREN, B.W., GOODMAN, N., WEISSENBACH, J., HAWKINS, T.L., FOOTE, S., PAGE, D.C., and LANDER, E.S. An STS-based map of the human genome, Science **270** (1995) 1945-1954.

[6] DIETRICH, W., KATS, H., LINCOLN, S.E., SHIN, H.S., FRIEDMAN, J., DRACOPOLI, N.C., and LANDER, E.S. A genetic map of the mouse suitable for typing intraspecific crosses, Genetics **131** (1992) 423-447.

[7] KIJAS, J.M.H., FOWLER, J.C.S., and THOMAS, M.R. An evaluation of sequence tagged microsatellite site markers for genetic analysis within *Citrus* and related species, Genome **38** (1995) 349-355.

[8] THOMAS, M.R. and SCOTT, N.S. Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs), Theor. Appl. Genet. **86** (1993) 985-990.

[9] LAVI, U., AKKAYA, M., BHAGWAT, A., LAHAV, E., and CREGAN, P.B. Methodology of generation and characteristics of simple sequence repeat DNA markers in avocado (*Persea americana* M.), Euphytica **80** (1994) 171-177.

[10] MURRAY, M.G., and THOMPSON, W.F. Rapid isolation of high molecular weight plant DNA, Nucleic Acids Res. **8** (1980) 4321-4325.
[11] RONGWEN, J., AKKAYA, M.S., BHAGWAT, A.A., LAVI, U., and CREGAN, P.B. The use of microsatellite DNA markers for soybean genotype identification, Theor. Appl. Genet. **90** (1995) 43-48.
[12] LANDER, E.S., GREEN, P., ABRAHAMSON, J., BARLOW,. A., DALEY, M., LINCOLN, S., and NEWBURG, L. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations, Genomics 1 (1987) 174-181.
[13] STAM, P., and VAN OOIJEN, J.W. JoinMap™ version 2.0: Software for the calculation of genetic linkage maps, CPRO-DLO, Wageningen, (1995).
[14] VAN OOIJEN, J.W., and MOLIEPAARDM, C. MapQTL ™ version 3.0: Software for the calculation of QTL positions on genetic maps, CPRO-DLO, Wageningen (1996).
[15] AKKAYA, M.S., SHOEMAKER, R.C., SPECHT, J.E., BHAGWAT, A.A., and CREGAN, P.B. Integration of simple sequence repeat DNA markers into a soybean linkage map, Crop Sci. **35** (1995) 1439-1445.
[16] DE-VICENTE, M.C., and TANKSLEY, S.D. Genome-wide reduction in recombination of backcross progeny derived from male versus female gametes in an interspecific cross of tomato, Theor. Appl. Genet. **83** (1991) 173-178.
[17] GROOVER, A., DEVEY, M., FIDDLER, T., LEE, J., MEGRAW, R., MITCHEL-OLDS, T., SHERMAN, B., VUJCIC, S., WILLIAMS, C., and NEALE, D. Identification of quantitative trait loci influencing wood specific gravity in an outbred pedigree of loblolly pine, Genetics **138** (1994)1293-1300.
[18] LEONARDS-SCHIPPERS, C., GIEFFERS, W., SCHAFER-PREGL, R., RITTER, E., KNAPP, S.J., SALAMINI, F., and GEBHARDT, C. Quantitative resistance to *Phytophthora infestans* in potato: A case study for QTL mapping in an allogamous plant species, Genetics **137** (1994) 67-77.
[19] JANSEN, R.C. Controlling the type I and type II errors in mapping quantitative trait loci, Genetics **138** (1994) 871-881.
[20] LAVI, U., LAHAV, E., GENIZI, A., DEGANI, C., GAZIT, S., and HILLEL, J. Quantitative genetic analysis of traits in avocado cultivars, Plant Breeding **106** (1991) 149-160.

DNA FINGERPRINTING OF SUGARCANE FOR DETECTING MOLECULAR EVIDENCE OF SOMACLONAL VARIATION

M. RAMOS LEAL, E. CANALES, R. MICHEL, O. COTO, M.T. CORNIDE
National Center for Scientific Research,
Havana, Cuba

XA9848411

Abstract

Molecular characterisation of eight sugarcane mutants which tolerate 1% NaCl, was performed by means of restriction fragment length polymorphism (RFLP) analysis at the genomic level, using a group of molecular probes for ribosomal DNAs and two others, Δ-pyrroline-5-carboxylate reductase (ΔP5CR) and ATPase, probably related to salinity stress tolerance. Results showed the molecular evidence of genetic changes in comparison to the susceptible donor variety and the possible presence of more than one mechanism to tolerate salt stress.

1. INTRODUCTION

Plants vary in their ability to withstand and survive in saline environments. The possibility of using somaclonal variation for obtaining new tolerant genotypes (via in vitro selection) has offered a new approach to focus on this problem. Sugarcane is the most important crop in Cuba. At the same time, very important areas are affected by high levels of salinity in soil.

A group of salt tolerant mutants has been obtained, derived from a donor variety highly susceptible to salt stress [1]. Agromorphological, physiological and biochemical analyses have confirmed the results obtained in field trials concerning the high level of salt tolerance of these new genotypes. Nevertheless, taking into account the discrepancies about the existence of somaclonal variation, during the development of this contract (1995-1997), our first approach was to evidence molecular changes induced by somaclonal variation. We have detected the possible role of several genes, probably involved in the resistance response to osmotolerance (for review, see [2]), by means of restriction fragment length polymorphism (RFLP) analysis and established several genetic inferences.

2. MATERIALS AND METHODS

2.1. Plant material

A group of eight salt tolerant mutants (Table I) was obtained and analyzed by RFLP patterns. The highly susceptible donor plant (C 87-51) was used as standard control.

TABLE I. GENOTYPES EMPLOYED FOR THE MOLECULAR CHARACTERIZATION

Highly susceptible donor variety	Salt tolerant mutants			
C 87-51	CC 03-84	CC 54-84	CC 59-84	CC 70-84
	CC 19-84	CC 56-84	CC 07-84	CC 74-84

2.2. Genomic DNA analysis

Genomic DNA was isolated using the procedure of Doyle and Doyle [3]. The genomic DNA was digested with the enzymes *Eco*RI, *Eco*RV, *Bam*HI and *Hind*III, AND electrophoresed according to Sambrook *et al.* [4]. The DNAs were transferred to positively-charged nylon membranes (Amersham), according to Southern [5].

2.3. Hybridization

As heterologous probes, we used the maize ribosomal DNA probes (rDNA GL-1 and rDNA GL-26) kindly supplied by G. Beaulieu (Univ. Washington); Δ-pyrroline-5-carboxylate reductase (ΔP5CR) and ATPase, kindly supplied by R. Slocum and C. Leaving, respectively. DNA probes were labelled with alpha-^{32}P-dATP by random primer. After hybridization, the membranes were washed and exposed to X-ray film.

Statistical analysis was performed by means of the factor analysis of correspondences (FAC) (STATITCF version 4.0). For each enzyme-probe combination, the observed polymorphic fragments were transformed into a 1 (present) and 0 (absent) matrix.

3. RESULTS

The autoradiograph of the blotted DNAs exposed to the ribosomal probes visualized several major bands.

3.1. rDNA GL-26

The hybridization of *Hind*III and *Eco*RI-digested DNA detected differences for the somaclonal mutants studied. Various RFLPs are observed, indicating heterogeneity in the salt tolerant mutant population obtained. *Hind*III-digested DNA showed the absence of the lower band in four mutants. *Eco*RI digestion gave three bands, showing the absence of the second uppermost band in four mutants.

3.2. rDNA GL-1

*Hind*III digestion of the sugarcane DNA gave five fragments, identical in length for all individuals, except for genotypes CC 70-84 and CC 74-84. *Bam*HI digests showed clear differences between the susceptible donor variety and the somaclones. The mutants contained several extra fragments in the three uppermost bands, absent in the donor variety. *Eco*RV digestion also showed differences in the two uppermost bands. *Eco*RI digestion gave five fragments and the main difference is signalled by an extra fragment present in almost all new genotypes, but absent in the donor variety.

3.3. ΔP5CR

Concerning the autoradiograph of the blotted *Hind*III-digested DNAs exposed to the ΔP5CR probe, the lanes 3, 5 and 7 have differences in the second band in comparison to the donor variety and also the lanes 3 and 7 regarding the band 5. *Bam*HI digestion presented a few differences between the donor susceptible variety and three somaclones. *Eco*RV digestion gave four fragments. Bands 3 and 4 were absent in the genotypes: CC 07-84, CC 70-84 and CC 74-84. *Eco*RI digestion gave three fragments identical in length for all nine individuals.

3.4. ATPase

*Hind*III digestion of the sugarcane DNA gave four fragments identical in length for all individuals, except for genotype CC 19-84. *Bam*HI digests showed differences between the donor variety and the somaclones CC 03-84, CC 19-84, CC 59-84 and CC 70-84, due to the absence of two bands. *Eco*RV digestion also showed absence of bands in three mutants. *Eco*RI digestion gave three fragments and the main difference is signalled by absence of the lower fragment.

The factor analysis of correspondences (FAC) provided a location of the mutants in relation to the susceptible donor variety (Fig. 1). Two groups were observed: one group which contains the mutants CC 19-84, CC 56-84, CC 59-84 and CC 07-84, CC 70-84 and CC 74-84. The other group is formed by the donor variety C 87-51 and the mutants CC 03-84 and CC 54-84. Moreover, the three axes express around 62% of the total variability.

4. DISCUSSION

The use of rDNA probes evidenced the presence of genetic changes induced via somaclonal variation, which can be useful for salinity stress tolerance. Proline accumulation is one of the most important osmoprotectants in response to salt stress. Taking into account that ΔP5CR is the last enzyme in proline synthesis [2], differences in the ΔP5CR patterns among the mutants and the donor variety confirmed alterations in the proline synthesis response [1]. Plasma membrane ATPase activity has shown changes in response to salt stress. The ATPase patterns could suggest the possible role of the transport-associated function to salinity stress.

Our next step will be to finish with at least three additional specific probes, probably closely related to salinity stress (osmotin, heat shock gene and betaine aldehyde dehydrogenase) and to establish inferences related to genetic distances among the mutants and the susceptible donor variety.

A new approach for this contract will be to produce progeny, combining one of the salt tolerant mutants with the donor variety. Mutants can be considered as near isogenic lines (NILs), in relation to the donor susceptible variety. In this sense, study can be directed toward the evaluation via bulked segregation analysis (BSA).

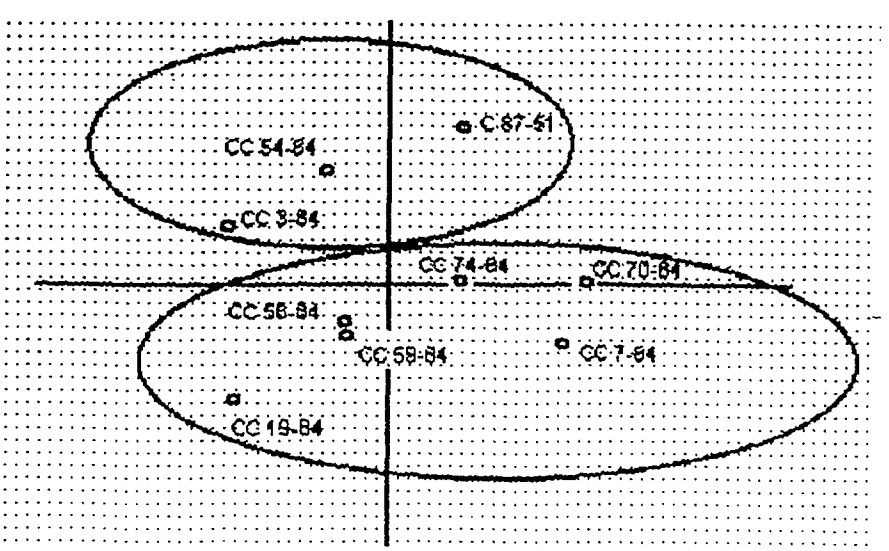


FIG. 1. Distribution of sugarcane mutants and the donor variety in planes (1,2) of a FAC using RFLP data.

ACKNOWLEDGEMENTS

This work was supported in part by an IAEA Contract No. 8137/RB to MRL. The authors wish to thank Drs. G. Beaulieu, Univ. of Washington; R. Slocum, Univ. of Massachusetts and C. Leaving at North Carolina State Univ., for kindly supplying the molecular probes used in this work.

REFERENCES

[1] DIAZ, P., MARIBONA, R.H., CORNIDE, M.T., COTO, O., MICHEL, R., and LEONARD, H. Obtainment of salt tolerant somaclones of sugarcane, Preliminary report to the Acad. Sci. of Cuba (1996).

[2] WINICOV, I. "Gene expression in relation to salt tolerance", Stress-induced gene expression in plants, Chap. 3 (BASRA, A.S., Ed.), Harwood Acad. Pub., Switzerland (1994) 61-85.

[3] DOYLE and DOYLE, J. A plant DNA mini- preparation, Plant Molec. Biol. Reporter **1** (1983) 19-21.

[4] SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).

[5] SOUTHERN, E., Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol. Biol. **98** (1975) 503-509.

[6] HANSON, A.D., MAY, A.M., GRUMET, R., BODE, J., JAMIESON, G.C., and RHODES, D., Betaine synthesis in chenopods: localization in chloroplasts, Proc. Natl. Acad. Sci. **82** (1985) 3678-3682.

THE EXAMINATION OF *HEVEA BRASILIENSIS* PLANTS PRODUCED BY *IN VITRO* CULTURE AND MUTAGENESIS BY DNA FINGERPRINTING TECHNIQUES

F.C. LOW, S. ATAN, H. JAAFAR
Biotechnology and Strategic Research Division,
Rubber Research Institute of Malaysia,
Kuala Lumpur, Malaysia

Abstract

Rubber (*Hevea brasiliensis)* plants derived from anther and ovule culture as well as gamma-irradiated plants were examined by several DNA marker techniques. These include restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), sequence tagged microsatellite sites (STMS), DNA amplification fingerprinting (DAF) and amplified fragment length polymorphisms (AFLPs). Compared to control plants produced by vegetative propagation (cutting and budding), plants produced by *in vitro* culture appeared to have a reduction in the number of rDNA loci. Two RAPD protocols were compared and found to be similar in amplification of the major DNA bands. After confirmation that the RAPD method adopted was reproducible, the technique was applied to the present studies. Eight out of the 60 primers screened were able to elicit polymorphisms between pooled DNA from *in vitro* culture plants. Variations in DNA patterns were observed between pooled DNA samples of anther-derived plants as well as between anther-derived and ovule-derived plants. Comparisons of RAPD patterns obtained between anther-derived plants exposed to increasing dosages of gamma-irradiation with non irradiated anther-derived plants revealed distinct DNA polymorphisms. The changes in DNA profiles did not appear to be correlated to the dosage of irradiation. Since somaclonal variation was detected, it was difficult to identify changes which were specifically caused by irradiation. Application of the STMS technique to tag microsatellite sequences $(GA)_n$, $(TA)_n$ and $(TTA)_n$ in the hydroxymethylglutaryl coenzyme A reductase-1 (hmgr-1) gene failed to detect differences between plants derived from anther and ovule culture. Although restriction endonuclease digestions with methylation sensitive enzymes suggested that four *in vitro* culture plants examined exhibited similar digestion patterns as the controls, a change in cytosine methylation in one anther-derived plant was detected. Examination of individual *in vitro* culture plants by the DAF technique revealed genetic heterogeneity among these plants. Differences in DNA profiles between anther-derived and ovule-derived plants were also detected. In general, more somaclonal variations were detected in anther-derived than ovule-derived plants. When the DAF technique was applied to DNA obtained from leaves of irradiated budded stumps, DNA profiles were shown to be different from non irradiated controls. Whilst DNA patterns of samples irradiated at the same dosage displayed similar DNA patterns, these varied with increasing dosage. Examination of *in vitro* culture plants by AFLPs confirmed earlier results that somaclonal variations were present in *Hevea.*

1. INTRODUCTION

Hevea brasiliensis Muell Arg., a member of the *Euphorbiaceae* family, is an important economic crop of Southeast Asia. *H. brasiliensis* is traditionally cultivated for the production of natural rubber, but more recently, it has emerged as a valuable renewable source of timber. Because planting of unselected seedlings yields uneconomic plantations, all commercial cultivation is carried out through vegetative propagation, that is by budding a desirable trunk clone to a seedling root stock. For further enhancement of productivity, a further budding of a desirable crown (canopy) clone to the trunk is often practised, resulting in a three-part tree [1]. Budding however, is a skilled and labour intensive exercise. *In vitro* culture was envisaged as an alternative method for mass propagation to circumvent the requirement for skilled labour as well as an avenue for *Hevea* crop improvement. Tissue culture of *Hevea* was initiated nearly four decades ago [2,4]. Although early success was limited [5], plants produced from *in vitro*

culture have been established in field trials in China and Malaysia. As far as we are aware, these *in vitro* culture plants have not been examined at the DNA level.

The present study was undertaken to determine whether *Hevea* plants produced by *in vitro* culture were true to type (i.e., genetically identical) *via* DNA fingerprinting techniques. At the same time, DNA fingerprinting would also be employed as an early selection criterion for improved characteristics after mutagenesis through gamma-irradiation. Several DNA fingerprinting techniques were utilized. These included a hybridization-based technique namely restriction fragment length polymorphisms (RFLPs) [6] and polymerase chain reaction (PCR)-based techniques such as random amplified polymorphic DNA (RAPD) [7-8], sequence tagged microsatellite sites (STMS) [9-10], DNA amplification fingerprinting (DAF) [11] and amplification fragment length polymorphism (AFLP) [12].

2. MATERIALS AND METHODS

2.1. Plant materials

A random selection of 59 *in vitro* culture plants which were derived from both anther and ovule culture of *H. brasiliensis* clone GL1 were examined. These plants were 36 months old at the initiation of this investigation and were already field planted at the Rubber Research Institute of Malaysia Experiment Station at Sungei Buloh. Plants of the same clone, but propagated vegetatively by cutting and bud-grafting, acted as controls.

2.2. Production of mutagenic plants by gamma-irradiation

Anthers of *H. brasiliensis* clone RRIM 600 were gamma-irradiated at 1-4 gray at the therapy unit of the Malaysian Institute for Nuclear Technology (MINT). The irradiated anthers were then cultured *in vitro* for plantlet production [13]. Leaves from two irradiated (anther-derived) plants at each dosage were sampled and their DNA fingerprints were compared with non-irradiated *in vitro* culture plants as controls.

Similarly, budded stumps of *H. brasiliensis* clone RRIM 905 were gamma-irradiated at 5-25 Gy at MINT as described [14].The irradiated budded stumps were then planted out in the field. Leaves which developed subsequently were harvested and used for DNA fingerprinting studies. DNA profiles from bifoliate leaves obtained from six RRIM 905 gamma-irradiated budded stumps were compared with DNA from bifoliate leaves from two non-irradiated budded stumps of the same clone as well as regular trifoliate leaves from untreated controls.

2.3. DNA and RFLP methods

DNA samples were isolated by the procedure of Low *et al* [15], with adaptations from Institut fur Genbiologische Forschung (IGF), Berlin. Quality of the isolated DNA was ascertained by analysis in agarose gel electrophoresis as well as its susceptibility to digestion by *Eco*RI. DNA for RFLP analysis was digested overnight at 37°C with various restriction enzymes according to the manufacturer's instructions. Restriction enzyme digestion was carried out at a concentration of 10 units of restriction enzyme per μg of DNA, in the presence of 5mM spermidine and 100 μg/ml casein. Other RFLP methods were similar to those described earlier [16,17].

Simple sequence oligonucleotides were generated by concatemerisation of the basic repeating unit to sequences of several kilobases by PCR and used as probes for RFLP analysis [18]. The clone pTa71 which contains a 9 kb *Eco*RI fragment of ribosomal DNA (rDNA) from wheat, *Triticum aestivum* [19] was recloned into pUC19 and was a generous gift from Flavell and O'Dell (John Innes Centre, Norwich, UK). This was used as a hybridization probe on digested DNA of *in vitro* culture plants.

2.4. PCR-based fingerprinting techniques

All PCR-based marker techniques, except AFLP, were carried out as described [17]. Examination of genomic DNA from *in vitro* culture and control plants by the RAPD technique was carried out with two different protocols [7,20]. All amplifications were preceded by "hot start" and executed with random hexamer primers (Operon Technologies, USA). Amplification products were analyzed after electrophoresis in 2% agarose gels in 1xTBE buffer as well as in 10% denaturing polyacrylamide gels containing 1.6M urea in 1xTBE followed by silver nitrate staining.

Amplification of microsatellite regions by STS was carried out according to Low *et al* [21] with primers flanking the regions of interest [17].

The DAF technique was carried out according to the procedure of Caetano-Anolles *et al* [11] and random hexamer primers, in various combinations of primer-pairs were tested for their ability to elicit DNA polymorphism as described [17].

2.5. AFLP analysis

Hevea DNA samples were digested with *Eco*RI and *Mse*I for AFLP analysis which was carried out by the radioactive γ -^{33}P-ATP procedure [18] using an AFLP Analysis System 1 kit (Gibco BRL, USA).

3. RESULTS

3.1. Production of mutagenic plants from gamma-irradiated anthers

A total of 2281 anthers were irradiated with gamma-rays at between 1-4 Gy. *In vitro* culture of the irradiated anthers yielded 573 embryoids, representing 47% embryogenesis. Twenty-seven plantlets were produced, with eight plants being planted in the field. DNA was isolated from leaves of these plants for DNA fingerprinting analysis.

3.2. Polymorphisms between gamma-irradiated and non irradiated budded stumps

Normal *Hevea* plants have trifoliate leaves. Occasionally, transient emergence of bifoliate leaves could be observed without treatment of the plant. These bifoliate leaves would revert to their regular trifoliate form after a time, without treatment. The reason for this transient expression is unclear and remains a topic of interest.

DAF profiles of normal, non-irradiated plants which had trifoliate leaves were shown to differ from those rarer, non-irradiated plants which had bifoliate leaves (Fig. 1). Similarly, bifoliate leaves derived from gamma-irradiated budded stumps displayed a different DNA fingerprint from those obtained from bifoliate leaves of control, non-irradiated plants. Though

DNA profiles varied with increasing dosage of irradiation, they were essentially similar, at the same dosage (5 Gy)

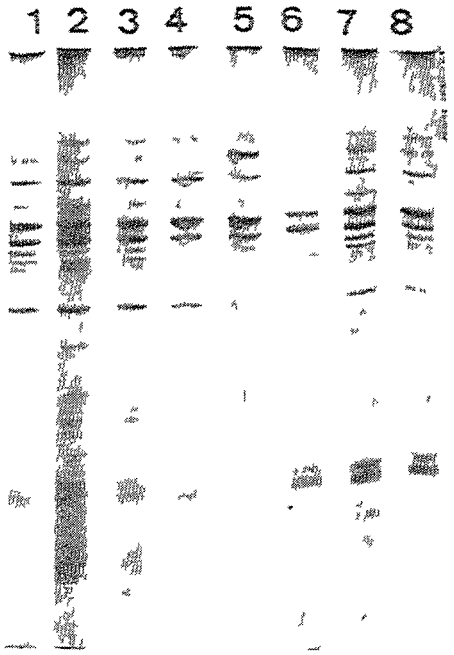


FIG 1 DNA amplification fingerprinting (DAF) profile of budded stumps after exposure to increasing dosages of gamma-irradiation 1 In vitro anther-derived plant EAC 1/7 (non-irradiated in vitro control), 2 Bifoliate leaves from gamma-irradiated budded stump (5 Gy), 3 Bifoliate leaves from gamma-irradiated budded stump (5 Gy), 4 Bifoliate leaves from gamma-irradiated budded stump (10 Gy), 5 Bifoliate leaves from gamma-irradiated budded stump (15 Gy), 6 Bifoliate leaves from gamma-irradiated budded stump (25 Gy), 7 Bifoliate leaves (non irradiated control), and 8 Trifoliate leaves (non irradiated control)

3 3 Methylation and reduction in rDNA copy number

DNA obtained from *in vitro* culture plants was digested separately with various restriction enzymes such as *Alu*I, *Hae*III, *Taq*I, *Hpa*II, *Mbo*I and *Msp*I as well as in combinations such as *Alu*I+*Hae*III Hybridization with a rDNA probe, pTa71, revealed that DNA from *in vitro* culture plants was digested more extensively with *Alu*I than DNA from control plants (Fig 2) Similar results were obtained when pTa71 was hybridized with DNA digested with *Hae*III as well as *Alu*I+*Hae*III in combination No differences in hybridization results were observed when pTa71 was probed with *Hpa*II and *Msp*I digested DNA from regenerated plants (data not shown)

Sixteen oligonucleotide probes were synthesized by concatemerisation of the basic di-, tri- and tetramers to several hundred basepairs (bp) in length In order to evaluate their methylation status, some of these SSR oligonucleotide sequences were used as hybridization probes on digested DNA from a small sample of regenerated plants Hybridisation with $(GACA)_n$ on DNA digested with *Taq*I produced similar discreet bands with DNA from control and *in vitro* culture plants However, one of the *in vitro* culture plants (EAC 4/91) appeared to have a more distinct fragment of approximately 2 kb than the others (*Fig. 3).* Hybridization with the same probe on DNA digested with *Hpa*II and *Mbo*I, on the other hand, elicited a polymorphism between *in vitro* culture plant EAC 4/91 and the other samples examined (*Fig. 3*) Whilst hybridization signals were similar between DNA from the control and the four regenerated plants, these were different in the case of the *in vitro* culture plant EAC 4/91 in both enzymic digests

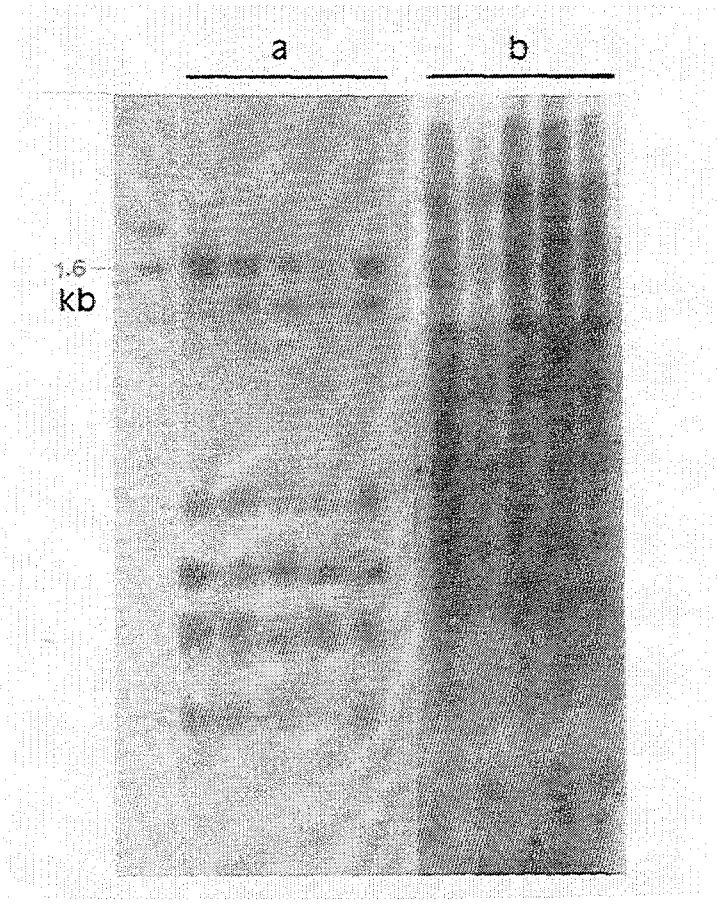


FIG. 2. A reduction in rDNA loci number as revealed by pTa71, after hybridisation with AluI-digested DNA from (a)five in vitro culture and (b) five control plants.

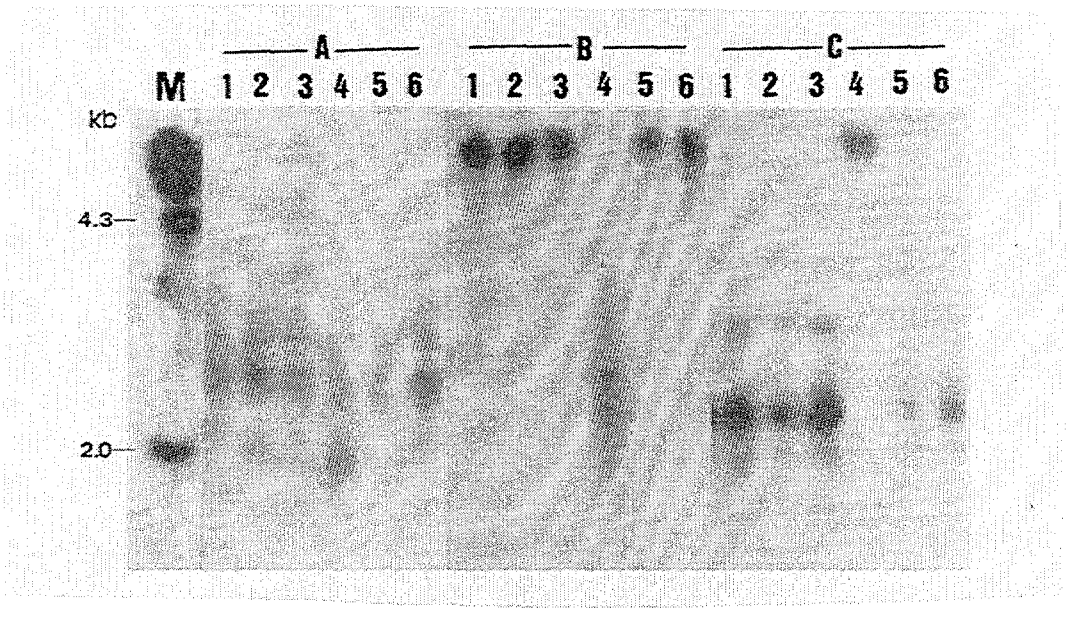


FIG. 3. Polymorphism as revealed by SSR oligonucleotide probe $(GACA)_n$ after hybridisation with DNA digested with (A) TaqI (B) HpaII and © MboI. 1: Control (cutting); 2: In vitro culture plant IVP 1; 3: In vitro culture plant IVP14; 4: In vitro culture plant EAC 4/91; 5: In vitro culture plant EAC 18/90; 6: In vitro culture plant EAC 34/90.

3 4 RAPD polymorphism in tissue culture plants

With the incorporation of "hot start", RAPD results were more reproducible and the number of amplified bands was also reduced Reproducibility of the RAPD technique after the above modifications was demonstrated when five sets of duplicate samples of genomic DNAs, obtained from independent extractions were amplified by the RAPD technique and subjected to polyacrylamide gel electrophoresis (PAGE, Fig 4) Comparison of the two RAPD protocols [7,20] demonstrated that all major bands were similarly amplified However, because of shorter cycling times, the protocol of Koller [20] was adopted in all subsequent RAPD experiments

Eight out of the 60 random primers screened were able to elicit polymorphism between *in vitro* culture plants As a quick screen to detect somaclonal variations, the population of *in vitro* culture plants was divided into groups of nine plants Anther-derived plants were grouped separately from ovule-derived plants DNA isolated from these plants was pooled according to these groups and pooled DNA profiles examined DNA polymorphisms between pooled DNA from anther-derived plants were detected as well as between anther-derived and ovule-derived plants (data not shown).

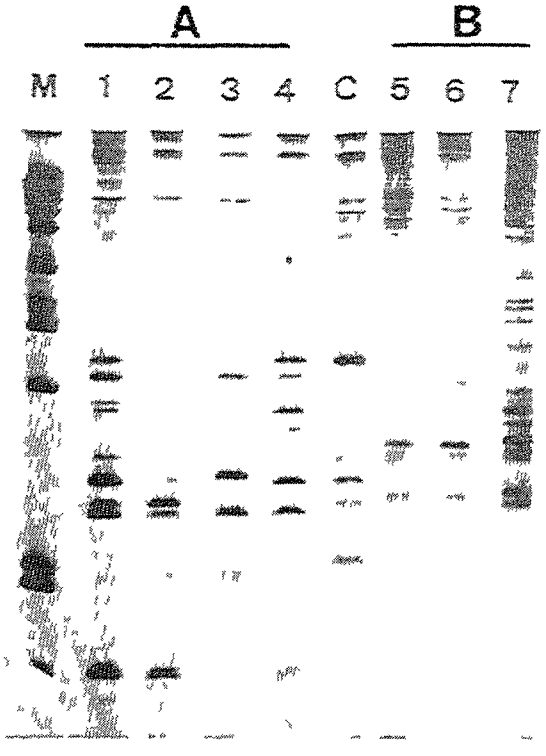


FIG 4 RAPD polymorphisms ((A) Primer Op-F07 and (B) Primer Op-F08) between control and anther-derived plants after exposure to increasing dosages of gamma-irradiation (1-4 gray) 1 γ -irradiated anther-derived plant (1 gray), 2 γ -irradiated anther-derived plant (2 gray), 3 γ -irradiated anther-derived plant (3 gray), 4 γ-irradiated anther-derived plant (4 gray), 5 γ -irradiated anther-derived plant (2 gray), 6 γ -irradiated anther-derived plant (3 gray), 7 γ -irradiated anther-derived plant (4 gray), C Control (non-irradiated anther-derived plant), M Mol wt marker, 1 kb ladder.

Comparison of RAPD patterns obtained after amplification with primer Op-F07 between anther-derived plants exposed to increasing dosages (1-4 gray) of gamma-irradiation with non-irradiated plants from the same source (anthers) revealed distinct DNA polymorphisms (Fig 4). DNA fingerprints between the irradiated materials appeared to vary in number and size of DNA fragments. With increasing dosage of irradiation, large DNA fragments appeared to be replaced by several shorter ones.

3.5. DAF polymorphism in tissue culture plants

Twenty hexamer primers in 190 combinations of primer-pairs were tested for their ability to elicit DNA polymorphisms. Of these, only two were found to be informative. As described above (Section 3.4.), samples of pooled DNA were used. With primer-pair (OpA4/OpA19), the DNA profiles between these groups were nearly indistinguishable. On the other hand, DNA profiles of individual plants were more diverse. Anther-derived plants tended to be more polymorphic than ovule-derived plants.

3.6. STMS within the HMGR-1 gene

Twenty seven combinations of forward and reverse primers were used to tag the microsatellite region $(GA)_8$ within the hydroxymethylglutaryl coenzyme A reductase (hmgr-1) gene in tissue culture plants. Even though many fragments were amplified within this locus, no polymorphism was detected. Separate digestion of these amplified products by *Alu*I and *Hae*III failed to yield any polymorphic fragment. Similarly, no polymorphism was detected at the $(TA)_n$ and $(TTA)_n$ loci.

3.7. AFLP between *in vitro* culture plants

Four *Eco*RI primers, namely, *Eco*RI+AAC, *Eco*RI+ACG, *Eco*RI+ACT, *Eco*RI+AGG and eight *Mse* I primers were used in combination, resulting in 32 primer-pair combinations to detect somaclonal variations among a random sample of ten *in vitro* culture plants. Depending on the primer pair combination used, polymorphic bands were obtained in all the regenerated plants, in comparison to the control (data not shown). Polymorphisms were scored as presence or absence of bands, without consideration for band intensity.

4. DISCUSSION

4.1. Production of mutagenic plants by gamma-irradiation of budded stumps

The differences in DNA profiles between bifoliate and trifoliate normal leaves are not surprising, since these were phenotypically different. However, the differences in DNA profiles between non irradiated and irradiated bifoliate leaves (Fig. 1) are interesting, as these would reflect changes as a consequence of gamma-irradiation. The observation that DNA profiles were similar after exposure to the same dosage of gamma-irradiation (5 Gray), suggested that the observed changes in DNA pattern were consistent and that the DAF technique was reproducible. Although variations in DNA profiles with increasing dosage of gamma-irradiation was demonstrated, there was an absence of a distinct trend in these differences, making it difficult to assign specific DNA fragments to dosage.

4.2. Methylation and reduction in rDNA copy number

The status of DNA methylation in regenerated plants compared to control plants was studied using the high copy number probe pTa71, as well as oligonucleotide probes such as $(GACA)_n$ on DNA which was cut by methylation sensitive enzymes. Comparison of pTa71 hybridization results (Fig. 2), suggested that there was a reduction in the number of rDNA loci in plants produced from cell culture. The reduction in rDNA loci in *Hevea* could be a result of the minimization of gene redundancy, whilst maintaining the gene product [21] or culture-induced [22]. Though reduction in rDNA loci number had been reported in other plants [21-24], the phenomenon is unknown in *Hevea* and may not have adverse effects [25].

Since DNA from *in vitro* culture plant EAC 4/91 was cleaved equally by both *Msp*I and *Hpa*II, in contrast to DNA from the control and other *in vitro* culture plants, this suggested the absence of methylation in cytosine in the recognition sequence, as revealed by probe pTa71. A reduction in DNA methylation in regenerated plants is not unusual and had been reported [27-28].

Polymorphism between *in vitro* culture plant EAC 4/91 and the other samples examined, as revealed by hybridization of $(GACA)_n$ on DNA digested with *Hpa*II and *Mbo*I (Fig. 3) was unrelated to DNA quality, since all samples could be digested equally by *Taq*I. Results after *Mbo*I and *Taq*I digestion suggested the presence of hydroxymethylated cytosine in plant EAC 4/91. Spectroscopic and chemical analysis would however, be needed to confirm the above, but are beyond the scope of the present study. Tissue culture-induced changes in DNA methylation have been reported and were attributed to the wide range of changes that occur after propagation of tissue culture plants [21]. Changes in DNA methylation were reported to be frequent in maize callus, regenerated plants and the progeny of such plants [27]. The extent of DNA methylation could vary with the plant and could also fluctuate with time in the same plant, as seen in carrot root explants [28]. It remains to be determined whether DNA methylation changes in *Hevea* are reversible and would have adverse effects with time.

4.3. RAPD polymorphism in mutagenic and *in vitro* culture plants

The demonstration that all major bands were amplified similarly by the two RAPD protocols tested provided confidence in the reproducibiltiy of the RAPD technique (data not shown). This was further confirmed when 5 sets of duplicate DNA samples produced similar amplification results (data not shown).

Since polymorphism in RAPD fingerprints were detected between irradiated and non irradiated control *in vitro* plants (Fig. 4), the observed changes were probably caused by gamma-irradiation, rather than the culture method. However, because somaclonal variations had been demonstrated in the present study, and these could not be differentiated from mutagenesis, it was difficult to ascertain the origin of these changes. With increasing dosage, large DNA fragments appeared to be replaced by shorter ones, indicating possible cleavage of long DNA by gamma-irradiation.

4.4. DAF polymorphism between *in vitro* culture plants

The lack of polymorphism when pooled samples of DNA from tissue culture plants were compared with control, suggested that either somaclonal variation was absent, or was present at a very low level. However, the diverse DNA profile obtained when DNA profiles of 48 *in vitro* culture plants were compared individually, as well as with control vegetative plants

suggested the presence of somaclonal variations. Since DAF profiles from individual anther-derived plants were more polymorphic than individual ovule-derived plants, it appeared that anthers have a greater propensity for somaclonal variation than ovules. This finding, though new in rubber had been reported in other plants [22-28]. The polymorphic bands detected in *Hevea* appeared to be unusually large in contrast to those reported elsewhere [29-30]. Preliminary examination of DAF polymorphic bands in *Hevea* suggested that the short fragments (less than 1.0 kb) were less polymorphic than the longer ones.

4.5. STMS within the HMGR-1 gene

The lack of STMS polymorphism within the hmgr-1 gene between control and regenerated plants is not unexpected since only a very minute region of a specific gene was examined. Failure to elicit polymorphism after separate digestion by *Alu*I and *Hae*III of the amplified fragments indicated that neither insertion/deletion nor point mutation [31] were present among the tissue culture plants within that gene. Being a latex-enriched [32] key enzyme of rubber biosynthesis, differences in hmgr-1 gene are expected to be less in leaves than in latex.

4.6. AFLP between *in vitro* culture plants

All 10 regenerated plants displayed polymorphism when compared to control, suggesting that somaclonal variations exist in *Hevea* (data not shown). This is in confirmation to results from other fingerprinting techniques discussed above. The frequency of somaclonal variation appeared to be high, as estimated by AFLP (data not shown).

REFERENCES

[1] YOON, P.K. R.R.I.M. Crown budding trials, Planters' Bulletin of the Rubber Research Institute of Malaya **92** (1967) 240-249.

[2] BOUYCHOU, J. G. La culture *in vitro* des tissues d'*Hevea, Proc. Rubb. Conf. Bogor, 1952. Arch. Rubb. Cult. Extra* **2** (1953) 50-53.

[3] MUZIK. T.J. Studies on the development of the embryo and seed of *Hevea brasiliensis* in culture, Lloydia. **19** (1956) 86-91.

[4] CHUA, S.E. Studies on tissue culture of *Hevea brasiliensis*. I. Role of osmotic concentration, carbohydrates and pH values in induction of callus growth in plumule tissues from *Hevea* seedlings, J. Rubb. Res. Inst. Malaya **19** (1966) 272-276.

[5] PARANJOTHY, K. and GHANDIMATHI, H. Tissue and organ culture of *Hevea*. Proc. Int. Rubb. Conf. 1975 Kuala Lumpur **2** (1976) 59-84.

[6] BECKMANN, J.S. and SOLLER, M. Restriction fragment length polymorphisms in genetic improvement:Methodologies, mapping and costs, Theor. Appl. Genet. **67** (1983) 35-43.

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

[8] WELSH, J. and McCLELLAND, M. Fingerprinting genomes using PCR with arbitrary primers, Nucleic Acids Res. **18** (1990) 7213-7218.

[9] EPPLEN, J.T. On simple GATA/GACA sequences: a critical appraisal, J. Hered **79** (1988) 409-417.

[10] TAUTZ, D. Hypervariability of simple sequences as a general source for polymorphic DNA markers, Nucleic Acid Res. **17** (1989) 6463-6471.

[11] CAETANO-ANOLLES, G., BASSAM, B.J. and GRESSHOFF, P.M. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers, Biotechnology **9** (1991) 553-556.

[12] VOS, P., HOGERS, R., BLEEKER, M., RIJANS, M., Van der LEE, T., HORNES, M., FRIJTERS, A., POT, J., PELEMAN, J. KUIPER, M. and ZABEAU, M. A new technique for DNA fingerprinting, Nucleic Acids Res. **23** (1995) 4407-4414.

[13] HAFSAH JAAFAR, RADZIAH, N.Z., LOW, F.C. and SAFIAH ATAN Mutation breeding of *Hevea brasiliensis*: Use of *in vitro* technology to produce disease-resistant mutants - Preliminary results. Proc. Asia-Pacific Conf. on Plant Physiol. II, Kuala Lumpur, Aug. 20-22, 1996. (In press)

[14] HAFSAH JAAFAR and RAMLI OTHMAN Mutation breeding of Hevea brasiliensis - 1: Radio sensitivity test of different plant materials, Int. Symp. on Contribution of Plant Mutation Breeding to Crop Improvement, Vienna. Extended Synopsis. 145-146 (1990).

[15] LOW, F.C., SITI ARIJA MAD ARIF, CHOW, K.S, WAN ABD. RAHAMAN WAN YAACOB and GALE, M. Restriction fragment length polymorphisms as probes to plant diversity. Conservation of Plant Genetic Resources Through *In Vitro* Methods (ISBN 967-99915-2-0), FRIM/MNCPGR , Kuala Lumpur, (1990) 110-117.

[16] LOW, F.C. and GALE, M.D. Development of molecular markers for *Hevea,* J. nat. Rubb. Res. **6** (1991) 152-157.

[17] LOW, F.C., SAFIAH ATAN, HAFSAH JAAFAR and H. TAN Recent advances in the development of molecular markers for *Hevea* studies, J. Nat. Rubb. Res. **11** (1996) 32-44.

[18] SAFIAH ATAN, LOW, F.C. and SALEH, N.M. The construction of a microsatellite-enriched library, J. nat. Rubb. Res. **11** (4) (1997) 247-255.

[19] GERLACH, W.L. and BEDBROOK, J.R. Cloning and characterization of ribosomal RNA genes from wheat and barley, Nucleic Acids Res. **7** (1979) 1869-1885.

[20] KOLLER, B.A., LEHMANN, J.M., McDERMOTT and GESSLER, C. Identification of apple cultivars using random amplified polymorphic DNA markers, Theor. Appl. Genet. **85** (1993) 901-904.

[21] SMULDERS, M.J.M., RUS-KORTEKAAS, W. and VOSMAN, B. Tissue culture-induced DNA methylation polymorphisms in repetitive DNA of tomato calli and regenerated plants, Theor. Appl. Genet. **91** (1995) 1257-1264.

[22] CULLIS, C.A. Quantitative variation of ribosomal RNA gene in flax genotrophs, Heredity **42** (1979) 213-217.

[23] LARKIN, P.J., RYAN, S.A. BERTELL, R.I.S. and SCOWCROFT, W.R. Heritable somaclonal variation in wheat, Theor. Appl. Genet. **67** (1984) 443-455.

[24] LANDSMANN, J. and UHRIG, H. Somaclonal variation in *Solanum tuberosum* detected at the molecular level, Theor. Appl. Genet. **71** (1985) 500-505.

[25] KAEPPLER, S.M. and PHILLIPS, R.I. Tissue culture-induced DNA methylation variation in maize, Proc. Natl. Acad. Sci. USA **90** (1993) 8773-8776.

[26] BROWN, P.T.H., GLOBEL, E., LORZ, H. RFLP analysis of *Zea mays* callus cultures and their regenerated plants, Theor. Appl. Genet. **81** (1991) 227-232.

[27] ARNOLDT-SCHMITT, B. Rapid changes in amplification and methylation pattern of genomic DNA in cultured carrot explants (*Daucus carota L*), Theor. Appl. Genet. **85** (1993) 793-800.

[28] CAETANO-ANOLLES, G. MAAP: a versatile and universal tool for genome analysis, Plant Mol. Biol. **25** (1994) 1011-1026.

[29] HE, C., PRAKASH, C.S. and JARRET, R.L. Analysis of genetic diversity in a sweet potato (*Ipomea batatas*) germplasm collection using DNA amplification fingerprinting, Genome **38** (1995) 938-945.

[30] TRAGOORUNG, S., KANAZIN, V. HAYES, P.M. and BLAKE, T.K. Sequence tagged sites facilitate PCR for barley genome mapping, Theor. Appl. Genet. **84** (1992) 10002-1008.

[31] CHYE, M.L., TAN, C.T. and CHUA, N.H. Three genes encode 3-hydroxymethylglutaryl coenzyme A reductase in *Hevea brasiliensis*:hmg-1 and hmg-3 are differentially expressed, Plant Mol. Biol. **19** (1992) 473-484.

GENETIC TRANSFORMATION OF DECIDUOUS FRUIT TREES CONFERRING RESISTANCE AGAINST DISEASES

E.L. MANSVELT, T. GLYN-WOODS, L. WATTS, A. RABIE
Agricultural Research Council, Infruitec

M. APPEL, D.U. BELLSTEDT
Biochemistry Department, University of Stellenbosch

Stellenbosch, South Africa

Abstract

Long breeding cycles make cultivar development a lengthy process in deciduous fruit species. Gene transfer is, accordingly, a goal with significant commercial value. In many plant species, especially in woody plants, a prerequisite for genetic engineering is the ability to regenerate plants from transformed cells. Development of single cell regeneration is the first step towards exploration of gene transfer techniques. In this investigation media for plum and apple leaf disk regeneration were developed. Transformation experiments were performed. The vector EHA105 containing the *gus*-intron gene was found to be effective for gene transfer. Induction of the *virG* genes with aceto-syringone did not enhance transformation. Cefotaxime that was supplemented in the plum selection medium to suppress the *Agrobacterium* vector seriously inhibited leaf disk regeneration. However, in apples it was not detrimental. With further apple transformation experiments, factors such as preculturing, age of leaves, sucrose and cefotaxime concentrations did not increase the transformation efficiency of the marker gene.

The harpin protein, essential for the pathogenicity of *Pseudomonas syringae* pv. *syringae* which incites bacterial canker of stone fruit, was amplified and cloned into an expression vector. The fusion protein was purified. This will be used in future studies to elucidate the host-pathogen interaction, and to identify antibacterial genes.

1. INTRODUCTION

Diseases of stone and pome fruit induce losses probably exceeding $ 25 million (U.S.) annually in South Africa. The severity of the damage varies from subtle, almost undetectable effects to rapid death of many trees in some nurseries and orchards.

Bacterial canker of stone fruit caused by *Pseudomonas syringae* pv. *syringae* appears to be more severe in South Africa than elsewhere in the world. The reason for this is obscure, but we suspect that a combination of several predisposing components, particularly climatic and soil factors, favors disease expression. Most researchers regard *P. syringae* as a weak pathogen that causes disease only when the host is stressed. The organism is an excellent opportunist by virtue of its ability to colonise the foliar surface epiphytically and then to spread through the tree. Systemic invasion is of particular concern when one considers that deciduous fruit trees are propagated by grafting vegetative material onto rootstocks [6].

In the past, programs to control bacterial canker of stone fruit have met with mixed success, as can be expected if the subtle nature of the disease and the excellent ability of the pathogen to survive on the surface and inside the host are considered. Moreover, some of the predisposing factors can not be remedied, and future control strategies will have to rely on the selection and breeding for disease resistance.

Locally, apple growing is the largest deciduous fruit industry. Apple cultivars vary widely in their susceptibility to scab, powdery mildew, *Phytopthora*, *Rosellinia*, and stem canker causing fungi. Fungal diseases can be prevented by spraying with fungicides up to fifteen times per year. This means high costs for the grower (usually about 10 % of the non-labour costs) and a serious burden for the environment.

The production of transgenic plants is expected to become an important tool for genetic improvement of horticultural crops. The transformation of plants depends on two essential requirements: the ability to stably introduce a desired gene into the plant genome, and the ability to regenerate a fertile plant from the transformed cells.

Our objectives were the following: to develop tissue culture techniques for regenerating single cells from deciduous fruits, to transform (*Agrobacterium*-mediated) deciduous fruit cultivar tissues with marker genes, and to identify and isolate anti-bacterial gene(s).

2. MATERIALS AND METHODS

2.1. Development of regeneration and transformation techniques

2.1.1. Plant material and growing conditions

Shoots of the cultivars and rootstocks used in this study were collected from greenhouse-grown plants. The *Prunus* rootstocks used were Mariana and F 12/1 (Mazzard), and the apple rootstocks were M. 109, M. 111 and M. 7. The scions used were *Prunus salicina* cv. Casselman and *Malus domestica* cvs. Granny Smith and Royal Gala. They were surface-disinfected with sodium hypochlorite (2% v:v) that contained a drop of Tween 20 per liter, dipped for 30 seconds in 70% ethanol, then thoroughly rinsed with 3 changes of sterile distilled water, and were established on their maintenance medium.

Shoots were subcultured every 4 weeks. Proliferating cultures were maintained under white fluorescent lights at 24°C (16-hr photoperiod).

For leaf disk regeneration studies, explants from each of the different plant sources were excised and sliced perpendicularly to the mid-vein. They were placed horizontally with the abaxial surface in contact with the medium. Apple explants were incubated for two weeks in the dark at 24°C, and then transferred to a growth chamber at 24°C with dimmed fluorescent daylight. Plum leaf disks were transferred directly to white fluorescent light, unless otherwise described.

2.1.2. Maintenance medium

The maintenance medium for *Prunus* explants consisted of MS salts and vitamins [13] supplemented with 3% sucrose, 0.29 μM gibberillic acid (GA3), 0.49 μM indole-butyric acid (IBA) and 2.22 μM 6-benzyladenine (BA). The medium was solidified with 0.7% agar (Merck), and the pH was adjusted to 5.5 before autoclaving. The maintenance medium for apples have been described [7].

2.1.3. Regeneration medium

For single cell regeneration, the following medium components were examined: Basal salts and vitamins of MS and N6 [5] medium; sucrose or sorbitol; IBA or 1-naphthalene-acetic acid (NAA); and thidiazuron (TDZ). The medium was solidified with 7 g/L agar, 2 g/L gelrite, or 50 g/L corn starch. Medium (25 mL) was dispensed into 90 x 15 mm petri dishes. After the explants were transferred, each petri dish was sealed with a strip of parafilm.

2.1.4. Optimisation of transformation

Four *Agrobacterium tumefaciens* strains were used: A281 (p35S-intron-GUS), C58 (pGV2260), EHA105 (pMT1), and LBA4404 (pAL4404). The C58 and LBA4404 strains were provided with the binary expression vector pBI121. The binary expression vectors of A281 and EHA105 were derived from pBIN19. Both contain the NPII gene for plant selection and the kanamycin gene for bacterial selection, but A281, which contains the 35S-intron-GUS-35S-3', differs from EHA105 which contains 35S-intron-GUS-OCS-3'. Furthermore, EHA105 has the pTiBo542 *VirG* fragment on pMT1.

The *Agrobacterium* strains, cultured in YEP medium (supplemented with kanamycin), were harvested and adjusted to an OD of 0.3 at 550nm. Each strain was suspended individually in sterile distilled water. Leaf explants were inoculated with the bacterial strains by placing them in the bacterial suspensions for 20 min at 22°C, and were blotted onto sterile filter paper. They were co-cultivated on their respective co-cultivation media for two to three days before being transferred to their selective media.

The simplified induction medium (SIM) of James and Dandekar [7] was used to induce the *VirG* genes of the *Agrobacterium* strains which were incubated at 24°C for 5 hr before use. In apples, preculture periods of 0, 3, 5, 7, 9 and 12 days of leaf disks on regeneration medium before transformation were performed. The age of leaves (two, three or four weeks), sucrose (2 or 3%) and cefotaxime (250 or 400 µg/mL) were tested to increase tranformation efficiency.

2.1.5. Gus assays

Leaves were assayed for expression of the *gus*-genes following the histochemical staining procedure of Jefferson [8]. Staining was overnight at 37°C. The number of transformed calli on selective media were determined 3 weeks after infection by counting the blue calli. Leaves of plants growing on selective medium were tested nine weeks after transformation.

2.1.6. Experimental design, data collection and analyses

A completely randomised block design was used for all experiments with each experiment having two replications. A replication consisted of a petri dish containing twelve leaf explants. Leaf disk regeneration is expressed as the percentage of leaves that regenerated shoots. Data were recorded after 40 days of culture. Percentage data were subjected to statistical analyses.

2.2. Amplification, cloning and expression of the harpin encoding gene of *P. syringae* pv. *syringae* NV

2.2.1. Amplification

A polymerase chain reaction (PCR) was performed, using genomic DNA of *P. syringae* pv. *syringae* NV as template and primers based on the terminal sequences of the hrpZ2 gene of *P. syringae* pv. *syringae* 61 [1]. Bio-X-Act DNA polymerase (Bioline, contains 3'-5' proofreading activity) was used in this reaction.

2.2.2. Cloning strategy

The *P. syringae* pv. *syringae* NV PCR product was purified from a 1% agarose gel run in TAE and cloned into the pMOSBlue vector (Amersham) according to the manufacturer's instructions (A/T cloning strategy). The recombinant plasmid (pBNV1) was used to transform competent *E. coli* JM109 cells. The transformation reaction mixture was plated out on LB-agar containing 50 μg/mL ampicillin, 50 mg/mL X-gal and 0.1 M IPTG. Plates were incubated for 16 hours at 37°C. Randomly chosen white colonies were picked up, resuspended in distilled water and lysed by boiling. The crude supernatants obtained after centrifugation were subjected to a PCR, using the same primers and conditions as for the original amplification reaction, but using a DNA polymerase without proof reading activity. The PCR products were run on a 1% agarose gel in TAE.

Plasmid DNA was isolated from an overnight culture (LB + 100 μg/mL Amp) of *E. coli* JM109 (pBNV1). This plasmid, as well as the plasmid pMAL-c2 (New England Biolabs), was digested with *Eco*R1 and *Pst*1 in SuRE cut buffer H (Boehringer Mannheim) for 90 min. at 37°C. The reaction products were run on a 1% agarose gel in TAE. The linearized plasmid from the pMAL-c2 digest and the 1 kilobase (kb) fragment from the pBNV1 digest were purified from the gel. These DNA fragments (molar insert:vector = 9:1) were ligated for 90 min. at 37°C, using the Fastlink kit (Epicentre Biotechnologies). The recominant vector (pMNV1) was used to transform competent *E. coli* TB1 cells. The transformation reaction was plated out on LB-agar containing 100 μg/ml ampicillin. Plates were incubated for 16 hours at 37°C. Randomly selected clones were transplanted to LB-agar plates supplemented with 100 μg/mL Amp, X-gal and IPTG as before and grown for 16 hours at 37°C. The success of the subcloning experiment was evaluated by plasmid isolation from overnight cultures of randomly selected white colonies, followed by digestion with *Eco*R1 and *Pst*1 as described before.

2.2.3. Expression and purification of recombinant harpinNV

E. coli TB1 (pMNV1) was grown in liquid culture (Rich Broth and 100 μg/mL Amp) at 37°C to an OD (600nm) of 0.5. Expression of the recombinant protein was induced by incubation of the cultures for a further 2 hours after the addition of IPTG to a final concentration of 0.3 mM. Cells were harvested by centrifugation, resuspended in column buffer (20 mM TrisHCl, 0.2 M NaCl, 1 mM EDTA) and incubated overnight at -20°C in the presence of 2 mM PMSF. Cells were defrosted in cold water, sonicated in pulses of 15 s each for 6 min at 4°C. The supernatant obtained after centrifugation of the sonication mixture contained the soluble proteins of the recombinant bacterium, including the overexpressed maltose binding protein (MBP)-harpinNV fusion. The fusion protein was separated from the other soluble proteins by affinity chromatography using an amylose column. The fusion protein was eluted from the column in column buffer with 10 mM maltose.

In order to cleave the recombinant harpinNV from MBP, the column eluate was incubated with factor Xa (1 μg per μg fusion protein, as determined by the Bradford method) for 60 hours at room temperature. The recombinant harpinNV was precipitated from the cleavage reaction by the addition of saturated ammonium sulphate to a final saturation value of 25%. The reaction mixture was incubated at 4°C for 45 min and centrifuged at 13000 x g for 30 min at 4°C. The pellet (containing only the recominant harpinNV) was dialysed against 200 volumes 10 mM MES (pH 6.3) containing 1 mM PMSF and stored at 4°C. All expression and purification steps were evaluated on 10% SDS-PAGE, followed by Coomassie staining.

3. RESULTS

3.1. Single cell regeneration from *Prunus* explants

In a preliminary experiment, leaf explants were tested for shoot regeneration on N6 medium containing 5.4 μM NAA and various concentrations of TDZ (Table I). Of the two rootstocks and one cultivar tested, "Mariana" showed regeneration.

Based on the combination of NAA and TDZ from which the highest regeneration were obtained, IBA was substituted for NAA at equal concentration. Results from this experiment indicated that IBA was superior for induction of plant regeneration in Mariana (data not shown). However, no regeneration was observed for "Mazzard" or "Casselman".

In a subsequent experiment the shoot regeneration capacity of cultured leaf explants of "Mariana" were tested on twelve recipes consisting of 2, 3, or 4% sucrose or sorbitol solidified with gelrite or agar which were tested under three light regimes (Fig. 1). The Shapiro-Wilk test for non-normality showed no evidence against normality (P=0.33) [14]. Therefore, no transformation was needed.

Significant interaction was found between the recipe and light regime. Shortfall tests were performed to test the best recipe within each light regime [16]. From these data, it was found that N6 medium supplemented with 18.16 μM TDZ, 5 μM IBA, 2% sorbitol, and solidified with gelrite gave the best response if grown in a light of 50 μmol m s, 16 hr photoperiod at 24°C (Fig. 1).

TABLE I. ADVENTITIOUS SHOOT FORMATION ON LEAF EXPLANTS OF *PRUNUS* ROOTSTOCK MARIANA CULTURED ON N6 MEDIUM SUPPLEMENTED WITH NAA AND VARIOUS CONCENTRATIONS OF TDZ

TDZ (uM)	No. of leaf explants	Percentage regeneration
19.8	183	9.29
22.7	213	3.28
25.6	217	1.84
28.4	259	5.41

All media described above were used for leaf disk regeneration studies of Mazzard cherry rootstocks and cv. Casselman Japanese plums. Although lots of calli were found, shoot culture regeneration from leaf explants only occurred erratically (data not shown).

3.2. Single cell regeneration from apple leaf explants

Initially, published recipes were tested. Higher numbers of plants developed on media supplemented with TDZ, and the higher the concentration, the more distorted the plants appeared. The leaves of plants from the BA-containing medium were larger and fewer. Therefore, in the case of Royal Gala, a MS-based medium supplemented with 3 mg/L TDZ was found to be optimal for leaf disk regeneration (data not shown).

The effect of the gelling agent and the carbohydrate source was investigated to improve leaf disk regeneration. Table II shows the best leaf disk regeneration responses obtained with 2 cultivars and 3 rootstocks tested on nine different media. For example, on medium 6 all the Royal Gala explants regenerated, and the highest numbers of shoots were found. The regeneration of M.7 was poor and plants developed after 3 months only.

3.3. Transformation

Medium 4 (Fig. 1) was used in co-cultivation and selection studies of Mariana leaf explants. Cefotaxime (400 μg/L) was added to the medium to contain *Agrobacterium* growth. This seriously inhibited leaf disk regeneration.

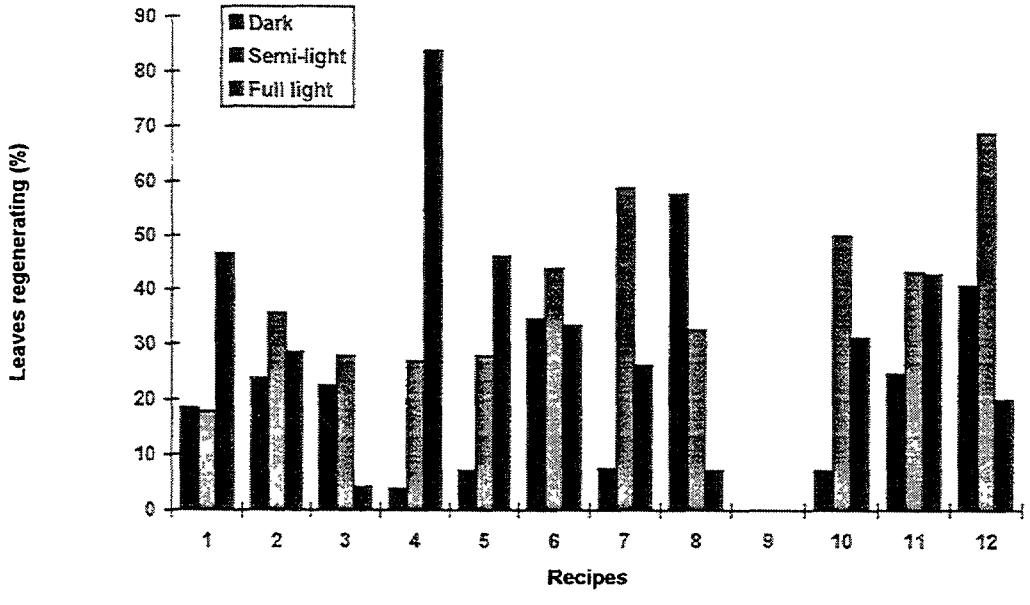


FIG. 1. Effect of several media (recipes) on the shoot regeneration from leaf disks. The number of leaves responding are expressed as percentages. Recipes were prepared as follows: supplemented with sucrose (1-3 and 7-9) or sorbitol (4-6 and 10-12) at 2% (1, 4, 7, and 10), 3% (2, 5, 8 and 11) or 4% (3, 6, 9 and 12). Media 1-6 were solidified with gelrite, and 7-12 with agar.

TABLE II. LEAF DISK REGENERATION STUDIES OF APPLE CULTIVARS AND ROOTSTOCKS. ONLY THE BEST RESPONSES ARE SHOWN

Plant	Medium*	% Leaf regeneration	Plants/leaf mean
Granny Smith	3	44	1.37
M.7	4	33	1
M.109	4	77	7.85
M.111	7	59	2.93
Royal Gala	6	100	20.58

* Media 1-3, 4-6 and 7-9 were solidified with agar, cornstarch or gelrite respectively, and contained sucrose (1, 4 and 7), glucose (2, 5 and 8) or sorbitol (3, 6 and 9)

3.3.1. Choice of *Agrobacterium* vector, and induction of VirG gene

To optimize gene transfer in deciduous fruit crops, we have investigated the effect of aceto-syringone on strain virulence of four *Agrobacterium* vectors. Transformation was influenced by the different *Agrobacterium* strains. Of the range of vectors tested, strain C58 gave higher frequency of transformation for most of the host plants, and strain A281 was unsuitable for transforming the plants studied. The presence of aceto-syringone in SIM before inoculating and co-cultivating the *Agrobacterium* vectors did not enhance the efficiency of transfer of the GUS genes. Stable expression of GUS was highest in apples.

The vectors showed differences in GUS expression (Fig. 2). Two of the vectors (A281 and EHA105) can only be expressed in transformed plants. By quantifying the blue pigments, EHA105 gave the best results.

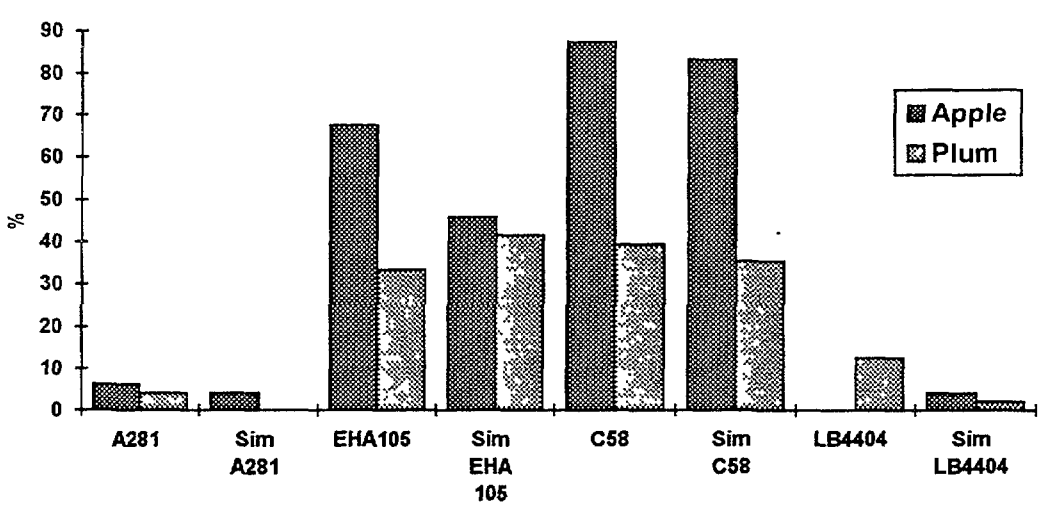


FIG. 2. Percentages of leaves showing gus expression Assays for stable expression was performed 3 weeks after inoculation. Agrobacterial strains labelled with SIM were incubated in the simplified induction medium containing aceto-syringone to induce the virulence genes [7].

3.3.2. Optimisation of transformation

Transgenic plants showing *gus* activity were obtained from 2-, 3- and 4-week old plants at a rate of 1 out of 400 leaves which were inoculated with EHA105. This translates to a transformation efficiency of 0.4%.

3.4. Amplification, cloning and expression of the harpin encoding gene of *P. syringae.* pv. *syringae* NV

A single PCR product, similar in size (1 kb) to the *hrpZ2* gene, was amplified from the genome of *P. syringae.* pv. *syringae.* This 1 kb band was successfully amplified from selected *E. coli* JM109 (pBNV1) clones, indicating that the PCR product was successfully cloned. The 1 kb fragment was also obtained with *Eco*RI/*Pst*I digestion of DNA from selected *E. coli* TB1 (pMNV1) clones, indicating that the subcloning experiment was successful. Using Rainbow Molecular Weight Markers (Amersham), the size of the purified recombinant harpinNV was estimated at 37 kDa. This correlated well with the size of the *P. syringae* pv. *syringae* 61 harpin (34.7 kDa, as determined by mass spectrometry).

4. DISCUSSION

Long breeding cycles make cultivar development a lengthy process in deciduous fruit species. Gene transfer is, accordingly, a goal with significant commercial value. In many plant species, especially in woody plants, the ability to regenerate plants from transformed cells is essential for genetic engineering. Development of a regeneration protocol is, therefore, a first step towards exploration of gene transfer techniques.

In most plants, *Agrobacterium*-mediated transformation of leaf discs followed by direct plant regeneration with minimal callus formation is the most common procedure. In *Prunus* spp. plant regeneration from hypocotyl slices [12], cotyledons [10] and embryos [15] of *Prunus* has been reported. These tissues are available only at certain times of the year, and moreover, the commercial cultivar desirability is lost, seriously limiting their usefulness for transformation. Only caulogenesis was reported from *Prunus* leaf disks [4].

We developed a medium which allows 80% shoot regeneration from plum leaf explants. To our knowledge, this is the first report of shoot regeneration from plum leaf disks. The dominant transportable sugar in *Prunus* spp. which is sorbitol [3] increased the percentage leaf explant regeneration on medium 4 in full light as illustrated in Fig. 1. However, if the light intensities were changed, this medium gave poor results. Furthermore, no pattern was established for the type of solidifying agent used, nor percentage of sugar included. Therefore, no generalisations can be made for future experiments.

The antibacterial genes will be used in gene transfer studies. However, additional genes need to be identified and isolated. Screening plant extracts for antibacterial activity has identified many potentially useful compounds, but it has seldom led to identification of the associated plant genes. Focusing on plant molecular signals during early stages of infection has been more successful; many research groups are targeting host genes involved in pathogen recognition/signal transduction of inducible defence products, such as phytoalexins, pathogenesis-related proteins, and structural proteins [9]. Several examples exist for applying this strategy against fungal pathogens, including *Rhizoctonia solani* [2]. The harpin protein which is essential for pathogenicity of the bacterial canker pathogen is a prime candidate for such studies.

Recent advances have led to the identification, and subsequent gene cloning, of several peptides with antibacterial properties in arthropods and some mammalian species. These peptides work independently, or in concert, to kill a range of important Gram-positive and -negative bacteria. Their activities vary, but several appear suitable against plant pathogens.

In general, three broad categories of antimicrobial properties exist: insect immune proteins, animal defence proteins; and lysozymes, followed by examples of natural or synthetic antibacterial genes that have been cloned, modified, and introduced for expression in plants.

The vigour of apple cultivars *in vitro* was severely decreased due to the presence of firestop, an antibiotic, which was routinely used to eliminate bacterial contamination in the mother cultures. Novobiocin was found to be superior as an antibiotic treatment. We also found an increase in plant growth as a result of this antibiotic (data not shown). Increase [11] and decrease [18] in plant growth as a result of treatment with antibiotics has been described. The increase in plant growth could be due either to a direct physiological effect of the antibiotic, or to inhibition of contaminants in the plant tissue cultures.

After the quality of the mother material was improved, leaf disk regeneration experiments were optimised for "Royal Gala" (Table 2). This supports the results obtained in other studies that Royal Gala is highly regenerative [17]. Leaf disk regeneration for apples will have to be investigated further, because optimal media remain to be developed for each cultivar and rootstock.

In this study, formation of adventitious shoots on leaf disks occurred with or without an intermediate callus stage depending on the genotype. The main factors affecting morphogenesis were TDZ concentration, the vigour of and presence of bacteria in mother plant material. The regeneration capacity increased substantially after shoot tip cultures were routinely grown on medium containing novobiocin. It is critical for leaf disk regeneration studies that mother plant material should grow vigorously.

The transformation efficiency was influenced by the use of different *Agrobacterium* strains. Of the range of vectors tested, the strain C58 showed higher frequency of *gus* expression. These results are misleading, because this vector carries the *gus* gene only, and it is not possible to distinguish between *gus* expression by the bacteria or the plant. Strain A281 is not suitable for transforming the plants studied. The *Agrobacterium* vector EHA105 gave the best results in gene transfer studies. Transgenic plum and apple calli were obtained (Fig. 2). Transgenic Royal Gala plants were found at a rate of 1 plant per 400 leaves treated.

The presence of aceto-syringone in SIM before inoculating and co-cultivating *Agrobacterium* vectors with the different leaf explants, preculturing of leaf disks, age of leaf explants, sucrose and cefotaxime concentrations die not did not influence the transfer of *gus* genes. The transformation efficiency in all combinations was 0.5%.

We shall continue our investigations to optimize conditions for increasing the single cell regeneration of apple and plum cultivars and rootstocks, and to increase transformation efficiency in these plants.

REFERENCES

[1] APPEL, M., MANSVELT, E.L. and BELLSTEDT, D.U. "Production of a harpin elicitor by *Pseudomonas syringae* pv. *syringae* isolated from a nectarine tree", *Pseudomonas syringae* Pathovars and Related Pathogens (Proc. Fifth International Conference Berlin, Germany, 1995).

[2] BROGLIE, K., CHET, I., HOLLIDAY, M., CRESSMAN, R., BIDDLE, KNOWLTON, C., MAUVAIS, C.J. and BROGLIE, R. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*, Science **254** (1991) 1194-1197.

[3] COFFIN, R., TAPER, C.D. and CHONG, C. Sorbitol and sucrose as carbon sources for callus culture of some species of the Rosaceae, Can. J. Bot. **54** (1976) 547-550.

[4] DECLERCK, V. and KORBAN, S.S. Influence of growth regulators and carbon sources on callus induction, growth and morphogenesis from leaf tissues of peach (*Prunus persica* L. Batsch), J. Hort. Sci. **71** (1996) 49-55.

[5] FASOLO, F., ZIMMERMAN, R.H., and FORDHAM, I. Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars, Plant Cell Tissue and Org. Cult. **16** (1989) 75-87.

[6] HATTINGH, M.J., ROOS, I.M.M. and MANSVELT, E.L. Infection and systemic invasion of deciduous fruit trees by *Pseudomonas syringae* in South Africa, Plant Disease **73** (1989) 784-789.

[7] JAMES, D.J. and DANDEKAR, A.M. Regeneration and transformation of apple (*Malus pumila* Mill.), Plant Tissue Culture Manual B8 (1991) 1-18.

[8] JEFFERSON, R.A. Assaying chimeric genes in plants: The GUS gene fusion system, Plant Mol. Biol. Rep. **5** (1987) 387-405.

[9] LAMB, C.J., RYALS, E.R., WARD, E.R., and DIXON, R.A. Emerging strategies for enhancing crop resistance to microbial pathogens, Bio/Technology **10** (1992) 1436--1445.

[10] LANE, D.W. and COSSIO, F. Adventitious shoots from cotyledons of immature cherry and apricot embryos, Can J. Plant Sci. **66** (1986) 953-959.

[11] LEIFFERT, C., CAMOTTA, H., and WAITES, W.M. Effect of combinations on micropropagated *Clematis*, *Delphinium*, *Hosta*, *Iris*, and *Photinia*, Plant Cell, Tissue and Organ Culture **29** (1992) 153-160.

[12] MANTE, S., MORGENS, P.H., SCORZA, R., CORDTS, J.M. and CALLAHAN, A.M. *Agrobacterium*-mediated transformation of plum (*Prunus domestica* L.) hypocotyl slices and regeneration of transgenic plants, Bio/Technology **9** (1991) 853-857.

[13] MURASHIGE, T. and SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiological Plantarum **15** (1992) 473-479.

[14] SHAPIRO, S.S. and WILK, M.B. An analysis of variance test for normality (complete samples), Biometrika **52** (1965) 591-611.

[15] SVIRCEV, A.M., BIGGS, A.R. and MILES, N.W. Peach regeneration from callus derived from embryos of selected cultivars, Fruit Var. J. **47** (1993) 13-18.

[16] VAN AARDE, I.M.R. Pivotal quantities for the substitution values of various entries, each in place of its best contender, given the standard analysis of variance model, Biom. J. **36** (1994) 673-687.

[17] YAO, J.-L., COHEN, D., ATKINSON, R., RICHARDSON, K. and MORRIS, B. Regeneration of transgenic plants from the commercial cultivar Royal Gala, Plant Cell Reports **14** (1995) 407- 412.

[18] YOUNG, P.M., HUTCHINS, A.S. and CANFIELD, M.L. Use of antibiotics to control bacteria in shoot cultures of woody plants, Plant Science Letters **34** (1984) 203-209.

APPLICATION OF MOLECULAR MARKERS IN GERMPLASM ENHANCEMENT OF CASSAVA (*MANIHOT ESCULENTA* L. CRANTZ) AND YAMS (*DIOSCOREA* SPP.) AT IITA

H.D. MIGNOUNA, R. ASIEDU, A.G.O. DIXON,
J. TONUKARI, N.Q. NG, G. THOTTAPPILLY
International Institute of Tropical Agriculture,
Ibadan, Nigeria

M. KNOX, T.H.N. ELLIS
John Innes Centre,
Norwich, United Kingdom

Abstract

The genetic variation among 28 varieties of cassava (*Manihot esculenta* L. Crantz), collected from different parts of the Republic of Benin was determined using random amplified polymorphic DNA (RAPD) markers. A set of ten primers out of the one hundred that were screened, detected polymorphisms. Thirty-five cassava landraces from three countries of West Africa, along with five improved varieties and one genetic stock (58308), were analysed using both microsatellite markers and nine selected random primers which generated fifty-four polymorphic markers. Based on the unweighted pair group method with arithmetic averages (UPGMA) and Principal Component Analysis (PCA), six major groups of clusters were identified among the forty one genotypes. Clone 58308, the original source of resistance to African Cassava Mosaic Disease (ACMD) in IITA's cassava breeding program, and TMS 30572, an improved cultivar derived from clone 58308, were found in the same cluster group. All 34 of the landraces that are known to be resistant to ACMD were genetically distant from 58308 and TMS 30572. A diallel mating programme has been initiated to elucidate the genetics of these new sources of resistance to ACMD and determine their complementarity as well as allellism for resistance. A set of eight random primers for RAPD and two combinations of enzymes and specific primers for AFLP were used to generate DNA fingerprinting of twenty varietal groups among the 32 described for cultivated yams in the region. The results obtained confirm that a given varietal group is a mixture of different genotypes. The molecular taxonomy of 30 accessions of cultivated yams, *D. rotundata* and *D. cayenensis*, and 35 accessions of wild yams from Nigeria was established using RAPD and microsatellite markers. The cultivated yams separated into two distinct groups corresponding to the two species. *D. rotundata* genotypes showed relationship to the wild species *D. abyssinica* and *D. praehensilis*, whereas *D. cayenensis* genotypes were related to the wild species *D. burkilliana*. IITA is currently developing molecular markers to tag genes conferring resistance to major diseases and pests of both cassava and yams. Molecular markers linked with these genes will then be used for marker assisted selection.

1. INTRODUCTION

Cassava (*Manihot esculenta* Crantz) and yams (*Dioscorea* spp) constitute the mainstay for millions of people world-wide particularly in the tropics. Production in Africa of the major staples is constrained by pests and diseases such as African Cassava Mosaic Disease (ACMD), cassava bacterial blight (CBB), cassava anthracnose disease (CAD), cassava green mite(CGM), yam anthracnose, yam viruses and nematodes.

Improved cassava varieties bred by the International Institute of Tropical Agriculture (IITA) and resistant to the ACMD and CBB have been adopted by many farmers in West and Central Africa. These varieties have been derived from one source of resistance to ACMD

(clone 58308). In the quest to diversify the sources of resistance, IITA has assembled several African landraces of cassava which show tremendous variation for resistance to diseases and pests. It is important to determine the genetic diversity and establish heterotic patterns among this collection using new tools provided by molecular biology.

Over the years, IITA has assembled and conserved over 2800 accessions of yams germplasm collected from West and Central Africa. In order to optimize the use of this germplasm for yam improvement, it is important to characterize, evaluate and assess the genetic diversity of the germplasm. Establishment of the phylogenetic relationships between cultivated and wild yams will expand the gene pool available for yams improvement.

2. MATERIALS AND METHODS

2.1. Benin cassava germplasm

The cassava clones used in this study were collected from farmers' fields in at least eleven locations in the southern part of the Republic of Benin (between latitudes 60N and 90N) where cassava is cultivated. Twenty eight major varieties were selected based on morphological differences and maintained in the screenhouse of the Germplasm Resources Unit at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. DNA was extracted from plants grown from stem cuttings in pots.

2.2. West African ACMD resistant landraces

DNA was extracted from forty-one genotypes of cassava comprising 35 local landraces collected in Nigeria, Republic of Benin and Togo; five improved clones and one genetic stock were grown in the experimental field of the Root and Tuber Improvement programme (TRIP), IITA, Ibadan, Nigeria.

2.3. DNA isolation

DNA was isolated from five grams of young cassava leaves according to a modification of Rogers and Bendich's method [1] as described by Tonukari *et al.* [2].

2.4. DNA amplification

DNA was diluted to the final concentration of 1ng/µl and used for polymerase chain reaction (PCR) amplification. Primers were 10-mer oligonucleotides purchased from Operon Technologies (Alameda, CA, USA). Amplifications were performed in a Perkin- Elmer 9600 thermocycler using 25µl of reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.5% Tween-20, 3 mM $MgCl_2$, 0.25 uM of each deoxyribonucleotide (dNTPs), 0.2µM of random decamer primer, 5 ng of template DNA and 2 units of Taq DNA polymerase from Promega Corporation (Madison, WI, USA). The amplification profiles were an initial denaturation step at 94°C for 3 min, followed by 45 cycles at 94°C for 1 min, 36°C for 1 min, 72°C for 2 min and finally at 72°C for 7 min. PCR amplification products were separated on 1.4% agarose gel and fragments were viewed under the ultra-violet transilluminator after ethidium bromide staining. DNA fragments were scored as present (+) or absent (-) for each genotype.

2.5. Data analysis

The data were first subjected to principal component analysis (PCA) using the statistical analysis system (SAS) [3]. The most informative randomly amplified polymorphic DNA (RAPD) markers were then used to determine the genetic distance between each pair of genotype according to Nei and Li [4]. The generated genetic distances were then used to draw a dendrogram based on the complete clustering method of the computer software NTSYS-pc package for numerical taxonomy and multivariate analysis version 1.8 [5].

2.6. Yam germplasm from West and Central Africa

Guinea yams (*Dioscorea rotundata* and *D. cayenensis*) accessions collected from Nigeria (numbering 42), Benin Republic (67), Cameroon (79), Ghana (32), Togo (34), C™te d'Ivoire (81), Burkina -Faso (12), and Guinea Conakry (34) were planted in an experimental field in Ibadan in a complete randomized block design. Morphological characters of the shoot at both juvenile and adult stages were scored using the International Board for Plant Genetic Resources (IBPGR) descriptor list [6]. Leaf extracts of each accession was analysed for four isozyme systems (malate dehydrogenese (MDH), 6-phosphogluconate dehydrogenase (6-PGD), shikimate dehydrogenase (SKDH) and phosphogluco-isomerase (PGI) using polyacrylamide gel electrophoresis [7] and enzyme activity staining [8].

DNA was extracted and polymorphism was detected using RAPD technique according to the method described by Mignouna *et al.*[9]. For AFLP fingerprinting, 0.5 μg of genomic DNA was digested with a hexacutter (*Eco*RI) and a tetracutter (*Mse*I) restriction enzyme and site specific double stranded adapters were ligated to the restriction fragments. The gel analysis was carried out as described by Vos et al.[10]. Thirty accessions of cultivated Guinea yams and 35 accessions of wild *Dioscorea* species collected from Nigeria were used in phylogenetic studies.

3. RESULTS AND DISCUSSION

3.1. Diversity studies on Benin cassava germplasm

One hundred random oligonucleotide primers (Operon Technologies sets A, B, D, E and O) were evaluated for their ability to prime PCR amplification of cassava genomic DNA. In a preliminary survey, genomic DNA of two of the accessions, TMe 1900 and TMe 1921 collected from Ahingodo and Bante, respectively, were used as templates. Nineteen of the 100 primers were able to amplify the genomic DNA, giving reproducible RAPD amplification patterns with individual fragments that stained intensely. Ten of these were chosen for the whole experiment and used to amplify the DNA of the various accessions.

Ten informative oligonucleotide primers detected polymorphisms among the 28 cassava accessions. The primers revealed a total of 78 clear and easily scorable bands twenty five (32%) of which were polymorphic among the cassava accessions studied. The number of bands per primer ranged from 6 to 12. The size of DNA bands that were produced in the PCR reactions ranged from 300 to 2100 bp, but most of the bands were between 500 and 1000 bp. None of the primers was specific for any of the accessions. However, primers OPE-03 and OPE-04 produced 480 bp and 1200 bp bands, respectively, in TMe 1862 and TMe 2052 only. Also a 920 bp band was generated in TMe 1876 and TMe 2052 only by primer OPF-01, while a 900 bp band generated by OPO-20 was specific only to TMe 1918 and TMe 1921. Data from the scores were subjected to unweighted pair group method with arithmetic averages

(UPGMA) analysis Jaccard's similarity coefficient ranged from 100% for the most closely related accessions to approximately 40% for those most distantly related, with a mean of 69% With the PCR conditions tested, TMe 1876 showed the highest number of polymorphic bands while TME 1906 showed the least None of the primers could provide enough markers to discriminate between all the cassava populations Identical RAPD patterns using the ten primers were observed for four pairs of the accessions, TMe 1900 and TMe 2055, TMe 1918 and TMe 1921, TMe 1972 and TMe 2056, and TMe 2059 and TMe 2066, having 100% Jaccard's similarity coefficient in each case Also, TMe 1937 and TMe 1951, as well as TMe 1906 and TMe 1911, showed 95% and 94% similarity respectively The similarities between accession TMe 2052 and accessions TMe 1906, TMe 1900 and TMe 2001 are 40%, 42% and 42% respectively Based on the average cluster analysis, the overall genetic relationships among the investigated accessions is illustrated in the dendrogram in Fig 1 The accessions can be categorised into 6 groups at 70% Jaccard's similarity coefficient level

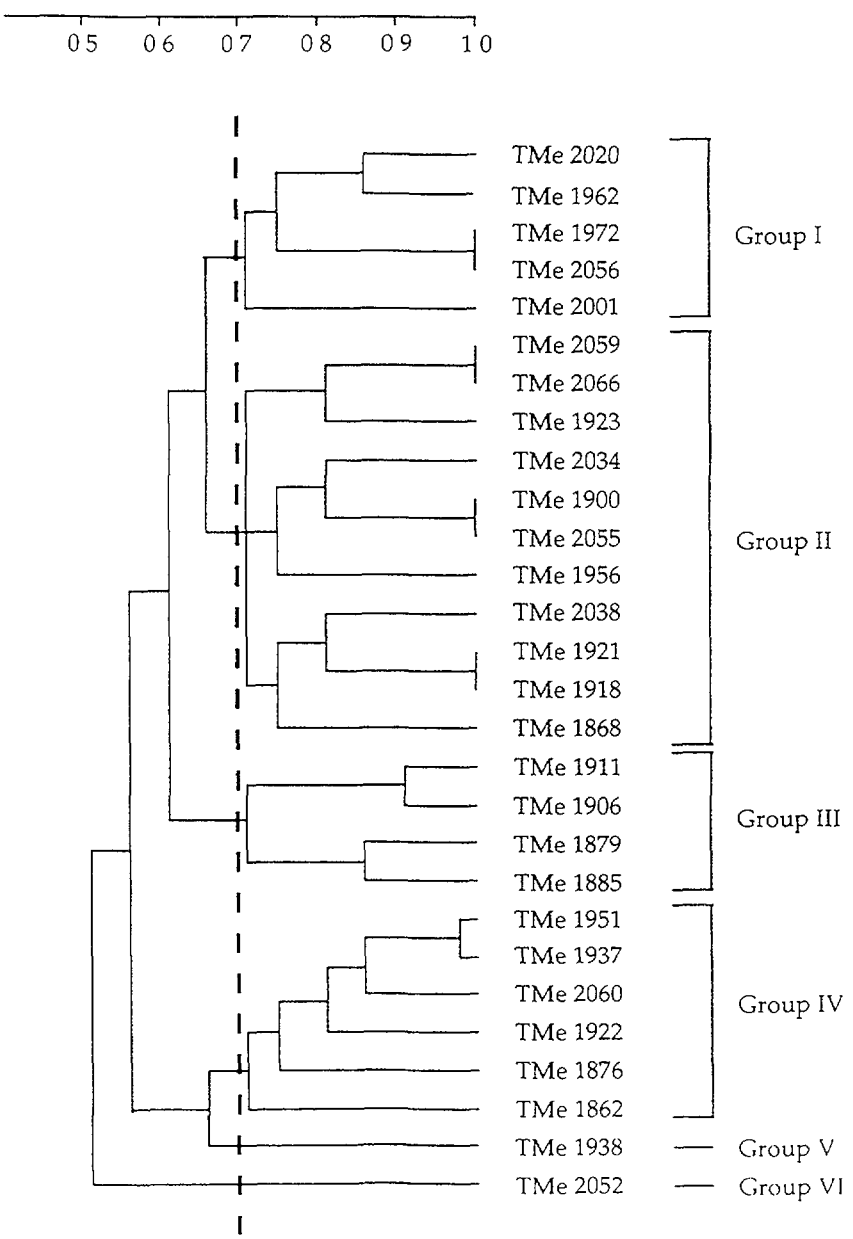


FIG 1 Average linkage cluster analysis of 28 Republic of Benin cassava accessions constructed from a pair-wise comparison of RAPDs between accessions by the Jaccard similarity index and UPGMA

3.2. West African landraces resistant to ACMD

Nine random primers selected from a preliminary survey gave RAPD fragments ranging in size from 300 bp to 2500 bp. The amplified fragment sizes generated in the analysis are similar to those previously reported in cassava germplasm [11]. The number of amplified fragments of the cassava germplasm varied according to the tested primers, the average number being eight. Among the total of 74 amplified fragments, twenty were monomorphic and were not considered in the statistical analysis. The remaining 54 polymorphic fragments were used in principal component analysis and 31 were finally selected to effectively discriminate the cassava genotypes.

The dendrogram constructed by hierarchical cluster analysis based on the genetic distance is shown in Fig. 2. At the 60% similarity level, six groups of clusters could be easily identified. The first group of clusters consisted of three landraces from Togo (Toma 378, Toma 393 and Toma 26) and ten from Nigeria (Bida1, Tokunbo, Oko-Iyawo, Power, Akinarinde, 2nd Agric, Bagi wawa, Abbey-Ife, Atu, and Lapai-1). In comparison to the susceptible check, Isunikankiyan, with an ACMD severity score of four (1=no symptom, 5= severe damage), the genotypes in this group of clusters were highly resistant to ACMD under heavy infection pressure in the field at Ibadan and Ubiaja.. The second group of clusters, genetically distant from the above group, consisted of seventeen genotypes: four from Togo (Toma 37, Toma 75, Toma 76 and Cameroon); three from Benin (RB 92/0188, Cap 96064 and Ben 86052); and ten from Nigeria comprising eight landraces (Ofege, Akano, MS 6, TME 1 (Antiota), MS 20, Obasanjo, Olekanga and Alice local). The grouping of the two improved clones with TME 1 is not surprising as they are derivatives of open-pollinated seeds from the landrace. The third group of clusters consisted of three genotypes. In this group, the genetic stock, 58308, clustered with improved clones, TMS 30572 and TMS 92/0326. The resistance to ACMD widely used in IITA's cassava breeding program was derived from 58308 which is also a parent of TMS 30572. TMS 92/0326 was selected from progenies generated on an isolated hybridization plot which had only TME 1 (Antiota) and TMS 30572. These genetic similarities are in agreement with their pedigree relationships. The fourth group comprised five genotypes: four from Benin (RB 89/509, 92/0099, RB 92/0116 and RB 92/0123) and one from Togo (Gbazekoute). The fifth group consisted of two genotypes: the landrace Isunikankiyan from Ibadan, which has been used widely in the breeding program over the years as an ACMD susceptible check and also a source of low cyanogenic potential and mealiness of the boiled storage roots; and TMS 30001, a resistant improved clone. Previous research on the genetics of ACMD resistance showed polygenic inheritance [12]. Therefore, the lack of a few genes or some allelic forms of genes controlling resistance could have given rise to the susceptible phenotype observed in the local variety Isunikankiyan which, though susceptible, tolerates the disease. The sixth group consisted of a lone landrace from Nigeria which was very resistant to ACMD.

The present study showed that several landraces are genetically distant from 58308 and TMS 30572. Cassava, being an open-pollinated and heterozygous plant, recombines on farmers' fields and also outcrosses to related species, resulting in greater genetic variability. Farmers' selection for adaptation to local conditions and utilization has taken place and numerous varieties have emerged in Africa which are good sources of resistance to diseases and pests, have good food quality traits, and are adaptable to the farming systems [13]. These genetically distant groups of landraces may carry other sources of genes for resistance to ACMD. This information is currently being used in the IITA cassava breeding program for studying the molecular genetics of ACMD resistance. The new sources of resistance are being

used in the hybridization program with a view to diversify the resistance to ACMD such that the resulting improved genotypes would prove difficult for the virus to circumvent

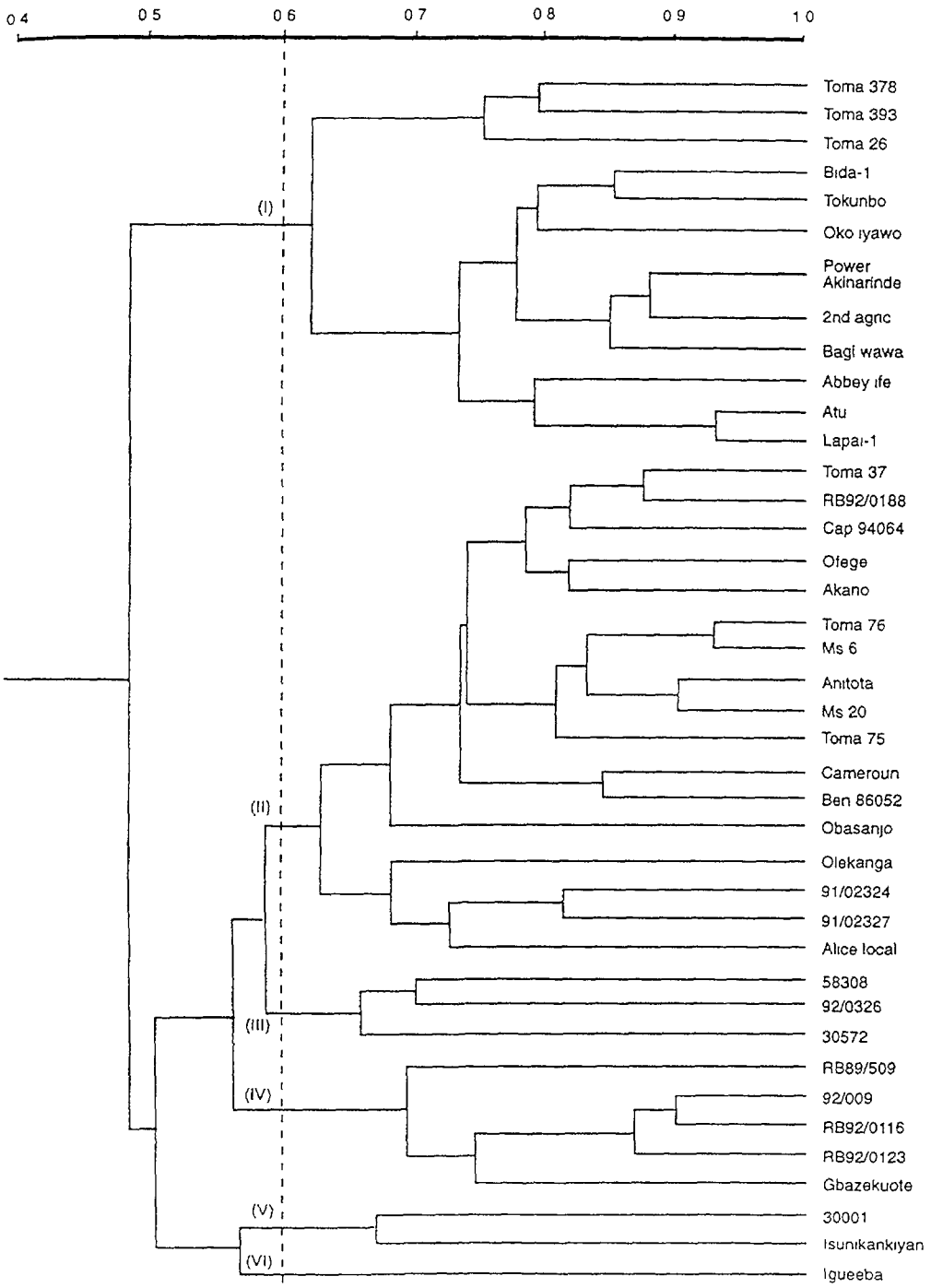


FIG 2 Dendrogram showing the genetic relationships for 41 cassava varieties constructed from RAPD markers based on UPGMA Scale shows the genetic distance derived from Jaccard's coefficient of similarity The indicated groups (I to VI) were formed at 60% similarity

It is also evident from this study that varieties in particular farmers fields may not be unique. This is so because there is a high degree of turnover in cassava genotypes grown by farmers, who are continually introducing new genotypes with desired attributes from neighboring villages, regions and countries [14]. Hence such genotypes may not be unique to those particular villages or countries in West Africa where the landraces were collected.

3.3. Genetic diversity in Guinea yams

Based on morphological data, the 446 accessions of cultivated yams used in the present study could be divided into 34 varietal groups. Among these varietal groups, eighteen were described previously from the materials collected in C™te d'Ivoire and two from Benin Republic [14]. Fourteen new varietal groups were identified in the germ plasm.

Although isozyme markers provided good genetic markers for yam varietal group identification, the level of intra-varietal polymorphism was low. Moreover the cost of isozyme assays was relatively high. Hence, RAPD markers were used to analyse the thirty-four varietal groups identified. The RAPD markers showed great variability between the 34 varietal groups identified. Some of the markers appeared to be variety-specific which could be used for identification and classification of Guinea yams in West and Central Africa.

3.4. Phylogenetic relationships between wild and cultivated yams of Nigeria

A similarity matrix based on Jaccard's coefficients was used to establish the level of relatedness between the cultivated and wild accessions of *Dioscorea* species (data not shown). From this matrix the mean genetic distances between species were computed (data not shown). The similarities among some accessions within *D. cayenensis*, *D. rotundata*, *D. praehensilis*, and *D. burkilliana* were 100 percent which suggest that they are identical genotypes and therefore might be duplicates. The estimates of similarity ranged from 0.48 between *D. rotundata* (6) and, *D. burkilliana* (59) to 100 between some accessions of *D. rotundata*, *D. abyssinica* and *D. praehensilis*. The molecular marker data identified two major groups of species at 0.70 level of similarity on the UPGMA dendrogram (Fig. 3). The first group comprises the cultivated accessions of *D. rotundata* and wild species *D. abyssinica*, *D. praehensilis* and *D. liebrechtsiana* and the phenotypically intermediate types between *D. praehensilis* and *D. liebrechtsiana*. The second group comprises the accessions of *D. cayenensis* and those of the wild species *D. burkilliana*. In this study the accessions of cultivated white yam, *D. rotundata*, displayed fifteen genotypes which were separated into three groups. The major group, which comprises twelve different genotypes, was closely related to the wild species *D. praehensilis*. One genotype which showed intermediate morphological characteristics between *D. abyssinica* and *D. rotundata* was closely related to the wild species *D. abyssinica*. The third group of *D. rotundata* genotypes occupied an intermediate position between the two wild species *D. abyssinica* and *D. praehensilis*.

In order to identify which wild species shared exchanged genetic information with the three intermediate genotypes of *D. rotundata* (12,14,15), the PCA was undertaken. The first two principal components which accounted for 51% of the variation effectively discriminated the species. Among the intermediate genotypes, *D. rotundata* (14), genotype was a hybrid between *D. abyssinica* and *D. rotundata* while the other *D. rotundata* (12,15) genotypes were identified as hybrids between *D. rotundata* and *D. praehensilis*.

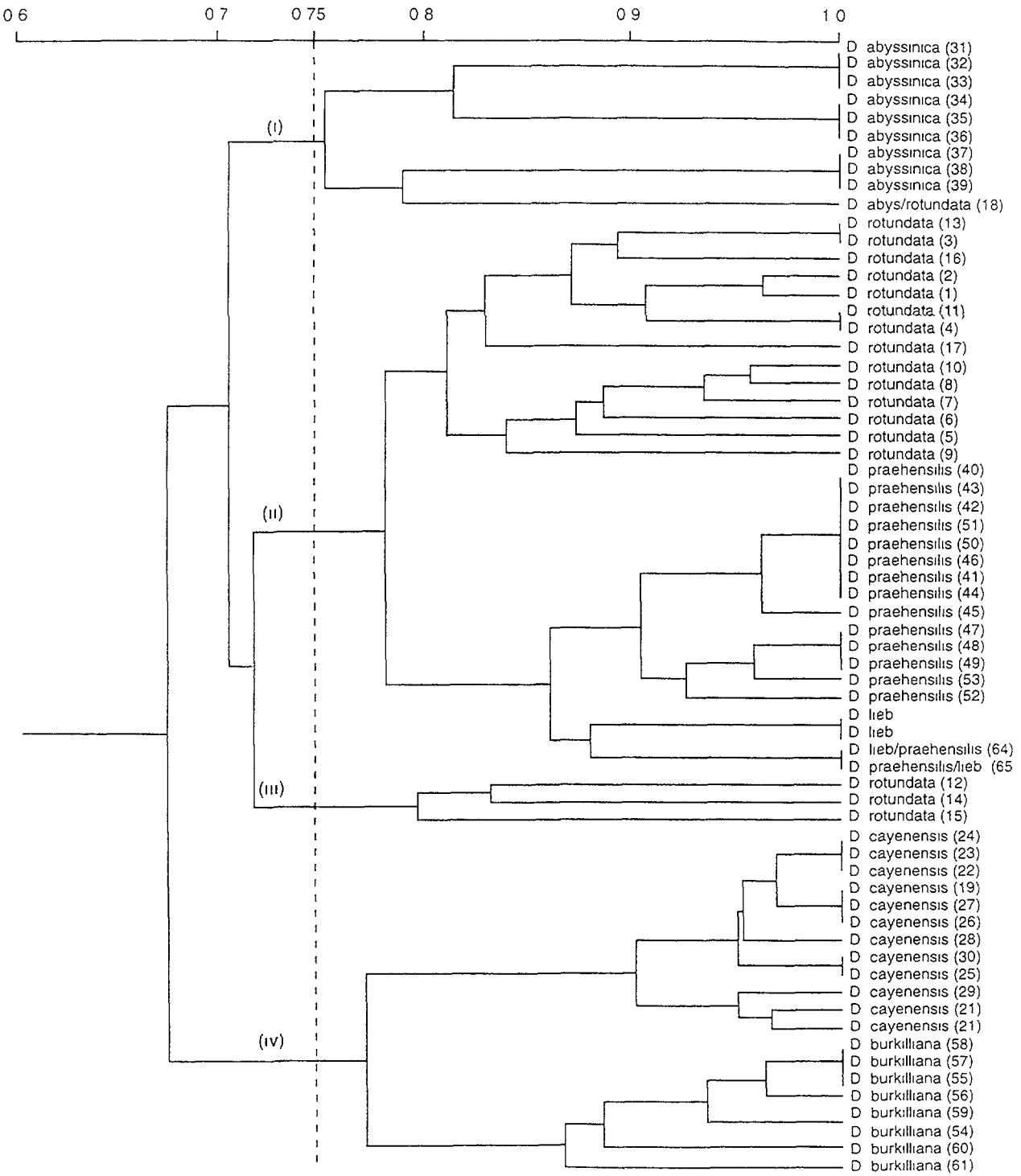


FIG 3 Dendrogram of Dioscorea spp Based on average linkage cluster analysis Axis on top indicates genetic distance.

To further examine the relationship between the species, the phylogenetic analysis using the parsimony method was inferred. The same general pattern of relationships between the genotypes was obtained whereby all the identified genotypes of the cultivated yams described as *D. cayenensis* were found closer to *D. burkilliana,* whereas the majority of *D. rotundata* genotypes were closer to the wild species *D. praehensilis* and *D. liebrechtsiana.* These relationships indicate that the genotypes of *D. rotundata* from Nigeria analyzed in the present study likely resulted from multicrosses between these three species *D. abyssinica, D. praehensilis* and *D. liebrechtsiana* whereas the *D. cayenensis* genotypes could have exchanged genetic information with *D. burkilliana.*

4. CONCLUSION

The different groups of cassava landraces highly resistant to ACMD are genetically distant from the widely used source of resistance and could possess other genes and mechanisms of resistance to ACMD.

A diallel mating programme is being carried out to elucidate the genetics of these sources of resistance to ACMD and determine complementarity of the relevant genes as well as allellism for resistance. Mapping populations are being developed for studies on molecular genetics of resistance to ACMD and identification of reliable molecular markers for use in marker-assisted selection. To complement this effort, other important traits related to quality of the storage roots (e.g. cyanogenic potential, culinary attributes) will soon be subjected to similar molecular analysis in the quest for further genetic improvement.

A total of 34 varietal groups were identified in yam germplasm from West and Central Africa, fourteen of which had not been described previously. RAPD analysis was very useful for the assessment of genetic diversity of yams but AFLP analysis detected more polymorphism than RAPD and thus could be even more efficient for genetic analysis and gene mapping of yams. These techniques are being applied to generate a linkage map of yams (*D. rotundata* and *D. alata*).

ACKNOWLEDGEMENTS

The work on yams was funded by the Gatsby Charitable Foundation, UK as one of the six projects jointly carried out by IITA and the John Innes Centre, UK.

REFERENCES

[1] ROGERS, S.O., and BENDICH, A.J. Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol. Bol. **5** (1985) 69-76.

[2] TONUKARI, N.J., THOTTAPPILLY, G., NG, N.Q., and MIGNOUNA, H.D. Genetic polymorphisms of cassava (*Manihot esculenta* Crantz) within the Republic of Benin detected with RAPD markers, African Crop Science Journal, (submitted) (1997).

[3] SAS Institute user's guide: statistics version. Release 6.03. SAS Institute, North Carolina, U.S.A. (1989) 1028 pp.

[4] NEI, M., and LI, W.H. Mathematical model for studying genetic variation in terms of restriction endonucleases, Proc. Natl. Acad. Sci. USA **76** (1979) 5269-5273.

[5] ROHLF, F.L. NTSYS-pc Numerical Taxonomy and Multivariate Analysis System. Exeter Publishing, New York (1989) 213pp.

[6] HAMON, P., and TOURE., B. Les ignames cultivees du complexe Dioscorea cayenensis-rotundata de Côte d'Ivoire. Inventaire et description des cultivars traditionnels. Catalogue publie par la faculte des Sciences et Techniques et International Board for Plant Genetic Resources, Rome, Italy, (1986) 63pp.

[7] HUSSAIN, A., BUSHUK, W., RAMIREZ, H., and ROCA, W. Identification of cassava (*Manihot esculenta* Crantz) cultivars by electrophoretic patterns of esterases enzymes, Seed Sci. Technol. **15** (1987)19-21.

[8] VALLEJOS, E. "Enzyme activity staining", Isozymes in plant genetics and breeding. Part A, (TANKSLEY, S.O., and ORSTON, Eds.). Elsevier Science Publishers B.V. Amsterdam (1983) 469-515.

[9] MIGNOUNA, H.D., ASIEDU, R., CHIKALEKE, V., HAHN, S.K., and THOTTAPPILLY, G. "Genetic diversity and phylogenetic relationships in the Genus Dioscorea using PCR-RAPD", Proceeding of the International Symposium on "l' Afrique face au défi des biotechnologies vegetales: cas de l'igname, Abidjan du 19 au 23 avril. Programme et résumés: N 026 (1993).

[10] VOS, P. HOGERS, R. BLEEKER, M., REIJANS, THEO van de L., HORNES, M., FRIJTERS A., POT, J., PELEMAN, J, KUIPER M., and ZEBEAU, M. AFLP: a new technique for DNA fingerprinting, Nucleic Acids Research **23** (1995) 4407-4414.

[11] MARMEY, P., BEECHING, J.R., HAMON, S., and CHARRIER A. Evaluation of cassava (*Manihot esculenta* Crantz) germplasm collections using RAPD markers, Euphytica **74** (1994) 203-209.

[12] HAHN, S.K., TERRY, E.R., and LEUSCHNER K. Breeding cassava for resistance to cassava mosaic disease, Euphytica **29** (1980) 673-683.

[13] MAHUNGU, N.M., DIXON, A.G.O., and MKUMBIRA, J. Breeding cassava for multiple pest resistance in Africa, African Crop Science Journal **2** (1994) 553-562.

[14] NWEKE., F.I, DIXON, A.G.O., ASIEDU, R., and FOLAYAN, S.A. Cassava varietal needs of farmers and the potential for growth in Africa. Collaborative Study of Cassava in Africa. (COSCA) Working Paper No. 10. Resource and Crop management Division, International Institute of Tropical Agriculture, Ibadan Nigeria. (1994) 239 pp.

[15] HAMON, P., and TOURE, B. Characterization of traditional yam varieties belonging to the *Dioscorea cayenensis-rotundata* complex by their isozymic patterns, Euphytica **46** (1990) 101-107.

MOLECULAR PHYLOGENY OF MANGROVES IV. NATURE AND EXTENT OF INTRA-SPECIFIC GENETIC VARIATION AND SPECIES DIVERSITY IN MANGROVES

A. PARIDA, M. PARANI, M. LAKSHMI, S. ELANGO, N. RAM, C.S. ANURATHA
M.S. Swaminathan Research Foundation, Taramani,
Madras, India

Abstract

Mangroves occupy estuarine ecosystems in the tropical regions of the world. Despite their highly productive nature and the protective roles they play in the coastal region, the ecosystem as a whole is under severe threat due to various climatic and anthropogenic factors. Therefore, the need for conservation of mangroves is widely emphasised. However, information on existing genetic diversity based on which a strategy for genetic conservation is to be drawn is not available for mangroves. This is primarily because conventional genetic analysis is difficult in these species for various reasons. Therefore, as an aid to our on-going conservation programme, efforts were made to assess the nature and extent of diversity in a number of mangrove species of the Indian coast using molecular markers. The nature and extent of intra-population diversity in sixteen mangrove species and detailed analysis of inter-population genetic polymorphism in four species, *Acanthus ilicifolius, Excoecaria agallocha, Avicennia* spp and *Rhizophora* (species and hybrid), is reported in the present communication.

1. INTRODUCTION

1.1. Coastal ecosystem: problems and prospects

The coastal ecosystem, that suffers from the twin problems of low productivity and uncertain yield, is an important part of the natural base of our country. Since these regions form vital bridges between the terrestrial and aquatic ecosystems, their preservation is essential to maintain the ecological balance and biodiversity. Despite their ecological and economic significance, the effects of current resource use practices evident both in the inland and coastal areas have rendered various forms of stresses on the coastal ecosystem. Increasing soil erosion and water pollution caused by intensive farm practices in the inland areas that gets transported through the river and canal systems are quite adversely affecting the coastal systems. Moreover, the sea water intrusion and the attendant soil and water quality problems caused by the ground water depletion have already started threatening the very sustainability of agricultural systems in the Saurashtra region of Gujarat and Tanjavur region of TamilNadu. At the same time, it is anticipated that by the year 2000, the human population living within 60 km of the shoreline will grow by over 50 per cent. Many of the world's poor are crowded in the coastal area and coastal resources are vital for the security of their livelihood. Above all, the problem of a rise in sea level, expected to be on the order of 8-29 cms due to global warming by 2025, makes the need to take concrete steps to ensure the sustainable management of the coastal ecosystem all the more urgent.

1.2. Mangroves

The coastal ecosystems are characterised with various physical, climatic and anthropogenic pressures. The most productive vegetation in the coastal regions are a group of specialised tree species, the mangroves, growing in the inter tidal and estuarine areas and are subject to high physical stress conditions such as high salinity and water logging. It is one of the most productive ecosystems of the world. It supports various micro-organisms and other

invertebrate species. They are the primary nursery ground for a number of commercial shrimp, crab and fish species. The total fish catch in the mangrove swamps account for about 68 per cent of total world production. They also stabilise shoreline and provide protection from tidal bores, ocean currents and storm surges. In addition, these plant species possess valuable characters for potential use in developing novel genetic material for adaptation to the ever fluctuating climatic extremes of the coastal zone.

Mangroves are very specialised forest ecosystems of the tropical and subtropical coastal regions of the world bordering the sheltered seacoasts and estuaries. Mangrove forests have valuable natural resources with high productivity and, hence, are often over-exploited for various purposes. The magnitude of the threat to this ecosystem is on the rise as more and more mangrove forests throughout the world are being used for agriculture, wood resources and forest products [1]. The increasing dependency of the local population on the mangrove species for various products and usage further threatens the sustainability of the system. Constant environmental and anthropogenic pressures on the ecosystem have led to a rapid decline of many natural populations of different species. This is evident from the fact that the once widely prevalent mangrove genus *Rhizophora* is now at the verge of extinction [2].

India, with a coastline of about 7500 km, represents 8 per cent of the world mangrove area [3]. However, in terms of species composition, it is represented by 60 species belonging to 42 genera and 29 families which are predominantly distributed along the eastern coast of India and that account for about 82% of Indian mangroves [4]. An alarming rate of depletion in the mangrove areas is being seriously felt in India; it has been reported that there has been about a 25% reduction in the mangrove forest cover along the Indian region during the last 25 years. This necessitates effective action plans to conserve and restore this valuable ecosystem.

Given the ecological and economic importance of the mangrove vegetation, and the considerable threat both in terms of increasing population pressure in the coastal areas and the exposure to anticipated climatic changes in the region, a concerted and well visualised action plan must be undertaken for the conservation, restoration, exploitation for useful traits and the rational use of these unique genetic resources.

1.3. Mangroves: current status and new approach

Despite the economic and ecological significance of the mangroves, experimental studies in this group of plant species have almost been completely neglected for a long time. Available reports on mangroves are highly fragmentary and no worthwhile information is available to account for species identity, relationship and evolution among the mangrove species. Based on the available information it is not possible to partition the observed variation into environmental and heritable components. As is well known, the phenotype is the result of intricate interaction of the genotype and the environment. The highly fluctuating environmental conditions in the coastal region inhabited by the mangroves influence, to a large extent, the phenotypic variation within and between the species. Hence, species identification and delimitation, based on quantitative characters and phenotypic markers, have resulted in the confusing taxonomic status of the mangrove species. It was in this context, that molecular marker based analyses of genetic material (DNA/RNA) or gene products (proteins/isoenzymes) were considered as the best means for studies related to identification, assessment of diversity, elucidating species relationship and depicting phylogenetic trends within and between the mangrove species of Indian region.

Unlike morphological markers, molecular markers are stable and are not prone to environmental influences and precisely portray the genetic relationship between plant groups [5,6,7,8] and hence are widely used in genetic resource characterisation and conservation. There are several marker systems available now, including the random amplified polymorphic DNA (RAPD) [9], restriction fragment length polymorphism (RFLP) and DNA fingerprinting, that have proved to be of invaluable assistance in molecular genetics, applications to plant breeding, phylogenetic analysis and marker-aided selection of traits. It is hoped that the advances in the field of molecular biology will contribute immensely to the genetic improvement of major crop and non-crop species, thereby, ensuring better productivity to meet the challenges of the growing population.

TABLE I. GEOGRAPHIC LOCATION AND PHYSICAL CHARACTERISTICS OF THE STUDY SITES

Name of the study site	Location	Latitude & longitude	Annual rainfall (mm)	Salinity (ppt)	Soil pH	Dominant soil type
Gulf of Mannar	Eastern Coast	8° 47'N 79° 14'E	900	7.0-17.0	6.0-7.5	Sandy
Muthupet	Eastern Coast	10° 46'N 79° 52'E	1280	6.0-18.0	5.9-7.3	Fine sand and clay
Pichavaram	Eastern Coast	11° 27'N 79° 47'E	1300	3.0-27.0	6.2-8.0	Fine sandy-clay
Ennore	Eastern Coast	13° 30'N 80° 15'E	1200	5.3-13.4	6.2-7.7	Sandy-silt
Coringa	Eastern Coast	16° 30'N 82° 20'E	1150	5.0-30.0	7.5-8.4	Clayey-silt
Bhitarkanika	Eastern Coast	20° 40'N 86° 52'E	1125	2.0-21.0	6.7-7.8	Fine silt/ clay
Calicut	Western Coast	11° 55'N 75° 81'E	1380	6.0-18.0	6.2-7.4	Sandy and clayey soil
Goa	Western Coast	17° 08'N 73° 52'E	890	2.0-35.0	6.8-7.7	Lateritic rock and clay
Ratnagiri	Western Coast	17° 08'N 73° 19'E	946	10.0-24.0	5.2-7.6	Lateritic rock with gray soil
Bombay	Western Coast	19° 25'N 73° 05'E	700	6.0-15.0	7.7-8.3	Sandy-clay
Karaikal	Eastern Coast	10° 78'E	1300	12-21	6.1-7.5	Clayey, dry and compact
Adyar	Eastern Coast	13° 00'N 80° 15'E	1250	6.1-19.5	6.6-7.3	Fine sand and silt
Pondicherry	Eastern Coast	12° 08'N 79° 52'E	1250	10-25	5.8-7.6	Fine sand

1.4. Species under investigations

Mangroves are the most diverse group of species with preferential adaptation to varying climatic and edaphic conditions. Therefore, species composition and relative abundance varies to a large extent in a given population. They are wide spread all along the coast line of the Indian region. Populations for the present study were collected from both eastern and western coasts of India. Samples of individual species were collected depending on their occurrence. In addition, some of the associated species and epiphytes were also included in the present study. A detailed account of the geographical location and physical characteristics of the study sites are given in Table I.

1.5. Intra-specific genetic variation

A preliminary study on the quantification of intra-specific variation in terms of polymorphic RAPD loci was conducted in a number mangroves species (Table II). The details of the species investigated for intra-specific genetic polymorphism using RAPD markers is given in Table II. Among theses species, *Acanthus ilicifolius, Excoecaria agallocha* and *Avicennia marina* were chosen for detailed analysis of the nature and extent of genetic polymorphism in different populations of the species collected from different parts of the Indian coast. These genotypes were analysed with both RAPD and RFLP. A detailed population study on *Rhizophora* species was done earlier [10] and the parentage of a *Rhizophora* hybrid found in India has been established using RAPD markers and RFLP analysis of the mitochondrial genome using maternal DNA specific probes [11].

TABLE II. EXTENT OF GENETIC POLYMORPHISM BASED ON RAPD PROFILES

Species	No. of populations analysed	No. of plants	No. of primers	No. of ampli-fication products	Per cent polymor-phism
Acanthus ilicifolius	8	48	18	86	7.3
Bruguiera cylindrica	1	16	7	58	10.6
Ceriops decandra	1	15	6	66	11.6
Excoecaria agallocha	6	36	16	149	65.0
Lumnitzera racemosa	1	17	8	48	11.2
Rhizophora apiculata	1	20	12	67	11.1
Rhizophora mucronata	1	25	12	75	12.3
Avicennia marina	10	200	17	172	76.7
A. officinalis	1	20	16	115	32.3
A. alba	1	12	12	111	37.8
Heretiera fomes	1	12	12	96	23.5
Xylocarpus granatum	1	15	16	82	12.6
Sesuvium portulacastrum	1	10	12	46	9.6
Sueda maritima	1	15	10	74	12.6
Nypa fruitcans	1	10	10	112	14.3
Salicornia bracheata	1	20	12	96	12.6

2. MATERIALS AND METHODS

DNA isolation and PCR amplification for RAPD were carried out according to the methods detailed earlier [12]. DNA extraction for all the investigated species were done using the leaf tissues, except for *Rhizophora*, for which petals were used. Southern transfer and hybridization were done according to the methods in Sambrook *et al.* [13]. Statistical interpretation was carried out using Nei's index [14] and unweighted pair group method with arithmetic averages (UPGMA) analysis [15].

3. RESULTS

3.1. *Acanthus ilicifolius*

Acanthus is the only genus in the family Acanthaceae that has representatives in mangrove communities. *A. ilicifolius* is typically a low woody herb that owes its ability for vegetative spread to its reclining stems so that it forms large patches by vegetative means. The epidermal glands in the leaves secretes salt which enable the plant to thrive in water with above normal salt concentration.

Plant samples from eight distinct populations along the eastern (Pichavaram, Karaikal, Pondicherry, Muthupet & Bhitarakanika) and western (Goa, Ratnagiri & Calicut) coasts of India were collected and assayed for RAPD and RFLP markers. Six randomly selected genotypes from each population were included in the present study. Geographical location and physical charactereistics of these populations are given in Table I. The samples were initially screened for intra-population variation using RAPD markers generated by 15-18 primers. In total, 69 to 78 RAPD loci were scored in each population. Pair wise comparison within the populations using dissimilarity index and clustering by UPGMA was carried out. Based on this preliminary anlysis using RAPD markers, one representative genotype from each population was selected. Thirteen random primers amplified 73 fragments, of which 22 were polymorphic in at least one pair wise comparison. The RAPD profiles of one representative sample from each population is given in Fig. 1. Two primers gave monomorphic banding pattern. Other primers produced a number of fragments common to all the genotypes. However, population specific bands were also observed enabling identification of the genotype from individual populations with one or two primers [16].

For RFLP analysis within populations, the same genotypes were digested with three restriction enzymes, *Eco* RI, *Eco*RV and *Hind*III, and hybridised with two PCR amplified fragments and three pUC18/*Pst*I genomic DNA library clones of *A. ilicifolius* as probes. Polymorphism in RFLP within populations ranged narrowly between 3.1 (Pichavaram) and 9.1 (Karaikal) per cent. Since the level of intra-population variation was less, five out of the eight populations were selected for inter-population study. One genotype from each of the five populations was selected at random and the DNA was digested with the three enzymes used for intra-population study and probed with the same probes. A total of 96 RFLP fragment were observed and of which 44 were polymorphic. The relationship between the populations was analysed using the 22 polymorphic RAPDs and 44 RFLPs. The two eastern populations formed a distinct cluster separated at a dissimilarity coefficient (DC) of 0.28, and the three populations from the weatern coast were separated from this cluster at a DC of 0.36, 0.39 and 0.42 [16].

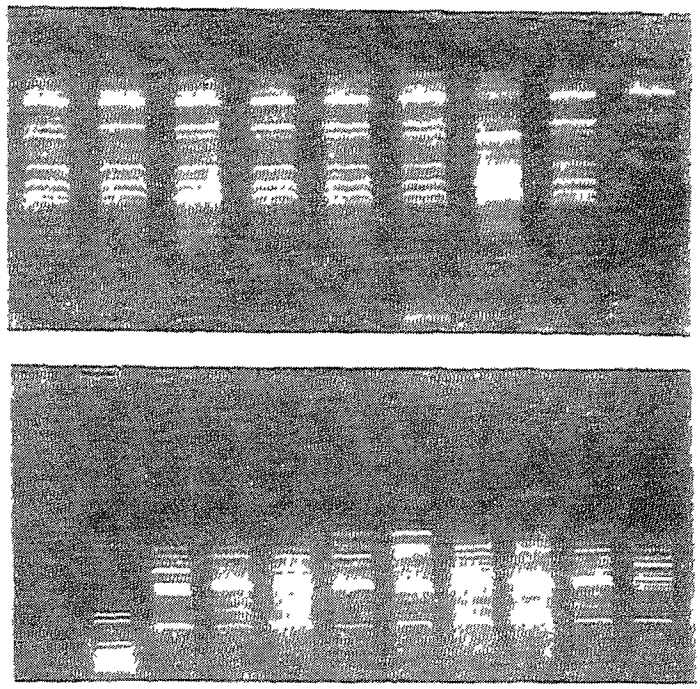


FIG. 1. Interpopulation RAPD profile in Acanthus ilicifolius

3.2. *Excoecaria agallocha*

Among the 5000 odd species of the family Euphorbiaceae, only *Excoecaria agallocha* is found in the mangrove habitats. This species is generally considered as a mangrove associate with landward habitation where there is low salinity. However, this species was also observed well inside the mangrove forest along with *Avicennia marina* which is a widely saline tolerant mangrove species. It is a dioecioous species and thus cross-fertilization is the rule.

Six different populations of this species (Pichavaram, Pondicherry, Bhitarakanika, Adyar, Ratnagiri & Goa) were sampled to assess the extent of genetic polymorphism using RAPD markers. The geographical location and physical characteristics of the populations are given in Table I. Six genotypes from each population were selected at random and leaf samples were collected for the present study. Total genomic DNA was isolated and a single primer assay was done with 16 primers. Out of 149 amplified DNA fragments, 97 were polymorphic (65%) in at least one pair-wise comparison. One genotype from each population was selected at random and RFLP anlaysis was done in 15 enzyme-probe combinations using the genomic clones from other mangrove species like *Avicennia marina* as probes. Seventy-four per cent of the RFLPs was found be polymorphic across the six populations. The high degree of polymorphism encountered in this species could be attributed to its outbreeding nature.

Male and female plants from each population were subjected to RAPD analysis using 20 primers to find out whether the polymorphism was dependant on sexual differences. No discrete differences were observed in the amplification products of male and female plants amplified by as many as sixteen primers. Although differences in the profiles were observed for the other four primers, in all, about 92% of the amplified fragments were common to both male and female genotypes.

In addition, the usefulness of two primer assay in genetic analysis of mangroves was examined in *E. agallocha.* Six primers were assayed individually, and then in all possible combinations of two primers. It was observed that the two primer assay products were the simple addition of those observed in the two corresponding single primer assays. Neither additional bands nor loss of bands were observed in the two primer assay. However, additional numbers of primers and combinations have to be done before reaching any firm conclusion.

3.3. *Avicennia* spp

Avicennia L. is a pantropical, exclusive mangrove genus of eight species, occupying diverse mangrove habitats. The genus was initially included in the family Verbenaceae but its plaement as a separate family, Avicenniaceae, is now generally accepted. We have almost no knowledge of intra-specific variations in any of the *Avicennia* species based on non-morphological characters like biochemical and molecular markers. Therefore, the nature and extent of intra-specific variation was analysed in three species of *Avicennia, A marina, A. officinalis and A. alba,* and the inter-specific relationship between them was established using DNA markers (RAPD and RFLP).

Ten ecogeographically distinct locations, along the eastern (Gulf of Mannar, Muthupet, Pichavaram, Ennore, Coringa & Bhitarkanika) and western coast (Calicut, Goa, Ratnagiri & Bombay) of India were chosen (Table I) and twenty individuals of *A. marina* from each location, eighteen individuals of *A. officinalis* from Pichavaram and ten individuals of *A. alba* from Coringa were selected at random for molecular analysis. Intra-population analysis in *A. marina* revealed varying degrees of genetic polymorphism in their RAPD profiles. Polymorphism was highest in the Muthupet population (38.9%) followed by Bhitarkanika, Pichavaram, Goa, Gulf of Mannar and Coringa populations. The Ennore population exhibited the lowest polymorphism of 17.8 per cent. This population represents back water mangrove comprised of patches of *A. marina.* When clustering was done, all the populations formed two distinct clusters with varying numbers of individuals.

Therefore, for inter-population study, one genotype from each cluster of individual populations (i.e. two individuals from each population and twenty individuals in total) was selected at random, and subjected to RAPD and RFLP analyses. Variation in RAPDs was studied using fifteen primers. For RFLP analysis DNA from the various genotypes was digested with *Eco*RI and *Hin*dIII and probed with twelve genomic clones. The genomic clones were obtained from a *Pst*I/pUC genomic library of *A. marina* and A. *officinalis.* In total 172 RAPDs were amplified, out of which 132 were polymorphic (76.7%). RFLP analysis of the same genotypes showed 66 per cent polymorphism. This indicated higher genetic divergence between the populations than within the populations [17]. The cluster diagram for inter-population relationship is shown in Fig. 2. RAPD analysis of 18 genotypes of *A. officinalis* and 11 genotypes of *A. alba* revealed 32.3 and 37.8 per cent polymorphism respectively. Clustering based on dissimilarity coefficient showed two distinct clusters in both the species. The cluster diagram for *A. officinalis* is shown in Fig. 2.

For inter-specific study, one individual from the major cluster of *A. officinalis* (Pichavaram), *A. alba* (Coringa) and *A. marina* (Pichavaram) was randomly selected to represent the respective species. RAPD analysis of the three samples was done using all the primers used for the inter-population study in *A. marina.* For RFLP analysis, the samples were digested with *Eco*RI, *Hind*III, *Taq*I and *Sau*3AI and probed with 24 genomic clones. Genetic distance as measured by similarity percentage in RAPDs and RFLPs showed that the widely

distributed *A. marina* is more closely related to *A. alba* than to A. *officinalis*, as shown in Fig 2

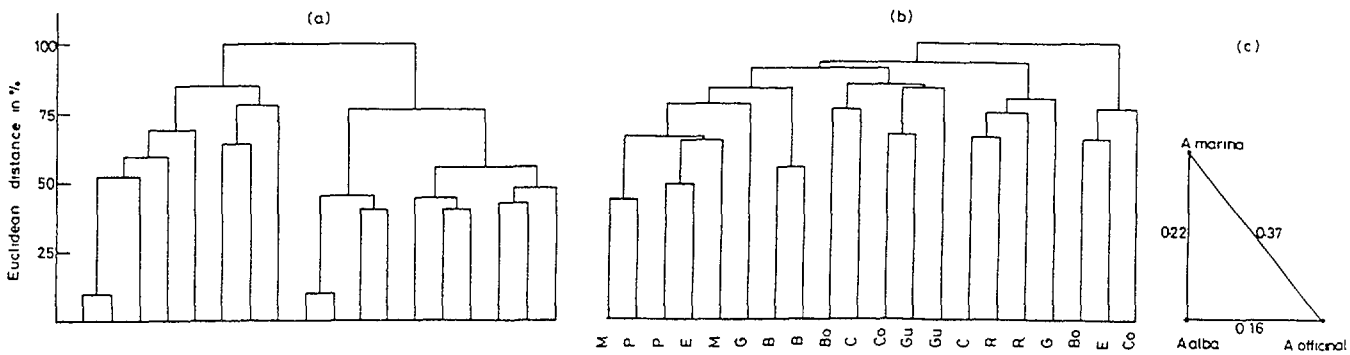


FIG 2 a) Intra-population dendrogram constructed based on 31 variable RAPDs of 18 genotypes of A officinalis collected from Pichavaram population; b) Inter-population dendrogram constructed based on 132 RAPDs and 62 RFLPs of A marina The populations sampled were from Gulf of Mannar, Gm, Muthupet, M, Pichavaram, Ennore, E; Coringa, Co, Bhitarkanika, B, Calicut, C, Goa, Go, Ratnagiri, R, and Bombay, Bo © Figure drawn on arbitrary scale based on genetic distance showing the inter-specific relationship among A marina, A officinalis and A alba

3 4 *Rhizophora*

There are six species and three putative hybrids reported in *Rhizophora* [18] *Rhizophora* x*lamarckii* Montr is one of the putative hybrids between *R. apiculata* and *R. stylosa* Griff found in New Caledonia, Papua Guinea and Queensland [18,19,20] Initially, the *Rhizophora* hybrid found in the Pichavaram Mangrove Forest has been identified as *R.* x*lamarckii* [21,22] based on morphological features and co-occurence of the putative parents However, parentage of this hybrid was disputed for the reasons that *R. stylosa* does not occur in the Pichavaram Mangrove Forest and morphological features of the *R.* x*lamarckii* described by Duke and Bunt [20] differed with that of the hybrid present in this area [23] In this regard, the molecular markers can establish not only the parentage, but also the maternal parent, which is almost impossible with morphological markers

In the present study, RAPD patterns of genomic DNA (using 25 primers) and RFLPs of mitochondrial DNA (digested with three enzymes) using a mitochondrial genome specific probe, atp 6 (from maize), were used to identify the parentage of the *Rhizophora* hybrid The combined RAPD profiles of two species, *Rhizophora apiculata* and *R mucronata* had 96 5 per cent similarity with that of the hybrid, indicating that these two species are the probable parents RFLPs for the mitochondrial genome were readily observed, and the RFLP profile of

R. apiculata was exactly similar to that of the hybrid, establishing its maternal status for the hybrid [11].

From the results, it was concluded that the *Rhizophora* hybrid found in the Pichavaram Mangrove Forest is a cross between *R. apiculata* and *R. mucronata* and that the former was the female parent of the hybrid under study. Based on the results, using the RAPD fingerprints of the *Rhizophora* species and the hybrid established in the present study as a reference, the DNA isolated from the self-sown seedlings of *Rhizophora* are being analysed to identify the hybrid genotypes for *in situ* conservation. RAPD analysis which is simple, fast and easy to perform on large numbers of samples was found to be more useful than other marker systems for this purpose.

4. SPECIES DIVERSITY

The origin and distribution of mangrove is well documented. They occupy the latitudinal range between 32N and 38S, and are found mostly on the eastern boarder of continents. This restricted distribution is due to sensitivity of mangroves to frost and cold temperature [24]. The two distinct patterns of geological distribution is said to have been caused by continental rearrangements and plate tectonics.

Mangroves consist mostly of taxonomically unrelated plant species, nevertheless have similar physiognomy, and physiological and structural adaptation to the essential characteristics of the habitat as a result of convergent evolution [25]. As already mentioned, the eastern coast of India (including the Andaman and Nicobar islands) accounts for about 82% of the mangrove area in this country. The number of species found in major mangrove formations of the eastern coast also varies to a greater extent. For example, while greater numbers of diverse species are present in places like Pichvaram, Bitarkanika and Sunderbans, relatively few species are found in Coringa, and pure stands of *Avicennia* are observed in Muthupet (Tamil Nadu).

Therefore, understanding the intra-specific diversity in individual species (as described above for a few species), the physical distribution range of the species, their association with mangroves and mangrove associates, the genetic relatedness among these taxonomically related species and the causative genetic and environmental factors limiting the species richness are essential to design an effective long-term conservation strategy for the mangrove ecosystem as a whole. Yet, the study will not be complete without looking into the micro-organisms and the faunal community of the ecosystem. Studies on these aspects will be reported elsewhere.

To start with, a detailed study using molecular marker was taken up to establish the genetic relatedness among the major mangove genera. Initially 21 different species belonging to fourteen genera were analysed. It was observed that different species of the same genus did not show much variation, and formed discrete clusters. Therefore, the study was extended to include additional genera by utilising only one species in a genus. In total, 22 genera including three monocots (two mangrove genera and one non-mangrove genus) two mangrove epiphytes, 4 mangrove associates and *Lycopersicon* as an out group were analysed. The genotypes were analysed for RAPDs using fifteen primers and for RFLPs in 21 probe-enzyme combinations. Details of the species relationships shall be reported at a later date.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

The present analyses were carried out as a part of our ongoing anticipatory research programme to consolidate genotypes capable of adapting to the ever-fluctuating climatic conditions in the coastal region. The results have undoubtedly helped us to understand the nature and extent of diversity in the Indian mangrove species both at the intra-population and inter-population levels. This has helped in selecting priority areas and sampling strategies for conservation and improvement. Molecular marker based analysis has also contributed immensely in elucidating confusing taxonomic status and establishing phylogenetic trends in mangroves. These analyses have opened up new avenues of research in utilising potential genetic material for combating present and anticipated problems in the coastal region. Isolation and characterisation of salt tolerant genes and their transfer to economically important species of the coastal region is among the utmost priorities.

ACKNOWLEDGEMENT

We are grateful to Prof. M.S. Swaminathan for his valuable suggestions. The financial assistance from Department of Biotechnology, Govt. of India; International Atomic Energy Agency and International Plant Genetic Resources Institute is gratefully acknowledged.

REFERENCES

[1] SNEDAKER, S.C. Mangroves: their value and perpetuation, Nat. and Res. **14** (1978) 6-13.

[2] BLASCO, F. "Outlines of ecology, botany and forestry of the mangals of the Indian subcontinent", Ecosystems of the World, vol. 1. Wet Coastal Ecosystems (V.J. CHAPMAN, Ed.) Elsevier Scientific, Amsterdam, (1977) 242-258 pp.

[3] ANONYMOUS. Status Report Mangroves in India, Ministry of Environ. and Forest, Govt. of India (1987).

[4] UNTAWALE, A.G. Mangroves of India: Present status and multiple use practices, UNDP/UNESCO Regional Mangrove Project, (1985) 67 pp.

[5] POWELL, W. "Plant genomes, gene markers and linkage maps", Biotechnology and Crop Improvement in Asia, (MOSS, J.P., Ed.) ICRISAT, India (1992) 297-322 pp.

[6] BROWN, A.H.D. Enzyme polymorphism in plant populations, Theor. Pop. Bio. **15** (1979) 1-42.

[7] BECKMANN, J.S., and SOLLER, M. Restriction fragment length polymorphism in plant genetic improvement of agricultural species, Euphytica **35** (1986) 35-44.

[8] TANKSLEY, S.D., YOUNG, N.D., PATERSON, A.H., and BONIERBALE, M.W. RFLP mapping in plant breeding: New tools for an old science, Bio/Technology **7** (1989) 257-264.

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

[10] SUBRAMANIAN, N. Studies on genetic diversity in some *Rhizophora* species. Ph.D thesis, Centre for Advanced Studies in Botany, University of Madras, Madras, India (1993).

[11] PARANI, M., RAO, C.S., MATHAN, N., ANURATHA, C.S., NARAYANAN, K.K., and PARIDA, A. Molecular Phylogeny of Mangroves III. Parentage analysis of a *Rhizophora* hybrid using Random Amplified Polymorphic DNA and Restriction Fragment Length Polymorphism Markers, Aquatic Botany (in press) (1997).

[12] PARANI, M., and PARIDA, A. Allozyme and RAPD based genetic polymorphism analysis in *Tylophora indica*. J. Biochem and Biotech, 7 (1997) 29-33.
[13] SAMBROOK, J., FRITSCH, E.F. and MANIATIS, T. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Lab Press.U.S.A (1989)
[14] NEI, M. and LI, W. A mathematical model for studying genetic variation in terms of restriction endo nucleases, Proc Natl Acad Sci, USA **76** (1979) 765269.
[15] SNEATH, P.H.A., and SOKAL, R. Numerical Taxonomy, Freeman, San Fransisco, CA. (1973).
[16] LAKSHMI, M., RAJALAKSHMI, S., PARANI, M., ANURATHA, C.S., and PARIDA, A. Molecular Phylogeny of Mangroves I. Use of Molecular Markers in Assesing Intra-specific Genetic Variability in the Mangrove Species *Acanthus ilicifolius* (Acanthaceae). Theor. Appl. Genet. **94** (1997) 1121-1127.
[17] PARANI, M., LAKSHMI, M., ELANGO, S., NIVEDITA, R., ANURATHA, C.S., and PARIDA, A. Molecular Phylogeny of Mangroves II. Intra- and Inter-specific variation in *Avicennia* revealed by RAPD and RFLP markers, Genome **40** (1997) 487-495.
[18] TOMLINSON, P.B. The Botany of Mangroves, Cambridge University Press. Cambridge, (1986) 317-360 pp.
[19] BANERJEE, L.K. New distributional records of some mangrove species from Orissa coast, J. Bombay Nat. Hist. Soc. **83** (1986) 271-273.
[20] DUKE, N.C., and BUNT, J.S. The genus *Rhizophora* (Rhizophoraceae) in North eastern Australia, Aust. J. Bot. **27** (1979) 657-658.
[21] LAKSHMANAN, K.K., and RAJESWARI, M. Distribution of organic contents in the leaves of *Rhizophora* species, Proc. 70th Ind. Sci. Cong. 3, India (1983).
[22] MUNIYANDI, K., and NATARAJAN, R. Incidence of seedling formation in *Rhizophora lamarckii* Montr. in Pichavaram mangrove, Tamil Nadu, India. J. Bombay Nat. Hist. Soc. **82** (1985) 441-442.
[23] KATHIRESAN, K. *Rhizophora annanmalai*: A new species of mangroves. Environment and Ecology 13 (1995) 240-241.
[24] WALTER, H. Climate. In V.J.Chapman (ed.) Ecosystems of the World: Wet Coastal Ecosystems, Vol. I. Elsevier, New York 1977 54-56 pp.
[25] YANNEY EWUSIE, Elements of Tropical Ecology: Heinemann Educ. Books. London. 1980.

IMPROVEMENT OF BANANA THROUGH BIOTECHNOLOGY AND MUTATION BREEDING

P.S. RAO, T.R. GANAPATHI, V.A. BAPAT, V.M. KULKARNI, P. SUPRASANNA
Nuclear Agriculture and Biotechnology Division,
Bhabha Atomic Research Centre,
Trombay, Mumbai, India

XA9848416

Abstract

Protocols were standardized for *in vitro* propagation of several elite and diverse banana accessions using shoot tip explants. Tissue culture raised plants were field planted at multiple locations. Studies were undertaken for the induction of mutations using multiple shoot cultures of six selected cultivars, Shreemanti (AAA), Basrai (AAA), Lal Kela (AAA), Rasthali (AAB), Karibale Monthan (ABB) and a wild diploid (BB). These shoot cultures were irradiated at different doses of gamma rays (0-100 Gy) and subcultured thrice (up to M_1V_3) to separate chimeras, followed by induction of rooting (M_1V_4). In general, the rate of multiplication had a negative association with the dose of gamma rays. Enhanced multiplication of shoots was noticed at lower doses. The proliferation of shoots was arrested beyond 50 Gy and a dose of 70 Gy was completely lethal for all the genotypes studied. The rooted plantlets were hardened in the green house and in the early stages of field growth, a few cholorophyll and morphological variants have been noticed. Preliminary studies have been made with DNA samples of different varieties and variants for DNA quality and restriction digestion. Studies are underway to characterize these using PCR based methods.

1. INTRODUCTION

Banana is an economically profitable fruit crop with a large in-country consumption and a considerable export potential. The world production of banana is about 68.5 million tonnes but only 7 million tonnes are in world export trade. In India, banana is grown on 3.96 million hectares with a total production of 10.4 million tonnes. Several cultivars of banana are cultivated in the country among which, Dwarf Cavendish and Robusta are predominantly grown because of higher yields, resistance to strong winds and short cropping duration besides yielding a good profit margin [1]. The fruit has good nutritive value with high carbohydrates (22.2%), fibre (0.84%) and protein (1.1%) with less fat (0.2%) and water (75.7%). Banana is a long duration crop of about one and a half years. Edible triploid bananas are vegetatively propagated by suckers as viable seeds are generally not produced. The production of suckers varies in different genotypes ranging from five to ten plants per year. Crop productivity and maturity is dependent on the size and age of suckers and uneven maturity extends the duration by three to four months. Suckers may also carry soil nematodes or disease causing organisms, such as bunchy top virus or leaf spot, thereby affecting the production of the crop considerably.

In vitro propagation of banana through shoot tip cultures is useful in the rapid multiplication of desirable disease free clones. In addition, careful selection and updating of mother plants results in improved crop yield [2]. New and effective means of propagating bananas would be advantageous over the conventional use of sucker material, for germplasm maintenance, exchange and transportation [3,4].

Mutation breeding *in vitro* is a powerful tool for the induction and selection of desirable mutants which can be utilized in banana improvement [5]. Mutagenic treatments on banana plants have been reported and *in vivo* sucker material has proved to be less effective and yielded low

mutagenized material for further screening [6,7]. On the contrary, *in vitro* mutagenesis employing multiple shoot cultures has several advantages: a high shoot multiplication ratio resulting in efficient chimera separation; a drastic reduction in time and space requirements; ease of treatment with mutagens and subsequent leaching off of chemical mutagens; and optional facility for *in vitro* selection against various stresses.

The genetic system of *Musa* is complicated owing to the inherent problems of sterility, heterozygosity and polyploidy in most of the clones. Asexual behavior is often an inseparable barrier in using cross breeding as a tool for genetic improvement. Mutation induction, using physical as well as chemical mutagens, is useful for the induction of genetic variability. DNA fingerprinting is a highly sensitive technique, useful for the detection and characterization of genetic variability and also for identification of various genotypes, at the molecular level [8]. Notably, the polymorphism measured through this technique is free from any environmental interference and can be repeated with precision.

At the Bhabha Atomic Research Centre (BARC), cell and tissue cultures of different cultivars of banana have been established and an *in vitro* system for propagation via shoot tip culture has been standardized. Technical know-how for the production of banana plants through micropropagation has been transferred to user agencies which have undertaken commercial production of plants for distribution to farmers. Linkages have been established with agricultural research stations and universities for conducting field trials of tissue culture developed plants. Protocols have been optimized for encapsulation of shoot tips for the production of synthetic seeds [9] and for low cost methods for *in vitro* propagation [10]. The radiosensitivity of *in vitro* shoot cultures has been documented in different genotypes. Induction and establishment of embryogenic cell and callus cultures and conversion of somatic embryos into plantlets has been achieved. Different plant growth regulators, dessication, encapsulation and stress treatments are being investigated to enhance the percentage of plant conversion.

Efforts have been made for collecting germplasm from different geographical regions and, to date, the germplasm collection comprises 26 accessions (Ambat Velchi, Ardhapuri, Bheemkel, Basrai, Chakkera Kela, Elakkibale, Grand Naine, Hajari, Hatti, Karpoorvalli, Karibale Monthan, Lal Kela, Lokhandi, Monthan, Mutheli, Nendran, Poovan, Rajeli, Rasthali, Robusta, Safed Velchi, Shreemanti, Trikoni, Williams and two Wild diploids).

In this article, results on *in vitro* propagation of different cultivars, effects of gamma irradiation on *in vitro* cultures of six selected genotypes and molecular studies for fingerprinting, conducted as a part of the Coordinated Research Project (CRP) of International Atomic Energy Agency (IAEA), Vienna, on the 'Mutation breeding and related biotechnologies for banana improvement, are described.

2. MATERIALS AND METHODS

Plant materials for starting tissue cultures were collected from known and reliable sources for the varieties listed in Table I.

2.1. Establishment of shoot tip cultures and plant regeneration

Shoot tips were isolated from suckers by removing the sheathing leaf bases and were established in liquid MS medium [11] supplemented with 5 mg/L benzyl adenine (BA). After three weeks these were transferred to semi-solid medium comprising of 2 mg/L BA and 30

mg/L adenine sulphate and 3% sucrose. Each shoot tip produced three to five shoots within a span of three to four weeks.

TABLE I. GENOMIC STATUS OF VARIOUS BANANA CULTIVARS

No.	Cultivar	Genome	No.	Cultivar	Genome
1.	Ambat Velchi	AAB	14.	Mutheli	AAB
2.	Ardhapuri	AAA	15.	Nendran	AAB
3.	Basrai	AAA	16.	Poovan	AAB
4.	Chakkerakela	AAA	17.	Rajeli	AAB
5.	Elakkibale	AB	18.	Rasthali	AAB
6.	Grand Naine	AAA	19.	Robusta	AAA
7.	Hajari	-	20.	Safed Velchi	AB
8.	Hatti	-	21.	Shreemanti	AAA
9.	Karpuravalli	ABB	22.	Trikoni	-
10.	Karibale Monthan	ABB	23.	Williams	AAA
11.	Lal Kela	AAA	*24.	Bheemkel (Assam)	BB/BBB
12.	Lokhandi	-	*25.	*Ensete superbum* (Deccan)	-
13.	Monthan	ABB	*26.	Wild diploid (Karnataka)	BB

* Wild species, - not known

Multiplication of shoots was carried out by isolating individual shoots and subculturing those monthly on the same medium until a sufficient number of shoots were obtained. The individual shoots isolated from these multiple shoot cultures were used for various experiments. All the experiments were conducted under controlled conditions of light (1000 lux), temperature (25 $\pm$ 2°C) and relative humidity (65%). To regenerate complete plantlets, the shoots were isolated individually from multiple shoot cultures and transferred to MS medium with NAA (1 mg/L) + 0.1% activated charcoal. Well grown, rooted plantlets (7-9 cm in length) were carefully removed from the culture vessel and the roots were washed thoroughly in tap water to remove the traces of nutrients. The plantlets were transplanted in the greenhouse in polybags containing a 1:1 mixture of unsterilized garden soil and farm yard manure (FYM).

2.2. Irradiation experiments

From each of the six genotypes chosen, twelve individual shoots (3-4 cm) were cultured on half strength MS medium (agar 0.8%) and were then exposed to gamma rays in a ^{60}Cobalt irradiator at 0, 10, 20, 30, 40, 50, 60, 70, 80 and 100 Gy at 20 Gy/min. Subsequently, the leafy portion of these irradiated individual shoots was excised and the shoot tips alone were recultured on the fresh half strength MS medium. After 30 days of culture, the percentage of shoot survival was calculated from the number of shoots which re-exhibited growth. The data were analysed and linear regressions were worked out as per Singh and Choudhary [12].

Multiple shoot cultures (three to five cultures / treatment, each with four to six shoots) of all the genotypes were irradiated at 0, 10, 20, 30, 40, 50, 60, 70 and 100 Gy doses with gamma rays at 20 Gy/min. These were immediately subcultured (M_1V_0) onto multiplication medium. Further subculturing was performed at an interval of 30 days, up to M_1V_3, in order to separate chimeras. At every stage of subculture, the multiplication ratios (from previous stage

to next stage) were calculated by dividing the 'number of cultures after subculturing' by the 'number of cultures before subculturing'.

Individual shoots from M_1V_3 were isolated and cultured on the rooting medium (MS with NAA 1 mg/L) to obtain M_1V_4 population of rooted plantlets. After 45-50 days, the M_1V_4 plantlets were transferred to perforated polythene bags containing a 1:1 mixture of soil and FYM, and hardened in the green house for 3 months followed by field planting.

3. RESULTS AND DISCUSSION

3.1. Establishment of *in vitro* regeneration system

3.1.1. Establishment of shoot tip cultures

The fresh shoot tips produced two or three shoots in the span of about three to four weeks when split longitudinally into two halves and subsequently transferred to semi-solid MS medium containing BA (2mg/L) and adenine sulphate (30 mg/L). Upon subculturing these shoots, each one produced an additional four to six shoots in the subsequent subcultures. The average multiplication ratio was found to be about 5 for varieties such as Basrai, Shreemanti and Lal Kela, whereas Rasthali, Poovan and Karibale Monthan required a little higher concentration of BA in the multiplication medium to obtain the multiplication ratio of 5 (data not shown). This indicated genotypic variations in the case of the multiplication ratios *in vitro* and hence, whenever required, the protocols were modified to optimize *in vitro* regeneration.

3.1.2. Plantlets' formation and their establishment in the soil

Shoots showed considerable elongation on medium with 1 mg/L NAA and two to four roots with laterals were noticed within three weeks and the frequency of complete plantlet formation was nearly 100%. The plantlets were hardened in the green house for about two months and the survival percentage ranged between 95-100%. Subsequently the hardened plants were planted in the field.

3.1.3. Evaluation of micropropagated plants

To date, more than 20000 plants have been regenerated in test tubes and around 4500 plants were planted at multiple locations in the states of Maharashtra and Gujarat for field trials. In almost all the places, the tissue culture raised plants showed vigorous growth, early maturity and marginal increase in bunch weight with better quality fruits. In an experiment conducted at the Research and Development farm of Gujarat State Fertilizers Company, Baroda, the data suggested an early maturity by about six weeks and an increase in yield of 33 percent, in the tissue cultured plants of cv. Basrai when compared to the control.

3.2. Studies on *in vitro* mutagenesis

Banana cultivation is seriously hampered by viral and fungal diseases warranting an intensive programme for the diversification of existing clones and breeding superior cultivars. However, problems like parthenocarpy, heterozygosity and difficulties in obtaining viable seeds, have become limiting factors to conventional breeding. In this direction studies were undertaken for induction of mutations using gamma rays, in six elite cultivars: Shreemanti, Basrai, Lal Kela, Rasthali, Karibale Monthan, and a wild diploid from Karnataka state.

3.2.1. Selection of explant material

Use of established *in vitro* multiple shoots as the initiation material for mutagenic treatment was found to be better than *in vivo* suckers or freshly cultured shoot tips from field grown plants, since it saved much effort in the establishment of cultures and reduced the chances of contamination and the initial number of shoot tips irradiated.

3.2.2. *In vitro* multiplication of six selected cultivars for irradiation

In vitro multiple shoot cultures could be easily established from the shoot apices of all the genotypes used. However, multiplication ratios were influenced by the genomic composition. In general, Lal Kela (AAA) and the Wild Diploid (BB) clone exhibited higher multiplication ratios (4.0 and 4.4 respectively) whereas Rasthali (AAB) and Karibale Monthan (ABB) exhibited low multiplication ratios (2.5 and 1.8 respectively) (Table II).

3.2.3. Effect of irradiation on individual shoots

It was observed that the irradiated individual shoots could be scored with precision as live or dead, only after excision of the leafy portion. The linear regression coefficients of dose (Gy) on the survival percentage of shoots ranged between -0.86 to -0.75 (Fig. 1). The negative values of the regression coefficients indicated that shoot survival and irradiation had an inverse association. The lethal dose 50% (LD50%) ranged from 30 Gy (Rasthali, AAB) to 52 Gy (Lal Kela, AAA), with an average of about 40 Gy (Fig 1). Doses beyond 70 Gy were completely lethal. Hence in further experimentation 0 and 100 Gy doses were included as the standard and lethal controls respectively.

TABLE II. THE GENOTYPES USED FOR ^{60}CO GAMMA IRRADIATION

Name of genotype	Genomic background	Salient features
BASRAI	AAA	A clone from Cavendish Dwarf.
SHREEMANTI	AAA	A selection from Basrai with increased height and yield.
LAL KELA	AAA	Tall, highly preferred for taste and attractive red fruit skin.
RASTHALI	AAB	Tall, short fruits with fresh yellow coloured skin and delicious taste.
KARIBALE MONTHAN	ABB	Tall, keeping quality is the main advantage.
WILD DIPLOID	BB	A seeded clone from Karnataka State, is currently in cultivation because of tolerance to BBTV.

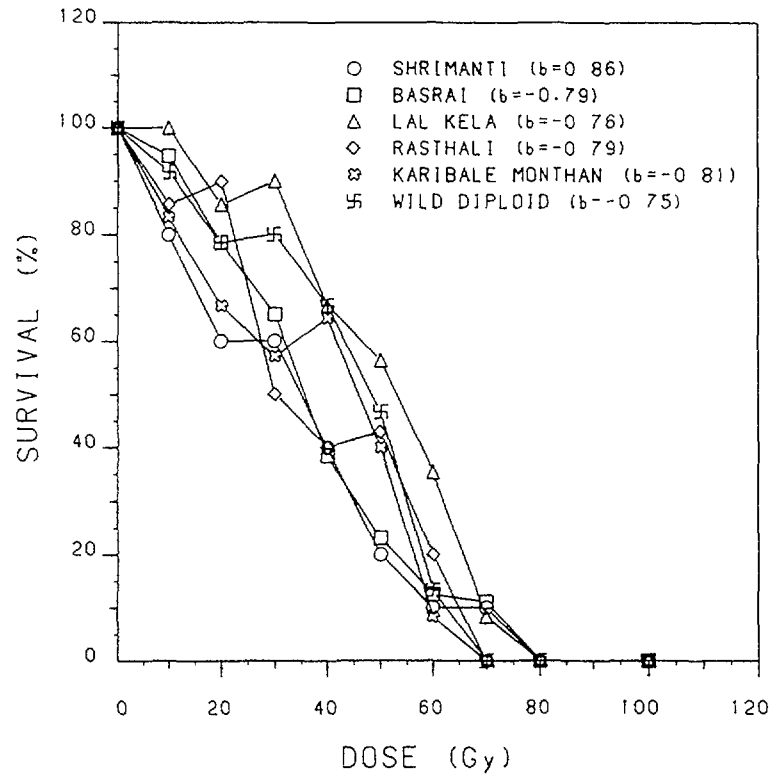


FIG 1. Effect of gamma irradiation on % survival of in vitro individual shoots of banana.

3 2 4 Effect of irradiation on multiple shoot cultures

In general, the shoot multiplication ratio decreased with increasing amount of irradiation; however, the data showed considerable variation (Table III). Furthermore, this variation increased with advancing subculture cycle (Fig. 2). Interestingly, the multiplication ratios were found to be enhanced at lower doses (Shrimanti and Rasthali, both at 10 and 20 Gy, and Basrai at 20 Gy) (Table III) The proliferation of the multiple shoots was arrested beyond 50 Gy except cv. Shrimanti, 60 Gy) and a dose of more than 70 Gy was lethal for all the genotypes studied At higher dose (50 Gy), cv Lal Kela exhibited numerous clusters of minute shoot buds.

Individual shoots of M_1V_4 populations could easily be rooted on medium with NAA (1 mg/L). Thick and dark coloured roots were noticed in the case of Shrimanti (10 Gy) (Fig. 3a). A shoot culture from cv Shrimanti exposed to 60 Gy was isolated which neither produced multiple shoots nor any roots, and where new leaves appeared very slowly (Fig 3b)

3 2 5 Field evaluation of M_1V_4

A total of 6300 rooted plantlets were generated from the *in vitro* irradiation experiments and hardened in the green house In the initial growth phase under field conditions, some plants of Shrimanti (50 Gy) expressed white spots on the first few leaves (Fig 3d) compared to control (Fig 3c) Leaf variants were also observed in the case of Karibale Monthan (20 Gy) (Fig 4b) Plants with a bilateral arrangement of leaves (traveler's palm-like) were noticed in case of Basrai (30 Gy) (Fig 4c) In cv Basrai (10 Gy), a single plant has flowered in less than 6 months (Fig 4d) These M_1V_4 plants are now being observed for their field performance and the variants will subsequently be re-evaluated for confirmation of the variations noticed

TABLE III. EFFECT OF ^{60}CO GAMMA IRRADIATION ON MULTIPLICATION RATIOS OF *IN VITRO* CULTURES OF BANANA

Multiplication ratio

Genotype	Generation	Dose (Gy)								
		0	10	20	30	40	50	60	70	100
SHREEMANTI	$M_1V_0 \rightarrow M_1V_1$	3.2	4.8	4.5	2.5	2.7	2.1	2.3	1.0	0.0
(AAA)	$M_1V_1 \rightarrow M_1V_2$	-	2.9	3.2	2.2	2.3	3.5	2.5	1.0	0.0
	$M_1V_2 \rightarrow M_1V_3$	4.0	3.8	5.8	3.9	5.3	4.6	5.5	0.0	0.0
	$M_1V_3 \rightarrow M_1V_4$	3.0	2.7	2.4	5.1	3.7	2.4	4.0	0.0	0.0
BASRAI	$M_1V_0 \rightarrow M_1V_1$	-	4.5	5.5	1.0	1.0	-	-	0.0	0.0
(AAA)	$M_1V_1 \rightarrow M_1V_2$	3.0	4.1	4.6	2.8	2.3	-	-	0.0	0.0
	$M_1V_2 \rightarrow M_1V_3$	4.8	4.7	5.1	4.4	4.1	-	-	0.0	0.0
	$M_1V_3 \rightarrow M_1V_4$	3.3	1.5	2.1	2.3	2.7	-	-	0.0	0.0
LAL KELA	$M_1V_0 \rightarrow M_1V_1$	4.0	3.4	2.9	3.3	1.6	3.8	1.0	1.0	0.0
(AAA)	$M_1V_1 \rightarrow M_1V_2$	-	3.5	2.5	2.3	1.9	2.8	0.0	0.0	0.0
	$M_1V_2 \rightarrow M_1V_3$	3.6	3.6	2.7	2.7	3.4	2.5	0.0	0.0	0.0
	$M_1V_3 \rightarrow M_1V_4$	2.6	1.4	2.3	3.4	5.6	3.0	0.0	0.0	0.0
RASTHALI	$M_1V_0 \rightarrow M_1V_1$	-	4.5	5.7	3.3	3.2	1.8	1.0	0.0	0.0
(AAB)	$M_1V_1 \rightarrow M_1V_2$	2.2	1.8	2.1	1.8	1.7	2.6	0.0	0.0	0.0
	$M_1V_2 \rightarrow M_1V_3$	2.1	2.0	4.5	1.3	1.5	6.0	0.0	0.0	0.0
	$M_1V_3 \rightarrow M_1V_4$	2.5	2.8	4.5	0.0	0.0	0.0	0.0	0.0	0.0
KARIBALE	$M_1V_0 \rightarrow M_1V_1$	1.8	3.7	2.2	3.2	1.0	1.2	1.0	0.0	0.0
MONTHAN	$M_1V_1 \rightarrow M_1V_2$	1.3	1.7	1.5	2.0	1.0	2.0	0.0	0.0	0.0
(ABB)	$M_1V_2 \rightarrow M_1V_3$	1.0	2.3	1.5	1.0	0.0	1.0	0.0	0.0	0.0
	$M_1V_3 \rightarrow M_1V_4$	1.0	2.9	1.1	1.0	0.0	0.0	0.0	0.0	0.0
WILD DIPLOID	$M_1V_0 \rightarrow M_1V_1$	3.5	4.0	3.3	2.5	1.0	0.0	0.0	0.0	0.0
(BB)	$M_1V_1 \rightarrow M_1V_2$	2.5	2.8	2.3	1.9	1.3	0.0	1.0	0.0	0.0
	$M_1V_2 \rightarrow M_1V_3$	4.0	3.2	2.7	3.3	1.0	0.0	1.0	0.0	0.0
	$M_1V_3 \rightarrow M_1V_4$	4.4	2.5	3.0	3.1	6.0	0.0	0.0	0.0	0.0

- contaminated, 0.0 necrosed.

In the present study, *in vitro* multiple shoot cultures of six banana cultivars belonging to different genomic groups (AAA, AAB, ABB and BB) were irradiated with gamma rays and it was observed that cultivars with a hybrid genome (AAB - Rasthali, ABB - Karibale Monthan) were more vulnerable to irradiation when compared to cultivars with a single genome (AAA - Lalkela, BB - Wild diploid).

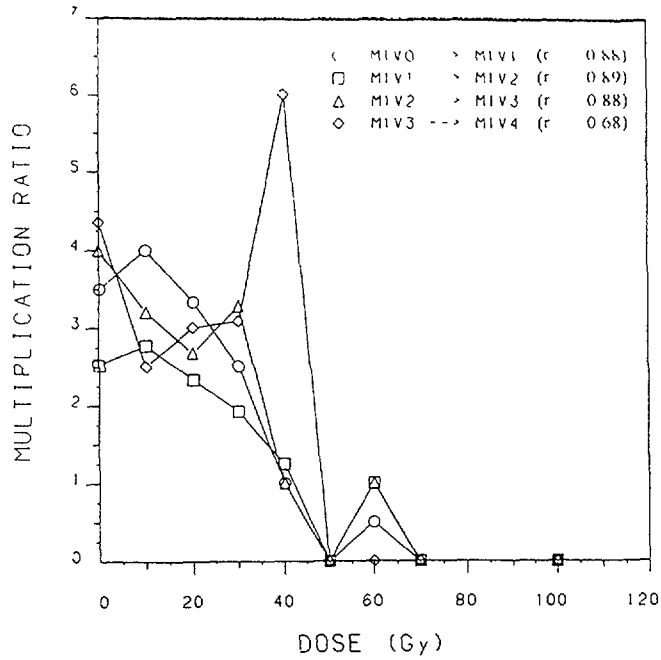


FIG. 2. Effect of gamma irradiation on multiplication ratio oversubcultures in Wild Diploid banana (BB).

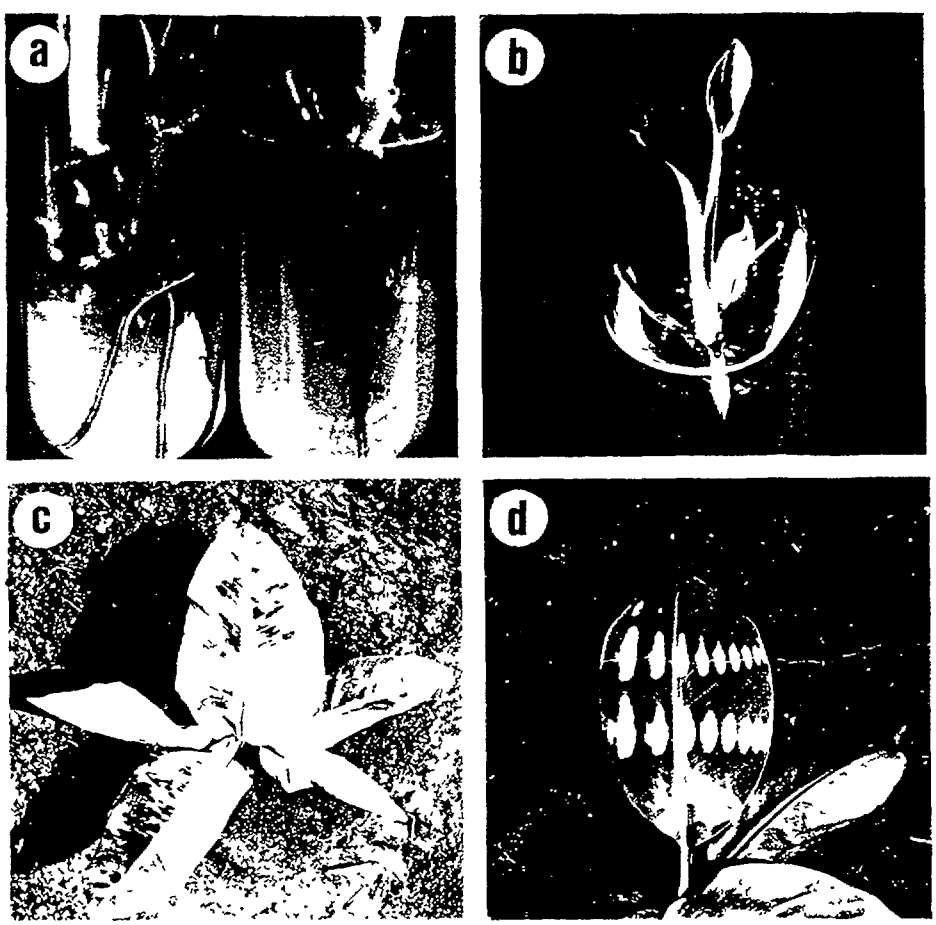


FIG. 3. Examples of phenotypic effects of irradiation a. left cv Shrimanti, control, normal roots, right, thick and dark roots with 10 Gy ;b. A shoot culture of cv. Shrimanti (60 GY) which produced neither multiple shoots nor roots; c Shrimanti (control), d White spots on the leaf (cv Shrimanti, 50 Gy)

A high multiplication ratio in both AAA and BB genomes and a lower ratio in the hybrid (AAB and ABB) genomes indicated that the genotype had an important role to play in the proliferation of *in vitro* shoots Vuylsteke [2] has summarized results of the effect of genotype on multiplication rates and clearly highlighted differential multiplication rates in different genotypes In our studies, the low multiplication ratios were mainly due to apical dominance of several individual shoots which strongly resisted production of multiples Certainly, the lower *in vitro* multiplication ratios may affect efficient separation of chimeric regions Therefore, it was felt that the multiple shoot cultures with at least moderately high multiplication ratios (3 0 or more) be established, otherwise subculturing of mutagenized populations would be required up to M_1V_6 or M_1V_7

FIG 4 a The split bunch observed in tissue culture derived plant of cv Rasthali, b left Control right, Leaf variant in cv Karibale Monthan (20 Gy), c Bilateral leaf arrangement (Traveler's palm like) in cv Basrai (30 Gy), d Early flowering plant (6 months) in cv Basrai (10 Gy)

It is necessary to understand the lethal effects on individual shoots before irradiating multiple shoots. Excision of the leafy portion of the shoots, after irradiation allowed precise scoring of live and dead cultures. This was because of unmasking of dead shoot tips by surrounding leaves, which if not excised, remained green for a longer period and thus dead shoots could wrongly be scored as live. The lethal effects on individual shoots were in conformity with Broertjes [13] who reported that mutation frequency increased with increasing dose (linearly with X or gamma rays) but survival and regeneration capacity decreased with increasing dose. In the case of banana, Yang *et al.* [14] obtained a very high correlation coefficient (0.97) between dosage and explant death for irradiation of dissected shoot tips, but the coefficient was only 0.18 when intact shoot tips were used.

In the present study, LD 50% was found to be 40 Gy on an average. Similar observations have been reported by Yang *et al.* [14] and Smith *et al.* [15]. It was observed that cvs. with hybrid genomes (Rasthali, AAB and Karibale Monthan, ABB) were more vulnerable whereas cvs. with single genome (Lal Kela, AAA and Wild Diploid, BB) were less sensitive to irradiation. Novak *et al.* [7] also have reported genotypic differences for the LD50%, 20 Gy being the least for diploid (AA) clone SH-3142.

The effect of irradiation on multiple shoots was characterized by a drop in multiplication ratios, 70 Gy and more being totally lethal. Yang *et al* [14] were of a similar opinion but Silayoi *et al.* [16] have reported only 30 Gy as a lethal dose. Such variation could be due to genotypic differences. Two additional features were: enhancement in multiplication ratio at lower dose (10 and 20 Gy) than was also observed by De Guzaman *et al.* [17] and Siddiqui *et al.* [18], and occurrence of a large amount of variation in multiplication ratios (see Fig. 2). Thus the *in vitro* proliferation of irradiated multiple shoots appears to be complex, affected by one or more of several factors: irradiation dose, imbalanced distribution of chimeric and non chimeric sectors resulting in differential growth rates, genotype, apical dominance, subculture cycle and its duration, and somaclonal variation.

In our studies, we compared different extraction methods for obtaining pure and high molecular weight DNA and subsequently, the total genomic DNAs were extracted from nine different genotyes (including a wild diploid and four samples obtained from field grown plants in the mutation breeding trial) using a modified CTAB (Cetyl trimethyl ammonium bromide) method. The DNA samples could be digested with *Eco*RI and *Bam*HI indicating that these are amenable to restriction digestion. A set of ten oligonucleotide DNA primers has recently been received from the IAEA through Dr. P.M. Gresshoff. These will be tested for their ability to elicit DNA polymorphism.

4. CONCLUSIONS AND PROSPECTS

Our studies have led to the collection of banana germplasm from different geographical regions and the development of *in vitro* systems for use in the selection of desirable clones through mutation breeding. Field observations have been made on certain morphological variants and evaluation of these is underway. Molecular studies have been initiated in the aspects of DNA isolation, restriction digestion and RAPD analysis.

The prospects of using biotechnological approaches like protoplast culture and fusion, and genetic transformation with novel, useful genes for banana improvement are promising [19]. Efficient *in vitro* systems like shoot tip cultures and embryogenic cultures are being worked out for genetic transformation, selection and regeneration of transformants. With the advent of particle bombardment using the biolistic system and the *Agrobacterium* mediated

transformation method, it is now possible to develop transgenic plants and studies have already shown success in obtaining transgenic plants of banana [20,21]. Since banana is the common man's fruit and also a staple food for the developing countries, major efforts should be directed for its overall improvement, especially for incorporating useful genes for: disease resistance; insect/pest resistance; enhanced fruit quality and shelf life; and pharmaceutically important proteins and vaccines. In coming years, it is hoped that biotechnological tools will be successfully employed in the genetic improvement of banana.

REFERENCES

[1] SINGH, H.P. "Country paper report on banana and plantain - India", Banana and Plantain R & D in Asia and the Pacific, INIBAP (1990) , pp. 161-185.

[2] VUYLESTEKE, D.R. Shoot tip culture for the propagation, conservation and exchange of *Musa* germplasm, Practical Manual for Handling Crop Germplasm *in vitro* **2**, IBPGR, Rome, Italy (1989).

[3] RAO, P.S., GANAPATHI T.R., SUPRASANNA, P., and BAPAT, V.A. Encapsulated shoot tips of banana: a new propagation and delivery system, InfoMusa **2** (1993) : 4 - 5.

[4] RAO, P.S. Biotechnological approaches for increasing plant productivity. AsiaTech. **3** (1994) 32-36.

[5] NOVAK, F.J., BRUNNER, H., AFZA, R., MORPURGO, R., UPADHYAY, R.K., VAN DUREN M., SACCHI, M., HAWZ, J.S., KHATRI, A., KAHL, G., KAEMMER, D., RAMSER, J., and WEISING, K. Improvement of *Musa* through biotechnology and mutation breeding. In: Biotechnology Applications for Banana and Plantain Improvement Proc. Of the Workshop. INIBAP (1993), pp 143-158.

[6] Personal communication, Banana Research Station (Jalgaon), Mahatma Phule Agricultural University, Rahuri, Maharashtra, India.

[7] NOVAK, F.J., AFZA, R., VAN DUREN M., and OMAR, M.S. Mutation induction by gamma irradiation of *in vitro* cultured shoot-tips of banana and plantain (*Musa* cvs), Tropical Agriculture (Trinidad) **67** (1990) 21-28.

[8] MORELL, M.K., PEAKALL, R., APPELS, R., PRESTON, L.R., and LLOYD, H.L. DNA profiling techniques for plant variety identification, Australian J. Experimental Agriculture **35** (1995) 807-819.

[9] GANAPATHI, T.R., SUPRASANNA, P., BAPAT, V.A., and RAO, P.S. Propagation of banana through encapsulated shoot tips, Plant Cell Rep. **11** (1992) 571-575.

[10] GANAPATHI, T.R., MOHAN, J.S.S., SUPRASANNA, P., BAPAT, V.A. and RAO, P.S. A low-cost strategy for in vitro propagation of banana, Current Science **68** (1995) 646-649.

[11] MURASHIGE, T., and SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue culture, Physiol. Plant. **15** (1962) 473-497.

[12] SINGH, R.K., and CHAUDHARY, B.D. Biometrical Methods in Quantitaive Genetic Analysis, Kalyani Publishers, New Delhi. (1985) 318 pp.

[13] BROERTJES, C. "Induced mutation breeding in asexually propagated plants" Manual on Mutation Breeding, IInd edition, Technical Reports Series No. 119, IAEA, Vienna (1977) 159-166.

[14] YANG, P., LI, C., WEI, F., HUANG, W., ZHENG, J., HUANG, D., HE, R., GUO, J., HUANG, X., CHEN, B., and LIN, W. "Radiation mutation induction in 'China Tianbao' banana", *In vitro* Mutation Breeding of Bananas and Plantains, IAEA, Vienna (1995) 17-24.

[15] SMITH, M.K., HAMILL, S.D., and LANGDON, P.W. "*In vitro* mutation breeding for the development of bananas with resistance to Race 4, Fusarium wilt (*Fusarium oxysporum* f.sp.*cubense*)", *In vitro* Mutation Breeding of Bananas and Plantains, IAEA, Vienna (1995) 37-44.

[16] SILAYOI, B., WANICHKUL, K., KEAWSOMPONG, S., and SARADULDHAT, P. "Study on mutation breeding of banana, 'Kluai Khai'", *In vitro* Mutation Breeding of Bananas and Plantains, IAEA, Vienna (1995) 65-77.

[17] DE GUZMAN, E.V., DEL ROSARIO, A.G., and PAGCALIWAGAN, P.C. "Production of mutants by irradiation of *in vitro*-cultured tissues of coconut and banana and their mass propagation by the tissue culture technique", Induced Mutations in Vegetatively Propagated Plants II, IAEA, Vienna (1982) 113-138.

[18] SIDDIQUI, S.H., KHATRI, A., KHAN, I.A., NIZAMANI, G., and KHAN, R. "Improvement of banana (*Musa* cvs.) through *in vitro* culture techniques and induced mutations", *In vitro* Mutation Breeding of Bananas and Plantains, IAEA (1995) 25-30.

[19] HUGGAN, R. Are bananas catching up?, Biotech. Develop. Monitor **14** (1993) 14-16.

[20] SAGI, L., PANIS, B., REMY, S., SCHOOFS, H., DE SMET, K., SWENNEN, R., and BRUNO, P.A.C. Genetic transformation of banana and plantain *(Musa* spp.) via particle bombardent, Bio/Technology **13** (1995) 481-485.

[21] MAY, G.D., AFZA, R., MASON, H.S., WIECKO, A., NOVAK, F.J., and ARNTZEN, C.J. Generation of transgenic banana (*Musa acuminata* L.) plants via *Agrobacterium* mediated transformation, Bio/Techn. **13** (1995) 486-492.

THE USE OF PCR TECHNIQUES TO DETECT GENETIC VARIATIONS IN CASSAVA (*MANIHOT ESCULENTA* L. CRANTZ): MINISATELLITE AND RAPD ANALYSIS

XA9848417

N. PAWLICKI, R.S. SANGWAN, B. SANGWAN-NORREEL
Université de Picardie Jules Verne,
Amiens, France

N. KOFFI KONAN
ILTAB, Plan Division-MCR7, Scripps Research Institute,
La Jolla, California,
United States of America

Abstract

Cassava is an important tuber crop grown in the tropical and subtropical regions. Recently, we developed protocols for efficient somatic embryogenesis using zygotic embryos and nodal axillary meristems in order to reduce the genotype effect. Thereafter flow cytophotometry and randomly amplified polymorphic DNA (RAPD) markers were used to assess the ploidy level and the genetic fidelity of cassava plants regenerated by somatic embryogenesis. No change in the ploidy level of the regenerated plants was observed in comparison with the control plants. In the same way, monomorphic profiles of RAPD were obtained for the different cassava plants regenerated by somatic embryogenesis. The genetic analysis of calli showed only a few differences. Using two pairs of heterologous microsatellite primers developed in a wild African grass, a monomorphic pattern was also detected. Moreover, cultivars of different origins were also analysed using these PCR techniques. Our data from RAPD and mircosatellite analyses suggested that these techniques can be efficiently used to detect genetic variations in cassava.

1. INTRODUTION

Cassava (*Manihot esculenta* L. Crantz), one of the major sources of food in Africa [1], is generally propagated vegetatively, but efficient *in vitro* regeneration systems have recently been developed in order to improve cultivars of cassava via genetic engineering. Somatic embryogenesis is a routine way to regenerate shoots from various explants [2,3,4], but regeneration is often described as genotype-dependent. Recently, we developed protocols for efficient somatic embryogeneiss using mature cotyledons and organogenesis using zygotic embryos and nodal axillary shoot meristems [5,6], to minimise the genotype dependence. However, because of somaclonal variations induced by *in vitro* cultures described in other species [7,8], it is important to verify the genetic stability of *in vitro* regenerated plants in comparison with the mother plant.

Genetic variations can be expressed by variations in the ploidy levels, by structural changes in chromosomes, by the loss of one or several chromosomes, or by mutations at the gene level. Alterations in ploidy levels can be detected by karyotypic analyses of metaphase chromosomes [9] or by flow cytometry analysis [10].

However, chromosomal changes and DNA variations do not always reveal alterations in individual genes. Isozyme analysis can be used as a method to estimate genetic diversity of micropropagated plants but within cassava, low polymorphism is detected, and the technique is

not yet reproducible [11,12,13]. A precise determination of changes in a particular gene sequence resulting from tissue culture can be obtained by restriction fragment length polymorphism (RFLP) analysis [14,15,16]. However, this method is time consuming (five to six days are necessary to undertake the analysis) and is limited to the gene sequence used as the probe. The random amplified polymorphic DNA (RAPD) analysis, developed by Williams *et al.* [17], is a recent method that can be used to verify the genetic integrity of plants regenerated by *in vitro* cultures. Advantages of this method are the technical simplicity and the speed of analysis. Single oligonucleotide primers of arbitrary sequences can be used to detect RAPD sequences by the polymerase chain reaction (PCR).

In cassava, as almost no molecular genetic knowledge existed prior to this study, we were interested in verifying the ability of the DNA markers to detect and characterise genetic variation, induced either *in vitro* or naturally, in this important crop plant. We also studied the ploidy level of *in vitro* regenerated cassava plants and investigated whether DNA molecular differences existed within the cassava plants regenerated by somatic embryogenesis, when compared to the parental lines.

2. MATERIALS AND METHODS

2.1. Plant material

In vitro plants were regenerated by somatic embryogenesis from mature cotyledons according to the method described by Konan *et al.* [5]. The regenerated plants were individually indexed, and micropropagated by culturing nodes on an MS medium [18] supplemented with 3% sucrose. Plants were chosen at random for the ploidy and RAPD analyses. Callus culture were also initiated from internodes of the regenerated plants on an MS medium containing 1 mg/L BA, 0.1 mg/l NAA and 3% sucrose. Cassava plants, obtained from vegetative propagation of nodes, were kept in the greenhouse and used as controls.

2.2. Flow cytophotometry

For flow cytophotometry, leaves isolated from cassava plants regenerated by somatic embryogenesis and from greenhouse-grown plants, were chopped with petunia leaves (internal reference) using razor blades, following the procedure of Brown *et al.* [10]. The nuclei were isolated using 0.5 mL of a citric acid (0.1 M) solution containing 0.5% Tween 20. The samples were filtered through a 30 mm nylon gauze, and their volume adjusted to 1 mL. After an incubation at room temperature for 20-30 minutes, nuclei were centrifuged (92500 rpm, 5 min.) and resuspended in 0.2 mL of the citric acid / Tween 20 solution to which was added 1 mL of 0.4 M Na_2HPO_4 solution, pH 9.0 containing 5 mL/L DAPI (4',6-diamidino-2-phenylindole) for 15 minutes. Flow cytophotometry was performed using a Partec CAII flow cytometer (Maison-Alfort, France) and 5 x 10^3 to 10^4 nuclei were analysed per experiment. The distribution of nuclei fluorescence is represented as a histogram of DNA content in arbitrary units.

2.3. DNA amplifications and RAPD conditions

Genomic DNA was extracted from leaf explants of the control plants, each of the *in vitro* regenerated plants, and the calli, according to the method of Roy *et al.* [19] using hexadecyltrimethyl ammonium bromide (CTAB). The DNA was dissolved in TE buffer (10 mM Tris-Cl, 0.1 mM EDTA, pH 8.0) to a concentration of 100 ng/mL measured by a Hitachi spectrophotometer at a wavelength of 260 nm.

Twenty arbitrary decamer oligonucleotide primers (Kit OPK, Bioprobe, France) were used for RAPD analysis (Table I). Amplification reactions were carried out in 25 µl volumes containing: 75 mM Tris-Cl, pH 9.0; 20 mM $(NH_4)_2SO_4$; 0.01% Tween 20; 1.5 mM $MgCl_2$; 0.2 mM dNTPs (Eurogentec, Belgium); 0.5 mM primer; 1 unit Taq polymerase (Eurogentec); and 100 ng genomic DNA. The reaction mixture was overlaid with 50 µl mineral oil (Sigma), and the amplification reactions were perfomed using the Thermojet apparatus (Eurogentec) programmed for 4 min denaturation at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min annealing at 35°C, 2 min extension at 72°C, and a final extension cycle at 72°C for 6 min. The amplification products were analysed by electrophoresis in 2% agarose gels in TBE buffer at 2.5 V/cm, and detected under UV light after staining with ethidium bromide (0.5 mg/ml). All amplification reactions were repeated at least twice.

In addition to these RAPD primers, two pairs of primers designed for microsatellites of a wild African grass ("7" and "10", Garnier *et al.*[20]) were used under similar conditions, but with a "touchdown" program, as described by Brown *et al.* [21]. Such a program allows the amplification of discrete bands under low specificity on heterologous DNA.

3. RESULTS AND DISCUSSION

3.1. DNA content analysis

After somatic embryogeneis, all regenerated cassava plants were phenotypically normal, and the number of chromosomes of the regenerated plants was the same as the control plants (2n=36). Nevertheless, ploidy levels of cassava plants regenerated by somatic embryogenesis were studied by the analysis of nuclear DNA contents using the flow cytofluorometry technique. In the histogram, presented in Fig.1, the peaks 1 and 3, corresponding to cassava, indicate the existence of two populations of nuclei of differentiated cells, giving the position G0/G1 that is an indication of the 2C DNA content of these cells. In a population of 5000 to 10000 nuclei that have been analysed per treatment, 95%of the nuclei were at GO/G1 phase. Moreover, the absence of a shoulder on the peaks indicated a homogenous population of the nuclei in G0/G1, showing an absence of aneuploid cells.

During the analyses, petunia leaves were used as an internal reference (Fig 1., peak 2). The knowledge of the DNA content corresponding to the 2C value of petunia (2CX=2.85 pg of DNA per nucleus) allowed the estimation of the DNA content of the cassava leaf nuclei at 1.65 pg for stages G0/G1. This value appears to be similar (1.43 to 1.72 pg/2C for seventeen different cassava cultivars) to those reported by Arumuganathan and Earle [22].

TABLE I. SEQUENCE OF THE PRIMERS IN THE STUDY

Primer Number	Sequence	Primer Number	Sequence
OPK1	CATTCGAGCC	OPK11	AATGCCCCAG
OPK2	GTCTCCGCAA	OPK12	TGGCCCTCAC
OPK3	CCAGCTTAGG	OPK13	GGTTGTACCC
OPK4	CCGCCCAAAC	OPK14	CCCGCTACAC
OPK5	TCTGTCGAGG	OPK15	CTCCTGCCAA
OPK6	CACCTTTCCC	OPK16	GAGCGTCGAA
OPK7	AGCGAGCAAG	OPK17	CCCAGCTGTG
OPK8	GAACACTGGG	OPK18	CCTAGTCGAG
OPK9	CCCTACCGAC	OPK19	CACAGGCGGA
OPK10	GTGCAACGTG	OPK20	GTGTCGCGGAG

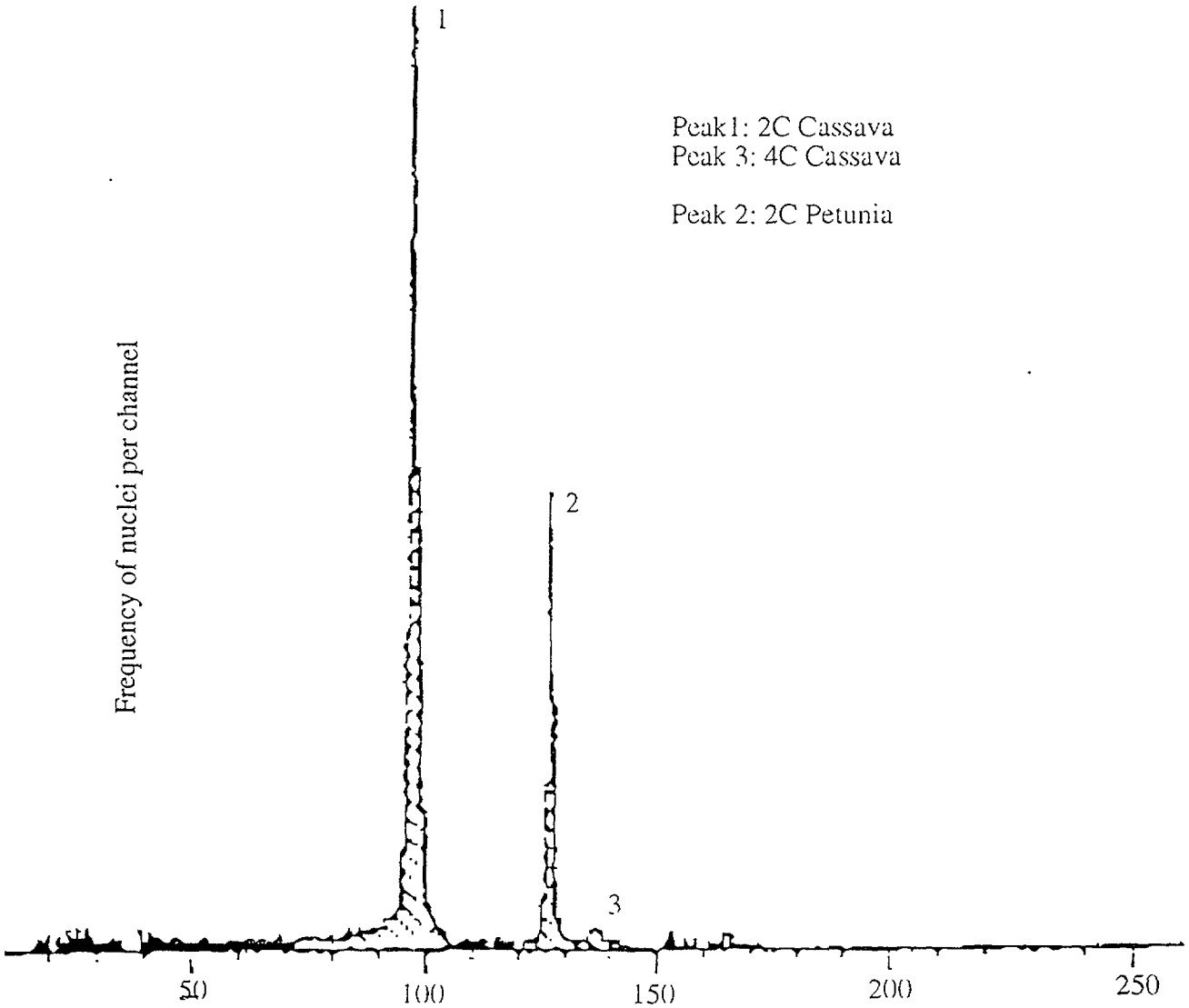


FIG.1. Histogram (logarithmic scale from the PARTEC CAII) of nuclear DNA from cassava (peaks 1 and 3) and petunia leaves (peak 2). Cassava leaves were isolated from somatic embryogenesis regenerated plants.

3.2. RAPD and microsatellite analyses

In order to look for eventual genetic differences between the tissue-culture-derived plants and the control plants, RAPD amplifications were also carried out. Indeed, it has been shown [17] that the RAPD technique is a fast and sensitive technique to study genetic diversity in plants.

Twenty different primers of arbitrary sequence were used for this study. Of these 20 primers tested within an individual plant, RAPD profiles (Fig.2) could be detected for most of them. Four primers (OPK 4, OPK 6, OPK 8 and OPK 15) revealed patterns difficult to interpret and for primer OPK 7 no bands were detectable after the ethidium bromide fluorescence staining (data not shown). These primers were not used in further experiments. For the other primers, a number of clearly defined major fragments were amplified, often against a background of less strongly amplified minor fragments. The amplified DNA fragments ranged from 200 to 2300 base pairs (bp) is size. The number of intense amplification bands produced by each primer varied from two (e.g. primer OPK 20) to six (e.g. primer OPK 9 or 17). This number is lower than was detected by Marmey *et al.* [23] during their evaluation of cassava germ plasm collections by RAPD (five to 24 primers). In the same way, a low number of amplification bands (ranging from one to thirteen) were found in tomato [24] and in soybean [17].

Using different primers, we amplified DNA from seven regenerated cassava plants and from one control plant. The results are given in Fig. 3. for some of the primers tested. Whichever primer was used, a monomorphic pattern was obtained for the different plants. The analyses were repeated with other (>25) regenerated plants and similar results were always obtained, indicating no variations at the DNA level in regenerated cassava plants.

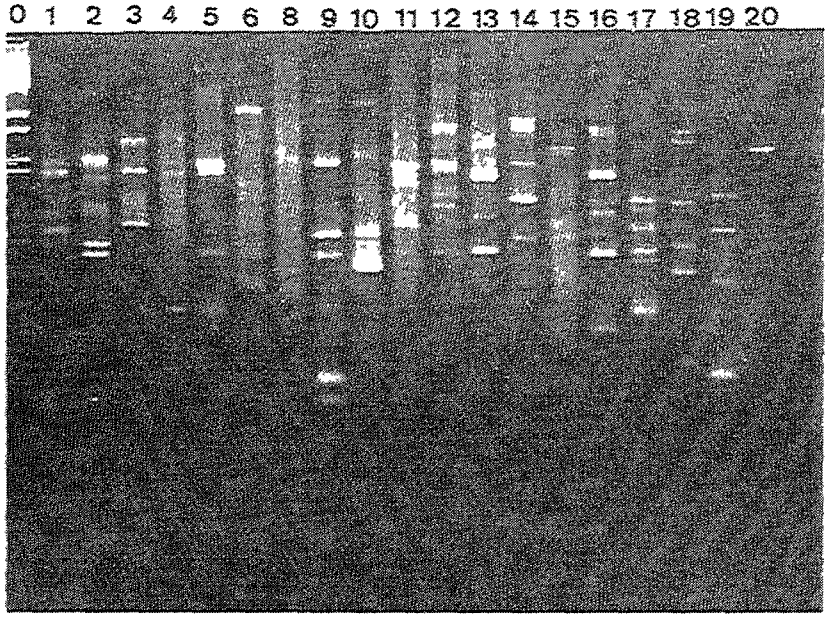


FIG. 2. Amplification products from a cassava control plant using nineteen different primers (Bioprobe, France); lane 0, size marker, λ DNA digested with BstEII.

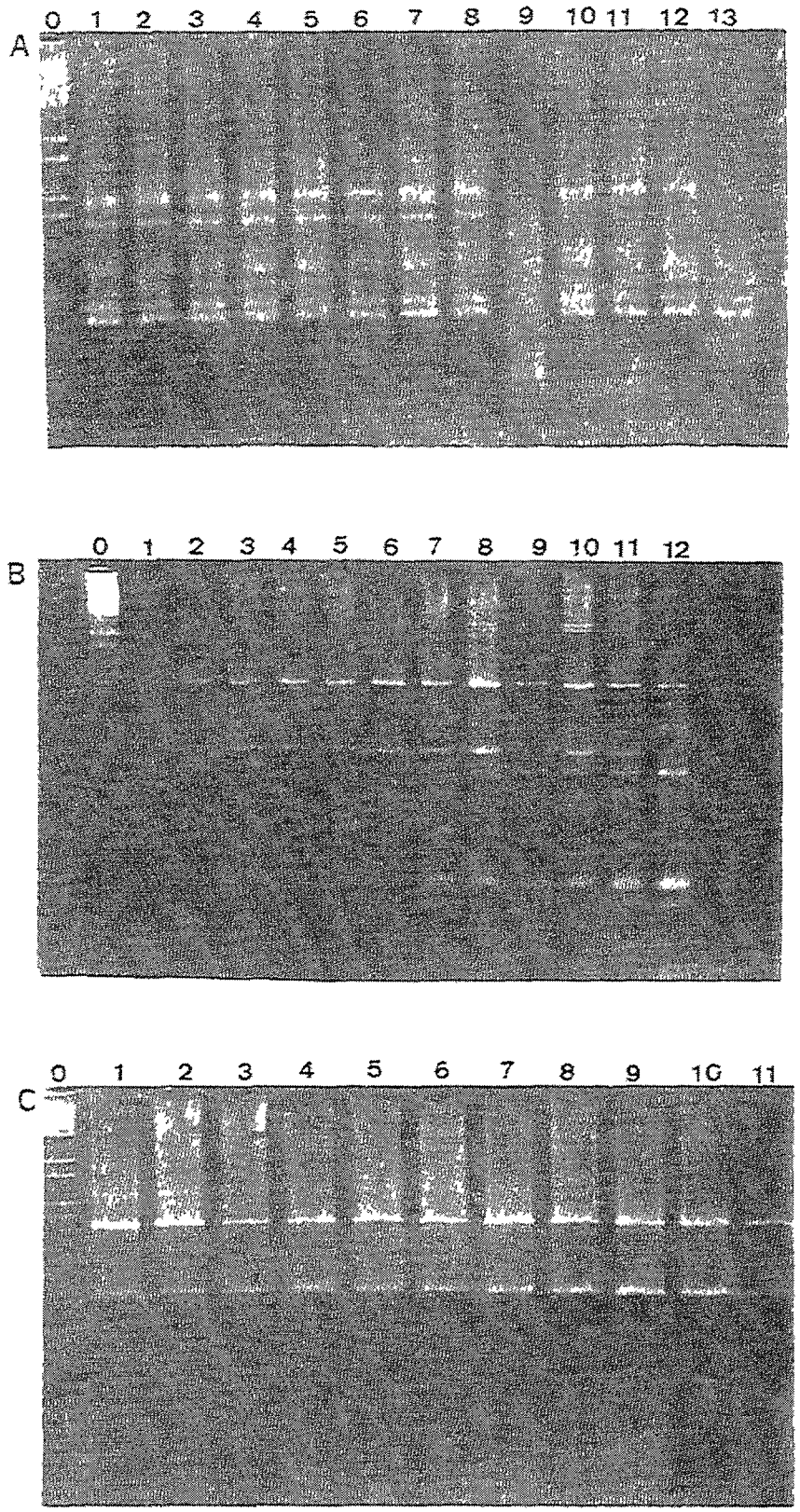


FIG. 3. RAPD profiles obtained with different primers: a) OPK2; lane 0, size marker, λ DNA digested with BstEII; lanes 1-7, regenerated cassava plants; lane 8, control plant; lanes 9-13, calli. b) OPK9; lane 0, size marker, λ DNA digested with BstEII; lanes 1-7, regenerated cassava plants; lane 8, control plant; lanes 9-12, calli. c) OPK13; lane 0, size marker, λ DNA digested with BstEII; lanes 1-6, regenerated cassava plants; lane 7, control plant; lanes 8-11, calli.

DNA was also isolated from calli obtained from internodes, and maintained on a medium supplemented with BA and NAA. Contrary to the results described by Brown *et al.* [25], we could amplify DNA from the calli without problems With primers OPK 5, OPK18 and OPK 19 (data not shown) and primers OPK 9 and OPK 13 no differences in the amplification bands were observed within the samples, or when compared with the regenerated plants (Fig. 3B, 3C). However, for primer OPK 2 (Fig. 3A), some samples showed differences For some of primers, the differences lie in the presence of new bands or in the absence of specific amplification products (lane 9), and for others in weaker signals for some markers (lane 13).

Hence, all tested primers revealed an identical number and size of amplification bands between the control plant and the cassava plants regenerated by somatic embryogenesis. Monomorphic probes were also described for the genetic analysis of micropropagated plants of *Populus* [26,27]. In that case, plants were propagated by axillary branching through a meristem culture, whereas in this case the explants underwent a differentiation to give somatic embryos Even if some reports described a genomic alteration in plants regenerated by somatic embryogenesis [25,28], our system of regeneration does not generate variation at the DNA level. With the heterologous microsatellite primers a monomorphic pattern was also detected (Fig. 4A and 4B).

In a study on date palm cultivar identification by RAPD, Corniquel and Mercier [29] did not detect variations in calli. In our experiments, a low level of polymorphism was found in only a few samples, the other samples not showing variations in the amplification bands between calli and regenerated plants. This polymorphism, detected only in a few calli, could be a reflection of somaclonal variation or could result from variation in the quality of DNA during the extraction.

In conclusion, our results show that the RAPD and microsatellite techniques could be used to determine the genetic variability of cassava plants.

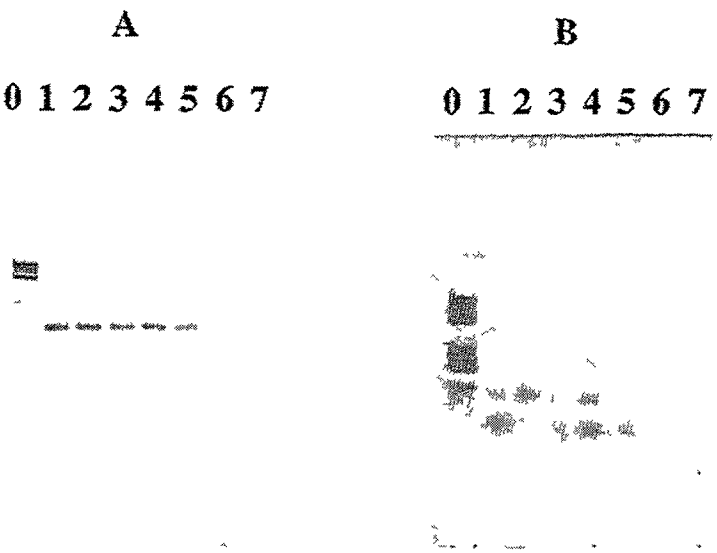


FIG. 4. Amplification products were detected by agarose analysis and BET staining. Lane 0, molecular weight marker, pBR322 digested with HaeIII; lane 1, cassava DNA control; lanes 2-5, four samples from regenerated plants. A) primers "7"; B) primers "10".

REFERENCES

[1] CORK, J.H. Science **218** (1982) 755-762.
[2] STAMP, J.A., and HENSHAW, G.G.Z. Pflanzenphysiol **105** (1982) 183-187.
[3] STAMP, J.A. An. Bot. **59** (1987) 451-459.
[4] SZABADOS, L., RODRIGO, H., and ROCA, W. Plant Cell Rep. **6** (1987) 248-51.
[5] KONAN, N.K., SANGWAN,R.S., and SANGWAN-NORREEL, B.S. Plant Cell Tis. and Organ Cult. **37** (1994) 91-102.
[6] KONAN, N.K., SANGWAN,R.S., and SANGWAN-NORREEL, B.S. Plant Breeding **113** (1994) 227-236.
[7] LARKIN, P.J., and SCOWCRAFT, W.R. Theor. Appl. Genet. **60** (1981) 197-214.
[8] DE KLERK, G.J. Acta. Bot. Neerl. **39** (1990) 129-144.
[9] KARP, A. Oxf. Surv. Plant Mol. Cell Biol. **7** (1991) 1-18.
[10] BROWN, S.C., DEVAUS, P., MARIE, D., BERGOUNIOUX, C., and PETIT, P.X. Biofutur **105** (1991) 2-16.
[11] HUSSAIN, A., BUSHUK, W., RAMIREZ, H., and ROCA, W. Seed Science and Technology **15** (1987) 19-21.
[12] RAMIREZ, H., HUSSAIN, A., ROCA, W., and BUSHUK, W. Euphytica **36** (1987) 39-48.
[13] LEFEVRE, F., and CHARRIER, A. Euphytica **66** (1993) 73-80.
[14] CHOWDHURY, M.K.U., and VASIL, I.K. Theor. Appl. Genet. **86** (1993) 181-188.
[15] VALLÉS, M.P., WANG, Z., MONTAVON, P., POTRYKUS, I., and SPANGENBERG, G. Plant Cell Rep. **12** (1993) 101-106.
[16] CLOUTIER, S., and LANDRY, B.S. *In Vitro* Cell Dev. Biol. **30** (1994) 32-39.
[17] WILLIAMS, J.G.K., KUBELIK, A.R., LIVAK, K.J., RAFALSKI, J.A., and Tingey, S.V. Nucl. Acids Res. **18** (1990) 6531-6535.
[18] MURASHIGE, T., and SKOOG, F. Physiol. Plant. **15** (1962) 473-497.
[19] ROY Theor. Appl. Genet. **85** (1992) 173.
[20] GARNIER, L., DURNAD, J., and DAJOZ, I. "Reproductive biology of the genus *Hyparhenia* in Lamto savannas : possible consequences on the structure and function of grass populations", Lamto, a tropical system (ABBADIE, L. and MENAUT, J.C., Eds.) Springer Verlag, 1996.
[21] BROWN, S.M., HOPKINS, M.S., MITCHELL, S.E., SENIOR, M.L., WANG, T.Y., DUNCAN, R.R., GONZALEZ-CANDELAS, F., and KRESOVITSCH, S. Theor. Appl. Genet. **93** (1996) 190-198.
[22] ARMUGANATHAN, K., and EARLE, E.D. Plant Mol. Biol. Rep **9** (1991) 208-218.
[23] MARMEY, P., BEECHING, J.R., HAMON, S., and CHARRIER, A. Euphytica **74** (1994) 203-209.
[24] FOOLAD, M.R., JONES, R.A., and RODRIGUEZ, R.L. Plant Cell Rep. **12** (1993) 293-297.
[25] BROWN, P.T.H., LANGE, F.D., KRANZ, E., and LÖRZ, H. Mol. Gen. Genet. **237** (1993) 311-317.
[26] CASTIGLIONE, S., WANG, G., DAMIANI, G., BANDI, C., BISSOFI, S., and SALA, F. Theor. Appl. Genet. **87** (1993) 54-59.
[27] RANI, V.R., PARIDA, A. and RAINA, S.N. Plant Cell Rep. **12** (1995) 101-106
[28] FLANDRE, F., and SANGWAN, R.S. PLANT CELL REP. (1996) submitted
[29] CORNIQUEL, B., and MERCIER, L. Plant Sci. **101** (1994) 163-172.

TOWARDS MAPPING THE *DIOSCOREA* GENOME

R. TERAUCHI*, G. KAHL
Biocentre, University of Frankfurt,
Frankfurt am Main, Germany

Abstract

Yams are important starchy tuber crops in (sub-)tropical countries of the world. Despite their importance in the regional economy, no serious attempt has been made toward their improvement. In order to obtain basic knowledge of the genetics of yams, we are trying to establish a linkage map of a wild yam species, *Dioscorea tokoro*. So far, six allozyme markers, six STMS markers and twenty AFLP markers have been identified. They will be used for linkage mapping of a population comprising 80 progeny obtained from a controlled cross.

1. INTRODUCTION

World yam production is about one-tenth of the total production of cassava and sweet potato [1]. Most of the yam crops are cultivated and consumed locally by subsistence farmers of tropical and subtropical regions around the world. Therefore, improvement of yam is important for enhancing sustainable agriculture in those regions where yams are cultivated. Despite its economical importance, so far no yam improvement has been carried out, apart from the selection of cultivars/clones currently under cultivation [2]. Breeding trials involving crossing experiments are not common [3,4], partly because of the dioecious nature, and partly because of the difficulty in crossing small flowers, of *Dioscorea*. Little information is available on the genetics of yams [4,5]. To improve our knowledge on yam genetics, we are presently constructing a linkage map of yam using a wild species, *Dioscorea tokoro* Makino.

2. MATERIALS AND METHODS

A wild yam species, *Dioscorea tokoro* Makino, belonging to the section *Stenophora* was chosen for linkage analysis. This species is widely distributed in East Asia, and has been used for population genetic studies [6]. It is a dioecious, diploid ($2n = 2x = 20$) species. Controlled crossing of *D. tokoro* is relatively easy. Under suitable conditions, a plant can grow from a seed to a mature plant within a year. These favorable features made *D. tokoro* an attractive model species for genetic studies on yams.

In 1995, we crossed, by hand pollination, a male (DT7) and female individual (DT5), both collected from the wild. This crossing gave rise to 200 seeds. Among them, 80 individuals were grown, and their sex, a single easily-scored morphological character, was determined when the flowers appeared. The leaves of the progeny were collected and used as source material for further genotyping. Parents used for the cross, as well as all the progeny, have been maintained through the years by vegetative propagation.

For linkage mapping, we have already developed six allozyme markers [6] and six STMS markers [7]. Variation in these genetic markers in the natural population of *D. tokoro* was studied. Additionally, we have started applying AFLP [8] markers, considering the high throughput of the method. Analysis of the segregation will be carried out by the pseudo-test cross strategy [9].

---

* Present address: Kyoto University, Kyoto, Japan.

3. RESULTS

Six allozyme markers (*Est, Got-1, Got-2, 6pgdh, Pgi* and *Skdh*) showed high genetic diversities (mean genetic diversity [10] was 0.38) [6]. Their segregation followed a simple diploid Mendelian fashion, although a segregation distortion was found in a locus *Pgi*. As expected, the STMS markers showed far larger genetic diversities (mean genetic diversity: 0.68) than the allozyme markers [7]. The higher genetic diversities imply a higher probability of the heterozygous state in *D. tokoro* genome. This high heterozygosity makes the application of the pseudo-test cross scheme [9] relevant for linkage mapping. These two subsets of markers are co-dominant markers, and should be useful for joining different family maps obtained with dominant markers, such as AFLP.

To further increase the number of useful genetic markers, we have started applying the AFLP [8] technique for yam genome mapping. First we have screened different primer combinations to see the levels of polymorphism detectable between the two parents used for crossing. Two commercially available kits for AFLP (AFLP analysis system I and II, recommended for organisms with genome sizes of 500 to 6000 Mbp/1C and of 100 to 500 Mbp/1C, respectively, Gibco BRL) were tested. These two systems are differentiated by whether the PCR primers have a 6-base selective anchor (system I), or 5-base selective anchor (system II) at their 3' ends. After applying 8 primer combinations for each kit, we have detected a total of 203 (52 polymorphic) fragments for kit I, and total of 432 fragments (81 polymorphic) fragments for kit II. Optimal results were obtained by kit II. This may reflect the relatively small genome size of yam species (555 Mbp/1C reported for *D. alata* [11]). The number of polymorphisms detected between the two parents is satisfactory, considering the possible number of primer combinations available.

Using two selected primer combinations (*Eco*RI-AC x *Mse*I-CAC and *Eco*RI-AA x *Mse*I-CTC), we studied the segregation of AFLP among progeny obtained by the controlled cross. A total of 26 polymorphic fragments were detected between the two parents (Table I). They could be grouped into five different categories in terms of presence or absence of the fragments in either parent and the segregation among progeny. Only the polymorphisms grouped in the categories 1 and 2 are useful for linkage mapping with the pseudo-test cross scheme [9]. Thus, 18 AFLP fragments remain for further linkage analysis. Considering the fact that two linkage maps are constructed, each corresponding to one parent, we should divide this number by two to represent the number of useful polymorphic fragments per linkage map. Therefore, we have 4.5 useful AFLP fragments for mapping per primer combination per parent, if the results obtained for the two experiments can be regarded as representative. This means only 25 AFLP experiments are needed to obtain 100 useful markers for linkage mapping, confirming a high throughput of the AFLP technique, and its applicability to obligately outcrossing species such as yams.

4. DISCUSSION

The co-linear arrangement of genomes (so-called synteny) has been reported in Gramineae and Fabaceae plants. There is a high probability that this is also the case among *Dioscorea* species. Therefore, the linkage map obtained for *D. tokoro* in this current study will be helpful to make linkage maps in other economically important yam species. To further facilitate the transfer of linkage information from *D. tokoro* to other species, selected polymorphic AFLP fragments would have to be cloned and sequenced. Development of

various new genetic markers have now made it possible to embark on the molecular-marker-based breeding in yams, a long neglected crop.

TABLE I. OCCURRENCES OF FIVE CATEGORIES OF AFLP'S OBSERVED AMONG PARENTS AND PROGENY OF *DIOSCOREA TOKORO*

Category	Female parent (DT5)	Male parent (DT7)	Segregation among progeny	No. of occurrences
1	+	-	Yes	10
2	-	+	Yes	8
3	+	+	Yes	5
4	+	-	No	2
5	-	+	No	1
Total No. of polymorphic fragments				26

(+: fragment present, -: fragment absent) ; The results for the two AFLP reactions (*Eco*RI-AC x *Mse*I-CAC and *Eco*RI-AA x *Mse*I-CTC) are pooled.

ACKNOWLEDGEMENTS

This research was supported by fellowship No. IV-1-7121-1028559 to RT from the Alexander-von-Humboldt Foundation, Germany, for which we express our cordial gratitude.

REFERENCES

[1] FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, Production Year Book, FAO, Rome (1987).

[2] HAHN, S.K., OSIRU, S.O., AKORODA, M.O., and OTOO, J.A. Yam production and its future prospects, Outlook on Agriculture **16** (1987) 105-110.

[3] SADIK, S., and OKEREKE, O.U. A new approach to improvement of yam, *Dioscorea rotundata*, Nature (London) **254** (1975) 134-135.

[4] ZOUNDJIHEKPON, J. First controlled progenies checked by isozyme markers in cultivated yams *Dioscorea cayenensis-rotundata*, Theor. Appl. Genet. **88** (1994) 1011-1016.

[5] MARTIN, F.W. Sex ratio and sex detemination in *Dioscorea*, Journal of Heredity **57** (1966) 95-99.

[6] TERAUCHI, R. Genetic diversity and population structure of *Dioscorea tokoro* Makino, a dioecious climber, Plant Species Biol. **5** (1990) 243-253.

[7] TERAUCHI, R., and KONUMA, A. Microsatellite polymorphism in *Dioscorea tokoro*, a wild yam species, Genome **37** (1994) 794-801.

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

[9] GRATTAPAGLIA, D., and SEDEROFF, R. Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross: Mapping strategy and RAPD markers, Genetics **137** (1994) 794-801.

[10] NEI, M. Analysis of gene diversity in subdivided populations, Proc. Natl. Acad. Sci., USA. **70** (1973) 3321-3323.

[11] ARUMUGANATHAN, K., and EARLE, E. D. Nuclear DNA content of some important plant species, Pl. Mol. Biol. Rep. **9** (1991) 208-218.

ABBREVIATIONS

AFLP	amplified fragment length polymorphism
ASAP	arbitrary signatures from amplification profiles
BAC	bacterial artificial chromosome
DAF	DNA amplification fingerprinting
DNA	deoxyribonucleic acid
LOD	log of the odds
MAS	marker assisted selection
PAGE	polyacrylamide electrophoresis
PCR	polymerase chain reaction
RAMPO	random amplified microsatellite polymorphisms
RAPD	randomly amplified polymorphic DNA
RCM	research co-ordination meeting
RFLP	restriction fragment length polymorphism
SCAR	sequence characterized amplified region
SSR	simple sequence repeat
STMS	sequence tagged microsatellite site
VNTR	variable number of tandem repeats
YAC	yeast artificial chromosome

LIST OF PARTICIPANTS

Burg, K.	Österreichisches Forschungszentrum Biotechnology Department A-2444 Seibersdorf, Austria
Gresshoff, P.	University of Tennessee Plant Molecular Genetics 269 Ellington Plant Sciences Building P.O. Box 1071 Knoxville, TN 37901-1071, USA
Kahl, G.	J.W. Goethe University, Biocentre Marie-Curie-Str. 9 60439 Frankfurt/Main, Germany
Lagoda, P.	CIRAD-BIOTROP Lab. AGETROP B.P. 5035 2477 Av. du Val de Montferrant 34032 Montpellier Cedex, France
Lavi, U.	Volcani Center Institute of Horticulture, ARO P.O. Box 6, Bet-Dagan, 50250, Israel
Leal, M.	National Center for Scientific Research Ave. 25 y 158, Cubanacán Apdo. 6880, Habana, Cuba
Low, F.C.	Rubber Research Institute of Malaysia 260 Jalan Ampang 50450 Kuala Lumpur, Malaysia
Mansvelt, L.	Agricultural Research Council, Infruitec Private Bag X5013 Stellenbosch 7599, South Africa
Parida, A.	M.S. Swaminathan Research Foundation Third Cross Road, Taramani, Institutional Area, Madras 600 113, India
Rao, P.S.	Biotechnology Division Bhabha Atomic Research Centre Government of India, Trombay Mumbai-400 085, India
Sangwan, R.S.	Univ. de Picardie Jules Verne Lab. Androgenese et Biotechnol. 33 Rue Saint-Leu 80029 Amines Cedex, France

Terauchi, R.
Kyoto University, Faculty of Science
Department of Botany
Laboratory of Plant Systematics
Kyoto 606-01, Japan

Weck, E.
(Scientific Secretary)
Plant Breeding and Genetics Section
Joint FAO/IAEA Division
International Atomic Energy Agency
Wagramerstrasse 5, P.O. Box 100
Vienna, A-1400, Austria