IAEA-TECDOC-1664

Physical Mapping Technologies for the Identification and Characterization of Mutated Genes Contributing to Crop Quality

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IAEA-TECDOC-1664

PHYSICAL MAPPING TECHNOLOGIES FOR THE IDENTIFICATION AND CHARACTERIZATION OF MUTATED GENES CONTRIBUTING TO CROP QUALITY

INTERNATIONAL ATOMIC ENERGY AGENCY
VIENNA, 2011

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FOREWORD

The improvement of quality traits in food and industrial crops is an important breeding objective for both developed and developing countries in order to add value to the crop and thereby increasing farmers' income. It has been well established that the application of mutagens can be a very important approach for manipulating many crop characteristics including quality. While mutation induction using nuclear techniques such as gamma irradiation is a power tool in generating new genotypes with favourable alleles for improving crop quality in plant breeding, a more thorough understanding of gene expression, gene interactions, and physical location will improve ability to manipulate and control genes, and directly lead to crop improvement. Physical mapping technologies, molecular markers and molecular cytogenetic techniques are tools available with the potential to enhance the ability to tag genes and gene complexes to facilitate the selection of desirable genotypes in breeding programmes, including those based on mutation breeding.

This Coordinated Research Project (CRP) on 'Physical Mapping Technologies for the Identification and Characterization of Mutated Genes Contributing to Crop Quality' was conducted under the overall IAEA project objective of 'Identification, Characterization and Transfer of Mutated Genes'. The specific objectives of the CRP were to assist Member States in accelerating crop breeding programmes through the application of physical mapping and complementary genomic approaches, and the characterization and utilization of induced mutants for improvement of crop quality.

The IAEA-TECDOC describes the success obtained in the application of molecular cytology, molecular markers, physical mapping and mutation technologies since the inception of the CRP in 2003. The CRP also resulted in two book chapters, 35 peer reviewed papers, 25 conference proceedings, one PhD thesis, and 22 published abstracts. In addition, thirteen sequences were submitted to the worldwide Genbank.The CRP was initiated by S. Nielen, continued by M. Miranda, both formerly of the Joint FAO/IAEA Division. The IAEA Officer responsible for this IAEA-TECDOC was Y. Lokko.

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SUMMARY

1. INTRODUCTION

Physical and molecular mapping techniques are tools that allow identification of landmarks on DNA including genes and provide new opportunities for the rapid advancement of marker-assisted selection in breeding programs including the and are applicable to gene manipulation for crop improvement. It has been well established that the utilization of nuclear techniques such as gamma irradiation is a very important approach for manipulating many value-added traits for improved crop production on a world-wide scale (for example, improved nutritional quality and abiotic stress tolerance). Understanding of gene expression, gene interaction, and physical location will improve the ability to manipulate and control genes, and directly lead to crop improvement.

Previously, various aneuploid, genetic, and mutation stocks (spontaneous and induced by irradiation or chemical treatments) have accelerated crop production. Molecular markers provide an unlimited number of polymorphisms for diverse crops enabling the construction of genetic and physical maps of phenotypic characters. On the other hand, physical mapping of value-added traits are target oriented. By integrating classical breeding with molecular marker and cytogenetic techniques, considerable progress has been made in manipulating genes and gene complexes for crop improvement. However, plant breeders are still not able to utilize large proportions of the publicly available genomic information and a better understanding of genome structures and variation (spontaneous and induced) to carry out the directed gene manipulation is required.

Following the complete genome sequencing for several plant species, vast amounts of DNA sequence information are now available, but the complexity in annotating these sequences and understanding gene interactions delays progress in utilizing them in plant breeding. The localization of cytogenetic and molecular markers on chromosomes and genomes is essential for physical mapping. Development of fluorescent staining methods and molecular cytogenetics tools (e.g. FISH, GISH, TUNEL test, COMET assay), high-resolution mapping and genotyping (e.g. RFLP, AFLP, SSR, SCAR, ISSR, IRAP, EST, SNP), and megabase technology (e.g. BAC libraries with deep genome coverage) add powerful new dimensions to chromosomal analysis, including assessing mutation induced changes.

1.1. Justification

The improvement of quality traits in food and industrial crops is one of the most important goals in plant breeding. Improved crop quality is considered to be of great economical value for both developed and developing countries and in the case of nutritional quality it will have a significant positive effect on human health. The use of induced mutagenesis is an efficient means of altering genes controlling quality trait. Physical mapping can also provide an effective means to manipulate various quality characters, including the transfer of genes between varieties and even species. The technology is applicable to seeded as well as non-seed propagated crops. A particular advantage is that genetic polymorphisms and large segregating populations are not required for physical mapping.

The IAEAs involvement in the development and coordination of the CRP was justified by:

1. The need to promote crop improvement with the production of quality trait mutants and to maximize the application of information, genetic, cytogenetic, and molecular stocks, linkage maps, and germplasm for crop improvement;
2. The need to foster relationships, transfer knowledge, technology, and genetic resources between all research groups for their mutual benefit between and among developing countries;
3. The need to generate, physically map and characterize quality trait variation with the aim of utilization;
4. The need to include physical mapping approaches currently not covered by other international programs;

5. The need to encourage distribution and dissemination of basic information, genetic stocks, cytogenetic and molecular techniques on an unrestricted basis;
6. The need to produce guidelines and publish methods on the application of molecular cytogenetics to plant breeding.

1.2. Objective

The objective of this IAEA coordinated Research project (CRP) on Physical Mapping Technologies for the Identification and Characterization of Mutated Genes Contributing to Crop Quality, aimed at enhancing Member States' capacity to identify, isolate, characterize and transfer agronomically useful genes produced through the application of induced mutagenesis, physical mapping and complementary genomic approaches, and the characterization and utilization of induced mutants for improvement of crop quality.

It was envisaged that the CRP would enable participants to:

- Establish, transfer and utilize quality genes and to promote the development of new crop improvement programs in the context of enhancing sustainable agricultural systems;
- Explore and develop genetic, cytogenetic, molecular and comparative genomic approaches to physical mapping of mutated crop genomes;
- Characterize by physical mapping new and/or existing radiation-induced mutations affecting quality traits;
- Develop, and apply to new crops, techniques for physical gene mapping through workshops and dissemination of generic techniques.

The expected research outputs were:

1. Quality-trait data generated by physical and genetic mapping associating cytogenetic markers with induced mutants.
2. Quality-trait data generated by physical and genetic mapping associating DNA markers with induced mutants.
3. Association of mutated genes with individual large insert clones (for example, BACs and arrays).
4. Quantifying the effects on genome structure of tissue culture and induction of mutations.
5. Identification of homology between the induced mutations and known genes in crops or model species.
6. Newly characterized lines with physically mapped genes or chromosome segments entered into national breeding programmes for quality.

2. IMPLEMENTATION OF THE CRP

Under this CRP, 10 funded research projects and 4 research agreements were initially supported from Argentina, Bulgaria, China, Czech Republic, Germany, Iceland, Pakistan, Poland, Ukraine, United Kingdom, and Viet Nam.

The main crop species included in the CRP represented various botanical and genetic classifications such diploids and polyploids, seed and vegetatively propagated, dicot and monocot, annual and perennial. The specific crops were *Musa* spp (banana and plantain); *Oryza* sp (wild and cultivated rice); rye, barley and wheat and their relatives; Perennial *Triticeae*; *Brassica*; quinoa; sugar beet and relatives; *Capsicum* spp (chilli peppers (hot and sweet); *Lycopersicon esculentum* (tomato and relatives) and cotton. Value-added quality traits that were assessed within the CRP were; bread making (quinoa and lymegrass); fruit colour and shape (tomato and chilli pepper); carotenes (tomato and chilli pepper); anthocyanins (chilli pepper and tomato); low phytic acid (rice); fibres (cotton); oils and fatty acids (Brassica); amylose and starch (rice); cold tolerance (rice); salinity (rice); acidity (rice, rye, wheat); male sterility (chilli pepper and tomato); plant architecture (chilli pepper and tomato); and yield.

The first Research Coordination Meeting (RCM) was held from 31 March – 4 April 2003 at the IAEA headquarters' in Vienna, Austria, to discuss the individual project activities, to adapt the work plans, and to facilitate possible collaboration between the research teams. To ensure that a platform of key technologies is available to all participants at the time of project initiation, a three-day workshop was held at the Agency's Laboratories in Seibersdorf. All ten contract holders attended the workshop with agreement holders P. Heslop-Harrison, University of Leicester, UK, and K. Anamthawat-Jonsson, University of Reykjavik, Iceland were resource persons. The workshop provided a hands-on opportunity for the participants to standardized protocols and techniques for their respective crops. Since some of the participants already had good experience with some of the techniques, it also functioned as a place for exchange of practical tips, which usually cannot be found in published papers.

The progress and achievements made in line with the project objectives were reviewed during the second RCM held in Reykjavik, Iceland, 22-26 August 2005, and at the third RCM in Cordoba, Argentina, 19-23 March 2007. Each RCM provided an opportunity to further strengthen collaboration between the research teams, discuss and critically asses individual work plans, and recommend suitable changes where required.

Following the success implementation of the CRP and the significant results obtained towards achieving the objects of the project, the CRP was awarded an extension up to 31 December 2008, to complete the characterization of stable mutant lines for incorporation into germplasm enhancement and breeding programmes. The final RCM of the CRP was held from 11–15 August 2008 at the IAEA Headquarters in Vienna, Austria, in conjunction with the International Symposium on Induced Mutation in Plants (ISIMP).

In addition to induced mutations used to generate the populations and tissue culture for propagation the CRP employed a range of molecular and cytogenetic technologies to address the problem and provided the counterparts the knowledge and skills on the use of these techniques.

2.1. Specific research achievements and recommendations

The main research outputs from this CRP are listed under the following five sections:

2.1.1. Quality-trait data generated by physical and genetic mapping associating molecular markers with induced mutants

Molecular markers (gene specific markers) for beta carotene content in sweet pepper were developed and incorporated into breeding programmes for marker assisted selection (MAS).

- Molecular markers (SSR and RAPD) associated with high Oleic acid content in mutant rape seed populations were identified for MAS.
- A preliminary genetic linkage map was developed and markers associated with approximately 16 QTLs for cotton fibre quality were identified to initiate marker-assisted selection (MAS).
- Three QTLs for cold tolerance in rice were identified and the associated markers will be developed for MAS.
- High inorganic phosphate gene in japonica rice (HIPj) was mapped on chromosome VI.
- Physical mapping of centromere associated markers (satellite and retrotransposons) were develop in sugar beet which can be used for the selection of interspecific hybrids.
- Rice-derived PCR-based markers in rye are being used in marker-assisted-selection (MAS) programs designed to transfer rye gene complexes into hexaploid wheat via wheat/rye hybridizations followed by backcrossing to wheat and selecting for the presence of a wheat plant phenotype and presence of the markers flanking the rye region of interest.

Recommendations:

- RAPD and anonymous markers should be converted into robust sequence tagged sites.
- Markers associated with specific quality traits should be used in breeding programmes to facilitate early selection if not already initiated.
- Additional markers should be identified to saturate chromosomal regions containing QTLs.

2.1.2. Association of mutated genes with individual large insert clones (for example, BACs) and arrays

Seven BAC clones was found suitable for use as cytogenetic markers in Musa. Preliminary results with FISH on meiotic pachytene chromosomes confirmed a possibility of using this approach for cytogenetic mapping in Musa.

- Selected BAC clones were physically mapped to integrate genetic linkage maps with chromosomes in sugar beet.
- BAC clones that are rich in unique sequences for the identification of individual chromosomes and chromosome arms in brassica and wheat).
- A rice BAC clone was used in high resolution mapping and characterisation of the aluminium tolerance gene complex *Alt3* gene complex in rye.
- Three rye BAC clones have been identified that hybridize to acid soil tolerant gene complexes from other species. These three rye BAC clones are being sequenced in order to establish the location and function of rye genes controlling acid soils tolerance.

Recommendations:

- Identifying BACs that contain relevant genes should be continued and integrated with publicly available genome sequences of crops.
- Microarray as a technology to identify genes has been superseded by other genomic tools (next-generation sequencing) and is no longer recommended for plants.

2.1.3. Quantification of the effect of tissue culture and induction of mutations on genome structure

- Assessment of DNA damage and repair in barley cells by COMET and TUNEL assay.
- DNA damage and repair due to irradiation was characterised in developing seedlings of barley.
- Cytological and physiological data on the effect of X ray irradiation in chilli peppers were obtained to facilitate the development of induced reciprocal translocation lines in chilli peppers.
- Cytological and physiological effects of irradiation were correlated degree of DNA damage assessed by the TUNEL test.

Recommendations:

- Wider use of TUNEL and COMET test to estimate DNA damage after irradiation and to select optimal radiation doses for mutant generation is recommended.

2.1.4. Homology between the induced mutations and known genes in crops or model species identified

- Mutated genomic segments in sweet pepper with homology to genes associated with beta carotene (*bCrtZ-A* ; *bCrtZ-B* ; *bCrtZ-C* ; *bCrtZ-D* ; *bCrtZ-E* ; *bCrtZ-D/C*) were isolated.
- Different mutant genes, (such as anthocyaninless of Hoffman (*ah*), anthocyanin without (*aw*), Baby leaf syndrome (*bls)* and positional sterility (*ps-2*)), encoding economic useful traits anthocyaninless and positional sterility were transferred into *Lycopersicon esculentum* lines.
- A mutation in the starch synthase IIa gene *(SSIIa)* associated with low gelatinization temperature in rice was identified.

- Fifty advanced mutant lines (M_5 generation) with substantially higher and lower fibre quality are available for functional genomic studies.

Recommendations:

- Agronomically useful genes identified in this CRP should be optimized for diagnostic markers in cross species applications.

2.1.5. Newly characterized lines with physically mapped genes or chromosome segments entered into national breeding programmes for quality

- Five sweet pepper mutant lines with high beta carotene (pro-vitamin A) content have been introduced into breeding programmes and are in advanced field trials in Bulgaria.
- Sweet pepper mutant lines with agronomically useful traits (earliness, fruit size, anthocyaninless, male-sterile) are being pyramided into mutants with high beta carotene content to develop new varieties in Bulgaria.
- Three mutant lines of tomato with high lycopene and beta carotene contents have been introduced into breeding programmes in Bulgaria.
- Male-sterile mutant lines of tomato and pepper for hybrid seed production in Bulgaria.
- Tomato and pepper with determinate habit (more suitable for mechanical harvesting) have been identified for breeding in Bulgaria.
- A translocated chilli pepper line (M_5) developed in Argentina with a conspicuous marker rearranged chromosome is available for field evaluation in Pakistan.
- Fifteen rapeseed mutant lines (M_6 generation) with high mono-unsaturated fatty acids (Oleic acid) and low undesirable fatty acid profile identified, isolated and field tested for agronomic performance in Pakistan.
- Three stable high yielding mutant lines of rapeseed with improved oleic acid content have been recommended for advanced yield trials in Pakistan.
- Seventy substitution lines (M_4 generation) with different fatty acid profiles have been developed for future utility in breeding programs in Pakistan.
- Five tetraploid cotton mutant lines with high quality fibre traits are in uniform yield trials in Pakistan.
- One mutant rice line with low gelatinization temperature, requiring less energy for cooking has been introduced into breeding programmes in China.
- Five rice mutant lines with varying levels of amylose (either low or high) for specific market demands have been introduced in breeding programmes in China.
- Eight rice mutant lines with low phytic acid content and enhanced bioavailability of minerals (based on preliminary animal test has been identified.

Recommendations:

- Counterparts are encouraged to exchange of useful mutant lines to other counterparts in the CRP or other Member States for breeding, in accordance with the respective national regulations on material transfer.

In addition to the five sections listed above, the CRP yielded a number of spin-offs:

2.1.6. Development and establishing of protocols in Member States

- Protocols based TUNEL and COMET test were developed to quantify the effect of radiation at the molecular and cellular levels in barley.

- A method for HPLC analysis of carotenoids was adapted for sweet pepper.
- Fluorochrome staining techniques, for comparative investigation of chromosome structure was applied in *Capsicum* species.
- A marker system based on SINEs for genetic mapping and evaluation of diversity in crops was developed and applied in sugar beet and potato.
- Platform for genomic analysis (bio-robotics) for automated handling of large genomic libraries developed.
- A rye BAC library approximately 6× in genome size with an average insert size estimated to be 130 kb was developed, and is being screened in an attempt to evaluate rye as a source for the map-based positional cloning of various abiotic genes and gene complexes for utilization in gene characterization and for cereal improvement.
- Targeted isolation and application of cytogenic markers (mini-satellites, dispersed repeats) from repeat enriched genomic libraries from *Musa sp* (8064 clones; 614 sequenced) and Beta *vulgaris* (2340 clones, 1763 sequenced).
- Flow-diagram for the identification of rye alien material in wheat background by FISH with genomic DNA and specific repetitive sequences was developed.
- Development and evaluation of molecular marker based screening method for drought tolerance in cowpeas collected from Ghana.

2.1.7. Germplasm evaluation and assessment of existing biodiversity

- Repetitive sequences including retroelements-like sequences were isolated from wild rice with the aim to study diversity and applied as diagnostic molecular markers.
- Molecular cytogenetic markers including repetitive DNA sequenced were developed for characterizing chilli pepper germplasm, identification of chromosomes and establishing chromosome homologies between *Capsicum* species.
- Phylogeny of the genus *Capsicum* and the geographic origin of domesticated chilli peppers were determined.
- Translocation lines in chilli peppers are available for studies on chromosome pairing and recombination.
- New chromosome markers for *Brassica* and *Chenopodium* species were identified for gene mapping and diversity studies in the two species.
- Estimation of genome size and localization of rDNA loci and nucleotide sequence analysis of ITS region in a set of banana species provided novel information about genetic diversity and phytogenetic relationships within the family *Musaceae*.
- A total of 100MB sequence data from Musa was obtained that will facilitate characterization of most of DNA repeats in the Musa genome and the isolation of new markers for diversity studies and to identify specific chromosomes.
- The autopolyploid or segmental allopolyploid genome of *Leymus* species was confirmed based on the isolation and characterization of Ns genome specific repetitive sequences from Leymus.
- Using genomic in situ hybridisation (GISH) and FISH of various genome and chromosome-specific probes, genome composition in *Triticeae* polyploids was identified.
- New Ns specific repetitive sequences were isolated for identification of individual chromosomes and chromosome segments in *Triticoleymus* and wheat breeding lines.
- Diversity of Iranian *Triticum tauschii* varieties was established by FISH, SSR and IRAP markers.

- Diversity and genome organization of a repetitive DNA element was studied in diploid, polyploidy and hybrid wheat and rye.
- Diversity of 20 Southern Indian banana varieties was established by inter-retro-element (IRAP) DNA markers.
- Retroelement diversity, evolution and function were studied in wild and cultivated banana and sugar beet.

Recommendations:

- Information from genome diversity studies of wild species should be used for the identification of novel quality traits and improvement of crops.

2.1.8. Dissemination of Information

The CRP also resulted in 2 book chapters, 35 peer reviewed papers, 25 conference proceedings, 1 PhD thesis, and 22 abstracts in published book of abstracts where produced under this CRP. In addition, thirteen sequences were submitted to the worldwide Genebank. The list of publications according to participating country is listed Annex I

2.2. General recommendations

- To facilitate selection of quality traits and the isolation of genes responsible for these traits, approaches combining a range of molecular tools some of which have been already employed in the CRP are needed. In addition, novel approaches using next generation of sequencing technologies, TILLING and DaRT markers should be considered either by outsourcing to laboratories or as services provided by the IAEA's Agriculture & Biotechnology Laboratory.
- The techniques available in the CRP should be further disseminated to other Member states use in their national crop improvement programmes through training.
- The techniques available in the CRP for the assessment of the existing genetic diversity as sources of useful genes should be utilised in member states to ensure a continued maintenance and improvement of the existing biodiversity.
- To further characterise useful quality trait genes from induced and natural mutants and to be transfer to cultivated crops as new varieties, another CRP on use of nuclear techniques physical mapping should be initiated.

3. CONCLUSION

To a large extent, the CRP achieved its objective by enhancing the capacities in the participating institutions, to identify, isolate, characterize and transfer genes conferring improved crop quality, through mutation techniques and other biotechnology methods (molecular cytogenetics and molecular markers). This is evident by the number well characterised advanced mutant lines of tomato, pepper, rice, oil seed rape and cotton, with improved quality traits (nutritional, organoleptic, processing, fiber-quality and abiotic stress tolerance), which were developed and the molecular markers associated with the traits identified. As the crop varieties used were targeted to improve their quality traits, the mutant lines selected in most cases are being used by breeders, to meet the demands for improved varieties with high yield, quality, and market-preferred traits.

In most cases, molecular and cyotogentic maps based on mutant germplasm were developed for further genetic studies of the affected traits. In the case of wild rice (China), lymegrass (Iceland) and Quinoa (Poland) the technique were applied to new or orphan crops.

Finally, this CRP added to the knowledge and use of induced mutations in crop improvement, marker technologies, genomics and cytogenetics among participating institutions.

COMPARATIVE PHYSICAL MAPPING OF REARRANGED AND NORMAL PLANT CHROMOSOMES BY HIGH-RESOLUTION FISH AND MEGABASE DNA TECHNIQUES

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Abstract

Repetitive DNA sequences form a major component of plant genomes and show often species-specific amplification, divergence and dispersion patterns along chromosomes. Repeats vary widely in size, type and copy number and are subject to rapid evolutionary changes. Our research is focussed on tandemly repeated DNA (satellites and minisatellites) and various types of transposable elements. In order to perform comparative genomic studies we have applied key technologies including construction and screening of large-insert libraries, analyses of the $c_0(t\text{-}1)$ DNA fraction and fluorescent in situ hybridization (FISH). We demonstrate the application of FISH for the physical mapping of repeats and genes, and for structural analyses of chromosome domains such as centromeres. Of particular interest are chromosomal mutations consisting of aberrations of alien chromatin or rearranged minichromosomes in sugar beet (*Beta vulgaris*).

1. INTRODUCTION

Nuclear genomes of higher plants vary enormously in size. A substantial proportion of this variation is due to polyploidy, and it is assumed that 50% or more of angiosperms are polyploids. However, although all plants at the diploid level require a similar number of genes and regulatory DNA sequences for physiological processes like germination, growth, flowering and reproduction, the total amount of the chromosomal DNA ranges from some 154 Mbp in the model species *Arabidopsis thaliana* L. up to more than 25000 Mbp in the timber species *Pinus elliottii* Engelm. var. *elliottii*. The differences in complexity of diploid nuclear genomes over several orders of magnitude [1] are caused by repetitive DNA.

The genus *Beta* belongs to the *Amaranthaceae* and includes the sections *Beta*, *Corollinae*, *Nanae* and *Procumbentes*. All cultivars such as sugar beet, fodder beet, table beet and chard are domesticated variants of *Beta vulgaris* L. and belong exclusively to the section *Beta*. Sugar beet is the only crop grown in temperate climates for the production of sugar and is also important for animal feeding and as a source for sustainable energy such as bioethanol.

The genus *Beta* provides a group of about 10 to 12 closely or distantly related species which represents an attractive set of plants for comparative studies of genome organization and evolution. Diploid forms of sugar beet have 2n=18 chromosomes and some 60% of its 758 Mbp genome [2] consist of repeated sequences [3]. The large scale genomic organization of the sugar beet genome has been well studied and numerous dispersed and tandemly repeated sequences have been localized along chromosomes [4-8]. In particular, the chromosome structure of sugar beet has been extensively investigated by fluorescent in situ hybridization and molecular methods to study the large scale organization of repetitive DNA. Major repeat classes of *B. vulgaris*, consisting of different satellites and retroelements have been physically mapped and incorporated into a plant chromosome model.

Interspecific crosses between sugar beet (*Beta vulgaris*) and wild beets of the section *Procumbentes* (*Beta procumbens*, *Beta webbiana* and *Beta patellaris*) enabled the selection of genotypes such as PRO1 and PAT2 containing small monosomic chromosome fragments derived from the wild beet species [9-11]. The stability of the chromosome fragments during mitotic cell division strongly suggests that they contain functional centromeric DNA sequences. Two BAC (Bacterial Artificial Chromosomes) libraries with deep genome coverage with more than 20 genome equivalents have been generated and represent a major resource for genome analysis of *B. vulgaris* outlined in various projects [12,13].

This paper is focussed on the molecular cytogenetics of *Beta* centromeric DNA, the characterization of different classes of transposable elements of *Beta vulgaris* L. and the genome-wide analysis of repetitive DNA isolated by a targeted approach.

2. MATERIALS AND METHODS

For comparative evolutionary studies, a collection of wild beet species and *Beta* cultivars has been established and maintained under glass house conditions.

A complementary set of methods such as automated DNA sequencing, high-resolution FISH, BAC analysis and many standard molecular techniques is used. Biorobotic techniques are applied for clone handling and replication. Furthermore, plasmid libraries consisting of 55000 clones with 0.5-2.0 kb inserts are available. Large and small insert libraries (BAC and plasmid clones) are spotted on high-density filters which are used for screening of repeated DNA sequences.

Positive BACs containing novel repeated DNA motifs are nebulised, subcloned and sequenced on an automated capillary sequencing machine followed by database searches and various bioinformatic analyses. After Southern hybridization, repeats are localized along *Beta* chromosomes by high-resolution fluorescent in situ hybridization. All methods have been described in detail in various publications of our group cited in this paper.

3. RESULTS AND DISCUSSION

3.1. Genome analysis platform

A current topic of research is the genome-wide molecular-cytogenetic characterization of repetitive sequences of the *B. vulgaris* genome. In particular, our research is focused on the analysis of retrotransposons and transposons as major factors affecting plant genome size and organization. Another research topic is directed to the molecular dissection of specific chromosome domains. These results are related to the evolution of sequence families, genomes and chromosomes and to species phylogeny. Furthermore, we aim for the identification and comparative analyses of novel repeat families and the isolation of a plant centromere towards the construction of plant artificial chromosomes.

In order to achieve these objectives we have introduced methods of high-throughput genome analyses. We have established biorobotic techniques for automatic handling of large-insert libraries each consisting of 86,000 ordered clones. These techniques include spotting of clones on high-density filters containing 27,684 colonies in duplicate on a single membrane, and replicating of BAC libraries. The genome analysis platform includes a facility for automated dispensing of liquid media and downstream applications such as DNA nebulisation for cloning, automated sequencing and high-resolution fluorescent in situ hybridization (FISH).

3.2. Characterization of centromeres

Repetitive DNA is a key component of plant centromeres which are essential domains of eukaryotic chromosomes. Although the long-range sequence organization apparently follows similar structural rules, the DNA composition of plant centromeres displays a high degree of variation, even between chromosomes of a single species. It has been shown that the centromeric DNA of most higher plants extends over several hundred kilobase pairs and is predominantly composed of various repetitive sequences such as satellite repeats and retrotransposons [14]. Apart from a few rice centromeres, the organization of plant centromeric DNA is still poorly understood.

An excellent experimental system for the isolation of a single plant centromere is provided by hybrids between cultivated and wild beets. These chromosomal mutants of *B. vulgaris*, designated PRO1 and PAT2 carry a monosomic chromosome fragment of *B. procumbens* and *B. patellaris*, respectively, which is efficiently transmitted in mitosis. Ambiguities in physical mapping due to allelic variations

are excluded as the centromeres can be analyzed on a monosomic minichromosome in a heterologous genetic background. In addition, genome-specific satellite DNAs that can unequivocally distinguish between *B. procumbens* and *B. vulgaris* chromatin are available.

By high-resolution multicolour-FISH on pachytene chromosomes and extended DNA fibres we analyzed the long-range organization of centromeres in the wild beet *B. procumbens* using a set of centromeric repetitive sequences. We developed a structural model of a plant centromere which has been compared with the putative centromere of the monosomic PRO1 and PAT2 chromososome fragments [12]. According to this model the two non-homologous satelite repeats pTS4.1 and pTS5 form large arrays directly adjacent to each other and represent the majority of the centromeric DNA. The *B. procumbens*-specific sequences pTS4.1 and pTS5 were used for the isolation of BAC clones on high-density filter grids (Fig. 1) to investigate the molecular structure of the centromeric DNA.

The mutant PRO1 carries a single *B. procumbens* chromosome fragment of approximately 6-9 Mbp and resembles a minichromosome which is functional in sugar beet. So far, 86 BACs were identified originating from the minichromosome centromere. Based on PCR assays, AFLP analyses, BAC fingerprinting and FISH, BACs were grouped into four classes which could be assigned to specific regions of the PRO1 centromere. Tandemly repeated DNA sequences are highly amplified at the centromere. FISH analysis has shown that the satellite arrays reside at the physical end of the minichromosome indicating an acrocentric position of the centromere, and it has been shown that the centromeric satellite arrays of the PRO1 minichromosome occupy approximately 340 kb [15]. The arrays are interspersed with other repetitive sequence elements, in particular Ty3-*gypsy* retrotransposons as revealed by FISH to extended chromatin fibres and BAC subcloning.

Restriction analysis of centromeric DNA by pulsed field gel electrophoresis showed that the PAT2 centromere has only a size of 50-70 kb. Therefore, the PAT2 system has been chosen for further analysis. Both PRO1 and PAT2 minichromosome centromeres consist mainly of satellite DNA families (pTS5 and pTS4.1) and rearranged Ty3-*gypsy*-like retrotransposons. Restriction analyses enabled the identification of 10 out of 97 BACs spanning the PAT2 centromere. A candidate BAC containing two large satellite arrays is considered as a candidate clone and currently prepared for sequencing. In order to investigate whether PAT2 centromere if fully functional in sugar beet and able to recruit centromeric proteins we aim to develop antibodies against kinetochore-associated proteins, in particular CenH3, which is a centromere-specific variant of histone H3. Selected BACs will be introduced into *B. vulgaris* callus cells by biolistic transformation and tested for stabilty and inheritance.

Fig. 1. Screening of pTS5-containing BACs on a high density filter containing 9256 clones spotted in duplicates.

3.3. Identification and molecular characterization of transposable elements

Transposable elements, in particular retroelements, are discrete components of plant nuclear genomes that can amplify and reinsert as novel copies at other genomic sites, thereby increasing the genome size and causing genetic diversity. Depending of the mode of transposition, either DNA transposable

elements (class II transposons) or retrotransposons, class I transposons) are differentiated. Both classes are characterized by extreme sequence heterogeneity. Retroelements are the major portion of the repetitive DNA and frequently make-up half of plant nuclear DNA. Major classes of retroelements are Ty1-copia-, Ty3-gypsy- and env-like retrotransposons as well as LINEs and SINEs.

3.4. Ty3-gypsy retrotransposons

The molecular investigation of centromic DNA revealed that retrotransposons are highly amplified in this chromosomal region of Beta genomes. In particular, Ty3-gypsy-like retrotransposons are very abundant in centromeres and dispersed throughout large satellite arrays forming islands of complex DNA organization. The complexity of the centromere is further increased as most Ty3-gypsy-like retrotransposons are rearranged, truncated or have a nested structure.

Two Ty3-gypsy-like retrotransposons originating from a PRO1 centromeric BAC have been isolated and characterized [16]. They were designated beetle 1 and beetle 2. Beetle2 has inserted in inverse orientation into the polyprotein region of beetle 1. beetle 1 and beetle 2 have a chromodomain in the C-terminus of the integrase gene and are highly similar to the centromeric retrotransposons (CRs) of rice, maize, and barley. PCR analysis using RNA as template indicated that beetle 1 and beetle 2 are transcriptional active. Based on the sequence diversity between the LTR sequences it was estimated that beetle 1 and beetle 2 transposed within the last 60,000 years and 130,000 years, respectively. The centromeric localization of beetle 1 and beetle 2, their transcriptional activity combined with high sequence conservation within each family indicate an important structural role in the centromeres of B. procumbens chromosomes. Fig. 2 shows the molecular characteristics and nested organization of beetle 1 and beetle 2.

Both retroelements were localized in the centromeric region of *B. procumbens* chromosomes by fluorescent in situ hybridization (Fig. 3). Therefore, they can be classified as centromere-specific chromoviruses.

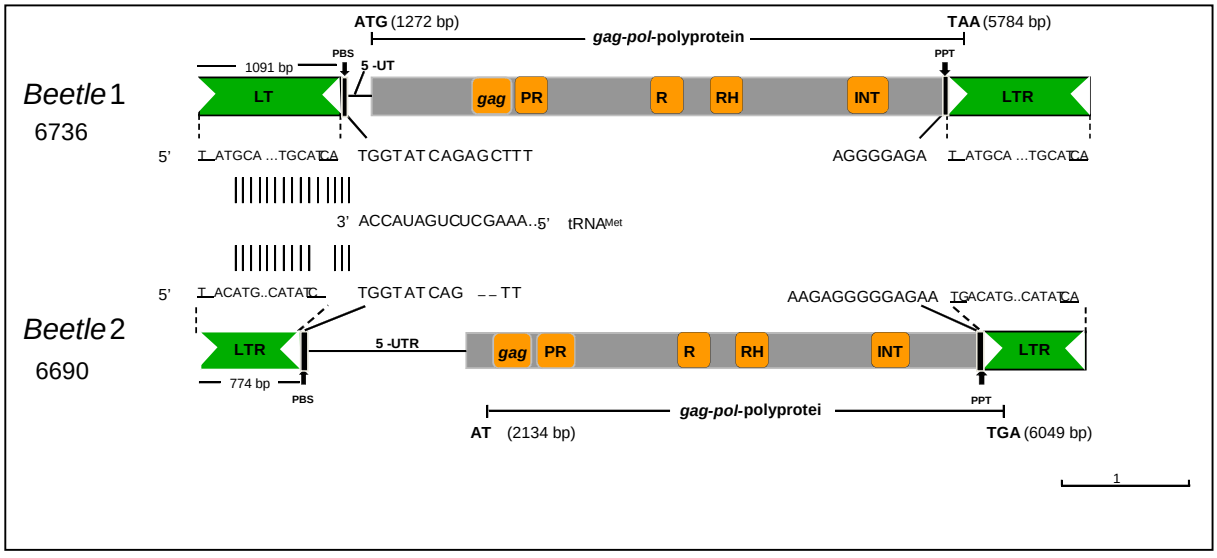

Fig. 2. Structure of the centromeric Ty3-gypsy retrotransposons beetle 1 and beetle 2 (schematic drawing to scale).

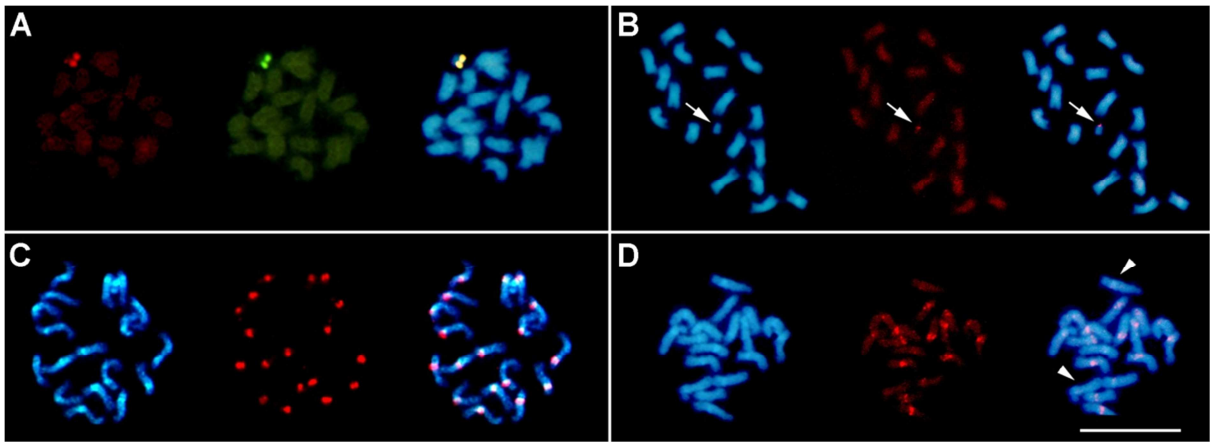

Fig. 3. Localization of beetle 1 and beetle 2 on prometaphase and metaphase chromosomes of B. procumbens and PRO1 minichromosome by fluorescent in situ hybridization (FISH). In each panel, the DAPI-stained DNA (blue fluorescence) shows the morphology of the chromosomes. Hybridized probes were detected with Cy3 (red fluorescence) and FITC (green fluorescence). The LTRs of beetle 1 (A) and beetle 2 (B) hybridize exclusively to the PRO1 minichromosome (arrows, red signals). Note that the pTS5 satellite family (green signals) co-localizes with beetle 1-positive regions (A). Yellow signals in the overlay of the beetle 1-LTR and the pTS5 satellite repeat originate from simultaneous hybridization (right in A). (C) The beetle 1-LTR (red fluorescence) exclusively hybridizes to the centromeric regions of all B. procumbens chromosomes. (D) The beetle 2-LTR strongly hybridizes to the centromeric regions, however not all centromeres could be detected (examples are marked by arrowheads). Scale bar in D: 10µm (Modified from Weber and Schmidt 2009).

3.5. LINEs and SINEs

A systematic search of *Beta* genomes has so far revealed many characteristic features of mobile DNA sequences. Non-LTR retrotransposons are evolutionary older than LTR retrotransposons, less characterized and divided into LINEs (long interspersed nuclear elements) and SINEs (short interspersed nuclear elements). The absence of LTRs makes is difficult to delimit the borders of the elements. The analysis of high density BAC filters resulted in the identification of the non-LTR retrotransposons BvL1, BvL2 and BvL3 which were studied in detail. For example, BvL2 is 6679 bp long and create target site duplication of 18 bp upon integration. Both BvL1 and BvL2 are characterized by a poly(A) tail at their 3`ends. LINEs of this type are characteristic for many plant genomes.

We have also identified a novel LINE type which is characterized by a substitution of the Zinc finger in ORF1 which is replaced by an RNA recognition motif. This RNA-recognition motif may play a role during reverse transcription. Database searches revealed the presence of this LINE variant in genomic sequences of many higher plant genomes.

Another type of non-LTR retrotransposons is represented by SINEs. Using a combination of methods we were able to identify many SINE families in public databases such as Genebank. The experimental approach is based on the design of tRNA-related query sequences which are used for successful SINE mining from database entries. This method is complemented by an in-house developed computer algorithm which is used to search in public databases. The analysis of diverged SINE families initiated the development of a PCR-based marker system based on family-specific primers. These markers are designated Inter-SINE amplified polymorphisms (ISAP) and can differentiate between *Beta* as well as *Solanum* genotypes and segregate in a Mendelian fashion.

3.6. Genome-wide analysis of repetitive DNA and targeted isolation of tandemly repeated sequences in *Beta vulgaris* and *Leymus triticoides*

In order to get a comprehensive insight into the repetitive DNA of the sugar beet genome, we have established a c_0(t-1) plasmid library. The isolation of c_0(t-1) DNA is based on differences of the re-annealing time of heat-denatured single stranded DNA. Formation of double stranded DNA by

controlled renaturation depends on the copy number; sequence motifs with repetitive DNA sequences show a faster renaturation.

The c_0(t-1)-library consists of 2304 clones which were completely sequenced. 1763 clones had inserts large enough for further analyses while the remaining inserts most likely represent very short microsatellite motifs which are highly abundant in the sugar beet genome. The size distribution of inserts is shown in Fig. 4.

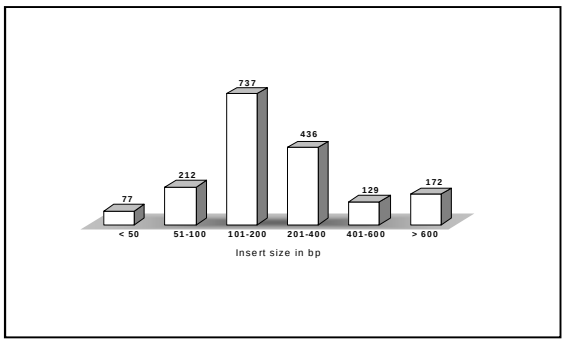

Fig.4. Distribution of insert sizes of 1763 clones of the c_0(t-1)-library. The average insert size is 250 bp.

In total, approximately 440kb of repetitive DNA have been cloned. Comparative inspection revealed that the most abundant sequence classes in the library belong to tandem repeats (Fig. 5). Further analysis resulted in the identification of 752 novel repeat which are currently analyzed in detail. Using bioinformatic software (Tandem Repeat Finder) we were able to isolate minisatellites which can rarely be isolated by other methods. So far, we have characterized 10 different minisatellite families with repeating units ranging from 10 to 96 bp. Most families have a dispersed organization along all *Beta* chromosomes, however, there is one minisatellite, which is amplified on a limited set of chromosomes only. This repeat can be used as a cytological marker for chromosome identification.

The molecular characterization of repetitive sequences is of central importance as repeat sequences can make up 50-90% of higher plant genomes. The group at the TU Dresden is partner in a national consortium aiming to sequence the sugar beet genome. Based on a physical BAC map sequencing is currently underway and detailed knowledge about the repeated DNA is crucial for genome annotation. The cloning of the c_0(t-1) DNA of plant genomes is an efficient technology to identify repetitive DNA and investigate the evolution of repeated sequences as has been shown in small grain cereals such as Leymus [17] where repetitive clones provide cytogenetic markers enabling also insight into the chromosome evolution.

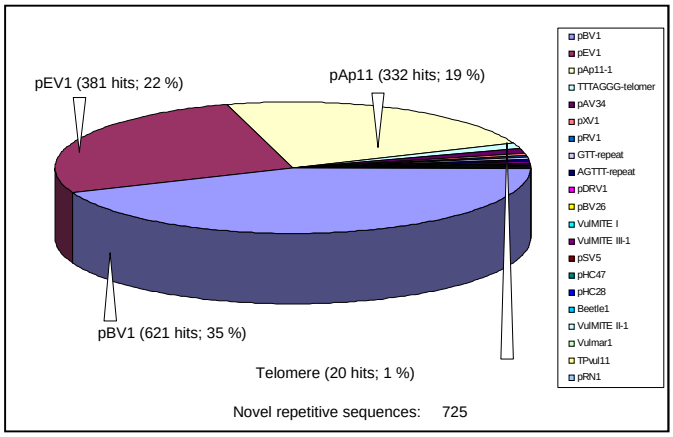

Fig. 5. Abundance of major repeat classes in the c_0(t-1) library.

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EXPLOITATION OF PHYSICAL MAPPING TECHNOLOGIES FOR BREEDING OF CANOLA MUTANTS IN OILSEED BRASSICAS

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Abstract

A mutant population of oilseed rape (*Brassica napus*) & mustard (*Brassica juncea*) consisting of 25,748 M_2 mutants developed and screened through non-destructive quality analysis using Near Infrared Spectroscopy (NIRS) for modified fatty acid profile. The genetic stability of mutant lines with desirable fatty acid profile ascertained in the $M_{2:4}$ population. The DNA molecular polymorphism survey was conducted using DNA extracted from stable mutant lines. A total of 80% of the SSR primers screened yielded amplification products in all the selected lines. The polymorphism for the mutated genetic makeup of selected mutant plants of *brassica* with respective initial parents were studied using fluorescence in situ hybridization (FISH) to characterize the distribution of rDNA probes. With modified fatty acid composition 14 brassica mutant lines of the $M_{2:5}$ generation were tested for yield performance under replicated yield trials for two consecutive years at Nuclear Institute for Food & Agriculture (NIFA). The yield and quality performance of these 14 mutant lines were also evaluated under diversified agro-climatic conditions across the country. All the brassica mutant lines confirmed the genetic stability in modified fatty acid composition and yield potential.

1. INTRODUCTION

One of the most important objectives in rapeseed and mustard breeding programme at the Nuclear Institute for Food & Agriculture (NIFA), Peshawar is genetic modification of seed quality by changing the proportion of fatty acids suitable for either nutritional or industrial purposes. Presently, the oil of indigenous rapeseed varieties cultivated in Pakistan is low for nutritionally desirable fatty acids such as oleic acid (18:1n-9) [1]. Contrary to the nutritional necessity of certain n-6 and n-3 polyunsaturated fatty acids (PUFA), rape and mustard oil containing 8-15% linolenic acid (18:3n-3) and are more liable to rapid oxidative damage than oils with little or no 18:3n-3. The oxidation of linoleate (18:2n-6) and linolenate are approximately 10 and 25 times higher respectively than that of oleic acid. To increase the oleic acid content and concomitantly, lower the level of PUFA, induced mutations through physical and chemical mutagenesis has been successfully used in conjunction with breeding methods and other advanced molecular techniques [2]. The objective of this project aimed at developing better quality mutant genetic stock in oilseed brassica.

2. MATERIALS AND METHODS

2.1. Plant materials and field experiments

2.1.1. Radiation treatment and M_1 generation

Rapeseed (*Brassica napus* L.) is characteristically grown as a winter sown crop in northern cool temperate areas of Pakistan such as Northern Punjab and North West Frontier Province. The rapeseed winter crop is drilled during the mid of September to mid of October and harvested in May. A high yielding *B. napus* variety Abasin-95 was used in this study. Uniform and healthy seeds (12% moisture) were exposed to gamma ray doses of 0.8, 1.0 and 1.2 kGy (approximately 500seeds/per dose) from a ^{60}Co source. The M_1 generation was planted in the field during the normal winter sowing. At maturity, the pods from the main raceme were collected from the M_1 plants and the seeds were bulked dose-wise.

2.1.2. Segregating M_2 and stable M_3/M_4 generations

The harvested M_2 seeds from M_1 plants were planted in separate blocks according to the doses in field and the segregating M_2 generation was raised to identify and select the mutant plants with modified fatty acid profile. The selected mutant plants were sown in plant to progeny rows in field as M_3 generation. The stable M_3 mutant lines for modified fatty acid composition promoted to M_4 generation for further field testing.

2.1.3. Assessment of genetic stability of advanced mutant lines

Genetically stability of selected 14 advanced M_5 mutant lines for modified fatty acid profile and agronomic performance were evaluated in preliminary replicated field trails at different agro-climatic regions of Pakistan. Data was collected on important agronomic traits including 50% flowering, plant height, pod length and number of branches recorded. At maturity, the seeds of five randomly selected individual mutant plants, entry-wise and replication-wise were harvested for biochemical analysis and seeds of the remaining plants were bulked entry-wise and replication-wise. The total seed yield and seed weight recorded accordingly. The quality analysis of seeds was determined using a non-destructive Near Infrared Reflectance Spectroscopy (NIR) System. All the collected data was subjected to principal component analysis and compared to a model PCA1 and or PCA2 data principal component analysis. The basic purpose of principal components is to account for the total variation forming a new set of orthogonal and uncorrelated composite traits. The first principle component has the largest variance; the second have a variance smaller than the first but larger than the third, and so on. Therefore, traits that included in the first components are considered the most important ones [3,4].

2.2. Seed quality analysis

2.2.1. M_2 generation screening

The fatty acid profile of the seeds (about 4g) of the individual harvested M_2 plant of generation were scanned on a monochromator (NIR Systems, model 6500 Inc., Silver Spring, MD) equipped with sample autochanger. For each sample the reflectance spectrum; (log 1/R) from 400 to 2500 nm was recorded at 2-nm intervals with ISI software, version 3.10 (Infrasoft International, Port Matilda, PA). The fatty acid profile quantified for oleic Acid (C18:1), linolenic Acid (C18:3) and erucic acid (C22:1) in whole seeds of the mutant plants [5,6].

The total oil content in intact seeds of each individual mutant plant of M_2 was also determined as mentioned above on NIR Systems, model 6500.

2.2.2. M_3/M_4 and advanced generation assessment

The fatty acid composition of bulked seeds of M_3/M_4 plant to progenies and advanced mutant lines of M_5/M_6 generations was estimated similar method as described above through NIRS.

The total oil content in intact seeds of M_3/M_4 plant to progenies and advanced mutant lines of M_5/M_6 generations was quantified through NIRS.

2.3. Fluorescence in situ hybridization (FISH)

Healthy and uniform seeds $M_{2:4}$ mutant plants of brassica were germinated and treated for fluorescence in situ hybridization (FISH) to characterize the distribution of rDNA probes for homologous sites on the chromosomes. The rDNA probes were labelled with digoxygenine or rhodamine and FISH was applied to mutant lines of brassica chromosomes according to Hasterok *et al.* [7]. The 5S rDNA probe was amplified and labelled with rhodamine- 4-dUTP from the wheat clone pTa794, using PCR with universal M13 sequencing primers. The 25S rDNA probe was obtained by nick translation with digoxygenin-11-dUTP of a 2.3 kb sub-clone of the 25S rDNA coding region of A. thaliana.

2.4. Molecular mapping

Seventy seven stable M_5 mutant lines of *brassica* from the four gamma irradiation treated *brassica* populations including both high oleic acid and low high oil content types were studied for induced genetic polymorphism. The mutant lines were raised in isolation, DNA was extracted from fresh leaf material of the selected mutant lines and initial parent line using phenol-chloroform protocol. A total 100 microsatellite primer sets were used to screen the polymorphism between initial parent, the segregating M_2 population and the M_5 stable mutant lines [9]. PCR amplification was achieved using a final reaction volume of 25 µl containing: 50ng of template DNA, 200µM of each dATP, dGTP, dCTP, dTTP, 50mM KCl, 10mMTris, 1.5mM MgCl2, 2.5 U of Taq DNA polymerase and 0.25 µM of primers and the final volume adjusted with distilled, sterile water (App. 1). The amplification was carried out in a GeneAmp PCR System 2700 (Applied Biosystem) programmable thermocycler. The program began with 2 min denaturation at 94°C, followed by 31 cycles of 1 min at 94ºC, 1 min at annealing temperature, 1 min elongation at 73°C and final extension at 73°C for 5 min. Depending on the primer pair, annealing temperatures were 60°C or 55°C. PCR-products were separated by 2% agarose or 10% polyacrylamide gel electrophoresis. The polymorphism between the initial parent with normal oleic acid and mutant lines with high oleic acid content were analyzed.

2.5. Seed yield

At physiological maturity, yield was determined in the individually harvested M_2 plants). The seeds of M_3/M_4 generations and advanced mutant lines in replicated field trials were harvested at maturity and seed yield recorded entry-wise.

3. RESULTS AND DISCUSSION

3.1. M_1 generation

Following gamma irradiation, a majority of plants had normal morphology. A few plants in each applied dose developed with severe growth retardation in both selected species of brassica. The frequencies of some morphologically different plants are given in Table 1. *Brassica napus* had a higher percentage of plants with changed morphology and sterility.

TABLE 1. EFFECTS OF GAMMA IRRADIATION ON M_1 SEEDLINGS - EMERGENCE, SURVIVAL AND PLANT MORPHOLOGY

Treatments	Seedlings %		Frequency (%) of plants with			
	Emergence	Survival	Small leaves	Short plants	Small/thin pods	Sterility
Brassica napus (mutants)	90.4	80.7	17.3	9.4	16.4	8.5
Brassica napus (control)	94.5	87.5	0	0	0	0
Brassica juncea (mutants)	94.6	83.1	9.8	4.5	20.4	6.5
Brassica juncea (control)	95.2	88.9	0	0	0	0

3.2. M_2 generation

The plant growth in the M_2 generation was mostly normal and fertile. A few plants were observed with retarded growth or sterility. About 25,748 individual healthy plants harvested at maturity. The species-wise selection details for both species given in Table 2.

TABLE 2. DOSE WISE COLLECTIONS OF M_2 PLANTS IN OILSEED BRASSICA

Species	0.8 kGy	1.0 kGy	1.2 kGy	Total
Brassica napus	4814	5024	5590	**15429**
Brassica juncea	3750	3885	2684	**10319**
Total	**8564**	**8910**	**8274**	**25748**

The fatty acids profile determined on NIRS of mature harvested seeds from M_2 individual plants of both species showed variability. The gamma irradiation of 1 kGy effectively induced variability for erucic acid ($C_{22:1}$) and linolenic acid ($C_{18:3}$) in *Brassica napus* mutant plants (Fig. 1). The *Juncea* spp. showed sensitivity to 0.8 kGy dose for oleic acid ($C_{18:1}$), linolenic acid ($C_{18:3}$) and erucic acid ($C_{22:1}$).

Selection and evaluation of the M_3 mutant lines for the field agronomic performance was also initiated in this generation. Based on modified fatty acid profile, 379 mutant plants of the two species, *Brassica napus* and *Brassica juncea* were selected to ascertain their genetic satiability in plant to row progeny test. A total of 185 *Brassica napus*, mutant plants and 194 mutant plants of *Brassica juncea* with modified fatty acid profile were planted (Table 3). The oleic acid can be increased with high oil content in brassica mutants by gamma radiation and the single mutant plant selection as shown in Fig. 2. Maximum oleic acid of more than 60% was observed in 20 M_3 mutant lines with an elevated oil content up to 52%.

TABLE 3. SELECTIONS OF M_3 LINES WITH MODIFIED FATTY ACIDS

Species	0.8 kGy	1.0 kGy	1.2 kGy	Total
Brassica napus	45	87	53	185
Brassica juncea	95	57	42	194
Total	140	144	95	379

3.2.1. Screening of mutants with modified fatty acids

All the collected 16,000 M_2 mutant plants screened for the mutated fatty acids (Erucic Acid $C_{22:1}$, Oleic Acid $C_{18:1}$, and Linolenic Acid $C_{18:3}$) through Near Infrared Spectroscopy (FOSS 6500 NIR System). *Brassica napus* expressed more induced mutations as compared to *Brassica juncea* for the fatty acid profile in intake seeds (Fig. 2 and Fig. 3). Erucic Acid ($C_{22:1}$) showcased highest mutation frequency than the rest of the fatty acids.

3.3. Field assessment of M_4 mutant lines:

A maximum seed yield of 803g $plot^{-1}$ (with unit plot size 2.5m2) was recorded in this field experiment of 38 advanced M_4 mutant lines. The oil content in seeds of mutant lines ranged from 32-47%. The oil yield $plot^{-1}$ (gm) of top yielding mutant lines with initial parent is presented in Fig. 3. The pattern of Oleic Acid and Linolenic Acid content in seed oil of these advanced mutant lines is presented in Fig. 4. A total of eight mutant lines confirmed with high Oleic Acid content (> 65%).

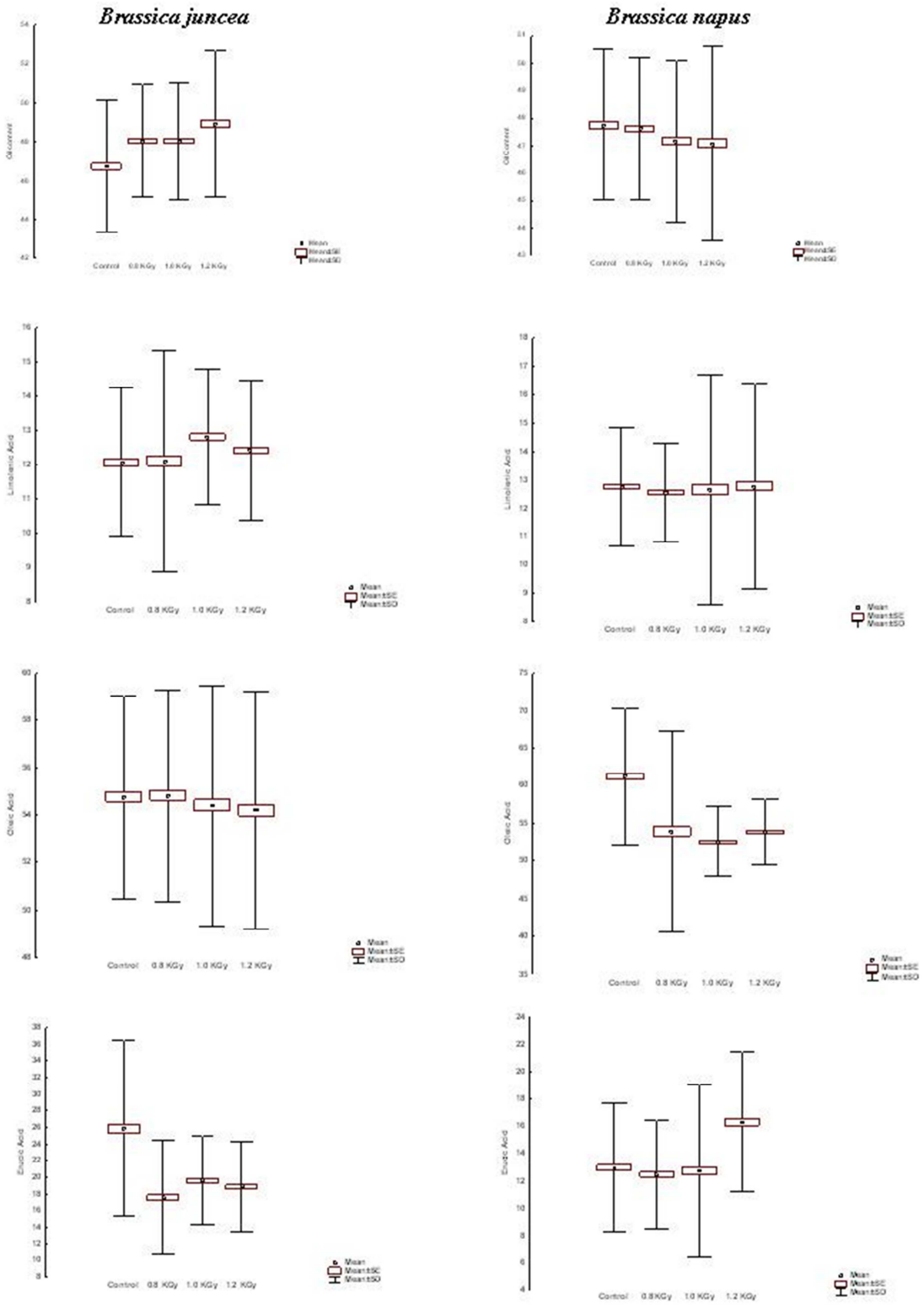

Fig. 1. Oil content and fatty acids of the control and M_2 mutant.

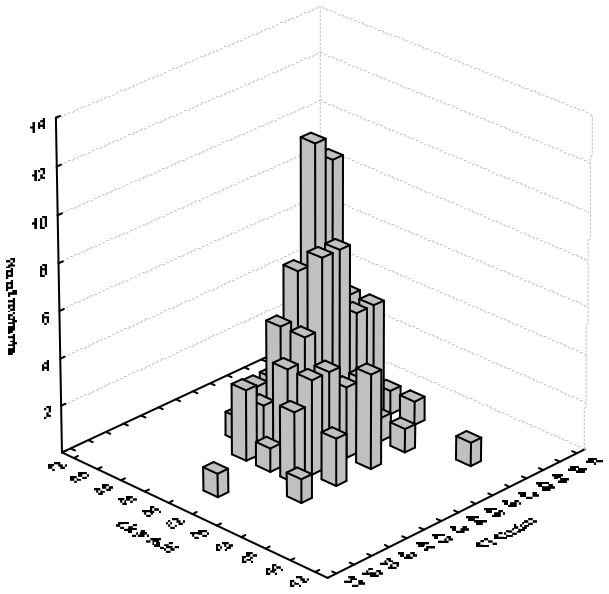

Fig. 2. M_3 generation mutants with high oleic and high oil content.

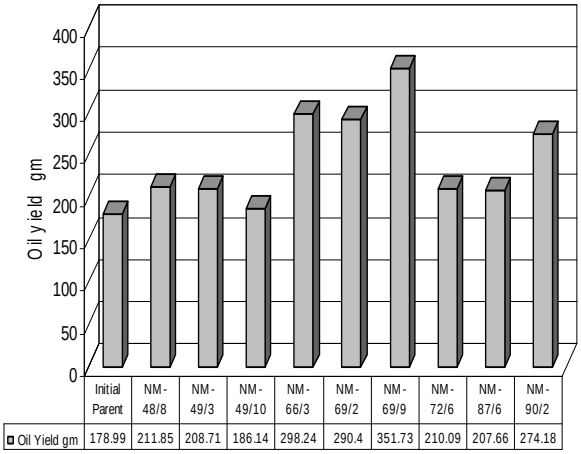

Fig. 3. Oil yield plot-1 (gm) of top yielding mutant lines in M_4 generation.

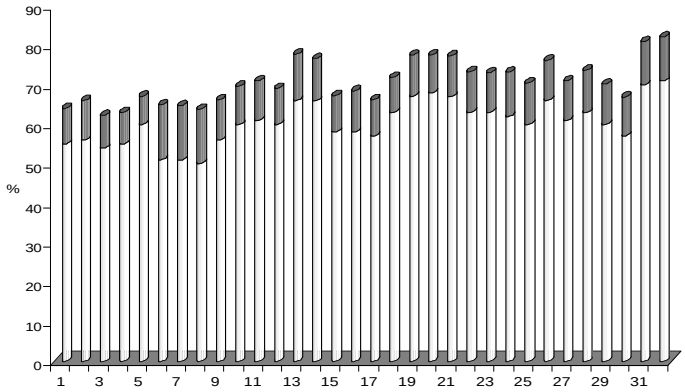

Fig. 4. Oleic acid and linolenic acid content (%) in M_4 mutants.

3.4. Genetic stability of M_5 mutant lines

The PCA of the performance of the mutant lines showed a consistence in their expression of the quality traits in the three locations. Mutants expression low erucic acid, the fatty acid responsible for the canola quality of the oilseed brassica species clustered in the lower quartile of PC1 and PC2 (Fig. 5). The low PC1 and PC2 values confirmed the genetical stability of the mutant lines for the modification in erucic acid content [6,7]. The mutants performed with confirmed consistency regarding the desirable monounsaturated fatty acid oleic acid at three different locations. The oleic acid expressed a high range of PC1 as well as high PC2 (Fig. 6). In case of linolenic acid PC1 for the three locations is high but PC2 for Centre and South zones were high and for North it was low. The PC2 for glocusinolate content in seeds of mutant lines was low at three locations but at Centre and South it was high for PC1 while at North PC1 was low. Total three mutant lines significantly out yielded the check variety Abasin-95. The six mutant lines produced low concentrations of undesirable erucic acid in their seeds as compared to check. Regarding oleic acid three mutant lines synthesized higher content than check while three mutant lines also produced high oil content as compared to check variety Abasin-95.

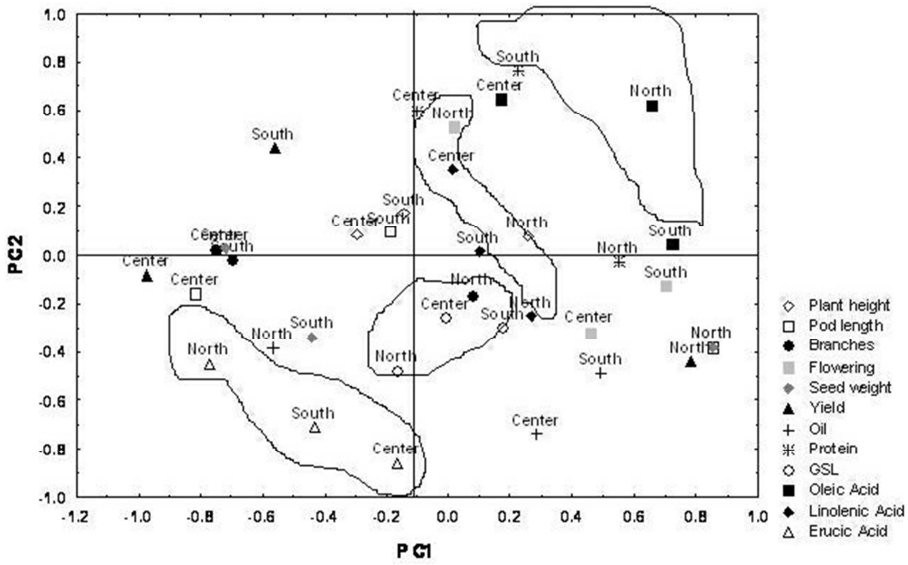

Fig. 5: Scatter plot of PC1 and PC2 for different yield and quality traits of stable mutant lines in zonal field trials.

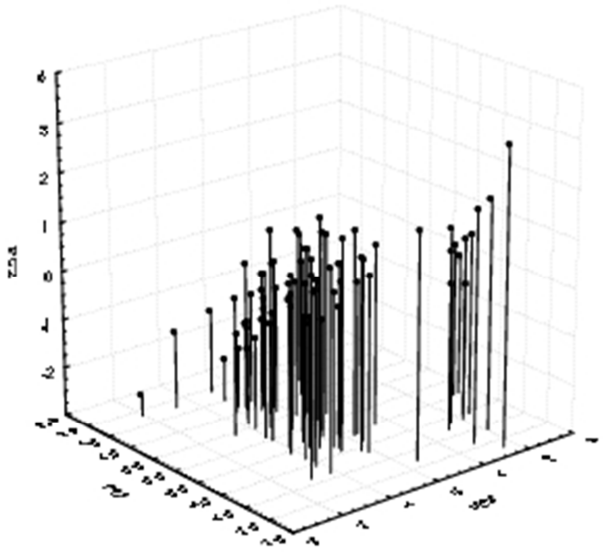

Fig. 6. Mutant plants with high oleic acid and oil content.

3.5. Fluorescence in situ hybridization (FISH) studies in mutants

The polymorphism of number, appearance and chromosomal localization of ribosomal DNA (rDNA) sites and in relation to anueploidy, induced mutations and mutated fatty acid profile in selected mutants of *Brassica napus* studied. FISH revealed frequent polymorphism in number, appearance and chromosomal localization of both 5S and 25S rDNA sites. The polymorphism was observed between intermutants (*i.e.* interindividual) of initial variety. A number of aneuploids (2n=35,36,37) were identified. The number of rDNA sites can differ significantly within mutants of the same initial parent with the same or variant chromosome number (aneuploids). A total of eight variant chromosomal types with ribosomal genes were indentified in the mutants. The extent of polymorphism is genome dependent but not dependent on the gamma radiation dose. Comparing the A and C genomes revealed the highest rDNA polymorphism in the A genome. The loci carrying presumably inactive ribosomal RNA genes are particularly prone to polymorphism [8].

3.6. Chromosomes typing

The FISH investigations demonstrated the utility of simultaneous use of 25S and 5S rDNA probes in the case of the mutants of Brassica species. The results of simultaneous FISH of 25S and 5S rDNA probes to the somatic metaphase chromosome complement of the initial parent are presented in Table 4. Ribosomal DNA sites effectively marked seven different chromosomal types in this genotype of the allotetraploid spp. of *Brassica napus* for A and C genomes. Chromosomal type I is the preserved chromosome of the A genome and bears the secondary constriction, and a large 25S locus closely linked to a smaller 5S site in its short arm. The nucleolar organising chromosome type VI of genome C is similar, but does not possess a 5S site in the short arm. Chromosome type II of the A genome is the only other chromosome to bear both 25S and 5S rDNA sites, which are in close apposition in the long arm. Chromosome type IV is found in the C genomes and having one proximal 5S rDNA site in the long arm. Chromosome type V is confined to the A genome, and is a small submetacentric with a terminal 5S rDNA site in the long arm. Chromosome type VII has a prominent, distal 25S rDNA site in C genomes, and chromosome type VIII of the A genome only bears a large proximal 25S rDNA site in its long arm. Double-target FISH with 5S and 25S rDNA probes enables the discrimination of a substantial number of chromosomes of the complement of the initial parent and the mutants under observation.

3.7. Frequency of changed cells

Variation in the frequency of changed cells has been observed in different mutant lines as compared to initial parent line (Table 5). In the case of initial parent line only 40% frequency of changed cells has been observed but in three mutant lines NM77, NM85 and NM90, a 100% frequency of changed cells were registered. The mutant line NM78 and NM81 produced 85 and 84% frequency of changed cells respectively. The lowest frequency of changed cells, 35%, was observed in the mutant NM80.

3.8. Chromosome numbers

The majority of the mutants had normal chromosome counts of 38 chromosomes. The three mutant lines namely NM80, NM81 and NM85 registered a variation in the chromosome numbers with 35-38 or 36-38 (Table 5).

TABLE 4. DIFFERENT TYPES & NUMBERS OF CHROMOSOMES OF BRASSICA

Chromosome type	I	II	IV	V	VI	VII	VIII
rDNA	5S+25S	5S+25S	5S	5S	25S	25S	25S
Genomes	A	A	C	A	C	C	A
Chromosome numbers	2	4	2	2	2	2	2

TABLE 5. CHANGED CELLS AND CHROMOSOMES WITH rDNA SITES

Genotypes	Frequency of changed cells (%)	2n	No. of chromosomes with rDNA sites		
			5S	25S	5S+25S
NM-47	40	38	4	5-7	6
NM-77	60	38	4	6-8	6-7
NM-78	100	38	4	6-7	7-8
NM-80	85	38	4	7-8	6
NM-81	35	35-38	4	5-7	6-8
NM-85	84	36-38	3-5	6-7	6
NM-90	100	35-38	3-4	7-8	5-7
NM-100	100	38	3-4	4-7	7-8
NM-47	10	38	4	7-8	4-6

3.9. Chromosome pattern of rDNA loci

Convincing variations for number of chromosomes in the different mutant lines with rDNA loci were observed (Table 5). The highest number of rDNA loci for 5S (3-5) was detected in the mutant line NM81 while mutant lines NM85 and NM90 produced 3-4 sites. The rest of the mutants counted with 4 rDNA loci as in the initial parent. In case of 25S rDNA loci all the mutants registered variations except the mutant NM80. Double targeted rDNA with variant loci at 5S and 25S were observed on the chromosomes of six mutants of *Brassica napus*. Only two mutants NM78 and NM81 remained in the range of initial parent.

3.10. Molecular characterization of mutant lines

The simple sequence repeats polymorphism survey was conducted using DNA extracted from total 77 representative plants of one control and three irradiated brassica populations (0.8., 1.0 & 1.2 kGy). The results of the polymorphism survey are summarized in Fig. 6. The 84 primer pairs produced amplification products from all used plants. All the SSR primers that yielded amplification products could discriminate between the high and low oleic acid plants (Fig. 4). However, none of the primer pairs tested was able to detect variation between the control & mutant plants or between the gamma irradiation doses [9].

ACKNOWLEDGEMENTS

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MARKER ASSISTED SELECTION FOR FIBER QUALITY IMPROVEMENT IN MUTATION BREEDING PROGRAMME OF COTTON

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Abstract

A total of 34 cotton genotypes were screened for fibre quality traits. Out of these, four genotypes; FH-883, FH-631S, CIM-707 (*Gossypium hirsutum* L., an allotetraploid species) and Ravi (*G. arboreum*, a diploid species) contrasting for lint quality traits were selected. The genotypes, CIM-707 (fibre length above 30 mm) and Ravi (fibre length 17 mm) were irradiated with gamma rays (125, 150, 200, 250 and 300 Gy), and were also treated with EMS (1, 1.5 and 2%). Both mutagens adversely affected the germination percentage of cotton seed. EMS-treated M_1 population was much better than irradiated M_1 population with respect to germination and plant growth. Moreover, ploidy level also affected the germination percentage. Staple length of M_2 plants derived from the Ravi variety was in the range of 13-17.5 mm, while of M_2 plants derived from CIM-707 was in the range of 22-31 mm. EMS and gamma irradiation can successfully be used for creating variation among cotton germplasm and hence mutation is a valuable tool for fibre quality improvement. After surveying 520 RAPDs and 435 SSRs, a genetic linkage map was constructed using 117 $F_{2:3}$ lines derived from a cross FH-631S x FH-883. Twenty loci were mapped into four linkage groups (LG) spanning around 230.2 cM with 5% of the cotton genome coverage. The average genetic distance was 11.5 cM between two adjacent loci. Low level of polymorphism between the two parents might be the result of narrow genetic base, as reported in multiple investigations. LG1 to LG4 were assigned to chromosome 20, 10, 18, and 15, respectively. QTLs for fibre traits were identified using SMA, IM and CIM at LOD > 2 with WinQTLCart. In total, 16 putative QTLs were identified for fibre traits including fibre length, fibre fineness, fibre strength, fibre length uniformity, short fibre index, fibre elongation, and fibre colour. Out of these, nine fibre QTLs were detected on A-subgenome, while seven on D-subgenome, which suggest that fibre traits result from gene interaction of both the subgenomes of cotton.

1. INTRODUCTION

Cotton is the leading fibre crop worldwide. Pakistan is the fourth-largest producer of cotton in the world. Cotton is vital for Pakistan's economy and its products share about 60% of foreign exchange. Cotton fibre quality is defined by the physical properties associating to its spinnability into yarn and contributes to textile performance and quality [1]. The most important fibre properties are associated with the length, strength and fineness (micronaire) [2]. The commercially grown cotton varieties have limited genetic diversity [3,4]. Several evolutionary bottlenecks have reduced the genetic variation in modern cultivated cotton. Further more, several decades of intensive breeding for better and improved cotton fibre traits has also narrowed the gene pool available for cotton improvement. Chemical mutagenesis has proven to be an important tool in genetic mapping of *Arabidopsis* genome [5]. Mutation breeding has been successful to increase genetic diversity in economically useful traits of many major crop plants such as rapeseed, sugar beets and rice [6-11].

Although historically, mutation played a marginal role in cotton breeding [12], however, in the last decade it is being successfully employed for improvement of different traits in cotton [13-17]. Induced mutagenesis using gamma irradiation has been employed for the creation of mutant tetraploid cotton and breeders have found mutants with improved earliness [13], enhanced phosphorous uptake, improved drought tolerance, photoperiod insensitivity, cytoplasmic sterility, fibre properties, increased lint percent and improvements in both earliness and lint yield [17]. Chemical mutagenesis has identified mutants in both *G. barbadense* and *G. hirsutum*. Cotton researchers were able to identify resistance to *Verticillium* wilt (dimethyl sulfate), found glandless mutants and improved fibre characteristics (ethylmethane sulfonate). Colchicine has also been used to create genetic variation in lint yield, lint percentage, fibre quality, partially naked seed and parthenogenesis. The treatment of cotton seed with colchicine in combination with gamma radiation or with gamma radiation alone resulted in increased chromosomal aberrations [17].

Molecular markers can accelerate breeding progress for complex traits such as fibre quality, which is otherwise difficult with conventional and mutation breeding procedures. Today, various marker techniques and breeding strategies have been employed to detect polymorphisms for mapping and analysis of quantitative trait loci (QTLs) [18]. The DNA markers currently available in cotton are based on restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), microsatellites/simple sequence repeats (SSRs), and single nucleotide polymorphisms (SNPs) [19]. Mutagenesis in combination with the application of modern genomic tools has the potential to rapidly increase the genetic variability in cotton for quality and yield improvement [17]. The main object of this research work was the improvement of cotton fibre quality through DNA markers in mutation breeding.

2. MATERIALS AND METHODS

2.1. Screening of cotton genotypes and mapping population for fibre quality traits

Thirty-four cotton genotypes collected from different cotton research institutes were screened at NIBGE for three main fibre quality traits (fibre length, fibre fineness or micronaire and fibre strength) quality traits during normal cotton growing season in 2002 (Table 1). Plant to plant distance was 30 cm while row to row distance was 75 cm. All the agronomic practices were kept similar among the three replicates. At crop maturity, bulk seed cotton was harvested from each line. After harvesting, seed cotton was ginned and the lint of these cotton genotypes was analyzed from Fibre Technology Laboratory. On the basis of these screening results cotton parents with extreme diverse quality fibre were selected for hybridization and induction of mutation.

Fibre length can be accurately determined by photoelectric measurement with fibrograph and high-volume instruments (HVI) [20]. Staple length is reported to the nearest 32nd of an inch or to the nearest millimetre (mm). For this experiment we measured fibre length in mm with fibrograph. Micronaire (Mic) has been the most widely used method of determining fibre fineness. Mic reading is a measure of resistance to airflow of a constant weight of fibres. We took micronaire reading as an indicator of fibre fineness. Bundle fibre strength is measured in grams-force tex^{-1} with HVI while in Pressley zero-gage it is reported as thousand pounds per square inch (tppsi), when relative humidity of testing room is adequately controlled [21]. We measured the fibre strength in tppsi.

Two *G. hirsutum* cotton genotypes FH-883 (30 mm fibre length) and FH-631S (23 mm fibre length) of contrasting for fibre quality traits were selected on the basis of their field performance. To develop a population for mapping QTLs associated with fibre quality traits, these two genotypes were crossed. The F_1 population was grown in green house and selfed to get F_2 seeds to develop the segregating F_2 population. During the next season, single rows of each individual F_2 plant were grown to obtain $F_{2:3}$ populations. Seed cotton was harvested from 117 $F_{2:3}$ cotton lines for the analysis of quality traits. Fibre analysis of 117 cotton lines of $F_{2:3}$ (FH631S × FH-883) population were performed with USTER High Volume Instrument (HVI), Fibre Technology Department, University of Agriculture Faisalabad (UAF), Pakistan, for eight fibre quality traits: fibre length (FL), fibre fineness (FF or Mic), fibre strength (FS), fibre length uniformity (FU), short fibre index (SFI), fibre elongation (FE), and fibre colour (Reflectance or Rd and Yellowness or +b).

2.2. Gamma irradiation and EMS treatment of selected cotton varieties

Three cotton genotypes, CIM-707 (32 mm extra long fibre), PBD-883 (30 mm long fibre) and Ravi (17 mm very short fibre) selected for quality traits on the basis of their previous performance in the field, were irradiated at different doses (125 to 300 Gy) using cobalt-60 as a source of gamma radiations(Table 2). Ethyl methanesulfonate (EMS) was also used as a chemical mutagenesis, to induce mutations in the three selected cotton genotypes (Table 3) with different doses (1 to 2% v/v). Safety measures were taken during the gamma irradiation and EMS treatments.

TABLE 1. COTTON GENOTYPES/ VARIETIES AND THEIR FIBRE TRAITS

S. No.	Cotton genotypes/ varieties	Breeding centre	Staple Length (mm)	Fineness (µg/i)	Strength (tppsi)
1	Qalandri	CRS, ARI, Tandojam	28.8	3.5	92.4
2	B-557	CRI, AARI, Faisalabad	27.0	4.6	92.3
3	MNH-93	CRS, AARI, Multan	28.2	4.7	94.1
4	NIAB-78	NIAB, Faisalabad	27.2	4.8	92.2
5	S-12	CRS, AARI, Multan	28.4	4.5	93.1
6	FH-87	CRI, AARI, Faisalabad	28.5	4.4	95.2
7	CIM109	CCRI, PCCC, Multan	27.2	4.3	91.3
8	RH-1	CRS, AARI, R. Y. K.	28.8	4.0	99.2
9	NIAB-86	NIAB, Faisalabad	28.9	4.4	94.1
10	BH-36	CRS, PCCC, Bahawalpur	28.2	4.3	87.5
11	NIAB-26	NIAB, Faisalabad	28.1	4.5	93.4
12	S-14	CRS, AARI, Multan	29.4	4.4	93.7
13	SLS-1	CRS, PCCC, Sahiwal	27.3	4.5	95.7
14	KARISMA	NIAB, Faisalabad	26.6	4.9	97.3
15	CIM1100	CCRI, PCCC, Multan	29.3	4.1	94.2
16	FH-634	CRI, AARI, Faisalabad	28.4	4.2	95.2
17	CIM448	CCRI, PCCC, Multan	28.3	4.3	94.0
18	CIM443	CCRI, PCCC, Multan	27.5	4.6	96.5
19	BH-118	CRS, PCCC, Bahawalpur	28.4	4.8	96.4
20	FH900	CRI, AARI, Faisalabad	26.6	4.6	92.6
21	FH901	CRI, AARI, Faisalabad	26.8	5.1	92.8
22	MNH552	CRS, AARI, Multan	27.4	5.3	96.4
23	CIM473	CCRI, PCCC, Multan	29.2	4.6	94.6
24	FH-631	CRI, AARI, Faisalabad	23.0	5.5	79.0
25	RH-114	CRS, AARI, R. Y. K.	26.7	5.1	87.5
26	VH-59	CRS, AARI, Vehari	26.0	5.6	85.5
27	NIBGE-1	NIBGE, Faisalabad	28.0	4.8	99.8
28	PBD-883	NIBGE, Faisalabad	30.0	4.4	97.0
29	CIM-707	CCRI, PCCC, Multan	32.1	4.3	97.3
30	ST-12	Exotic	26.6	5.1	83.4
31	DP-54A	Exotic	25.8	5.3	82.2
32	Coker-312	Exotic	27.5	4.91	90.0
33	Giza-66	Exotic	27.0	5.8	80.6
34	Ravi	CRI, AARI, Faisalabad	17.0	7.5	70.0

TABLE 2. COTTON GENOTYPES AND DOSE OF RADIATION

S. No.	Cotton genotypes	Species	Radiation Dose (Gy)	No. of seeds irradiated
1	CIM-707	*Gossypium hirsutum*	300	1000
2	CIM-707	*Gossypium hirsutum*	250	1000
3	CIM-707	*Gossypium hirsutum*	200	800
4	CIM-707	*Gossypium hirsutum*	150	800
5	PBD-883	*Gossypium hirsutum*	300	275
6	Ravi	*Gossypium arboreum*	200	1000
7	Ravi	*Gossypium arboreum*	150	1000
8	Ravi	*Gossypium arboreum*	125	1000

TABLE 3. SELECTED COTTON GENOTYPES AND EMS TREATMENT

S. No.	Cotton Genotypes	Species	EMS % age	No. of seeds treated
1	CIM-707	*Gossypium hirsutum*	2.0	1000
2	CIM-707	*Gossypium hirsutum*	1.5	1000
3	PBD-883	*Gossypium hirsutum*	2.0	175
4	Ravi	*Gossypium arboreum*	1.5	1000
5	Ravi	*Gossypium arboreum*	1.0	1000

2.3. Development of mutant cotton population for selection of desired mutants

M_1 seeds of the three cotton genotypes, viz., CIM-707 (32 mm), PBD-883 (30 mm) and Ravi (17 mm) that were EMS treated and irradiated at different doses using cobalt-60 as a source of gamma radiations was planted in NIBGE cotton fields during 2003 normal cotton season. Gamma irradiated and EMS treated M_1 seeds were sown along with controls. The EMS treated M_1 population was subjected to extensive selfing to obtain M_2 seeds and a large M_2 population (3039 M_2 plants) was sown during 2004. Two large EMS treated M_2 populations (one for *G. hirsutum*= 2000 M_2 plants and second for *G. arboreum*= 1039 M_2 plants) were developed to obtain maximum variations. M_2 Mutants contrasting for fibre length were selected and in successive years their M_3 to M_6 lines were developed.

Fibre analysis of selected mutants was conducted. However, in case of gamma irradiated M_1s not only germination was adversely affected but also observed significant delay in flowering. Therefore, a relatively small gamma irradiated M_2 population was planted in 2005. Its generations were not successfully advanced in successive years due to very low germination and poor seed setting each year.

2.3.1. DNA markers analysis, genetic mapping and identification of cotton fibre QTLs

DNA fingerprinting techniques (RAPD and SSR) were applied to find DNA markers and QTLs linked to fibre quality traits. DNA was extracted from the young leaves of the cotton parents (FH-883 and

FH-631S) and $F_{2:3}$ population using a modified CTAB (22). DNA concentration was measured with DyNAQuant 200 Fluorometer. The quantity of DNA was also compared with Quantification Standards, Phage λ DNA (GibcoBRL) on 0.8% agarose gel. Quality of DNA was checked by running 50 ng DNA on 0.8% agarose gel. Working dilutions of DNAs were prepared from stocks accordingly for RAPD and SSR analysis. Five hundred and twenty RAPDs and 435 SSRs (including 85 EST-SSRs) were surveyed on the two cotton parents. PCR amplifications were performed in Eppendorf mastercycler gradient, Germany. RAPD primers belonged to the series OPA through OPZ, with 20 primers in each series (Operon Technologies, Inc. USA). RAPD products were analyzed by electrophoresis on 1.2% agarose gel in 0.5× TBE buffer and detected by ethidium bromide (10 mg/ml) staining. SSRs of different series (BNL, CM, JESPR and MGHES -EST based SSR primers) were obtained from publicly available cotton microsatellite data (CMD) (www.cottonmarker.org) and synthesized from GeneLink, USA. MetaPhor agarose (Cambrex Corporation, USA) gels (4%) made in 1× TBE and stained with ethidium bromide were used for resolving SSRs. Polymorphic RAPD and SSR markers were surveyed on 117 $F_{2:3}$ (FH-631S × FH-883) cotton lines.

A preliminary genetic linkage map was constructed using the Mapmaker 3.0 software. Map units (cM) were computed by applying the Kosambi function. Linkage groups were identified at a minimum LOD 3.0 and a maximum distance of 37.2 cM. WinQTLCart 2.5 was used for QTL analysis using single marker analysis (SMA), interval mapping (IM) and composite interval mapping (CIM).

3. RESULTS AND DISCUSSION

3.1. Development of mutant population

Germination of irradiated and EMS-treated M_1 seeds was adversely affected. In another study, reduction in cotton seed viability of the EMS treated M_1 was less than 50% was reported [17]. Germination of CIM-707, PBD-883 and Ravi was affected with the increment in radiation dose (Table 4). Germination of EMS treated M_1 seeds of CIM-707 and Ravi was increased by decreasing the dose concentration of EMS. However, PBD-883 could not be evaluated because of failure in germination (Table 5).

The EMS-treated M_1 populations were much better than irradiated M_1 populations with respect to germination and plant growth. Furthermore, EMS treated M_1 of the diploid *G. arboreum* (2×) genotype (Ravi) was comparatively better than the EMS treated M_1 population from the tetraploid (4×) *G. hirsutum* genotypes (CIM-707 and PBD-883). A range of different mutants were obtained in the M_2 populations for staple length (Figs. 1 & 2). Staple length of selected mutant lines of *G. arboreum* ranged from 13 to 17.5 mm, while for the *G. hirsutum* mutant lines they ranged from 22 to 31 mm.

All these lines were advanced to M_6 for harvesting stable mutants. Few of these mutants have been utilized for doing crossing with the locally adapted material. Also, these mutants would be utilized for initiating functional genomic studies. All these resources would be available to the international cotton community for identifying the functions of various genes involved in fibre development and conferring high quality traits in cotton which would certainly set a stage for achieving sustainability not only in cotton production but would also way for breeding high quality lint**.**

3.2. Mapping population and molecular marker analysis

The intraspecific 117 $F_{2:3}$ lines exhibited significant variations for fibre traits based upon the family means. Among $F_{2:3}$ families, the range of fibre quality traits was 21.2 to 29.6mm for fibre length (FL), 3.6 to 6.2 reading for micronaire (FF or Mic), 14.5 to 28.9 g/tex for fibre strength (FS), 42.8 to 56.6% for fibre uniformity (FU), 7 to 31.6% for short fibre index (SFI), 4.9 to 8.2% for fibre elongation (FE), and for fibre colour 47.8 to 70.1 value for Rd and 4.9 to 12.4 value for +b.

TABLE 4. EFFECT OF GAMMA RAYS ON GERMINATION IN M_1 GENERATION

Treatment	Cotton Genotypes	Radiation Dose (Gy)	Germination % age
1	CIM-707	300	0.1
2	CIM-707	250	0.2
3	CIM-707	200	4.4
4	CIM-707	150	5.1
5	PBD-883	300	0.7
6	Ravi	200	0.00
7	Ravi	150	0.00
8	Ravi	125	10.8

TABLE 5. EFFECT OF EMS TREATMENT ON GERMINATION IN M_1 GENERATION

Treatment	Cotton Genotypes	EMS % age	Germination % age
1	CIM-707	2.0	8.60
2	CIM-707	1.5	10.3
3	PBD-883	2.0	0.00
4	Ravi	1.5	9.70
5	Ravi	1.0	11.2

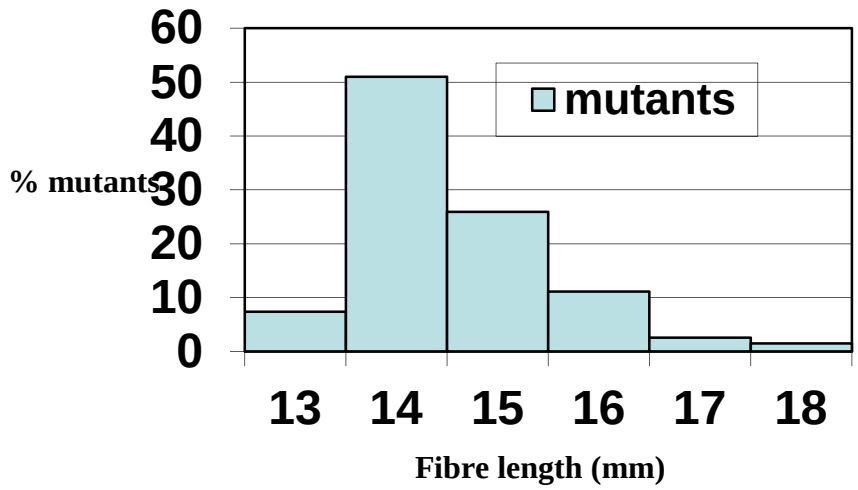

Fig. 1. Fibre length and M_2 mutants (G. arboreum).

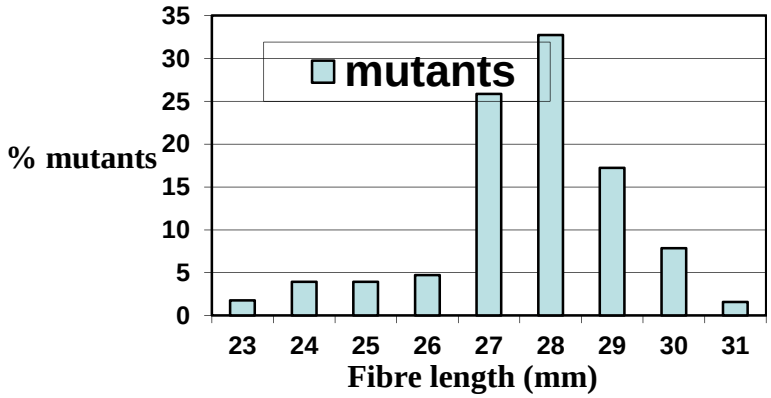

Fig. 2. Fibre length and M2 mutants (G. hirsutum).

Out of 520 RAPD primers, 506 amplified scoreable fragments while rest of the primers was poorly amplified. The total number of loci amplified was 2683 with an average of 5.3 loci per primer, ranging from 1 to 13 fragments per primer. Out of the 506 primers, eight primers were polymorphic between the two parents and amplified 10 polymorphic loci. Four hundred and thirty five SSRs including 85 EST-SSRs derived from fibre tissues were also surveyed on the cotton parents FH-883 and FH-631S. Four hundred and nine SSRs amplified 750 loci with an average of 1.8 loci per SSR. A total of 401 were monomorphic SSRs, while eight (2%) were polymorphic. Three primer pairs JESPR-152, JESPR-153 and MGHES-73 produced three, two, and two loci, respectively; yielding 12 polymorphic SSR loci with product size range of 85–420 bp. All the polymorphic RAPDs and SSRs were surveyed on the 117 lines of the $F_{2:3}$ population.

3.2.1. Genetic linkage map and identification of cotton fibre QTLs

A preliminary genetic linkage map of cotton was constructed with Mapmaker (version 3.0) using polymorphic molecular markers data of 117 lines of $F_{2:3}$ (FH-631S × FH-883) population. Twenty loci out of 22 RAPDs and SSRs were mapped into four linkage groups (LGs) (Fig. 3), while two markers were polymorphic but could not be assembled into any linkage group. The resulting genetic map spanned 230.2 cM with 5% of the cotton genome coverage. The average genetic distance was 11.5 cM between two adjacent loci. The number of markers placed on these linkage groups ranged from three to eight. Linkage groups were assigned to specific chromosomes of cotton using already known anchored and informative loci. LG1 was assigned to long arm of chromosome 20 in D sub-genome, while LG2, LG3 and LG4 were assigned to chromosome number 10, 18 and 15 respectively.

Analysis of cotton fibre related QTLs was conducted with WinQTLCart using the phenotypic and genotypic data of the 117 $F_{2:3}$ lines. QTLs for fibre traits were identified by performing SMA, IM and CIM at LOD > 2. All the QTLs were detected in linkage groups LG1 (Chr. 20) in the D subgenome and LG2 (Chr. 10) in A subgenome, and these two are also homologous chromosomes. The comparative positions of the fibre QTLs are presented in Fig. 4. SMA detected eight QTLs, 15 QTLs were identified with IM, while 10 QTLs were found with CIM analysis. Collectively 16 putative QTLs were identified, of which 12 were commonly found with at least any two of the procedures, while four QTLs were identified with IM or CIM only.

Two QTLs (L1s and L2s) for FL located on LG1 and LG2 were identified with SMA and the positions of L1s and L2s were 0.01 cM and 37.9 cM at LOD 2.52 and 2.25, respectively. Similarly two QTLs, L1i and L2i were detected for FL with IM at LOD 2.59 and 2.76, respectively. The phenotypic variances explained (PVE) were 11.5 and 16.6% for L1i and L2i, respectively. With the CIM analysis, one QTL (L1c) for FL explained 11.5% of the phenotypic variation at 2.6 LOD. SMA identified one QTL (F1s) for FF with position at 88.8 cM on LG1 and at LOD 2.47. Two QTLs designated as F1ia

and F1ib were found on LG1, with IM. Their PVE were 9.6 and 7.4% at LOD 2.21 and 2.66, respectively. With CIM, two QTLs F1c and F2c were associated with micronaire, located on LG1 and LG2 with PVE of 6.3 and 10.3%, and at LOD 2.46 and 2.17, respectively. QTLs S1s and S2s associated with fibre strength were detected with SMA at LOD 2.88 and 2.37, respectively. Position of S1s was at 0.01 cM on LG1, while S2s was at 21.3 cM on LG2. Three QTLs S1i, S2ia, and S2ib for FS, one on LG1 and two on LG2 were identified with IM at LOD 4.05, 2.75, and 3.16, respectively with PVE 16.5, 12.9, and 17.7%, respectively. Similar to S1i, CIM found one QTL (S1c) for FS at LOD 4.03 and 16.5% PVE.

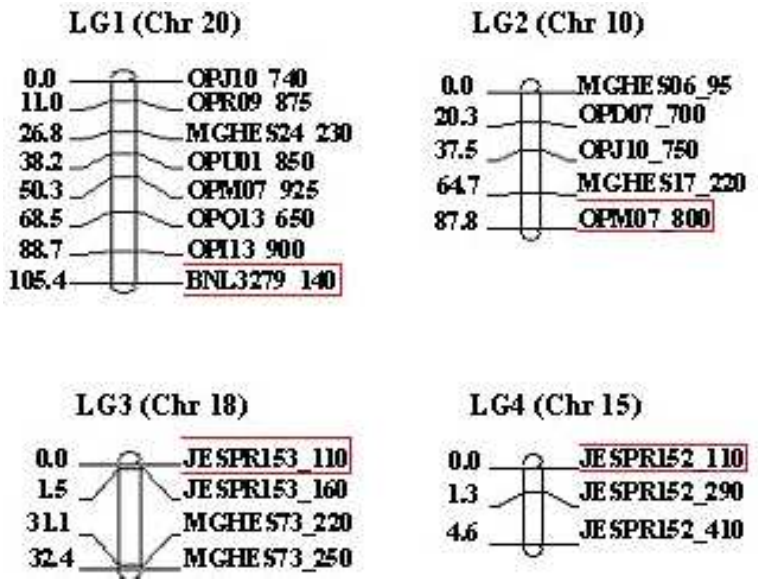

Fig. 3. Genetic linkage map constructed using (FH-631S × FH-883) $F_{2:3}$ intraspecific cotton (G. hirsutum) population. Marker positions are in centiMorgan (cM) with Kosambi function at min LOD 3 and max distance 37.2. The informative and framework loci that were already anchored to specific chromosomes of cotton are in boxes.

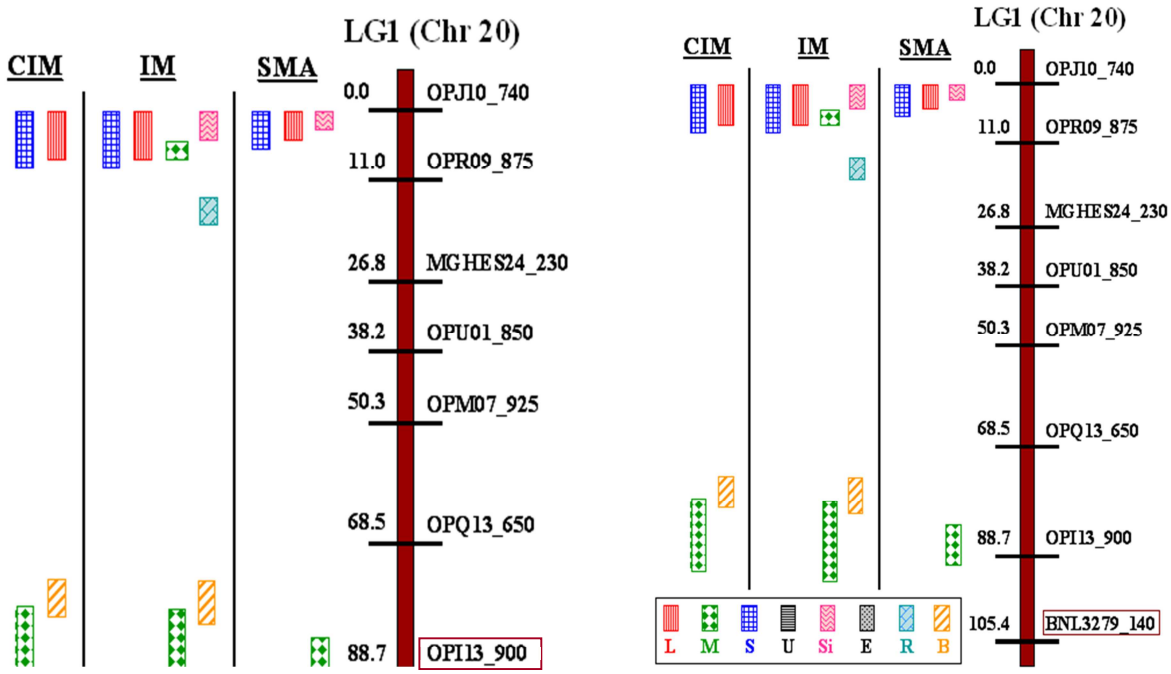

Fig. 4. Comparison of QTLs and their positions (cM) in linkage group 1 and 2 (LG1, LG2) for cotton fibre quality traits using SMA, IM and CIM. SMA: single marker analysis, IM: interval mapping, CIM: composite interval mapping, L: fibre length, M: fibre fineness (micronaire), S: fibre strength, U: fibre length uniformity, Si: short fibre index, E: fibre elongation, R: fibre colour (reflectance= Rd), B: fibre colour (yellowness= +b).

No QTL was detected for FU with SMA, however, one QTL (U2i) was found on LG2 with IM explaining 11.6% PVE at LOD 2.06. Similarly, CIM identified one QTL (U2c) identical to U2i in percentage PVE and LOD. With SMA, two QTLs Si1s at 0.01 cM with LOD 2.3 and Si2s at 37.9 cM with LOD 2.41 for SFI were mapped on LG1 and LG2 respectively. The IM analysis also revealed two QTLs (Si1i and Si2i) at about the same positions. The PVE of Si1i and Si2i were 9.4 and 14.6% at LOD 2.42 and 3.28. However, CIM analysis found only one QTL (Si2c) for short fibre content on LG2 with LOD score 3.28 and PVE 14.6%. No QTL was found for FE with SMA, however, one QTL (E2i) at 86.7 cM with LOD 2.28 was found on LG2 using IM procedure. With CIM analysis one QTL (E2c) for FE similar to E2i in position and LOD was identified. One QTL for reflectance (Rd) was detected with SMA on LG2 at 21.3 cM with 2.19 LOD score. Three QTLs, one (R1i) on LG1 and two (R2ia and R2ib) on LG2 were identified for Rd with IM. LOD score of R1i was 2.21 with PVE 12.1%. For R2ia and R2ib, LOD was 2.25 and 2.12, while their PVE were 12.5 and 9.6%, respectively. Similarly, CIM revealed two QTLs for Rd on LG2 identical to two QTLs found with IM. For yellowness (+b), one QTL was detected with IM, which was identical in position (81.3 cM) and LOD (2.15) to QTL found with CIM analysis.

4. CONCLUSIONS

Sensitivity level to EMS and gamma irradiation was variable for different cotton genotypes. CIM 707 and Ravi cotton varieties are more sensitive to gamma radiation than EMS treatment. The immediate effect of these mutagenic agents is obvious as reduced germination rate while gamma radiation also delays the flowering. EMS and gamma irradiation can successfully be used for creating variation among cotton germplasm. Both *G. arboreum* and *G. hirsutum* diverse mutant lines have been selected which have a range for fibre quality traits. The mutants with desirable traits are being used in cotton breeding programs for fibre quality improvement.

In the present study, level of polymorphism between two cotton parents (FH-883 and FH-631S) was 1.6% for RAPDs and 2% for SSRs, which was less than the expected. It may be due to narrow genetic base between the cotton genotypes. Factors such as introduction of high-yielding tetraploid cotton varieties in early 1970s from America; repetition of same gene pool in breeding programs; release of sister lines as different varieties; and a compulsion to breed for cotton leaf curl disease resistance by using limited resistant genetic resources contributed towards narrowing the genetic window in *G hirsutum* blood. Globally, many other researchers have reported such commonalities. Linkage map with less coverage of genome was possibly due to low polymorphism at DNA level between parents in the present study. Collectively 16 putative QTLs related to eight cotton fibre quality traits were detected. The co-localization of the QTLs for fibre traits was mostly in accordance with the observed phenotypic correlations. The QTLs detected in both the A and D subgenomes suggest that fibre-related traits result from gene expression and interaction between homologous A and D subgenomes.

ACKNOWLEDGEMENTS

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MUTAGENESIS AND PHYSICAL MAPPING OF GENES IN CROPS WITH SMALL CHROMOSOMES

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Abstract

The manipulation of quality genes in agronomical and economical important plant species requires well-established cytogenetic maps and detailed genome characterization. Advance cytogenetic molecular methods, especially fluorescence in situ hybridization (FISH) have proved to be helpful in detecting chromosome-specific tags. The introduction of new cytogenetic markers to karyotyping using FISH is necessary, especially in species, which chromosomes are inordinately small and morphologically uniform, such as *Brassica* and *Chenopodium* species. The application of rDNA as probes for FISH does not provide enough chromosome specific landmarks in *Chenopodium and Brassica.* More molecular markers are still needed for identification of chromosomes of investigated species. In this study, different DNA sequences: BAC clones, retroelements-like and transposon-like sequences were localized on chromosomes of the three species studied using FISH. A detailed characterization of chromosomal aberrations in *Hordeum vulgare* (2n = 14) cells was done by the identification of individual chromosomes involved in their formation with FISH. Simultaneous FISH with 5S and 25S rDNA and, after reprobing of preparations, telomeric and centromeric DNA sequences as probes, was used to compare the cytogenetic effects of different mutagens on root tip meristem cells of barley. This is the first application of more than 2 DNA probes in FISH experiments in order to analyze chromosomal aberrations in plant cells. A better knowledge of the correlation between the level of DNA breaks detected by TUNEL and comet assay and the frequency of chromosome aberrations could speed up evaluation of effectiveness of mutagenic treatment in barley root cells. The comet assay and TUNEL test can be used as a predictive test for the outcome of the CA after using physical mutagen. The potential usefulness of the analysis of the level of DNA breaks in embryo in order to speed up the evaluation the effectiveness of mutagenic treatment was proved.

1. INTRODUCTION

Chenopodium species have started to attract scientific attention because of their high nutritional value. Among them especially *C. quinoa* (quinoa) is becoming more and more popular as a crop for human food and animal feed. Quinoa seeds have a high nutritional value and a better amino acid balance than the proteins in most cereals. There is little information about the genome and karyotype of *Chenopodium* species, however the agronomical importance of these crops makes their chromosome mapping necessary. The small size and great number of quinoa chromosomes make cytogenetic analysis difficult.

The genus Brassica contains a number of important crop species, however molecular cytogenetic investigations are often limited to species of the classic 'U-triangle' [1]. The main diploid species in the triangle are: *B. campestris*, *B. nigra*, *B. oleracea*, which represent the A, B, and C genomes respectively. Others species in the triangle are allotetraploids, which arose by spontaneous interspecific hybridization from diploid ancestors, and contain a full chromosome set of both ancestral species. Since in the *Brassica* species both classes of rDNA are usually found in numerous loci situated in very different chromosomal locations, simultaneously FISH with 5S and 25S rDNA probes is a powerful tool for more detailed studies, regarding intergenomic and interindividual polymorphism studies, polyploidization events, as well the analysis of the behaviour of alien chromosomes during meiosis in monosomic addition lines (MALs), or to assay chromosome variation following *in vitro* culture. Although FISH with rDNA as probes enables the discrimination of a substantial number of chromosomes in *Brassica* species, still more cytogenetic markers are needed for a better identification of all chromosome pairs. The objective of this part of study was development of chromosome (-arm)-specific markers for *Brassica* and *Chenopodium* species for physical mapping of quality genes.

A wide range of chemical and physical agents are used to induce gene mutations in order to increase plant variability. Gene mutations induced by chemical and physical agents are accompanied by direct DNA damage and chromosome rearrangement. The majority of induced DNA breaks are repaired, but if not repaired or repaired improperly may lead to chromosome aberrations (CA). A better knowledge of the correlation between the level of DNA breaks and the frequency of chromosome aberrations should speed up evaluation of effectiveness of mutagenic treatment. The effect of mutagenic treatment can be determined directly on DNA level as a frequency of the DNA fragmentation estimated in a comet assay (single cell gel electrophoresis) and TUNEL test (Terminal transferase (TdT) mediated dUTP-digoxigenin/biotin Nick End Labelling). Most studies, which involved the comet assay used animal and human cells, however, recently some studies have been published on the use of the comet assay in plant systems [2,3]. The procedure of the comet assay, especially isolation of nuclei and electrophoresis conditions needs to be modified to each species. TUNEL test, based on labelling the 3'OH ends of DNA with fluorescein – conjugated dUTP by terminal deoxynucleotidyl transferase (TdT) mainly had found application in apoptosis studies, but it was adapted to the detection of DNA damage in mutagenesis [4,5]. A positive correlation between results of methods, which detect DNA fragmentation and CA frequency could speed up evaluation of effectiveness of mutagenic treatment, as analysis of CA is labour-intensive. Hartmann et al [6] demonstrated the usefulness of the comet assay as a screening test for the prediction of the outcome of the chromosomal aberration test in Chinese hamster cells and human lymphocytes.

The choice of proper dose of mutagen for treatment is very important for effectiveness of mutagenesis. The optimal dose of mutagen should reveal high mutagenic efficiency and not cause high sterility and a reduction of survival. There are differences in sensitivity among various species and varieties of the same species to a particular mutagen [7]. Cytogenetic tests of the frequency of chromosomal aberrations, as well as tests for somatic effects (germination dynamics, emergence reduction and stem growth reduction tests) are quick methods for the estimation of optimal doses of mutagen in M_1 plants.

Although structural chromosomal aberrations, which accompany gene mutations, can be detected with simple classical cytogenetic methods, physical mapping technologies and especially FISH, provides new tools for chromosomal aberrations analysis. The identification of chromosomes or chromosome arms is very helpful in the detection and detailed characterization of chromosome rearrangements. Additionally, one of the advantages of FISH technique is the possibility of detecting chromosome or chromosome fragments in interphase nuclei. Until now, FISH is not widely applied in plant mutagenesis for detection and precise localization of chromosome aberrations, because DNA probes required for particular plant species are limited. Nevertheless, there are some examples where FISH has been successfully used in analysis of chromosomal aberrations in plant cells [8,9].

Among various chromosomal aberration bioassays, the micronucleus test is widely recommended for the evaluation of the genotoxic effects of chemical and physical agents. Micronuclei could be results of acentric fragments, as well as whole chromosomes, which can be involved in micronuclei due to damaged kinetochores or spindle fibre defects. FISH with specific DNA probes can improve existing micronucleus test providing information on the mechanisms underlying the formation of chromosome aberrations [10].

The objectives of this part of study are as follows:

- The analysis of correlation between frequency of DNA breaks and frequency of chromosome aberrations after mutagenic treatment in order to assess the effectivity of the mutagens.
- The analysis of the involvement of barley chromosomes and chromosome arms in formation of chromosome aberrations following chemical mutagenic treatment. Simultaneous telomere, centromere- and 5S and 25S rDNA specific probes were used for fluorescent in situ hybrydization (FISH) in order to estimation of the frequency of micronuclei containing individual groups of chromosomes or chromosome arms in *Hordeum vulgare* cells treated by N-nitroso-N-methylourea (MNU) and maleic acid hydrazide (MH). The frequency of micronuclei with signals of used specific DNA probes were analyzed.

- The analysis of the frequency of DNA breaks by comet assay and TUNEL test after irradiation with different doses of γ ray in *H. vulgare* embryo cells in order to speed up evaluation of the mutagenic treatment by irradiation.

2. MATERIALS AND METHODS

2.1. *Chenopodium* species

Three *Chenopodium* species were investigated:

- *Chenopodium quinoa*, 2n=4×=36.
- *Chenopodium album* - 3 forms: diploid (2n=2×=18), tetraploid (2n=4×=36), and hexaploid (2n=6×=54).
- *Chenopodium berlandierii* (2n=4×=36), with 2 subsp. – cultivated *C. nuttaliae* (2 forms - Quelita and Huazontle), and berlandierii (wild cultivar).

Since the chromosomes spread is difficult to obtain from root tips, young leaves were collected for cytogenetic analysis based on mitotic chromosome preparations and extended DNA fibres (EDF). Leaves were pre-treated with 8-hydroksyquinoline for 4h in RT, fixed in methanol – glacial acetic acid (3:1) and stored in -20°C until use. Prior to use material was digested enzymatically and squash preparations were made in a drop of 60% acetic acid.

The procedure of extended chromatin fibre technique for *Chenopodium quinoa* was elaborated by modification of existing protocols for *Arabidopsis* by Fransz *et al*. [11]. Leaves of 2-week old *C. quinoa* plant were chopped in isolation buffer NIB (10 mM Tris-HCl pH 9.5, 10 mM EDTA, 100 mM KCl, 0.5 M sucrose, 4 mM spermidine, 1 mM spermine and 0.1% 2-mercaptoethanol). The homogenate was filtered through nylon mesh filter and then pelleted by centrifugation. The pellet was then resuspended in isolating buffer and the nuclei suspension was pipetted on one edge of object slide and air dried. The nuclei were then disrupted in STE lysis buffer (0.5% SDS, 50 mM EDTA and 100 mM Tris, pH 7.0) in room temperature for 45 s. The DNA fibres were stretched by tilting the glass slide and allowing the buffer to float downwards. After air-drying the slides were fixed in ethanol/acetic acid (3:1) for 2 min, air dried and store at 4°C until used.

Fluorescence in situ hybridization was applied according to the method described by Maluszynska and Heslop-Harrison [12] with minor modifications.

Different sequences were used as probes to FISH:

- 5S rDNA from *Triticum aestivum* [13] and 25S rDNA from A*rabidopsis thaliana* [14].
- two repetitive sequences isolated from *C. quinoa* genome: 12-13P and 18-24J.
- retroelement–like (22-19A, 15-5D, 21-5D) and transposon–like (20-20I) sequences from *C. quinoa* genome.
- dispersed repetitive sequences pTaq10.
- centromeric satellite pBV 1 (presents in section Beta).
- intercalary satellite pEV4 (presents in sections Beta and Procumbentes).
- subterminal satellite pAV34 (presents in all Beta sections and *Spinacia*).

2.2. *Brassica* species

Different species of *Brassicaceae* family were used for the studies. Seeds were obtained from botanical gardens, plant breeding stations, research centres and commercial sources.

Additionally, *B. rapa* - *B. oleracea* var. alboglabra MALS (2n=2×=20+1; AA+1C) monosomic addition lines (MALs) were used.

For *Brassica*, root tips were used for the cytogenetic analysis. Mitotic and meiotic chromosome preparations followed techniques described for *Arabidopsis* [12] with minor modifications.

Method of FISH was adapted with some modifications from Schwarzacher and Heslop-Harrison [16]. Different sequences were used as probes to FISH:

- 5S rDNA from *Triticum aestivum* pTa794 [13] and 25S rDNA from A*rabidopsis thaliana* [14].
- few BAC clones isolated from *B. oleracea* genome.

2.3. Mutagenesis

Seeds of the barley (*Hordeum vulgare*, 2n=14) variety 'Start' were used for mutagenesis with n-nitroso-N-methylourea (MNU) and maleic acid hydrazide (MH) and gamma irradiation. The barley seeds were pre-soaked in distilled water for 8 hours, then treated for 3 hours with 1; 2; 3 and 4 mM MNU solution or 1; 2; 3 and 4; mM MH solution, then washed 3 times in distilled water. Five doses of gamma-irradiation 150; 175; 200; 225 and 250 Gy were used to irradiate barley seeds. The determination of the doses of chemical and physical mutagens was carried out on the basis of germination and growth rate of M_1 plants. Two concentrations of chemical mutagens: 3 and 4 mM MH, 2 and 3 mM MNU and two doses of gamma rays: 175 and 225 Gy were selected for cytological analysis based on the mitotic index and frequency of chromosomal aberrations in root-tip meristems using Feulgen method.

Previously established TUNEL (according to the manufacturer's specifications of in situ Cell Death Detection Kit, Roche) and comet assay (using modified procedure according to Jovtchev *et al.* [17]) protocols for *Hordeum vulgare* root and embryo cells were used in this study. The results of the optimisation of duration of electrophoresis in comet assay showed that 14 min of electrophoresis should be used in further experiments. Then experiments to elucidate the correlation of comet assay and TUNEL test results to chromosomal aberrations test were carried out from 36 to 84 hours after treatment.

Fluorescence in situ hybridization was applied according to the method described by Maluszynska and Heslop-Harrison [12] with minor modifications. Two FISH experiments were applied on the same slides:

First one with:

- HT100.3 - telomere DNA isolated form *Arabidopsis thaliana* labelled with rhodamine-4-dUTP (Roche)
- CCS1 – centromere DNA isolated form *Brachypodium sylvaticum* labelled with digoxygenin-11-dUTP (Roche)

Second FISH experiment with:

- 5S rDNA isolated from *Triticum aestivum* - pTa 794 directly labelled with rhodamine-4-dUTP using PCR labelling kit (Amersham Life Sciences)
- 25S rDNA isolated from *Arabidopsis thaliana* labelled with digoxygenin-11-dUTP by nick translation (Roche).

The frequencies of micronuclei with specific DNA signals and without signals were calculated. The results of analysis were pooled for all concentrations of MH and MNU as well as post incubation times (36, 48, 60 h).

The protocols of TUNEL test and comet assay were also optimized for embryo cells and then level of DNA damage was estimated.

3. RESULTS AND DISCUSSION

3.1. Development of chromosome specific markers for *Chenopodium* species

To search for chromosome markers, the number and localization of rDNA loci were studied in *Chenopodium* species. FISH with 5S and 25S rDNA as probes has shown markers for only three pairs of *C. quinoa* chromosomes, whereas additionally two pairs are distinguish because of their size. The number of 5S and 25S rDNA loci was also examined in *C. album* forms: diploid, tetraploid and hexaploid. Three pairs of chromosomes could be distinguishing in diploid form of *C. album*, four, in the tetraploid form and six, in the hexaploid form. This study allowed the analysis of the evolutionary events of this species. The number of rDNA bearing chromosomes changed in correlation with the ploidy level only in the case of one 5S rDNA bearing chromosome type. The number of other 5S and 25S rDNA-bearing chromosomes in the polyploids was lower than expected. FISH with rDNA revealed the difference in the number of marked chromosomes in subspecies of *C. berlandierii*. The discrimination of three chromosome pairs was possible in subspecies *berlandieri*i, whereas four chromosome pairs could be easily distinguish in subspecies *nuttaliae* cultivar Quelita, and five in the cultivar Huauzontle. Summarizing, FISH with rDNA to chromosomes of *Chenopodium* species tagged a maximum 6 chromosome pairs, so ribosomal rDNA is an insufficient marker for their chromosomes.

To develop new chromosome specific markers for *C. quinoa* the distribution of mobile elements in its genome were analysed using FISH with retroelement-like sequences: 21- 5D, 15-5D, 22-19A and transposon like sequence: 20-20I. Analysed clones came from a *C. quinoa* genomic DNA library. After visualization and image capturing, slides were reprobed with rDNA. All retroelement-like sequences were seen as relatively weak, but with discrete hybridization signals. Two types of retroelement-like sequences (22-19A, 15-5D) were present on all eighteen chromosome pairs, whereas 21-5D hybridized only with twelve pairs. In all cases hybridization signals were preferentially localized in a pericentromeric region, but chromosomes with distal signal were also observed. A few chromosome groups could be distinguished basing on distribution pattern of analysed sequences. A characteristic banding pattern for one pair of chromosomes was observed when 15-5D and 22-19A were applied. Additionally three pairs of homologous chromosomes were distinguishable when these repetitive sequences were combined with 25S and 5S rDNA.

Similarly, a transposon-like sequence (20-20I) was detected on all chromosomes, mainly in the pericentromeric region. Two chromosome pairs with numerous small signals dispersed along the whole chromosome arms were present. In interphase nuclei signals of hybridization with all elements were present mainly in heterochromatic regions.

The chromosomal distribution of retroelements in plants shows high variability and depends on retroelement types and host genome organization. Ty1-copia retrotransposon were distributed uniformly along chromosomes of *Beta vulgaris* whereas LINEs showed an organization in discrete clusters [18] similar to this was observed in *C. quinoa* genome. Discrete clusters of Ty1-copia retroelements were observed on *Gossipium hirsutum* chromosomes but in this case signals were predominantly localized near the telomeres, whereas pericentromeric region were largely devoid of signals [19]. Centromeric localization of Ty3-gypsy-like retrotransposon was detected on chromosomes of *Beta vulgaris* [20]. Opposite to retroelements, reports on the chromosomal localization of class II transposons by fluorescent in situ hybridization are rare. Stagginus *et al*. [21] indicated that this type of repetitive sequences was distributed in discrete clusters within pericentric heterochromatin regions adjacent to euchromatic region on chickpea chromosomes. Some chromosomes possessed also loci in pericentromeric heterochromatin. Both on quinoa and chickpea chromosomes transposon sequences were largely excluded from the NOR and centromere region.

FISH conducted on mitotic chromosomes of *C. quinoa* indicated that hybridization signals of 5S rDNA overlap with 21-5D and pTaq10 signals what suggested that 5S rDNA arrays are not completely separated from these two disperse repetitive sequences. A higher mapping resolution was achieved by FISH to DNA fibres (EDF-FISH). After double FISH with 5S rDNA and repetitive sequences pTaq10 hybridization signals were observed as dots arranged in shorter or longer arrays. Signals for 5S rDNA created long tracks interrupted with gaps what indicated that 5S rDNA arrays are not homogenous in

quinoa genome, but they are interrupted by unrelated sequences. Comparison of 5S rDNA and pTaq10 localization revealed that these two sequences are mainly separated and very rarely hybridization signals for these sequences were interspersed. Hybridization signals for retroelement like sequence 21-5D, similarly to clone pTaq10, were present as arrays of different length. However tracks of 21-5D signals often co localized with hybridization signals of 5S rDNA. It suggested that interspersion of the 5S rRNA genes and retroelement like sequences occurs, and that they are not completely spatial separated.

Mapping resolution of FISH using metaphase chromosomes is limited to 2-5 Mbp. The use of extended DNA fibres enhances the physical mapping resolution to the extent of 2-5 kb. Because EDF-FISH display linear position of DNA sequences it is a very powerful method to analysed organisation and interspersion of DNA probes at the kilobase level [11,22]. EDF-FISH used in studies on detailed structural organization of centromers of *Beta* or maize revealed a complex structure of that region of chromosomes [20,23]. Preliminary results obtained for *C. quinoa* genome indicated that 5S rDNA loci do not only consist of arrays of the monomer, but their structure is more complex.

Genomic organization of a few transposable elements was also examined in genome of *Chenopodium* species: *C. berlandierii* (cultivated subsp. *nuttalliae* and wild subsp. *berlandieri*) and *C. album* (diploid and hexaploid form). Analyzed clones came from a *C. quinoa* cove. 'Real' genomic DNA library and included three retroelement like sequences (22-19A, 15-5D, 21-5D) and transposon–like sequences. (20-20I). Cloned DNA probes were labelled with digoxigenine-11-dUTP by PCR. Southern hybridization experiments were conducted using DIG High Prime DNA Labelling and Detection Starter Kit II (Roche). Our results indicated that only 21-5D retroelement-like sequence was present in genomes of all analyzed species. This cloned sequence was abundant in *C. quinoa* and *C. berlandieri* genomes, but less abundant in *C. album* genome. Each species was characterized by a different hybridization pattern. After FISH with 21-5D clone hybridization signals were observed on *C. berlandieri* chromosomes as week, but discrete signals localized mainly in the pericentromeric region of every chromosome. In *C. album* genome copy number of the clone was too low to be detected by FISH.

The retroelement-like sequences (22-19A, 15-5D) and transposon-like sequences (20-20I) were present in *C. quinoa* and *C. berlandieri* genomes but not in *C. album*. These sequences were present in a fewer copy numbers in *C. berlandieri* than *C. quinoa*. It was especially easy to notice in case of 22-19A retroelement-like sequences, which was observed as a very weak band in plants of *C. berlandieri* lines. FISH on *C. berlandieri* chromosomes exhibited hybridization pattern very similar to the results for *C. quinoa* but signal number and strength was reduced. Low copy number of 22-19A clone in *C. berlandieri* made the FISH analyze impossible. The hybridization pattern indicated that used clones were characterized by disperse organization in genomes of analyzed species what corresponds to results obtained earlier for *C. quinoa*. Disperse genomic organization exhibits many transposable elements for example LINEs and Ty3-gypsy-like elements in *Hordeum* genome or En/Spm-like transposon in *Cicer arietinum* genome [21,24].

Mobile elements in plants show high variability in chromosomal localization for example *Ty1-copia* retrotransposon were distributed uniformly along chromosomes of *Alium cepa* whereas retroelements belonging to *Ty3-gypsy* family were localized in grass centromers [25,26]. In *Chenopodium* genome transposable elements were localized in many small loci mainly in pericentromeric region. Similar distribution of retroelements exhibited *Beta vulgaris* [25,27]. Discrete clusters of DNA transposon were revealed within pericentric heterochromatin regions adjacent to euchromatic region on chickpea chromosomes [21]. Localization of transposable elements in *C. berlandieri* genome is similar in all chromosomes; therefore these sequences were not as good chromosome markers as rDNA, which allowed to distinguish a few chromosome pairs in karyotype of *Chenopodium* species.

In order to find markers for *Chenopodium* chromosomes and to study the phylogenetic relationship of two other repetitive sequences isolated from *C. quinoa*, showing homology to *Beta corroliflora and Drosophila* sequences, were also applied as probes to FISH. The results showed that both sequences exist in *C. quinoa* and *C. berlandieri* genome in high copy number. One of the sequences 18-24J, hybridized to all out of 36 chromosomes of *C. quinoa* and *C. berlandierii*, however the signals were

stronger on 18 chromosomes, thus confirming allotetraploid origin of this species. The clone 12-13P is localized on all chromosomes of *C. quinoa* and *C. berlandierii* in pericentromeric regions (however the signals are weaker on 18 chromosomes) thus confirming that both ancestral species of both species possess the same centromeric sequences. These results proved that *C. quinoa* and *C. berlandierii* may have the common ancestral species.

Moreover physical mapping of three repetitive sequences on chromosomes of *Chenopodium* species was done:

- centromeric satellite pBV 1 (presents in section Beta).
- intercalary satellite pEV4 (presents in sections Beta and Procumbentes).
- subterminals satellite pAV34 (presents in all Beta sections and Spinacia).

After FISH using different conditions (hybridization stringency, hybridization time) no signals were observed indicating lack of these sequences in *Chenopodium* genome.

3.2. Development of chromosome specific markers for *Brassica* species

FISH with rDNA enabled the discrimination of a substantial number of *Brassica* chromosomes and eight chromosomal types with ribosomal genes from *Brassica* species previously described by Hasterok et al. [28] was recently completed by analyzing numerous species of *Brassicaceae*. Additionally, the intergenomic and interindividual polymorphism regarding rRNA genes was shown. Comparison the A, B and C genomes revealed the highest rDNA polymorphism in the A genome.

Molecular cytogenetic analysis of meiosis in the species of *Brassica* using double-target FISH with 5S rDNA and 25S rDNA probes was the continuation of the previous study performed on somatic metaphase chromosomes [28,29]. As the individual rDNA-carrying bivalents are also identifiable by the same way, they can be easily tracked down during diakinesis and metaphase I at meiosis. This enables detailed study of chromosome pairing both in diploid and allotetraploid species of *Brassica* as well as *B. campestris* x *B. oleracea* monosomic addition lines (MALs), which were designed for the purpose of fine analysis of meiotic chromosome interactions between closely related species of that genus.

To find new markers for *Brassica* chromosomes the distribution of *B. oleracea* (2n=18, C genome) BAC clones (BoB21L05, BoB34L12, BoB39C15, BoB14006, BoB06N23, BoB45L02, BoB02E15) was analyzed using fluorescence in situ hybridization. The BAC clones come from a *B. oleracea* genomic DNA library at the TAMU BAC Centre, Texas, USA, and were also the subject of study by Howell *et al.* [30]. Simultaneously rDNA probes were used with BAC clones to compare the localization of investigated clones with known chromosome markers for *Brassica*. FISH was applied to the *B. oleracea*, *B. campestris* and *B. napus* chromosomes. The results of FISH revealed the diversity in the number and physical distribution of investigated clones in *B. campestris* and *B. napus* chromosomes. The BoB21L05 and BoB34L12 clones are interstitially located, as discrete signals on one pair of *B. oleracea* chromosomes. In other species these two clones are mapped on more than one pair of chromosomes: BoB21L05 clone on six chromosomes of *B. campestris* and eight chromosomes of *B. napus*; BoB34L12 clone on 12 and 24 chromosomes of *B. campestris* and *B. napus*, respectively. The BoB39C15 and BoB14006 BAC sequences occur as dispersed signals in all *B. oleracea* chromosomes, as we noted in earlier study, and hybridize to 16 and 18 *B. campestris* chromosomes, respectively. In *B. napus* both clones preferentially hybridize to *B. oleracea*–derived genome, distinguishing 18 of 38 chromosomes. Application of BoB06N23 allowed identification of one chromosomes pairs in *B. oleracea and B. napus* genome. In addition, one chromosome pair of *B. oleracea* could be distinguished using BoB02E15 clone.The application of investigated BAC sequences for clear characterization of remaining chromosomes revealed the signal co-existence of these sequences with reference to one of rDNA markers and also identification of known types of chromosomes. The part of BAC clones hybridizes to chromosomes without any marker, showing a new type of *Brassica* chromosomes. One of these sequences, the BoB21L05 BAC clone showed the terminal localization on some of *B. campestris* chromosomes, and terminal and interstitial localization

on some of the *B. napus* chromosomes. The presence of chromosome with terminal localization of 21L05 BAC clone in *B. napus* may confirm the membership of this chromosome to the genome A.

In the light of earlier study on genome in the *Brassica* allopolyploids, there were no possibilities to positive differentiation of A and C genome in allopolyploids nuclei [31]. The close homology between these two genomes did not allow for chromosome identification of A and C genome by use of GISH method. The BoB39C15 and BoB14006 BAC sequences allow tracking down of particular genomes in allotetraploid *B. napus*. Both clones enable the differentiation of C genome in this species, and application of this BAC system is effective techniques of genomes identification in *B. napus*.

As BoB14006 allows identification of all chromosomes of C genome, it was used with the marker 25S rDNA in the analysis of *Brassica* genomes, with suspected changes in *in vitro* conditions. Regarding the polyploidy existence in *in vitro* cultures of *Brassica* species, the application of BAC clones was essential in such investigations. BoB14006 BAC clone and 25S rDNA were used to probe chromosomes of diploid and polyploid cells of *B. oleracea*, *B campestris* and *B. napus* calli. In the *B. oleracea* callus lines, cells with different ploidy level were observed. In diploid cells, the BoB14006 BAC sequence occurred as dispersed signals with the same level of fluorescence intensity in all *B. oleracea* chromosomes. In the polyploid cells of *B. oleracea* callus, FISH with this BAC sequence also revealed the BAC signals in all chromosomes, however, interestingly in some chromosomes the fluorescence more intense than in diploid cells. These results may indicate an amplification of retrotransposon-type sequences, which are in BAC located, to a different extent in particular chromosomes.

The BoB14006 BAC sequence allows tracking down of ancestral genomes in allotetraploid *B. napus*. This clone enables the differentiation of C genome in this species and application of FISH with this BAC is an effective technique for genomes identification in *B. napus*. In diploid *B. napus* callus cells the investigated BAC clone hybridized to 18 chromosomes, originating from the C genome. In the octopolyploid cell of *B. napus*, FISH with BAC BoB14006 can also identify chromosomes belonging to *B. oleracea*-derived genome. These results indicate that during *in vitro* culture, polyploidysation concerns equally both genomes of allopolyploid. As rDNA enabled the discrimination of a substantial number of chromosomes of Brassica FISH with these sequences as probes was also used for identification of structural chromosome aberrations in metaphases and numerical aberrations in interphase cells.

To find new markers for *Brassica* chromosome and to distinguish the species the methods for the detection of methylated DNA and histones were applied. The immunostaining with monoclonal antibodies (anti-5`mc, anti-H3K4me2, anti-H3K9me2, anti-H3K9me3) to chromosome and interphase nuclei of *Brassica* species was applied. No specific banding pattern of methylated DNA was observed on metaphase chromosomes, however the differences in the localization and intensity of signals were seen between investigated species. 8-10 chromosomes of *B. campestris* were characterized by the stronger intensity of anti-5`mc signals. All *B. oleracea* and *B. napus* chromosomes have similar centromeric signals.

The signals of the anti-5`mc, anti-H3K4me2, anti-H3K9me2, anti-H3K9me3 in interphase nuclei were located mainly in heterochromatin region (chromocentres). The pattern of methylation of DNA molecules can be informative in distinguishing the *Brassica* species, rather than as chromosome markers.

3.3. Analysis of DNA damage induced by mutagenic treatment

Somatic effects seen as reduction of germination, emergence and growth reduction were observed for all mutagens treatments. Results of seed germination test did not show significant differences between MH and MNU action. MH did not change time of germination, but percent of germinated seeds after treatment decreased with increasing the of mutagen dose. Somatic effect of MH treatment was weaker than MNU treatment. Based on these results 3 and 4 mM MH, and 2 and 3 mM MNU were used in cytogenetic analysis. The chromosomal aberrations observed after chemical mutagens treatment were acentric fragments, and dicentric chromosomes, seen as bridges at anaphase of mitosis. The highest

frequency of aberrations was observed after 4 mM MH treatment and 3 mM MNU. The mutagen in applied doses did not significantly reduce mitotic activity of root meristems.

The frequency of chromosomal aberrations increased after MH and decreased after MNU treatment with the prolongation of post incubation time. These differences can be a result of a different phase of the cell cycle when applied mutagens act: MH acts in S-phase, whereas MNU in G2 [32].

The effect of gamma rays seen as a reduction of the number of germinated seeds was not significant. In contrast to germination, irradiation of the seeds caused high reduction of seed emergence. The growth reduction increased in proportion to gamma rays dose: the highest effect, about 25%, was generated after irradiation with 225 and 250 Gy whereas no reduction was observed after 150 Gy irradiation. On the basis of the analysis of somatic effects of irradiation, 175 and 225 Gy gamma irradiation were chosen for cytogenetic test. The frequency of chromosomal aberration (bridges and fragments) after 175 and 225 Gy irradiation, in 36 hours after treatment, of about 8%, was similar. Prolongation of post incubation time after treatment caused decreasing of the frequency of chromosomal aberration. Applied doses of mutagen did not cause significant changes in mitotic index.

In subsequent experiments the correlation between frequency of chromosomal aberrations induced by MNU, MH, gamma ray and DNA fragmentation detected by TUNEL and comet assay in barley was estimated. Each of three mutagens within the concentration range used in the experiments caused nuclear DNA damage in barley cells. MH and gamma irradiation induced higher level of DNA damage than MNU. In our studies, primary roots were treated at the embryo stage, as such low values of comet parameters could be used to indicate their lower sensitivity compared with roots treated at the seedling stage. In our studies, DNA damage observed in root cells of barley roots treated at the seedling stage with 2 mM MNU were similar with DNA damage observed with 1 mM MNU in another study [17]. It is also possible that most of DNA damage may have been repaired during 36 h between treatment and cell harvest. It may have been useful to take an earlier sample, so we optimized the procedure of comet assay for embryo cells. The highest values of TD were observed in 36 h of germination, and then the level of DNA damage within analysed recovery times is decreased. Similarly in previous studies, in barley roots treated with MNU in seedlings stage, showed significant reduction of DNA damage during recovery time was observed [17]. Nevertheless, in our present studies, we did not observe a complete repair of DNA damage in analysed post incubation times, even with comets values were over the control. Results of comet assay indicate that MH induced higher level of DNA damage, and/or more effective repair processes are activated than in case of MNU.

The analysis of the level of DNA damage expressed as TD caused by 175 and 225 Gy gamma irradiation in embryo cells showed that it increased from 30h to 36h of germination. It is well known that breaks present in DNA may indicate high damage and/or repair or misrepair processes [33]. Additionally, DNA breaks can be due to repair-mediated breaks still formed with high efficiency but not effectively sealed, which leads to increase level of DNA damage.

The present studies indicated a high correspondence between the results of comet assay and CA test after treatment with gamma ray. TD values and chromosomes aberration frequency changed in direct proportion with prolongation of post incubation time after irradiation. In contrast, no clear correlation between DNA fragmentation and CA was observed after treatment of barley seeds with the chemical mutagens.

Whereas DNA damage can be measured quantitatively in comet assay, the TUNEL test shows only the frequency of nuclei with DNA fragmentation. Similarly as in case of comet assay the correlation between DNA fragmentation analysed in TUNEL test and CA gave clearer results only in case of physical treatment. The frequency of cells with DNA fragmentation after gamma irradiation analyzed in TUNEL test decreased only during early germination, till 10h of post incubation. These results indicate that the comet assay and TUNEL test can be used as a predictive test for the outcome of the CA after treatment with a physical mutagen.

Analysis of the correlation between DNA synthesis and the level of DNA damage in barley cells after gamma irradiation with use of the simultaneous bromodeoxyuridine (BrdU) incorporation into DNA and TUNEL test was done. The results showed that the cells with DNA fragmentation after mutagenic

treatment could undergo DNA synthesis. The analysis of the frequency of nuclei with incorporated BrdU showed that gamma irradiation slightly decreased the rate of DNA replication, probably in order to repair processes.

FISH applied in this study allowed the analysis of the composition of micronuclei and a better understanding of the mechanisms of micronuclei induction by two the chemical mutagens MH and MNU which are characterized by different mechanism of action. MH is a clastogenic agents, leading to chromosome breaks, and it can cause spindle fibre defects, whereas MNU as alkylating agent mainly induces gene mutations. No differences were observed in the composition of the micronuclei after treatment with the mutagens. The micronuclei with telomere specific signals, micronuclei with centromere specific signals, micronuclei without any signals and micronuclei with telomere and centromere specific signals were observed with similar frequencies after MH or MNU treatment. Both mutagens frequently caused terminal deletions, as micronuclei with signals of telomeric DNA were most often observed. Similarly Jovtchev *et al* [34] has shown that most MNU-induced micronuclei revealed telomere specific signals, whereas the frequency of micronuclei with only centromere-specific signals was very low. Similarly, the analysis of the frequency of micronuclei with signals of the investigated DNA probes did not showed differences between the MH- and MNU- induced micronuclei. In subsequent experiments, the application of rDNA together with centromeric and telomeric DNA as probes for FISH, allowed more detailed analysis of the composition of the micronuclei, by the evaluation of involvement of specific chromosomes with 5S or 25S rDNA in the micronuclei formation.

The concentrations of mutagens, as well as a post incubation times used in the study did not influence the frequency of micronuclei with signals of the DNA probes used. As such all obtained data generated were pooled for both MH and MNU. Interestingly differences between the frequency of MH and MNU induced micronuclei with specific signals follow from the results. The micronuclei with signals of telomeric DNA and rDNA were the most frequently observed in both mutagens, however with a higher frequency after MH treatment (46%) than MNU (37%). Moreover, only 10% of MH-induced micronuclei are characterized by presence of telomere DNA sequences only, whereas almost 3 times more in case of MNU- induced micronuclei (28%). These results could indicate that in the case of MNU, a higher number of small distal acentric fragments (which are not including rDNA loci) are involved in micronuclei formation. In contrast, MH rather leads to large acentric chromosome fragments including rDNA loci, which are located in the interstitial regions or near the centromere.

In this study, the presence of micronuclei revealing centromeric and telomeric signals could indicate aneugenic action of the mutagens, which fit the expectations in the case of MH, but was not expected for MNU. It is related to the mechanism of action, in that MH can cause spindle fibre defects [17,35,36].

The application of FISH with few DNA probes, including reprobing, in detailed characterization of chromosomal aberrations in plants is not known. There are only few examples where rDNA sequences are used as probes in FISH, to distinguish between micronuclei of different origin and detailed characterization of other chromosomal aberrations [10,37].

As the quantification of micronuclei in plant cells is not very popular, results presented are very valuable in plant genotoxicity studies due to understand the mode of action of MN inducers.

4. CONCLUSIONS

The FISH with rDNA probes to chromosomes of *Chenopodium* species tagged a maximum of 6 pairs of chromosomes. Also the distribution pattern of retroelements-like sequences and transposon-like sequences coming from the *C. quinoa* cv. ‘Real’ genomic DNA library, did not provide sufficient and convenient chromosome markers. Only a few chromosome groups in *C. quinoa* are distinguished, based on distribution pattern of analyzed sequences. A significant progress was made using resolution extended DNA fibres technique: a co-localization of retroelements-like sequences with 5S rDNA in *C. quinoa* chromosomes was showed. In addition the application of the two other repetitive sequences

isolated from *C. quinoa* did not bring new chromosome markers however proved that *C. quinoa* and *C. berlandierii* may have the common ancestral species.

Similarly, beside rDNA more molecular markers are still needed for identification of all chromosome pairs of Brassica species. The different DNA sequences localize on chromosomes using BAC- FISH are good chromosomes marker. The physical mapping of few *B. oleracea* BAC clones enabled to distinguish new chromosomes types with specific distribution pattern in *B. campestris* and *B. napus* genomes. Surprisingly, using this technology, a progress has been made with respect to the identification of genomes in allotetraploid *Brassica napus*.

Cytogenetic effects of mutagenic treatment observed as chromosomal aberrations, such as fragments, bridges and micronuclei in root cells can be detected using simple cytogenetic methods such as Feulgen staining. Even though the availability of region and chromosome specific DNA probes in plants is still not as wide as for human, FISH improves the effectiveness of the assessment of the effects of mutagenic treatment. One of the advantages of FISH is the possibility to study chromosomal fragments in interphase nuclei. MN test combined with FISH made possible to explain their origin. Due to the possibility of distinguishing the majority of barley chromosomes by presence and specific localization of 5S and 25S rDNA, these sequences and additionally telomere- and centromere-specific probes were used as probes to FISH. Two FISH experiments (including reprobing) were used for identifying the specific chromosome or chromosome fragments involved in the micronuclei, induced by treatment with MH and MNU. Differences between the frequency of MH- and MNU-induced micronuclei with specific signals were observed. We showed that in case of MNU a higher number of small distal telomeric fragments (which are not including rDNA loci) are involved in micronuclei formation. In contrast, MH rather leads to large acentric chromosome fragments, including rDNA loci. Application of new plant chromosome markers as probes for FISH can make this method more valuable.

Molecular methods enable analysis of direct effects of mutagenic treatment. Quantitative analysis expresses by frequency of cells with DNA fragmentation is possible in TUNEL test. Comet assay make possible to estimate the level of DNA damage in one single cell. The comparison of DNA breaks and chromosome aberrations frequency in root cells enabled the description of the effectiveness of repair processes, then leading to CA. The potential usefulness of the analysis of the level of DNA breaks in embryo in order to speed up the evaluation the effectiveness of mutagenic treatment was demonstrated.

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MOLECULAR CYTOGENETICS OF TRITICEAE POLYPLOIDS: LYMEGRASS AND WHEAT X LYMEGRASS HYBRIDS

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Abstract

The aim of this project was to develop molecular cytogenetic markers for identification and characterization of genomes of perennial polyploids in the tribe *Triticeae* (*Poaceae*), especially *Leymus* and related genera. These markers will enable taxonomic classification of this plant group, will lead to a better understanding of genetic resources for wheat breeding, can be used for verification of chromosome transfers into wheat crops and in triteymus breeding lines suitable for cultivation in cold climates. Two types of repetitive sequences have been obtained in this project: (A) dispersed retroelement-like sequences from the tetraploid Alaskan lymegrass species *L. mollis* and the octoploid Icelandic/European species *L. arenarius*, all of which are highly specific to the N^sgenome of *Leymus* and related genera, and (B) sub-heterochromatic satellite tandem repeats from the tetraploid American lymegrass species *L. triticoides*, sequences which are prevalent in North American lymegrass species but not detectable in European and Asiatic lymegrass. Both types of Ns specific sequences can be used for accurate identification of lymegrass chromosomes in natural polyploids and in breeding materials.

1. INTRODUCTION

The genus *Leymus* (lymegrass) comprises about thirty polyploid, perennial grass species in the tribe Triticeae, family Poaceae. Cereal crops such as wheat, barley and rye are among the most important members of this tribe. *Leymus* has its main distribution in the temperate regions of Eurasia and North America. Its natural habitats range from coastal to inland areas, including diverse soil types and climatic conditions. Lymegrass is often a pioneer plant in an open or disturbed habitat, due to the ability of its extensive rhizome system to bind soil/sand as well as the plant's tolerance to environmental stresses such as salinity and drought.

Lymegrass is also known as having prolific growth and producing large and numerous seeds. Grains harvested from natural stands of lymegrass in south Iceland were used for human consumption (bread) and evidence of lymegrass cultivation was also found at sites associated with Norse settlements in North America. The possibility of domesticating lymegrass has been documented. The soil binding quality of lymegrass, together with its perennial habit, large seeds and tolerance to diverse environmental conditions, makes lymegrass attractive as a potential crop for farming in marginal habitats or in a sustainable, multi-species, and perennial system of future agriculture. Amphiploids have been developed from crosses between wheat and lymegrass (*Triticum* x *Leymus*) with an aim to increase agronomic quality and yield, hopefully making *Triticoleymus* a viable, perennial grain crop for sub-arctic regions [1-3]. Numerous *Triticoleymus* (triteymus) genotypes have been generated and different combinations of wheat and lymegrass genomes have been obtained. Some of these are annual fertile amphiploid lines deriving from crosses made in the seventies, whereas others are hybrids and hybrid derivatives containing uncharacterized intergenomic translocations and various chromosomal rearrangements [3-5].

In order to prepare for more targeted breeding strategies, these lymegrass hybrids and triteymus materials need to be examined. While wheat chromosomes are well characterized and mapped, lymegrass chromosomes are still relatively unknown. No reliable *Leymus* karyotypes, in which all chromosomes can be differentiated, have been made. The polyploid genus *Leymus* has only recently been confirmed as being auto- or segmental allopolyploid consisting of the basic N^sgenome [6-9], while in other cases *Leymus* is still considered being allopolyploids consisting of an unknown X^mgenome together with the N^sgenome from *Psathyrostachys*.

The aims of this project were therefore: A) To develop and use molecular cytogenetic markers for characterization of genomes of perennial polyploids in the tribe Triticeae (Poaceae), especially *Leymus* and related genera. These markers will enable genomic classification of this plant group, a possible taxonomic revision, and a better management of germplasm collections for wheat breeding. B) To develop and use molecular cytogenetic markers to accurately identify N^schromosomes and chromosomal regions, for identification of specific chromosome transfers into wheat and lymegrass chromosomes in triteymus breeding lines that are being developed for cultivation in colder regions such as Iceland.

2. MATERIALS AND METHODS

2.1. Plant material

Plant accessions used in this study were established from seeds, most of which were kindly provided by Dr Bjorn Salomon, from the Swedish University of Agricultural Sciences at Alnarp, Sweden. Accessions indicated with * were collected within this project in Iceland. Species indicated with ** were either collected by the author from outside Iceland or they were commercial cultivars. Triteymus amphiploids and wheat x lymegrass hybrid plants were developed prior to this project [3-5]. The plants were maintained in pots in growth rooms with 15°C and 16 h day-length. Young leaves were used for DNA extraction, whereas root-tips were collected for chromosome preparation. Plant species and breeding materials are:

- *Leymus* – Eurasian and Asiatic
    - *L. alaicus* 4× (H-10772: URS)
    - *L. chinensis* 4× (H-10776 & H-10777: Inner Mongolia & China)
    - *L. multicaulis* 4× (H-10789 & H-10790: URS & China)
    - *L. paboanus* 4× (H-10791 & H-10792: URS)
    - *L. pseudoracemosus* (H-10793: China)
    - *L. racemosus* 4× (H-10794 & H-10795: Ukrane)
    - *L. ramosus* 4× (H-10796 & H-10797: URS)
    - *L. secalinus* 4× (H-10799 & H-10800: Inner Mongolia & China)
    - *L. arenarius* 8× (Icelandic accessions)*
    - *L. karelinii* 8× (H-7548 & H-10787: China)
    - *L. angustus* 12× (H-10773 & H-10774: Turkey & China)
- *Leymus* – North American
    - *L. cinareus* 4× (H-10779 & H-10781: USA)
    - *L. innovatus* 4× (H-10785: Canada)
    - *L. mollis* subspecies *mollis* 4× (Is-Lm1: Alaska/Iceland*; H-10435: Greenland)
    - *L. salinus* 4× (H-10798: USA)
    - *L. triticoides* 4× (H-10801 & H-10802: USA)
- *Psathyrostachys* – Asiatic, 2×
    - *P. huashanica* (H-7002 or H-4387)
    - *P. fragilis* (H-4375)
    - *P. juncea* (H-7451 & H-10108)
    - *P. lanuginosa* (H-8803)

- Other species
    - *Hordeleymus europaeus* 4× (H-5029)
    - *Hystrix longearistata* 4× (H-10654)
    - *Elymus alaskanus* 4× (Is-9605)*
    - *Elymus caninus* 4× (Is-9601)*
    - *Elymus enysii* 4× (H-3153)
    - *Elytrigia repens* 6× (Is-9604, Is-G12, Is-N1, Is-N2)*
    - *Thinopyrum junceum* 6× (K-02: Spain)**
    - *Agropyron cristatum* (H-10154)
    - *Triticum aestivum* 6× (bread wheat cv. Chinese Spring)**
    - *Hordeum vulgare* 2× (barley)**
    - *Secale cereale* 2× (rye)**
    - *Triticoleymus* genotypes and breeding lines
    - Fertile 6× amphiploids containing 30 *Triticum* and 12 *Leymus* chromosomes.
    - Sterile *Triticoleymus* hybrids with perennial habit.
    - Semi-fertile backcrossed *Triticoleymus* derivatives that contain translocations.

2.2. Isolation and cloning of *Leymus*-specific DNA sequences from *L. mollis*

Isolation and cloning of *Leymus*-specific DNA fragments from *L. mollis* and *L. arenarius* were performed and described in Anamthawat-Jónsson and Bödvarsdóttir, 2003 [8]. Genomic DNA was isolated from fresh leaves of *L. mollis* (accession Is-Lm1) and used as probe in Southern genomic hybridization experiments, whereby the probe was allowed to hybridize to *Bam*HI, *Dra*I, *Eco*RI and *Hin*dIII digested genomic DNA of the probe species and many other *Leymus* and *Psathyrostachys* accessions in a varying level of hybridization stringencies, with or without blocking. The results were then examined and bands that were present essentially in *Leymus* species were identified. The restriction enzyme digested DNA samples were separated again by agarose-gel electrophoresis and the bands corresponding in size to those identified in Southern blots were excised, purified and cloned in pUC18 plasmids. The cloned fragments were screened with labelled total genomic *L. mollis* or *L. arenarius* probes by dot-blot hybridisation. Clones showing intense hybridisation signal, presumably containing repetitive DNA fragments abundant in the probe species, were then selected for further characterization and sequencing.

2.3. Isolation and cloning of *Leymus*-specific DNA sequences from *L. triticoides*

Isolation and cloning of *Leymus*-specific DNA fragments from *L. triticoides* were performed and described in Anamthawat-Jónsson *et al.,* 2009 [10]. A C_0(t-1) DNA plasmid library was generated from genomic DNA of American lymegrass species *L. triticoides* (accession H-10802). From over a thousand clones, probed by colony hybridization with labelled *L. triticoides* genomic DNA, about 10% of the clones showed strong signals. Ten plasmid clones showing the strongest signals were selected for further characterization by Southern blot hybridization and they were sequenced. The insert size of these clones ranged from 120 to 750 bp. The sequence comparison revealed that six clones partially overlapped and were members of a single DNA family, assigned Lt1 family. Using the sequence data of the aligned C_0(t-1) DNA clones, primers were derived from conserved motifs and full-length repeating units were isolated by PCR. Gel electrophoresis showed PCR products with a basic amplicon of approximately 380 bp and multimers thereof.

2.4. Fluorescent in situ hybridization (FISH)

Chromosome preparations were made from fixed toot-tips of plant accessions listed in section 2.1, using standard enzymatic squash method and were used in FISH experiments following the protocol previously described [11,12]. The DNA probes (selected clones from 2.2 & 2.3 above) were labelled directly, using a standard Nick translation method, with either green-fluorescing Fluorescein-12-dUTP (Enzo-Roche) or red-fluorescing SpectrumRed^{TM} dUTP (Vysis). The labelled probes were purified through ProbeQuant^{TM} G-50 Micro Columns (GE Healthcare), following the manufacturers protocol. The hybridization mix containing the labelled probe(s) was applied to the chromosome preparations, after which they were denatured at 89°C for 10 min in a PTC-100 Programmable Thermal Controller (MJ Research). The stringency of hybridization was about 75% in all experiments. After hybridization, the chromosomes were counterstained with blue-fluorescing DAPI (4, 6-diamidino-2-phenylindole, Sigma). The FISH signal on chromosomes was examined under a Nikon Eclipse 800 fluorescence microscope with 1000× magnification using appropriate filter sets, and the images were captured with a Nikon DXM 1200F digital camera.

3. RESULTS AND DISCUSSION

3.1. Molecular cytogenetic characterization of *Leymus mollis* sequences

Out of five repetitive DNA clones selected for further molecular characterization, three were found to be specific to *Leymus* and *Psathyrostachys*, i.e. pLm1, pLm44 and pLm53 (Table 1). None of these clones hybridised only to *Leymus*, but to both genera equally. These sequences are therefore referred to as *Leymus-Psathyrostachys* specific sequences.

The *Leymus-Psathyrostachys* specific sequences isolated here are not only present in the genome of these two genera in a relatively high copy number, but they are also dispersed throughout the whole genome. The FISH mapping experiments showed that all five clones (Table 1) hybridised to all chromosomes of *Leymus*, with uniform hybridisation signal along the chromosome arms except at centromeres, telomeres and nucleolar organizing regions. Examples of dispersed hybridisation patterns on chromosomes are shown in Fig. 1.

TABLE 1. *LEYMUS*-SPECIFIC SEQUENCES FROM *L. MOLLIS* AND *L. ARENARIUS*

Clone (in pUC18)	GenBank accession No.	Cloning site	Insert size (bp)	Hybridisation to *Leymus* and *Psathyrostachys*	Sequence type, based on Sequence homology search
pLm1	AY188516	*Hin*dIII	1,277	Relatively specific	*Gypsy*-type LTR retroelement (wheat, barley)
pLm44	AF493969	*Hin*dIII	1,164	Highly specific (most abundant)	*Gypsy*-type LTR retroelement (wheat)
pLm51	AY188517	*Hin*dIII	1,514	Not specific	*Gypsy*-type LTR retroelement (wheat)
pLm53	AY188518	*Hin*dIII	1,424	Relatively specific	*Copia*-type LTR retroelement (barley, rice)
pLa56	AY188519	*Eco*RI	1,514	Not specific	Retroelement-like sequence (Triticeae)

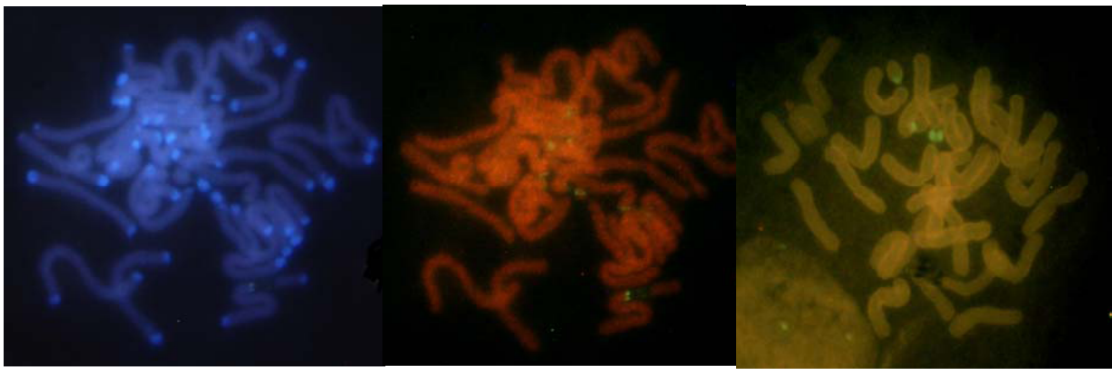

Fig. 1. Mitotic metaphase from root-tip of Leymus triticoides plant (2n = 28, tetraploid) showing DAPI-stained chromosomes (far left). Intense DAPI bands at sub-telomeric regions of most chromosome arms are common characteristics of this species. The same metaphase cell (centre) after fluorescent in situ hybridization (FISH) with red fluorescently rhodamine-labelled clone pLm44. Probe hybridization sites are distributed along chromosome arms of all chromosomes except at centromeres, telomeres, NORs and DAPI-positive sub-telomeric blocks. Mitotic metaphase from root-tip of a tetraploid Elymus plant (far right) after FISH with the same pLm44 probe under exactly the same hybridization condition. The N^S-genome specific probe does not hybridize at all to Elymus, whereas the rDNA sites (green fluorescently labelled) are positively localized on the chromosomes.

There is no differentiation in FISH labelling that indicates the existence of different ancestral genomes in *Leymus* (Fig. 1). In other words, all genomes of *Leymus* are N^S genomes. The *Leymus* species are therefore autopolyploids, or segmental allopolyploids if arising from hybridisation of sufficiently diverged N^S species. In fact it was suggested already in 1991 [13] that tetraploid *Leymus* species were segmental alloploid consisting of $N^S{}_1N^S{}_2$genomes and this was based on an analysis of genome-wide repetitive sequences. On the other hand, autotetraploid cytotypes, presumably having only N^S genomes, do exist in the diploid genus *Psathyrostachys* [14]. Interestingly, multivalent frequency in these cytotypes is low for autoploids, and this was suggested to be due to a pairing regulation mechanism. Multiple ploidy levels have been reported to occur frequently among *Leymus* species, possibly resulting from the union of unreduced gametes, for example *L. salinus* in North America has tetraploid, hexaploid and octoploid individuals [15], and the ploidy of Eurasian *L. angustus* seems to be variable ranging from 2n numbers of 28 to 84 [16]. Autopolyploidy, together with an effective genetic control of meiotic pairing, seems to have occurred and may have been a common event in the *Leymus* and *Psathyrostachys* evolution.

The N^S genome specific dispersed repeats isolated here have been used successfully to confirm the existence of N^S genome in *Leymus* and *Psathyrostachys* species from different geographical regions (listed in Table 1). Furthermore, species that were suspected to contain N^S genome were confirmed using these repeats as probes in FISH and Southern experiments. These are wood barley *Hordelymus europaeus* [17] and a few *Hystrix* species [18].

3.2. Molecular cytogenetic characterization of *Leymus triticoides* sequences

The C_0(t-1) sequences, represented by the Lt1-1 clone (Fig. 2), were mapped by FISH to the sub-telomeric chromosomal regions of *L. triticoides*, and co-localized with the bright DAPI-positive heterochromatic blocks, which are a distinctive feature of this *Leymus* species. These heterochromatic blocks (Fig. 1), although of varying sizes, were found on all or nearly all chromosomes of this tetraploid species (2n=4×=28): at 42-46 sites in accession H-10801 and 38-42 sites in accession H-10802, most often on both chromosome arms.

C_0(t-1) DNA fraction is an enriched source of highly and moderately repetitive DNA sequences. In plant species having a large genome, such as wheat and its relatives, this fraction is likely to contain a large proportion of repetitive DNA, and hence a convenient source of repetitive DNA sequences that can be used as chromosomal markers. Highly repeated satellite sequences for the identification of cereal hybrids and introgression lines and for in situ karyotyping have been isolated from C_0(t-1) DNA

fractions, relic DNA and ladder-like restriction fragments of genomic DNA, for example from rye *Secale cereale* [19], goatgrass *Aegilops speltoides* [20] and many other non-crop Triticeae species. The Lt1 satellite repeats obtained here are the first to be isolated from C_0(t-1) DNA fraction of *Leymus*.

Plant satellite sequences are often species- or genome-specific, and are therefore useful as diagnostic markers for rapid and reliable identification of species, including hybrids and natural allopolyploids. The large number of species- or genus-specific sequence families in higher plants indicates a rapid turnover of satellite DNA and hence can be characterized as rather fast evolving genome components [21]. Repetitive sequences that evolve with such a high rate can be used to differentiate related species. The Lt1 satellite repeats isolated here have been shown to be prevalent among American lymegrass species, while essentially absent in European and Asiatic species (unpublished results). Significant genetic distance between the American tetraploid *L. mollis* and the octoploid European lymegrass *L. arenarius*, which are nearly identical morphologically, was previously revealed by AFLP analysis and rDNA FISH [22]. This phylogeographical divergence may have had its cause in the different histories of plant survival and migration in the post-glaciation time.

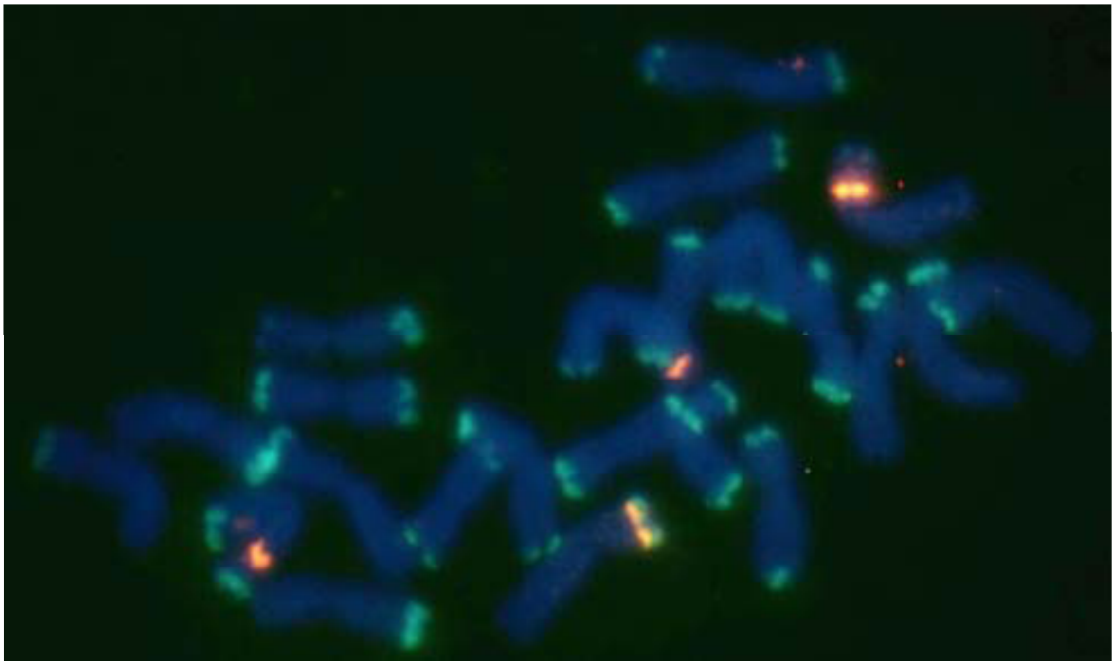

Fig. 2. Mitotic metaphase chromosomes from Leymus triticoides showing double FISH using Fluorescein-labelled Lt1-1 probe and SpectrumRed-labelled pTa71 (18S-25S ribosomal gene from wheat). The green signals of Lt1-1 colocalize with all DAPI-positive bands in this accession. The ribosomal gene mapping confirms the identity of Leymus chromosomes.

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DEVELOPMENT OF PHYSICAL CYTOGENETIC MAPS FOR BANANAS AND PLANTAINS

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Abstract

The aim of the research project carried out under the research contract was to unravel the long-range molecular organization of banana (*Musa* spp.) chromosomes and develop a fine physical cytogenetic map of this crop. During the project execution, new cytogenetic markers were isolated and used to characterize the banana karyotype. The analysis of nucleotide sequence of the ITS region was used to characterize genetic diversity and establish phylogenetic relationships within the family *Musaceae*. Moreover, massively parallel 454 sequencing was used for low depth sequencing of the nuclear genome of *M. acuminata* **'**Calcutta 4'. Most of all types of repetitive elements in the banana genome were characterized and a specific database of banana repetitive elements was created and used for analysis of sequence organization in already sequenced BAC clones. The 454 sequence data obtained in this work should facilitate annotation of nucleotide sequences during the ongoing banana genome sequencing project.

1. INTRODUCTION

bananas (*Musa* spp.) are giant perennial herbs growing mainly in developing countries of tropics and subtropics. The so-called desert bananas are palatable when eaten raw and constitute a major export commodity second only to citrus in terms of the world fruit trade. For many developing countries, bananas provide an essential source of foreign exchange. Nevertheless, only about 13% of banana production enters the world trades. Most of bananas are grown by small farmers for local consumption and eaten raw, cooked (plantains and cooking bananas) or fermented (beer banana). In terms of production as well as its gross value, banana ranks fourth (after rice, maize and wheat) in the world. By providing the staple diet for millions, banana production plays an important role in ensuring food security in many countries of Asia, Africa and Latin America. The production of bananas has been seriously threatened by an ever-increasing range of fungal, viral and insect diseases. In the absence of locally adapted resistant varieties, the diseases can only be controlled by extensive use of pesticides, which represents a considerable economic and environmental burden. Clearly, there is an urgent need for a wider diversity of genetically improved cultivars with disease resistance, increased yield, and adaptability to a wider range of environmental conditions.

Unfortunately, banana breeding has been complicated by the plant biology. While the wild bananas are diploid (2n=2×=22) and bear seeds, most of cultivated edible bananas are sterile triploid (2n=3×=33), produce fruits parthenocarpically and are multiplied vegetatively. Moreover, the origin of cultivated clones is not known. As a consequence, classical breeding endeavours achieved a limited success and no man-bred variety has been widely accepted until now. It is envisaged that mutation induction and genetic transformation will facilitate localization, isolation and transfer of genes controlling various characters, and aid in the development of new banana varieties resistant to diseases and with improved quality traits.

The objective of the research carried out under the research contract is to analyze the molecular organization of the banana (*Musa* spp.) genome, unravel the long-range molecular organization of its chromosomes, develop a fine physical cytogenetic map of banana and, if possible, integrate it with the existing genetic linkage map. The new information obtained, physical maps and cytogenetic markers would be used to characterize genetic diversity within the *Musa* genus and analyze karyotype changes in mutant banana stocks with altered chromosome numbers.

2. MATERIALS AND METHODS

2.1. Plant material

All banana plants were obtained as *in vitro* rooted plants from INIBAP Transit Centre (Katholieke Universiteit Leuven, Belgium). After transfer to soil, plants were maintained in the greenhouse.

2.1.1. Estimation of nuclear genome size

Approximately 50mg of midrib was cut from a young *Musa* leaf and transferred to a glass Petri dish. About 10mg of a young leaf of soybean (*Glycine max* L. cv. Polanka) with 2C = 2.5 pg DNA (Doležel et al., 1994) was added and served as an internal reference standard. The tissues were chopped simultaneously in 1ml of Otto I buffer (0.1 M citric acid, 0.5 % v/v Tween 20; Otto, 1990). Crude suspension of isolated nuclei was filtered trough a 50µm nylon mesh. Nuclei were then peleted (300 *g*, 5 min.), resuspended in 200 µl Otto I and incubated for 1hour at room temperature. Finally, 600 µl Otto II buffer (0.4 M Na_2HPO_4; Otto, 1990), supplemented with 50 µg/ml RNase and 50µg/ml propidium iodide (PI), was added. Samples were analysed using Partec PAS flow cytometer (Partec GmbH, Münster, Germany) equipped with 488-nm argon laser. The gain of the instrument was adjusted so that peak representing soybean G_1 nuclei appeared approximately on channel 200 on histogram of relative fluorescence intensity when using 512-channel scale. About 5,000 nuclei were analysed at rate 10 – 25 nuclei/sec. Three plants were measured per accession.

2.3. BAC library screening for 'low-copy' clones

Genomic BAC library of *Musa acuminata* cv. Calcutta 4 (C4BAM) was doubly spotted on one 22 × 22-cm Hybond N+ filter (AP Biotech) with the GeneTAC^{TM} G3 workstation (Genomic Solutions). Putative 'low-copy' BAC clones were selected based on weak Southern hybridization signals with genomic DNA of 'Calcutta 4', which was labelled with digoxigenin. Hybridization was done at 65°C overnight in 5 mL of hybridization buffer (5 × SSC, 2% blocking reagent 0.1% sodium N-lauroylsarcosin, 0.02% SDS) containing 400 ng of labelled probe. Stringency washing was done by incubation twice in 200 mL of 0.1 × SSC, 0.1% SDS buffer at 68°C. Hybridization signals were detected using anti-digoxigenin-AP (Roche Applied Science) and visualized after incubation with CDP Star chemiluminescent substrate (Roche Applied Science). BAC clones with very weak or no hybridization signals were selected for further work.

2.4. Selection of BAC clones after screening BAC pools with microsatellite markers

After growing overnight in 2YT medium supplemented with chloramphenicol (12.5 µg/mL) in 384-well plates, BAC clones from each of the 384-well plates were pooled, pelleted and resuspended in 4 mL of TE (10mM Tris, 1mM EDTA) buffer. Bacterial suspensions were lysed at 95ºC for 30 min, pelleted at 3,000*g* for 60 min and supernatant was diluted 25-fold by deionised water for PCR reaction. Primers specific for the sequences of microsatellite markers available in GenBank (GB codes: X87258 – X87265 and X90740 – X90750, Lagoda *et al*., unpublished) were designed using Primer3 software (Rozen and Skaletsky 2000). The PCR reaction mix (10 µL) consisted of 2 µL template pooled BAC DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 µM primers, and 0.5U of AmpliTaq DNA polymerase (Roche, Mannheim, Germany). PCR reaction was performed as follows: 30 cycles of 30 sec at 94°C, 30sec at 55°C, and 40 sec at 72°C, and final extension at 72°C for 10 min. Presence of PCR products was checked by electrophoresis on 1.5% agarose gel. Positive BAC clones were selected for further work.

2.5. Characterization of selected BAC clones for insert size

Individual BAC clones were cultured overnight in 1.5 mL LB medium supplemented with 12.5 µg/mL chloramphenicol. BAC DNAs were isolated using standard alkaline lysis method and digested to completion with *Not*I. DNA fragments were size separated by pulsed field gel electrophoresis in 1%

Gold SeaKem agarose (BMA) gel at 6V/cm, with a 1-40 sec switch time ramp, angle 120°, for 14 hr at 14.0°C in 0.5 × TBE buffer. The inserts of BAC clones was found to range from 60 kbp to 120 kbp.

2.6. BAC subcloning

In order to eliminate the negative effect of disperse repeats on localization of BAC clones; low-copy subclones were isolated from selected BAC clones. DNA of 'low copy' BAC clones was isolated using the Large-Construct Kit (Qiagen) and physically fragmented using a HydroShear DNA Shearing Device (GeneMachines). Fragments of 7 -10 kb were ligated using TOPO Shotgun Subcloning Kit (Invitrogen Life Technologies). Ligation mixtures were transformed into Electrocompetent TOP10 *E. coli* (Gibco BRL). 384 subclones from each of BAC clones were ordered in 384-well plates filled with the freezing medium (Woo *et al.* 1994), incubated at 37°C overnight and stored at -80°C. All BAC subclones were spotted onto a Hybond N+ filter and screened with labelled genomic DNA. Five subclones from each BAC showing weak signal after hybridization with labelled genomic DNA were randomly chosen for mapping using fluorescence in situ hybridization on mitotic chromosomes of *M. acuminata* 'Calcutta 4'.

2.7. Construction of a short insert DNA library enriched for repetitive DNA

Genomic DNA was extracted from fresh cigar leaves of *Musa acuminata* 'Calcutta 4', and sheared by sonication to fragments of 300-400 bp. In order to isolate genomic DNA fraction enriched in highly repetitive DNA, DNA fragments were denatured and reannealed to C_0t=0.1 and C_0t=0.05. After the reassociation, double-stranded DNA was separated using hydroxyapatite chromatography and cloned by blunt-end ligation to pBluescript II SK^+. The ligation mixture was used to transform *Escherichia coli* XL1-Blue MRF cells. C_0t libraries were constructed from 2,688 clones from C_0t=0.05 fraction, and 4,608 clones from C_0t=0.1 fraction. All clones from both C_0t libraries were spotted on two 8 × 12-cm Hybond N+ filters (AP Biotech) with the GeneTAC™ G3 workstation (Genomic Solutions). The filters were hybridized with probes for 45S and 5S ribosomal DNA (*Radka*1 and *Radka*2) and other known repetitive DNA sequences isolated from banana (Valárik et al. 2002) using the AlkPhos Direct Labelling and Detection System (Amersham). Hybridization signals were visualized after incubation with CDP Star chemiluminescent substrate (Roche Applied Science). 6,759 clones were identified as negative, indication high proportion of non-characterized and potentially repetitive DNA sequences.

2.8. Sequence analysis

DNA clones selected from C_0t libraries were sequenced at the Department of Plant Sciences, The University of Arizona (Tucson) and at The Institute for Genomic Research (Rockville). Sequence data were compared using the Dotter software (Sonnhammer and Durbin, 1995) and searched for homology to sequences in the GenBank database using BLAST 2.0.2 (Altschul *et al.*, 1997).

2.9. Genomic organization of tandem repeats and their copy number

Aliquots of genomic DNA of *M. acuminata* 'Calcutta 4' were digested using eight restriction endonucleases, *Rsa*I, *Hae*III, *Mse*I, *Alu*I, *Eco*RI, *Sma*I, *Sac*I and *Dra*I. BAC clones carrying 45S rDNA that were selected from the MA4 BAC library were digested using *Dra*I, *Sac*I and *Taq*I. Digested genomic DNA and BAC DNA were size-fractionated by 1.5% agarose gel electrophoresis, and transferred onto Hybond N+ nylon membrane (Amersham). Clones from C_0t-0.05 library containing tandemly organized sequence units (C427, 2F10, 4E2, C444 and 7D20) revealed by Dotter software were labelled using alkaline phosphatase (AlkPhos Direct Kit, Amersham) and used as hybridization probes of genomic DNA. The probes for C427, 2F10, 4E2, 18S rDNA and 26S rDNA respectively, were used for hybridization with digested DNA of BAC clones. The copy number of newly identified repetitive DNA sequences was estimated for the genomes of *M. acuminata* 'Calcutta 4' (AA genome) and *M. balbisiana* 'Tani' (BB genome). Serial dilutions of genomic DNA and PCR products of isolated repeats used as standards were dot-blotted onto Hybond-N+ membranes (Amersham). The PCR products were labelled using alkaline phosphatase and used as hybridization probes. Dots of genomic DNA and standards that gave the same intensity of hybridization signals were

identified after visual inspection. Copy numbers of individual probes were estimated assuming that 1pg of genomic DNA equals 0.978 x 10^9 bp (Doležel *et al.*, 2003).

2.10. Sequence analysis of the 45S rDNA locus and analysis of genetic diversity in *Musa*

DNA region containing ITS1 and ITS2 was amplified using specific primers ITSL and ITS4 (Nwakanma *et al.* 2003). Primer ITSL – 5´-TCG TAA CAA GGT TTC CGT AGG TG-3´ (Hsiao *et al.* 1994) is complementary to 18S rDNA and primer ITS4 – 5´-TCC TCC GCT TAT TGA TAT GC-3´ (White *et al.* 1990) anneals to 26S rDNA. The PCR reaction mix (25 µL) consisted of 10 ng of genomic DNA, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.25 µM primers, and 0.25U of AmpliTaq DNA polymerase (Finnzymes, Finland). PCR reaction was performed as follows: 30 cycles of 50 sec at 94°C, 50sec at 52°C, and 50 sec at 72°C, and final extension at 72°C for 10 min. Presence of PCR products was checked by electrophoresis on 1.5% agarose gel. PCR products were clean up using PCR Rapid Kit (Invitek, Germany) and used for direct sequencing and for cloning of the PCR products, respectively.

In the first step, PCR products of forty-eight ITS regions from different diploid banana genotypes were used for direct sequencing using dideoxy-chain reaction. Thirty-three genotypes produced readable ITS sequence with no polymorphism and fifteen genotypes produced unreadable highly polymorphic ITS sequences. PCR products of ITS region in highly polymorphic genotypes and hybrid banana clones were ligated into TOPO vector (Invitrogene, USA) and transformed into electrocompetent cells and four recombinant clones per individual genotypes were sent for sequencing. Nucleotide sequences were assembled using the Staden Sequence Analysis Package (Staden 1996) and further analyzed in BioEdit (molecular tools: Clustal W, DNA Maximum Likelihood programme, and Neighbor programme, http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Secondary structures of ITS regions were analyzed using Mfold (Zuker 2003).

2.11. Large scale sequencing

Intact nuclei of *M. acuminata* 'Calcutta 4' were isolated and used as template DNA for 454 sequencing. Sequencing was provided on GS FLX system and resulted in about 400 000 reads with average sequence length 200 bases (~ 100 MB). These amount of sequencing data represents 15% of nuclear genomes of *M. acuminata* 'Calcutta 4' (1C = 620 Mbp).

Duplicated sequences, specific linkers or primers used in sequencing reaction were removed from the dataset under the homology search using BLAST (Altschul *et al.*, 1997). BLAST program was also used for identification of other known banana DNA sequences deposited in the GenBank. Sequencing reads were assembled into contigs using cap3 program. The repeat reconstruction was done using TIGR Gene Indices clustering tools (TGICL), which have been optimized for 454 dataset (Macas *et al.*, 2007). All contigs were characterized by calculating their average read depth and genome representation, which were used for estimation of the copy number of assembled contigs (Macas *et al.*, 2007). All repetitive clusters that represent more than 0.01% of the nuclear genome of banana will be further characterized. Tandem Repeats Finder (Benson, 1999) and TRAP (Sobreira *et al.*, 2006) programmes were used for identification of tandem organized repetitive units.

2.12. Cytogenetic mapping using FISH and PRINS

Metaphase spreads were prepared from root tips of *M. acuminata* 'Calcutta 4' according to Doleželová *et al.* (1998). For FISH, inserts of selected DNA clones were labelled with digoxigenin-11-dUTP (Roche). FISH probe for 45S rDNA was obtained by labelling a *Radka*1 DNA clone containing 26S rRNA gene (Valárik *et al.*, 2002) with digoxigenin-11-dUTP or biotin-16-dUTP (Roche). 5S rDNA probe (*Radka 2*) was prepared from 400 bp insert of a part of the 5S rRNA gene (Valárik *et al.*, 2002). The sites of digoxigenin- and biotin-labelled probe hybridization were detected using anti-digoxigenin fluorescein (Roche) and streptavidine conjugated to Cy3 (Sigma), respectively.

PRINS was done according to Kubaláková *et al.* (1997). Briefly, the reaction mixture consisted of 0.1 mM dATP, dCTP, dGTP, and 0.01 mM Alexa Fluor 488-5-dUTP, 0.017 mM dTTP, 2.5 mM $MgCl_2$ and 3U/40µl of *Taq* polymerase (Finnzymes) in 1 x PCR buffer. Synthetic oligonucleotides specific for tandem repeats C427, 4E8 and 2F10, respectively were designed using the Primer3 software (Rozen and Skaletsky, 2000) and were used as primers (Table 1) at 1µM concentration. Temperature profile of the reaction consisted of denaturation at 92ºC for 1 min, primer annealing at 58ºC for 50 s and extension at 72ºC for 1 min.

Following FISH or PRINS, the preparations were counterstained with DAPI (0.2 µg/ml) and mounted in Vectashield antifade solution (Vector Laboratories). The preparations were evaluated using Olympus AX70 microscope and the images of DAPI, fluorescein and Cy3 fluorescence were acquired separately with a b/w CCD camera, which was interfaced to a PC running the ISIS software (Metasystems). The images were superimposed after contrast and background optimization.

3. RESULTS AND DISCUSSION

3.1. Characterization of a set of Musa species for genome size and the number and genomic distribution of rDNA loci

Nuclear DNA content and genomic distributions of 5S and 45S rDNA were examined in nineteen diploid accessions of the genus *Musa* representing its four sections *Eumusa*, *Rhodochlamys*, *Callimusa* and *Australimusa*, and in *Ensete gilletii*, which was outgroup in this study. The results showed that 2C nuclear DNA content ranged from 1.130 to 1.561pg in accessions representing the genus *Musa*. *E. gilletii*, which was the out-group in this work, had 2C DNA content of 1.210pg. Within the section *Eumusa*, the lowest nuclear DNA content was found in both accessions of *M. balbisiana* (2C = 1.130 and 1.133pg). The highest DNA content was found in *M. schizocarpa* (2C = 1.377 pg). An intermediate 2C DNA content (1.224-1.266 pg) was observed in *M. acuminata*. The differences between the three species of *Eumusa* were statistically significant. Although the differences between the accessions of *M. acuminata* were small (max. 3.4%), some of them were statistically significant as well.

Smaller interspecific variation of 2C DNA content was observed within the section *Rhodochlamys* (1.191-1.299 pg) but differences between some species were still statistically significant. The smallest range of nuclear DNA content variation (7.8%) was found between the species of *Australimusa*, with 2C value ranging from 1.435 to 1.547pg. The highest 2C nuclear DNA content in this study (1.561pg) was found in *M. beccarii*, the only representative of the section *Callimusa* in this study. Bonferroni's multiple comparison test revealed 10 groups distinguishable according to relative nuclear DNA content, three of them being represented by only one accession (*M. schizocarpa, M. ornata* and *M. textilis*). Five groups comprised representatives of at least two different sections; two of them involved accessions belonging to *Musa* and *Ensete*.

FISH with the probe for 45S rDNA revealed distinct hybridization sites on one pair of nucleolar organizing chromosomes in all accessions of *Eumusa* and *Australimusa*. In *Eumusa*, the sites of hybridization coincided with secondary constrictions of both chromosomes of the homologue pair. On the other hand, secondary constriction was not detectable on one of the homologues in all four accessions of *Australimusa*, indicating only one active nucleolar organizer region. A variable number of 45S rDNA sites were observed in the section *Rhodochlamys*. While three accessions possessed two sites (one chromosome pair), two accessions representing *M. ornata* were characterized by four 45S loci (two chromosome pairs). However, two additional loci were detected as very weak hybridization signals. They were located in the terminal position and did not coincide with secondary constrictions. *M. beccarii* was characterized by six sites of 45S rDNA genes (three chromosome pairs). Among the six strong hybridization clusters, only two coincided with the secondary constriction. The highest number (four pairs) of 45S rDNA loci was observed in *E. gilletii*. The intensity of the signals on different chromosome pairs differed, indicating a difference in the copy number of the 18S-5.8S-26S rRNA genes. A significantly larger variation was observed in the number of 5S rDNA loci. In the

Eumusa section, the number of 5S rDNA sites ranged from four to eight, five loci were observed in the seed-sterile clone 'Pisang Mas'. All *Rhodochlamys* accessions comprised two pairs of chromosomes bearing 5S rRNA genes except *M. velutina*, which had three chromosome pairs bearing 5S rDNA. In this case, two sites were major and four sites were minor, with significantly lower copy number. In contrast to a large variation in the number of 5S rDNA loci in other sections, all *Australimusa* accessions possessed four 5S rDNA sites. *M. beccarii* (*Callimusa*) and *E. gilletii* contained 5S rRNA gene clusters on five and six chromosomes, respectively. Two of the five 5S rDNA sites in *M. beccarii* were localized at terminal positions of chromosomes with interstitially localized signals of 45S rDNA.

3.2. Selection of 'low-copy' BAC clones based on hybridization with genomic DNA

Inserts of selected BAC clones were labelled with digoxigenin and used as probes for fluorescence in situ hybridization (FISH) on mitotic chromosomes of *M. acuminata* 'Calcutta 4' (Doleželová *et al.* 1998). Despite the expectations, even the use of pre-selected BAC clones, presumably containing least amounts of repetitive DNA, did not result in single locus signals. FISH with probes prepared from inserts of the BAC clones resulted in hybridization signals along all chromosomes. Alternatively, multiple sites of hybridization were observed resembling hybridization with tandem organized repeats. These results, together with the results obtained previously during the third year of project execution indicate that the pre-screening the BAC library with the genomic DNA and choosing for the clones with weak signals is not efficient to generate chromosome-specific cytogenetic markers. In order to eliminate the negative effect of disperse repeats on localization of BAC clones, low-copy subclones were isolated from selected BAC clones. However, none of the selected subclones could be localized. This could be due to the size of inserts which was probably too small to be localized on condensed mitotic chromosomes of banana. Therefore, another strategy based on selection of marker-tagged BAC clones was tested.

3.3. Selection of BAC clones after screening BAC pools with microsatellite markers

As the strategy based on screening the BAC library with the probe for the genomic DNA and selecting clones with the weak signals, was not efficient to generate chromosome-specific cytogenetic markers, we tested a different strategy based on PCR screening of the BAC pools with the primers specific for the sequences of microsatellite markers available in the GenBank (GB codes: X87258 – X87265 and X90740 – X90750). The positive BAC clones were isolated and used as probes for FISH on mitotic chromosomes of *M. acuminata* **'**Calcutta 4'. Twenty different BAC clones were isolated and used as probes for cytogenetic mapping. Out of them, six BAC clones gave weak discrete signals on one pair of mitotic chromosomes of *M. acuminata* **'**Calcutta 4' (unpublished).

In this work, the limited spatial resolution due to small size of compact mitotic metaphase chromosomes hampered a detailed analysis of genomic distribution of DNA sequences by FISH. In order to overcome this bottleneck, we have optimized a protocol for preparation of pachytene spreads in *Musa*. Preliminary results confirmed a possibility to perform high-resolution FISH o these preparations. The next step will be to localize selected BAC clones by FISH on pachytene chromosomes of *M. acuminata* **'**Calcutta 4'.

3.4. Isolation of new repetitive DNA sequences and analysis of their genomic distribution

Knowledge of repetitive DNA can facilitate mapping of important traits, phylogenetic studies and the tandem organized DNA repeats are useful cytogenetic markers. In this part of the work, we used reassociation kinetics to isolate and characterized the highly repetitive part of the banana genome. The so-called Cot-based cloning and sequencing approach was used to prepare two low-Cot libraries enriched on the highly repetitive DNA sequences. 614 of the Cot-clones were sequenced (sequences are deposited in the GenBank, GB codes: ED827164 – ED827777) and searched for homologies with the sequences deposited in the GenBank using BLASTn. Out of the 614 sequenced clones, 48% represented novel undescribed sequences. Up to 24.1% of the homologous sequenced clones showed

similarity to different types of retrotransposons (Fig. 1). Dot-plot analysis revealed that 14% of the sequenced clones contain tandem organized repeats. Out of these, three clones that carried different types of tandem organized units were selected for copy-number estimation and for cytogenetic localization using PRINS. One clone showed strong signal in the secondary constriction and weak signals in the centromeric regions of additional chromosomes. The other tandem organizes sequences were localized also in the secondary constriction and showed additional cluster signals on the other chromosomes of *M. acuminata* **'**Calcutta 4' (Hřibová *et al*. 2007).

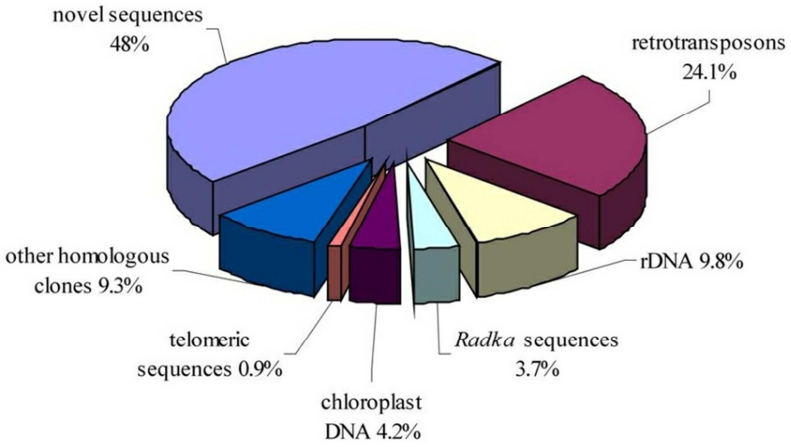

Fig. 1. Homology of DNA Clones isolated from the $C_0t \leq 0.05$ library to sequences deposited in GenBank. For details see Hřibová et al. (2007).

3.5. Localization of the new tandem repeats with respect to 45S rDNA

To confirm that the three tandem repeats constitute a part of the 45S rDNA unit that is localized in the secondary constriction, genomic BAC library of *M. acuminata* 'Calcutta 4' (MA4, Vilarinhos et al. 2003) was screened using a doubly spotted Hybond N+ filter (AP Biotech) with the GeneTAC^{TM} G3 workstation (Genomic Solutions). BAC clones carrying 45S rDNA were selected based on Southern hybridization signals with probes for 18S rDNA and 26S rDNA clones, which were labelled using alkaline phosphatase (AlkPhos Direct labelling, Amersham). Three BAC clones carrying 45S rDNA units (MA4_1P13, MA4_1J14 and MA4_1B22) were selected and digested using *Dra*I, *Sac*I and *Taq*I restriction endonucleases, size fractionated on the agarose gel and blotted onto a nylon membrane (Hybond N+). The membranes with restricted BAC clone were hybridized separately with the probes for C427, 4E2, 2F10, as well as 18S rDNA and 26S rDNA clones. Southern hybridization confirmed that the clones C427, 2F10 and 4E2 hybridized with the same band of the restricted BAC clones and indicated that the clones were parts of the 45S rDNA unit localized in the secondary constriction. For details see Hřibová *et al*. (2007).

3.6. Karyotype analysis

Cytogenetic analysis of the plant nuclear genome is one of the basic tools used in phylogenetic analysis. In order to characterize karyotype diversity in *Musa*, we elected to analyze two varieties of *M. beccarrii* (var. *beccarii* and var. *hottana*), section Callimusa. As described previously, *M. beccarii* var. *beccarii* consist of 18 chromosomes (2×=2n=18) with the 2C nuclear DNA content of 1.561 pg. We have found that the karyotype of *M. beccarii* var. *hottana* consists of 18 chromosomes (2×=2n=18) with different chromosome sizes and one chromosome pair carrying secondary constriction. As described previously, 45S rDNA is localized in the NOR and also in the centre of the two other chromosome pairs in the *M. beccarii* var. b*eccarii* (Fig. 2).

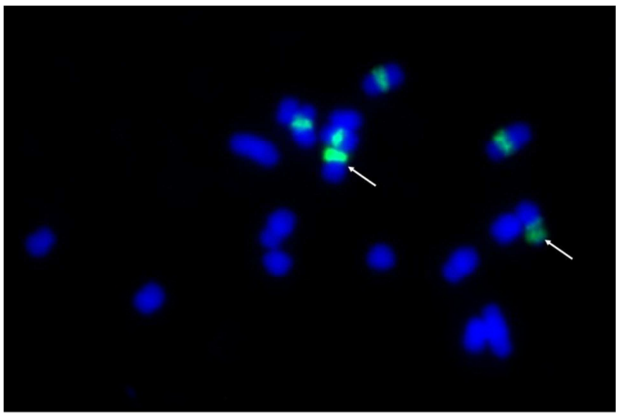

Fig. 2. Localization of 45S rDNA on mitotic chromosomes of M. beccarii var. beccarii. Probe was labelled by digoxigenin (green signals). The secondary constrictions are marked by arrows.

3.7. Evaluation of genetic diversity using repetitive DNA clones

Out of the set of 615 repetitive DNA clones that were isolated from the High-C_0t DNA library of *M. acuminata* Calcutta 4, a total of 576 repetitive DNA clones that represent various types of DNA repeats were selected. They were sent to the laboratory of Kornel Burg (Austrian Research Centers, Seibersdorf Research GmbH, Seibersdorf, Austria), where they are being used to assemble a boutique microarray.

3.8. Sequence analysis of the 45S rDNA locus and analysis of genetic diversity in *Musa*

The taxonomy and phylogenetic relationships within the genus *Musa* have never been fully resolved and remain a subject of debate. Recently, the internal transcribed spacer (ITS) region of the 18S-5.8S-26S nuclear rDNA has been utilized as a marker for phylogenetic analysis in many plant families. In this part of the research project, we focused on the ITS1-5.8S-ITS2 region of nuclear ribosomal DNA. The nucleotide sequences of the ITS regions were edited using BioEdit software and a phylogenetic tree was constructed based on the neighbour joining method. The tree rooted on *Zingiber* spp. supports the genus *Musa* as monophyletic group that is separated from genus *Ensete* and *Musella*. The genus *Musa* is divided into two distinct clades: a clade of *Callimusa* and *Australimusa* and a clade of *Eumusa* and *Rhodochlamys*, where the A genome is strictly separated from the B genome (unpublished data).

In order to obtain further insights into the structure of the 45S rDNA locus in *Musa* and isolate additional molecular markers suitable for analysis of genetic diversity, we selected three BAC clones from *M. acuminata* **‘**Calcutta 4’, *M. acuminata* **‘**Tuu Gia’ and *M. balbisiana* **‘**Pisang Klutug Wulung’ carrying 45S rDNA. These clones were fully sequenced and the sequence data are now being analyzed. We expect that markers developed from the external spacer will be useful for the analysis of genetic diversity in *Musa* as well as to study genomic constitution in interspecific hybrids.

3.10. Large scale sequencing

In this project, we used the 454 method for sequencing nuclear genome of *M. acuminata* **‘**Calcutta 4’. In one sequencing run provided by GS FLX system (Roche), we obtained almost 100 Mb of nucleotide sequence that represents 16% of the *M. acuminata* **‘**Calcutta 4’ genome. Until now, this is the largest amount of genomic sequence data available for *Musa*. Based on the experience with other species (Macas *et al.* 2007) we expect that this amount of sequence data will make it possible to capture most of the repetitive DNA in the genome of *M. acuminata*. The data are currently being analyzed using different bioinformatics tools. Various types of mobile elements and new tandem organized repeats were classified and characterized for copy number and genomic distribution (Fig. 3). The 454 sequence data will be also very important for annotation of nucleotide sequences that will be obtained during the ongoing banana sequencing project.

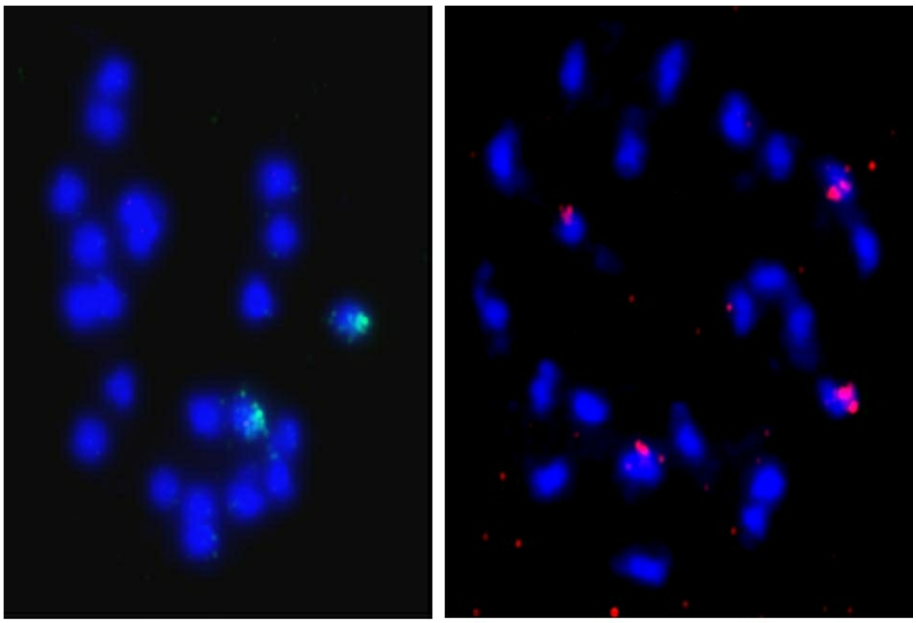

Fig. 3. Localization of tandem repeat CL18 (left) and CL 33 (right) on mitotic chromosomes of M. acuminata 'Calcutta 4'.

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MOLECULAR CHARACTERIZATION OF THE GRAIN GELATINIZATION TEMPERATURE TRAIT IN RICE *(ORYZA SATIVA L.)*

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Abstract

Gelatinization temperature (GT) is an important cooking quality character of rice grains. Low GT rice is preferred for easy cooking with less energy intake. The physical-chemical properties of a low GT mutant, induced by gamma irradiation, were investigated through molecular mapping. The gene controlling the GT was identified to be located on chromosome 6. Through candidate gene sequencing and expression analysis, the GT gene was identified to be the *SSIIa* gene, which affects GT through amylopectin structure. The $GC/TT_{2340\text{-}41}$ SNPs of *SSIIa* gene, which results in a *Leu* to *Phe* change, appeared to be the causative factor of the reduced *SSIIa* activity. Analysis of a set of 67 rice varieties showed the SNP GC/TT_{2340} of *SSIIa* play a role in GT and most low GT varieties have the homozygous SNP genotype of TT/TT.

1. INTRODUCTION

Gelatinization temperature (GT) is an important cooking quality character of rice grains [1]. It is the temperature at which starch granules irreversibly lose their crystalline order during cooking [2]. GT is usually indirectly tested by alkali digestibility following the method of Little *et al.* [3] using the alkali spreading value (ASV), and rice varieties are classified into low (ASV 6-7), medium (ASV 4-5) and high (ASV 1-3) GT types, approximately corresponding to GT of $<70^oC$, $70\text{-}74^oC$, and $>74^oC$, respectively [1].

Induced mutation has become an important source of genetic variations in plant breeding programs. According to the FAO/IAEA Mutant Variety Database (http: //www-mvd. iaea.org/MVD/default.htm), more than 2300 mutant varieties were developed worldwide during the past 30 years. More mutant lines, using either chemical or physical mutagens, are being developed for functional genomic studies and for elucidating the fundamental of important biological processes in rice [4]. In this study, physical-chemical properties of a low GT mutant, induced by gamma irradiation, was investigated together with genetic analysis, mapping and cloning of the mutant gene, in an attempt to disclose the fundamentals underlining the GT character at a new and different angle from previous studies.

2. MATERIALS AND METHODS

2.1. Plant materials and population development

Rice genotypes used in this study were: the low GT mutant rice line, cv, Huangyu B; low GT commercial varieties Niqingzhan (indica rice)' and 'Xiushui 110 (japonica rice), high GT restorer line of hybrid rice indica rice variety 'R3027'. The mutant variety Huangyu B was developed through gamma irradiation of an indica rice variety, cv, II32 B which is the maintainer line of the cytoplasmic male sterile (CMS) line II32A, and following screening for quality traits in the leaf colour mutants [5].

Field trials to develop segregating populations for high and low GT were carried out between 2001 to 2004 at the experimental farm of Zhejiang University on Huajiachi Campus, Crosses were made between the high and low GT genotypes and the F_1 plants were self-pollinations to develop the F_2 populations, which were and used for genetic analysis and molecular mapping of the low GT mutation.

2.2. Grain quality measurement

Apparent amylose content (AAC) was measured according to the Chinese national standard method NY147-88 (1988), based on iodine colorimetry [1]. The standard samples with AAC of 1.5%, 13.2%, 18.3% and 24.7% were provided by China National Rice Research Institute.

GT was measured by either alkali disintegration and/or differential scanning calorimetry (DSC). Alkali disintegration was done following the method of Little et al. [3]. Briefly: A total of from the parent lines and 30 from the F_2 progenies (from individual F_2 plants) were milled rice then treated in 1.7% KOH solution at 30^{o}C for 23 hrs, and ASV was recorded in a 2-7 scale.

DSC measurement was done with a thermal analyzer (Model 2920, TA Instruments, Newcastle, DE, USA). Rice flour (1.8mg each) was weighed into an aluminium pan, mixed with 12µl distilled water, and sealed. The pan was heated at a rate of 10^{o}C/min from 30^{o}C to 110^{o}C. Another sealed pan with 12µl of distilled water was used as a control. The onset (T_o), peak (T_p), conclusion (T_c) temperatures and enthalpy (ΔH) of gelatinization were calculated automatically by the Universal Analysis Programme, Version 1.9D, which comes with the instrument.

2.3. Chain-length profile analysis of amylopectin

Rice grains of II32 B and Huangyu B were dehulled using a Satake Dehuller (Satake Corporation, Japan), milled into milled rice using a Satake Test Mill (Satake Corporation, Japan) and ground into rice flour with a Cyclone Sample Mill, (UDY Corporation, USA). Rice flour was debranched as described by Umemoto *et al.* [6] and amylopectin chain distribution was determined by HPAEC-PAD as by Nakamura *et al.* at the laboratories of Nakamura, Akita Prefectural University, Akita City, Japan based on their previously described methods [7].

2.4. Genetic analysis of GT character

To test the mode of inheritance of the low GT mutation (dominant or recessive), reciprocal F_1 crosses of Huangyu B with II32 B and Huangyu B with R3027 were produced through artificial pollination and the hybrid seeds (at least 10 for each cross) were tested with alkali disintegration method. For the segregation analysis, F_2 plants of Huangyu B/II32 B and Huangyu B/R3027 were planted at single seedling per hill, the seeds ($F_{2:3}$) were harvested on individual plants basis. Thirty $F_{2:3}$ seeds each were analyzed, and F_2 plants were designated either as low GT homozygous (all seeds with ASV higher than 4), or high GT homozygous (all seeds with ASV lower than 4), or heterozygous (seeds with varying ASV from 2 to 7).

2.5. Molecular mapping of GT character

The two F_2 populations of the crosses Huangyu B×II32 B and Huangyu B×R3027 were used in linkage mapping. Genomic DNA was extracted using leaf tissues following a modified CTAB method [8]. DNA samples were quantified using Unican UV300 (Thermo Electron Corporation, Cambridge, UK) and each DNA sample adjusted to a final concentration to about 25ng/µl for SSR analysis. Approximately 50ng genomic DNA was used as a template in individual amplification reactions.

All parent lines, i.e., Huangyu B, II32 B, and R3027, were screened for 344 microsatellite loci that cover all 12 chromosomes of the rice genome with high density. Primer sequences for these microsatellites have previously been described [9], and were acquired from the Rice Sequence Information Resources (http://dna-res.kazusa.or.jp/9/6/05 /spl_table1/ table1.pdf). Amplification reactions were performed in 20µl volumes containing 1×PCR buffer, 400nM each primer, 200µM each dNTP, 2mM $MgCl_2$ and 1U Taq enzyme and the amplification conditions set according to Temnykh *et al.* [9]. To avoid any experimental error, microsatellite analysis for each locus was repeated twice. Amplified fragments were either on 3% low melting agarose gel (CAS#9012-36-6) or

on polyacrylamide gel, based on test of the parent II32 B and Huangyu B. Polymorphic loci between parents were used to analyze the F_2 genotype of plants of the homozygous and heterozygous plant types defined above.

To obtain SSR markers linked as closely as possible to the locus, new SSR markers were identified according to the maps publicly available at http://www.gramene.org, and were tested for polymorphism between the parent lines. The polymorphic markers for individual plants from the two populations were further analyzed .Since a major QTL responsible for ASV variation had been previously mapped in the *Wx* gene region [10] and our SSR mapping results also suggested a similar behaviour for the cross of Huangyu B/R3027, the Wx gene was also analyzed in the F_2 plants of this cross using the G/T SNP marker through PCR-AccI analysis [11]. The primer sequences were: *Wx*1 (forward): 5'-GCTTCACTTCTCTGCTTGTG-3' and *Wx*2 (reverse): 5'-ATGATTTAACGAGAGTTGAA-3'. The PCR and *Acc*I (Fermantas, Canada) digestion of the amplified DNA fragments was performed according to [11], and the products detected by electrophoresis in 2% agarose gels. The linkage and genetic distance analysis between the GT gene and SSR markers were performed using JoinMap3.0 programme [12].

2.6. Preparation and measurement of GBSSI and SSIIa proteins

Proteins tightly bound to starch granules from rice grains were extracted, separated by SDS-PAGE, and stained with a Coomassie Brilliant Blue R-250 [13]. The GBSSI protein was detected by staining the band in the gel and the SSIIa protein in the SDS-PAGE was determined by immunoblot analysis using polyclonal antiserum raised against SSIIa protein purified from rice endosperm [14].

2.7. Whole gene sequencing and comparison of SSIIa genes

According to the sequence of *SSIIa* gene of an indica variety Shuangkezao in Genbank (AY423717), five pairs of PCR primer were designed for amplifying the whole *SSIIa* gene (Table 1). Primers were designed in a way so that contiguous PCR products shared a common short overlapping region, which enables to build a complete contig for the *SSIIa* gene (Fig. 1).

Fig. 1. The structure of the coding region of SSIIa gene and the positions for each primer used for gene sequencing (ALKs) and SNP development (F/Rs). The numbers are the nucleotide position from the first ATG and open square boxes and black filled boxes are introns and exons of the SSIIa gene.

TABLE 1. PRIMERS USED IN THE SSIIA GENE AMPLIFICATION AND SINGLE NUCLEOTIDE POLYMORPHISM (SNP) MARKERS DEVELOPMENT

Primers	Sequence (5' to 3')	Annealing Temperature	Primer position（amplicon size）
For sequencing of *SSIIa*			
ALK1-F	GCATGGCAGACAAAGTAACTCG		-380-934
ALK1-R	CCTCTTCTACCGCAGGAATAGG	61°C	(1314bp)
ALK2-F	CTCTCCCCAAGCCTGACAAT		820-2017
ALK2-R	CCGCGTAATCACCGTACCT	57 °C	(1196bp)
ALK3-F	GAGGGACGGTAGTTACTTTTCG		1753-2955
ALK3-R	CTGCATCATGCCATTGTCTC	57 °C	(1198bp)
ALK4-F	AACTTTTGGACTGAAGCACACA		2532-3530
ALK4-R	GGGGTCGTACAGCTTGAAGT	57 °C	(1011bp)
ALK5-F	CATCATACGGGAGAACGACT		3665-+153
ALK5-R	AATACTCCCTCAACTCCACCA	57 °C	(985bp)
For development of SNP marker			
F7	CTGGATCACTTCAAGCTGTACGAC		3515-4348
R21	ACATGCCGCGCACCTGGAAA	55 °C	(832bp)
F22	CAAGGAGAGCTGGAGGGGGC		4330-+431
R1	GCCGGCCGTGCAGATCTTAAC	55 °C	(522bp)

PCR was conducted in a final volume of 100 µl, consisting 1×PCR buffer, 400nM each primer, 200µM each dNTP, 2mM $MgCl_2$ and 1 unit Taq enzyme. A special PCR buffer (2×GC buffer I, Takara, Japan) was used for the amplification of high GC DNA fragments. Amplified DNA fragments were cut from agarose gels and purified using a gel extraction kit (Vitagen, China). Purified DNA fragments were sequenced directly by Invitrogen Biotechnology Co., Ltd (Shanghai, China) with each sample's sequencing twice at least. Sequences of various amplicons were linked using ContigExpress. The full sequences of II32 B and the mutant were blasted against reported sequence of SSIIa genes in Genbank, and aligned for identification of SNPs and indel mutations using Clustal W1.8 [15].

2.8. Analysis of GC/$TT_{2340-41}$ SNPs of SSIIa gene

The primer sequences used for detection of the GC/TT_{2340} polymorphism are listed in Table 1. The genomic positions of these primers are shown in Fig. 2. GT measurement and DNA extraction, and PCR were carried out as described before. PCR products were separated on 1.5% agarose gels.

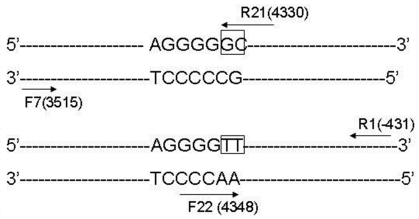

Fig. 2. The genomic position of GC/TT$_{2340}$ SNP primers.

TABLE II. THERMAL PROPERTIES OF RICE GRAINS OF THE GT MUTANT LINE AND OTHER VARIETIES

Genotype/Population	ASV‡	T_o (^{o}C)	T_p (^{o}C)	T_c (^{o}C)
Huangyu A	6.71±0.45	64.2±0.6	68.6±0.1	76.7±0.0
Huangyu B	6.83±0.38	64.1±0.7	69.0±0.4	77.8±0.8
II32 B	2.05±0.24	74.0±0.64	77.2±0.6	82.5±0.8
R3027	2.77±0.42	67.5±0.8	73.1±0.9	80.8±2.2
Niqingzhan	5.88±0.47	63.4±0.9	69.2±0.6	76.5±1.1
Xiushui 110	6.10±0.32	59.7±0.6	65.7±1.4	72.7±2.2
II32A×Huangyu B F_1	2.17±0.37	69.6±0.3	73.8±0.3	78.8±0.0
Huangyu A×II32 B F_1	3.18±0.92	68.9±0.3	73.2±0.0	78.7±0.5
LSD0.05	2.37	1.60	1.72	3.01
LSD0.01	3.59	2.43	2.61	4.56

‡ASV, alkali spreading value with a score scale of 2–7; To, Tp, Tc, are the onset, peak, and conclusion temperature of rice gelatinization analyzed using differential scanning calorimetry, respectively.

3. RESULTS

3.1. Thermal properties of Huangyu B

The ASV of Huangyu B reached 6-7, while that of II32 B was around 2 after 23hr treatment at 30^{o}C in 1.7% KOH solution (Table 2), indicating that the grain of Huangyu B could be more easily digested in alkaline solution than its parent This is confirmed by the DSC results in that, the mutant variety Huangyu B is about 6 ^{o}C lower than its parent II32 B in T_o, T_p and T_c, and ΔH values showed that Huangyu B requires 6.55 J/g less energy for gelatinization the (Table 2).

Further study showed that Huangyu B had similar digestibility to that of the low GT japonica variety, cv Xiushui110 and could be easily disintegrated than the low GT indica variety cv Niqingnian, although all three genotypes had similar ASV in 1.7% KOH solution.

3.2. Amylopectin chain length profile

Significant differences were observed in the chain length distribution of amylopectin molecules of II32 B and Huangyu B (Fig. 3). Huangyu B showed a higher ratio of chain with DP 6 to 10 and a lower ratio of chains with DP 11 to 24 than II32 B, but the longer chains with DP≥25 did not differ significantly. Consequently, the ratio of the short chains (DP≤10) to the short and the intermediate chains (DP≤24) of Huangyu B was higher (0.246) than that of II32 B (0.177).

3.3. Mode of inheritance of the GT mutation

The ASV of F_1 seeds of reciprocal crosses between Huangyu B and II32 B were all higher than that of II32 B, but slightly lower than that of Huangyu B (Table 1), which implied that to some degree, low GT (high ASV) was dominant to high GT (low ASV) but not completely. There was a clear dosage effect of this gene, where seeds with two high and one low GT copies (II32 B×Huangyu B) tended to be closer with high GT parent, while seeds with one high and two low GT copies (Huangyu B×II32 B) were similar to low GT parent (Table 3).

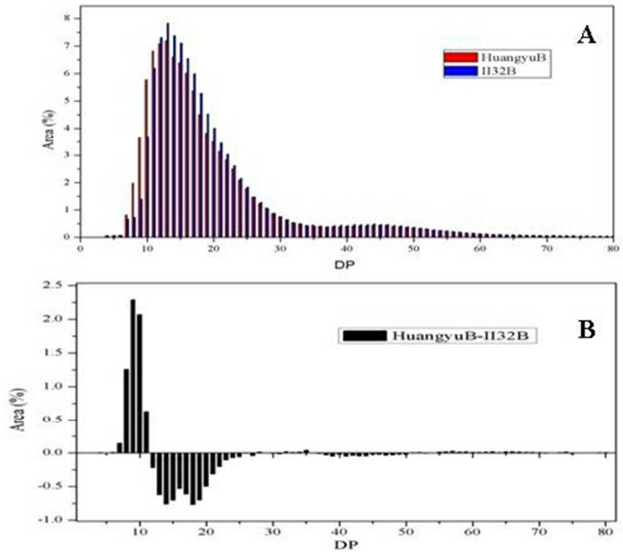

Fig. 3. Comparison of the amylopectin chain length profiles of rice endosperm from II32 B and Huangyu B, A: HPAEC–PAD analysis of debranched amylopectin from rice endosperm; B, difference in the chain-length distribution of amylopectin. DP: degree of polymerization.

TABLE 3. SEGREGATION MODE OF F_2 PLANTS BEARING $F_{2:3}$ SEEDS WITH DIFFERENT ALKALI DIGESTIBILITY

Crosses	HLASV	HEASV	HHASV	χ^2(1:2:1)	P
Huangyu B/II32 B	93	192	92	0.135	>0.9
Huangyu B/R3027	61	126	73	1.354	>0.5

HLASV, HEASV and HHASV are F_2 plants that produced $F_{2:3}$ seeds with alkali spreading values all below 3, ranging from 2 to 6-7 and above 4, respectively.

There were three distinct types of ASV variation $F_{2:3}$ seeds, which corresponded to the genetic make up of the F_2 plants. The first type of plants had $F_{2:3}$ seeds that were low in ASV, the majority of plants in this category had ASV less than 3, and thus those plants were recorded as high homozygous GT. The second type of plants had $F_{2:3}$ seeds that were high in ASV, usually higher than 5, these plants were recorded as low homozygous GT. The remaining plants produced $F_{2:3}$ seeds of different ASV ranging from 2 to 7, hence named as heterozygous GT. The segregation ratio of high homozygous GT: heterozygous GT: low homozygous GT fit a 1:2:1 in both populations (Table 3), which implied that the GT character was controlled by a single major gene.

3.4. Linkage analysis of the GT mutation

3.4.1 Huangyu B/ II32 B population

The polymorphic SSR markers between Huangyu B and II32 B were analyzed using a DNA pool of 10 F_2 plants producing $F_{2:3}$ seeds all of which had ASV<3.0. Potential markers co-segregating with the low ASV characteristic were further analyzed in 137 F_2 plants. As a result, the SSR markers RM402, RM276 and RM253 on chromosome 6 were found to be linked with alkali digestibility, with genetic distances of 2.59, 2.94 and 4.98 cM, respectively (Table 4). New SSR markers around the low GT locus were identified and synthesized according to the Gramene database (http://www.gramene.org/db) and used for further analysis (Table 4). The SSR marker RM7708 was found to be most closely linked with the gene for alkali digestibility in the Huangyu B/II32 B cross (Table 4).

3.4.2 Huangyu B/R3027 population

In a similar analysis of the F_2 population derived from the cross Huangyu B×R3027, the result was different from the population of Huangyu B×II32 B. Here we found a locus, which appeared to be responsible for the ASV variations in this population, but it is on the opposite side of the *SSIIa* gene to RM253. However, because most SSR markers between R3027 and Huangyu B were not polymorphic, initially we were only able to identify two SSR markers, i.e. RM314 and RM111 on chromosome 6. RM314 and RM111 were loosely linked with the locus that controlled the ASV variation, with genetic distances of 21.6 and 24.8 cM, respectively (Table 4). Since a major QTL for ASV had already been mapped to the *Wx* gene site [16] and our initial SSR mapping results also pointed to the same region, we further analyzed for a possible linkage between the *W*x gene and the gene for ASV variations in this F_2 population. Although, most of the homozygous F_2 plants had the parent allele combinations of the *Wx* gene and the gene for ASV, that is, the high AAC *Wx* allele was associated with the allele for high ASV, and low AAC *Wx* was associated with low ASV, as in the two parents, a very small number of recombinants with these two characters were also discovered. As a result, we identified that there is a genetic distance of 3.93 cM between the *Wx* and *ASV* gene (Table 4).

TABLE 4. GENETIC DISTANCE OF THE LOCI RESPONSIBLE FOR ALKALI DIGESTIBILITY FROM WX AND SSIIA GENES AND SSR MARKERS IN RICE CHROMOSOME 6

Gene/SSR marker	Reported genetic distance (cM)[a]	Gene/SSR polymorphism and genetic distance (cM)[b]			
		Huangyu B and II32 B		Huangyu B and R3027	
		Polymorphic	Distance	Polymorphic	Distance
Wx	22.0a; 8.2b	NT	NT	Yes	3.93
RM 204	25.1a; 12.9b	No	NA	No	NA
RM 225	26.2a	No	NA	No	NA
RM 314	33.6a	No	NA	Yes	21.8
RM 111	35.3a	No	NA	Yes	25.6
RM 253	37.0a	Yes	4.98	No	NA
RM 50	39.5a	No	NA	No	NA
RM 276	40.3a; 33.5b	Yes	2.94	No	NA
RM 402	40.3a; 34.3b	Yes	2.59	No	NA
RM 3370	35.8b	Yes	2.22	No	NA
RM 7708	35.8b	Yes	1.85	No	NA
SSIIa	37.3b	NT	NA	NT	NA

[a] Data with a mark ‘a’ are cited from the map of Rice Cornell SSR 2001; data with a mark ‘b’ are cited from the map of Cornell IR64/Azucena DH QTL 2001, both available on http://www.gramene.org/. [b] NT, not tested; NA, not applicable because of no polymorphism between the two parents; data are the genetic distances of the Wx and SSIIa gene and SSR markers from the gene responsible for the ASV differences in the given cross.

3.5. Concentrations of granule bound GBSSI and SSIIa

The concentrations of granule bound GBSSI and SSIIa expressed in the endosperm were measured to test their relationship to the gelatinization properties of the starch (Fig. 4). Similar amounts of GBSSI protein were present in the mutant Huangyu B as in the parent variety II32 B and another high GT variety IR36, whereas no significant amount of GBSSI protein were detected in the starch granules from R3027 or the japonica variety Kinmaze (Fig. 4A). On the other hand, very little granule-bound SSIIa was detected in starch granules of Huangyu B, R3027 and Kinmaze, but there were significant amounts in the II32 B and IR36 starch (Fig. 4B).

The differences between the amounts of GBSSI protein are consistent with the AAC data, since Huangyu B, II32 B and IR36 all had high AAC values whereas those for R3027, Xiushui 110, and Kinmaze were low AAC (see Table 2; for AAC/GT data see [17] for IR36 and Kinmaze. The differences in amounts of granule-bound SSIIa protein among the varieties tested were also consistent with the results of our ACL profile analysis and previous reports [14,18]. Also the higher amount granule-bound SSIIa protein is associated with lower ACR and consequently the starches have a higher GT [14].

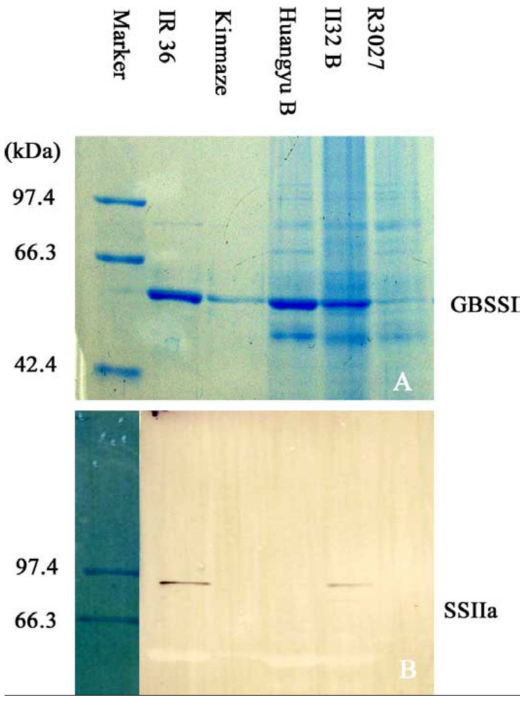

Fig. 4. Measurement of the GBSSI and SSIIa proteins bound to starch granules. Rice starch granules were prepared from various lines of rice, and proteins tightly bound to starch granules were extracted as described in [13]. The proteins were applied onto SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (A). The SSIIa protein in the SDS-PAGE gel was determined by immunoblot analysis using purified rice endosperm SSIIa (B). The amounts of protein applied in each lane were those included in 0.66 mg of starch granules.

3.6. SSIIa sequence and comparison

The whole *SSIIa* gene was successfully amplified using the PCR primers listed in Table 1. At the beginning, it continuously failed to amplify the first fragment which may have been due to the high GC content (up to 68.6%). Therefore, a special PCR buffer, 2×GC Buffer I (Takara, Japan), designed for amplification of high GC fragment, was used and proved useful.

Several SNPs were found between II32 B and Huangyu B, and more when they are aligned to Shuangkezao and Nipponbare (Fig. 5). Since the *SSIIa* gene is known to have 8 exons and 7 introns (Fig. 5), the implication of SNPs in the intron regions was therefore not pursued.

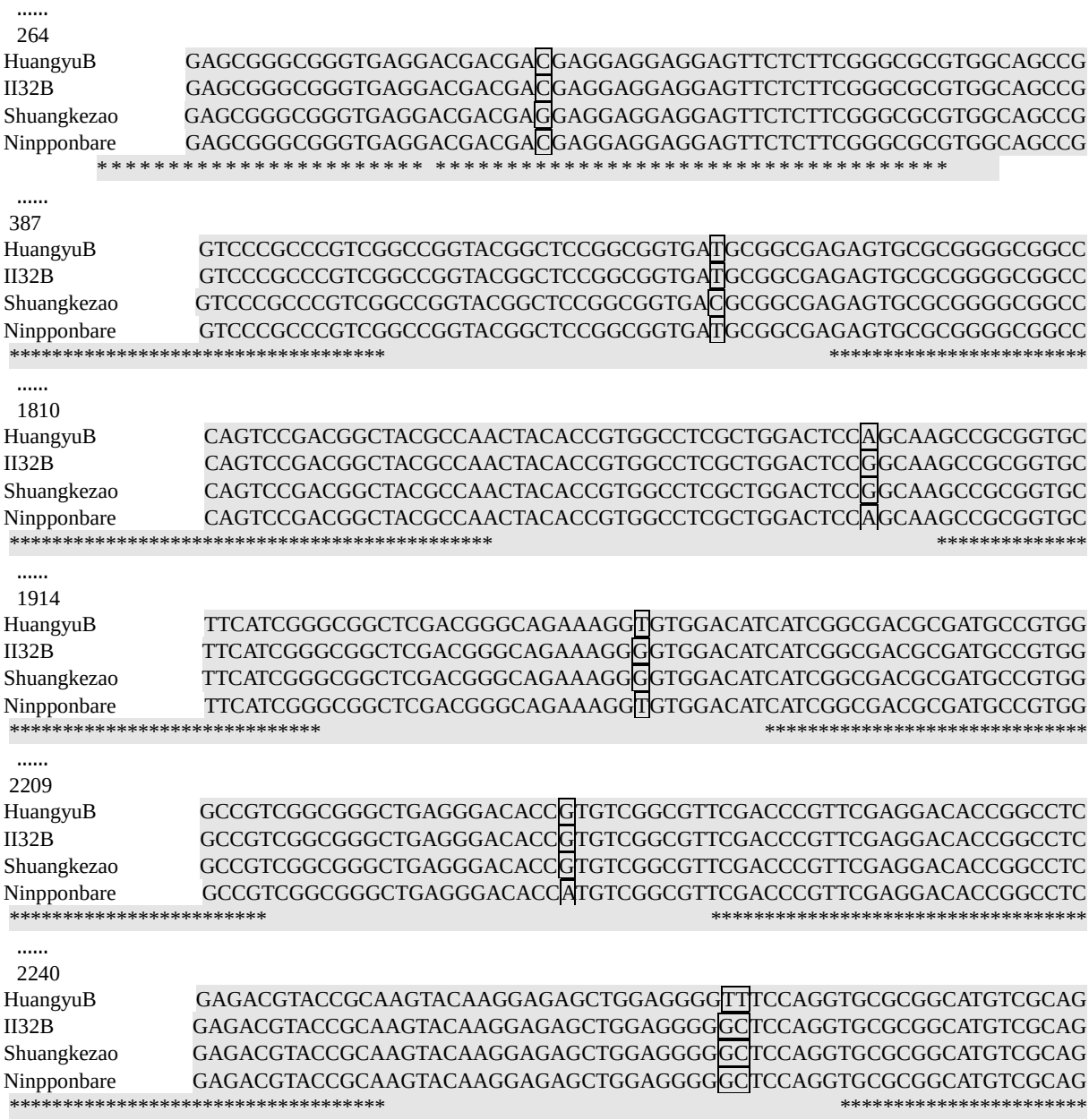

Fig. 5. The alignment of SSIIa sequences of Huangyu B and II32 B (this experiment) (part sequences), and with Nipponbare and Shuangkezao (from Genbank). '……' implicate the ellipsis sequences.

In the exons, four SNPs at nucleotide position of 1810 (G to A), 1914 (G. to C), 2340 (G to T) and 2341 (C to T) were found between II32 B and Huangyu B (Fig. 5), but it only translates into two amino acid changes at position 604 (Gly to Ser) and 781 (Leu to Phe) in peptide (Fig. 6).

When aligned with other reported sequences of the *SSIIa* gene of varieties with know GT values in Genebank, only the amino acid change of Gly_{604} to Ser_{604} seemed probably linked with the low GT characteristic of Huangyu B, because other amino acid changes are independent from the GT character. For example, generally high GT varieties have Leu_{781}, but the low GT variety Nipponbare also had Leu_{781} (Fig. 5) [18]. Nakamura *et al* [14] explained that the combination of Met_{737} and Leu_{781} had the same effect as Phe_{781} for a low GT type; our results could neither support nor reject this possibility.

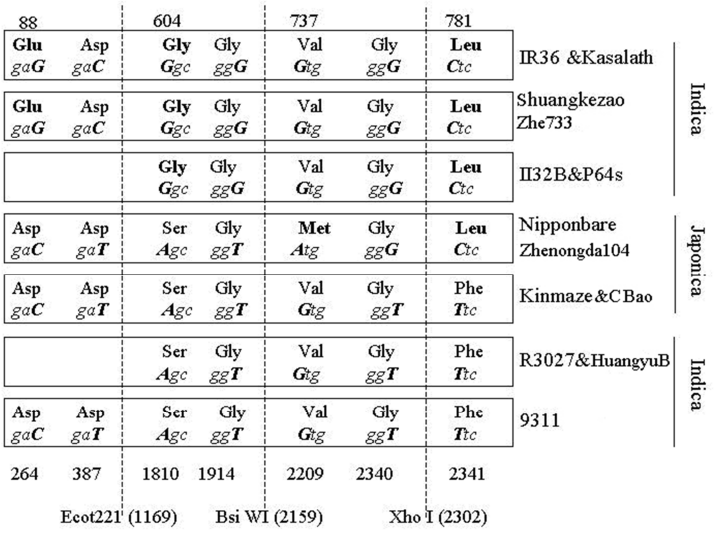

Fig. 6. Polymorphic nucleotide sites and deduced amino acid changes in the exons of SSIIa gene of rice varieties with different gelatinisation temperature (GT) characteristics. High GT varieties include II32 B (this experiment), IR36 (AB115917) and Kasalath (AB115918) [18], Zhe733, Shuangkezao; low GT rice include Huangyu B, R3027, P64s (this experiment) and C Bao (GQ151773.1) [19], Nipponbare (AB115915), Kinmaze (AB115916) [18], 9311 (AAAA02019632) and Zhenongda104 (AF419099).

3.7. GC/TT$_{2340\text{-}41}$ SNPs of SSIIa gene

3.7.1. Detection of GC/TT polymorphism

Polymorphic bands of PCR product could be easily separated on agarose gel and three genotypes could be identified, e.g., homozygous GC/GC and TT/TT and heterozygous GC/TT genotype were detected for the F_2 plants of Huangyu B×II32 B (Fig. 7).

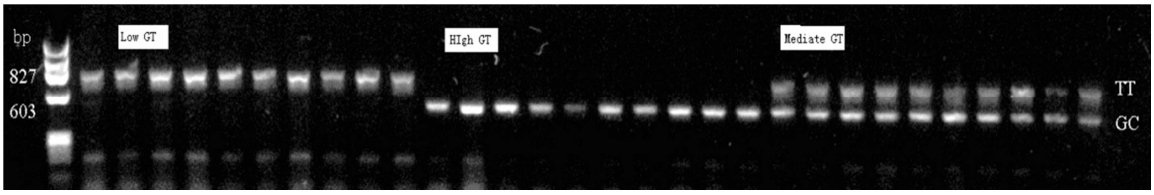

Fig. 7. Detection of SNP GC/TT$_{2340}$ on agarose gel and its segregation in F_2 population of cross Huangyu B/II32 B.

3.7.2. Co-segregation of GC/TT$_{2340}$ SNP with GT value

Huangyu B and II32 B have the genotype of TT/TT and GC/GC, respectively, corresponding to low and high GT value. In the F_2 population, three types of plant were identified, i.e., homozygous for GC (36 F_2 plants), homozygous for TT (36 F_2 plants), or heterozygous at the locus (23 F_2 plants). These three categories of plant perfectly co-segregated with $F_{2:3}$ seeds of high GT, low GT and segregating GT values (Fig. 7). This clearly demonstrated that the GC/TT$_{2340}$ SNP could be efficiently used for MAS for the GT character.

3.7.3. GC/TT genotype and GT value in conventional rice varieties

Sixty-seven rice varieties were analyzed for their SSIIa genotype using the above SNP GC/TT$_{2340}$ marker and for their GT based on alkali spreading value (ASV). The results were summarized in Table 5. It was very clear that most of the low GT varieties had the homozygous genotype for SNP TT/TT, while high GT varieties were homozygous for GC/GC. However, a few exceptions were also observed, for example, Iri371 had the SNP of TT/TT but had a high GT (ASV=2).

TABLE 5. THE GELATINIZATION TEMPERATURE AND THE SSIIA GENOTYPE OF THE SNP GC/TT_{2340} OF VARIOUS RICE VARIETIES

Cultivar	ASV	SSIIa SNP	Cultivar	ASV	SSIIa SNP
II32 B	2.00	GC	Jiazao106	3.50	GC
Iri371	2.00	TT	Zhongxuan #5	3.50	GC
Zhefu994	2.00	GC	Zhong 106	3.50	GC
Zhenong8010	2.00	GC	Zhong 86-44	3.67	GC
F89-70	2.00	GC	Zhefu 511	3.67	GC
Xieqingzao	2.10	GC	Luhongzao # 1	3.67	GC
Jiaxingxiangmi	2.17	GC	Zhongxuan# 2	3.83	GC
Jiayu948	2.17	TT	Zhongzu # 1	3.83	GC
Guichao13	2.17	GC	Zhe 852852	3.83	GC
Zhongfu 906	2.33	GC	Zhongzu # 4	4.00	GC
F8-1	2.33	GC	Zhongxuan 218	4.00	GC
Hongtu 31	2.50	GC	Jiayu 280	4.00	GC
Jiayu 253	2.50	GC	Zhongzao # 1	4.00	GC
Zhongxuan 181	2.50	GC	Zhongzao 18	4.00	GC
Xiangzaoxian #1	2.50	GC	Guanluai #4	4.00	GC
Zhefu726	2.50	GC	Zhufei # 10	4.00	GC
Jinzao47	2.67	GC	Jiazao 97-08	4.17	GC
Zhe733	2.67	GC	Zhefu 728	4.17	GC
Yuanfengzao	2.67	GC	Zhongxuan 214	4.33	GC
Erliuzhezao	2.67	GC	Zhenshan 97	4.33	GC
Zhefu 991	2.83	GC	Zaoxiangnuo	4.67	GC
Zhongxuan 78	2.83	GC	Zhongzao 22	4.67	GC
Ruanzhuke	3.00	TT	Zhongzao 21	5.33	TT
Taizao310	3.00	GC	Zhe 207	5.67	TT
Hangzao # 3	3.00	GC	Zhoushanhongmi	5.83	TT
Fuxian # 6	3.00	GC	Jiazao 312	6.00	TT
Ruike 26	3.00	GC	Jiazao 935	6.00	TT
Zhefu 802	3.00	GC	Xiangzaoxian 31	6.00	TT
Zaolian 31	3.00	GC	Zhou 903	6.00	GC
Sixian 213	3.17	GC	Zhongxuan 98	6.00	TT
Wuqing # 2	3.17	GC	Niqingzan	6.40	TT
Luqingzao # 1	3.17	GC	Huangyu B	6.67	TT
R3027	3.20	TT	Zaoyeqing	7.00	TT
Zhong 156	3.33	GC			

4. DISCUSSION AND CONCLUSIONS

Both classical analysis and recent molecular studies showed that GT is controlled by a major effect gene or plus a few genes with minor effect or QTLs [16,18-22]. Our study confirmed that the GT trait was also controlled by a single major locus in one population, but in a different population, GT maybe controlled by different loci.

Most studies [10,18,22,23] had mapped the major effect locus responsible for the GT character at the alk gene locus, which was first mapped on chromosome 6 [18], while other put it around the Wx gene locus [16] and [24]. It has be proposed that this discrepancy might result from the differences of mapping populations (indica/japonica vs indica/indica) used in those studies [18], but this explanation was not supported in recent studies.[23] which mapped the major QTL at the alk locus based on a DH population of two indica rice varieties. We used an induced mutant in this study and proved that the GT mutation was located on the same place as the alk gene in rice.

The amylopectin chains of degree of polymerization (DP) $\leq$12, 13$\leq$DP$\leq$24, 25$\leq$DP$\leq$37 and DP$\geq$37 correspond to A chains, B1 chains, B2 chains, B3 and longer chains, respectively [25]. The fine structure of amylopectin of Asian rice was largely classed into two classes, L-type and S-type. L-type amylopectin (Indica rice) has less short (DP$\leq$0) and more intermediate size 11$\leq$DP$\leq$24 than S-type (Japonica) [25]. Its been suggested that it is the fine structure of amylopectin that caused the differences of GT between indica and japonica rice [18], and further proposed that such difference was controlled by the alleles of starch synthesis IIa gene (SSIIa).

We found in this study that the value of ratio of amylopection chains of DP$\leq$10 to DP$\leq$24 (ACR) in Huangyu B (0.246) was higher than that of II32 B (0.177), which was consistent with that between japonica and indica rice in previous studies [26]. And we also mapped the GT mutation to the same place as the alk and SSIIa gene on chromosome 6. All these indicated that the GT mutation in Huangyu B was very probably resulted from a mutation of the SSIIa gene.

As reported previously, the SNPs GC/TT2340 could potentially explain the GT variation among rice varieties [14]. For marker-assisted selection (MAS), it is crucial to develop PCR-based markers that could differentiate SNPs among varieties. Therefore, primers were designed to generate a PCR-based SNP marker for SNP GC/TT2340. The efficiency of this marker was studied using about 50 rice varieties with different GT values and the segregating F2 population of the cross between II32 B and Huangyu B.

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PHYSICAL MAPPING AND RELATIONSHIP OF GENOME-SPECIFIC REPETITIVE DNA SEQUENCE WITH GENES CONTRIBUTING TO CROP QUALITY IN WILD RICE

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Abstract

We cloned AA genome-specific repetitive sequences from Dongxiang wild rice (*O. rufipogon*). Sequences analyzed results showed that some sequences encoded partial transposon protein. But most sequences belong to repetitive fragment, including *RIRE* family retrotranposons. Sequences of A8 and A15 belong to *RIRE3*, *RIRE2*, respectively; they were selected as probes for Southern hybridized with different genomes. The result showed that they dispersed high copies in the AA genome, but very low or no copies in the BBCC, CC, CCDD and EE genomes. The retrotransposon-specific primers were designed based of A8 and A15 to develop a novel molecular markers system, with operational principle similar to transposon display (TD) protocol. We found some subspecies-specific markers from this system. Regardless of the geographical regions of rice accessions used in this study, the characteristics of *indica* and *japonica* rice can be judged according to these subspecies-specific markers. Our results also revealed that genome-specific repetitive sequence from wild rice would be useful in developing species or subspecies-specific markers for research on genetic diversity and genetic evolution of *Oryza*. In addition, we also utilized SSR markers to map cold tolerance QTLs by root relative conductivity as a physiological index. The results indicated that there were three cold tolerance QTLs (*qRC-10-3, 4, 5*) on 10 chromosome of Dongxiang wild rice.

1. INTRODUCTION

Rice is one of the most important cereal crops in the world. It has become the model plant of monocot and current molecular biological research due to its relatively small genome size (430Mb), large number of related species and rich genetic resources. Recent development in various molecular techniques, allows studying the genetic variations of rice at the molecular level using DNA markers such as RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), SSR (simple repeat sequence), etc. The release of genome sequences from two subspecies (*japonica* and *indica*) has brought rice to the forefront of all genomic studies [1].

The genus *Oryza* includes 23 species, 20 of them being wild species. The chromosomes of genus *Oryza* consist of six basic genomes (AA, BB, CC, EE, FF, and GG) and three complex genomes (BBCC, CCDD, and HHJJ) [2]. Wild rice constitutes a unique and valuable natural gene pool for rice biotechnology and cultivars improvement. In order to use the valuable genes of wild rice in future breeding plan, it is interesting to isolate genome-specific repetitive DNA sequence which may be non-coding or with little effect on quality traits and to explore the relationship of this sequence with genes contributing to crop quality. It could allow us to distinguish the genomic composition for an unknown rice species and understand the evolution relationship among the rice species.

The major fraction of nuclear genomes is made up of repetitive DNA sequences in most plants. Repetitive DNA sequences include a group comprising DNA transposable elements (TE) with a dispersed organization on the chromosomes. The transposable elements can be divided into two classes [3]. Class I TEs include with long terminal repeat (LTR) and without LTR (non-LTR) retrotransposons. They integrate into host chromosomes via RNA intermediates by a mechanism that is dependent on reverse transcription, culminating in the production of highly abundant copies in plant genomes. Retrotransposons make up more than 50% of the maize genome [4] and more than 90% of the wheat genome [5]. Class II TEs transpose via DNA intermediates, usually resulting in relatively low copy numbers (usually <100 copies per genome) [6]. Regardless of its small genome, the *Oryza* species contains all classes of TEs [7] and the two classes of TEs have been found to contribute 19.9%

of the rice genome sequences [8]. During the past decade, a number of rice *RIRE* retrotransposon (*RIRE1* to *RIRE10*) with different LTR sequences and characters have been isolated from rice genomes [9,10]. Transposable elements are fundamental components of most plant genomes, which play critical effects on the formation, structure, variation and adaptive evolution of current genomes in plants [11,12]. Therefore, one of the main purposes of plant genomics is to understand completely the organization, structure, and impact of TEs activity during genome evolution for global genome regulation and phenotype variations [13].

Fluorescent in situ hybridization (FISH) provides scientists the opportunity to physically map for some genes or sequences and to identify the physical position of DNA or RNA fragment on the chromosomes of mitosis and meiosis or DNA fibre using the fluorescent labelled DNA or RNA fragments as probes. Recently, it has been proven to be effective in exploring differential chromosomal components, characteristics among different species in *Orzya* [14,15]. However, most studies so far have focused on chromosomal compositions of different rice species; genome-specific repetitive DNA sequences among different rice species have rarely been investigated.

The retrotransposon-based insertion polymorphisms (RBIPs) are very useful molecular marker in the studies of phylogeny and evolution in plants [3]. As the LTR terminal sequences are highly conserved between families, even intra-element recombination, resulting in the conversion of a full-length element to a solo LTR, either does not affect the marker band and can furthermore be exploited to produce a new marker band in RBIP. LTRs of *Ty1-copia* or *Ty3-gypsy* retrotransposons were usually utilized to the fingerprint marker system, which used to study genetic diversity in barley and wheat [16,17].

In this study, we cloned genome-specific retrotransposon fragments at first, and then used these sequences to develop the novel molecular markers based on simple modified TD protocol, and SSR markers were utilized to map cold tolerance QTLs in Dongxiang wild rice (*O. rufipogon*).

2. MATERIALS AND METHODS

2.1. Materials

We have collected 20 accessions of AA genome wild rice species from different parts of China to bulk up our research, including *O. rufipogon* (another name *O. spontanea*, Dongxiong wild rice in Chinese) which through our rice breeding and genetics programme, has previously been identified as cold-tolerant. The other genomes include three accessions of *O. punctata* (BB), two accession of *O. officinalis* (CC), and one accession of *O. minuta* (BBCC), *O .latifalia* (CCDD), *O. australiensis* (EE) and *O. brachyantha* (FF), respectively. In addition, we have selected about 60 cultivated rice species (*japonica* and *indica*) varieties with different agronomic characters.

All germplasms mentioned above were grown in the experimental field of Yangzhou University to produce hybrids between Dongxiang wild rice and cultivated rice Nanjing 11 (N11), and to multiply its offspring. The F1 of *O. rufipogon* X *O. sativa* was back cross with N11 (*O. sativa*) resulting in BC1, BC2 and BC3 (Table 1). The seeds of F2 and each back cross generations derived from all the crosses were harvested except for the F1 of *O. sativa* cross *O. latifalin*, which showed heavy sterile in F1 due to the presence of it's three different genomes (ACD).

TABLE 1. HYBRIDIZATIONS SCHEME IN THIS STUDY

Female parent	Male parent	F1	F2	BC1	BC2	BC3
O. rufipogon	*O. sativa*	√	√	√	√	(cross with N11)
O. sativa	*O. rufipogon*	√	√			
O. sativa	Weed wild rice	√	√			
O. sativa	*O. latifalin*	√				

2.2. Methods

2.2.1. Construction of a BAC library for wild rice

We isolated genomic DNA from young leaves of Dongxiang wild rice using the Dellaporta method [21], and a BAC library was constructed following the procedures of Georgi [18]. Genomic DNA was partially digested with *BamH*I at 37°C for 30min, 1% agarose in first pulsed-field electrophoresis to select fragments size in 200Kb. Second Pulsed-field electrophoresis was performed to collect over 100Kb DNA fragments. About 50~200ng selected DNA ligated with BAC vector (pBeloBAC11) by T4 DNA ligase at 16°C for overnight. Two microlitres ligation mixture was transformed in 20~25μl of *E. coli* DH10B competent cells (Gibco, BRL) according to its protocol. The white colonies were picked into 96-well plates with freezing medium and grown for 24h at 37°C, and then stored at -70°C.

2.2.2. Screening of the BAC library for positive BAC clones

The genomic DNA of *O. rufipogon* (AA genome) and *O. officinal* (CC genome) were labelled using HRP Labelling and Detection Kit (Pierce) as probes to hybridize with the BAC library. The clones that show strong hybridization signals when hybridized with *O. rufipogon* as probe and weak hybridization signals when hybridized with labelled genomic DNA of CC genome would be considered as the candidate clones and used in the proceeding study. These positive BAC clones may have genome-specific or *O. rufipogon* -specific repetitive sequences.

2.2.3. Cloning of genome-specific repetitive DNA sequences

We isolated the plasmid DNA from the candidate BAC clones using an appropriate method. The plasmid DNA were partially digested with the restriction enzyme *Sau3*A or mechanically cut down by syringe needle for subcloning. Digested fragments size in 1~2Kb in size were collected from 1% agarose gel, and then ligated with a *BamH*I-digested pUC19 vector. A total of 1μl ligation products were transformed into competent *E. coli* (DH5α) cells by electroporation. Transformed cells were grown on LB plates containing ampicillin (100 μg/ml), X-gal and IPTG. White clones were picked up and placed onto a fresh LB plate containing ampicillin. The plasmid DNA of selected subclones was confirmed by PCR amplification to check whether exogenous fragments transferred into vector and what size it is. The selected subclones were hybridized with labelled *O. rufipogon* DNA and CC genome DNA by Dot-blot hybridization to identify positive subclones again.

2.2.4. Southern hybridization and DNA sequencing

Southern hybridization was performed using DIG DNA labelling and detection system. The inserts of plasmid DNA of selected subclones were cut down with *BamH*I and separated from the vector in 3% agarose gel. The separated inserts were labelled as probes to hybridize with the genomic DNA of wild and cultivated rice germplasms to check genome-specific repetitive sequences. All genomic DNA samples were digested with five endonucleases (*Eco*RI, *Eco*RV, *Hind*III, *BamH*I, and *Dra*I), and separated on agarose gel and then transferred into a nylon membrane. The nucleotide sequences of repetitive DNA were determined on an Automatic DNA Sequencer (Model 373A). Blast analysis of cloned sequences was carried at the repeat database of NCBI (www.ncbi.nlm.nih.gov/BLAST) and TIGR (www.tigr.org/tdb/e2k1/plant.repeats/).

2.2.5. Development of molecular markers for fingerprinting, from cloned repetitive sequences

Previously described transposon display [19] and sequence-specific amplification polymorphisms [16] were modified for the molecular fingerprint analysis in *O. sativa.* The high copy character and conversation of LTR anchored primers were designed based of cloned *RIRE2* and *RIRE3* LTR sequences. All adaptor sequences (MPA-1,2), pre-amplification (MP-0) and selected-amplification primers refer to Lee *et al.* [20] (Table 1). The genomic DNA (500 ng) was fully digested with *Mse*I endonuclease, and the adaptor was linked with the digested DNA in a volume of 50μl at 16°C for 12hours. Pre-amplification was carried out with the MP-0 primer and with either anchored primer. The

final concentration of each ingredient in PCR was carried out with 0.5 μM of each primer, 0.25mM dNTP each, 1.5mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Takara) in a volume of 50μl. PCR reaction program was as follows: one cycle of 72°C for 2min and 94°C for 3min; 25 cycles of 94°C for 30sec, 56°C for 30sec, 72°C for 1min; and a final extension at 72°C for 5min before completion of the reaction.

For selective amplification, the pre-amplified products were diluted by 10-fold. 2μl of the dilution was mixed to final concentration with 0.5μM of Selected-amplification primer, 0.5μM of one of the anchors primers, 0.2mM dNTP each, 1.5mM $MgCl_2$, and 0.5 unit of *Taq* DNA polymerase in a total volume of 30μl. The PCR reaction program was as follows: one cycle at 94°C for 5min; ten 'touchdown' cycles of 94°C for 30sec, 64°C for 30sec, and 72°C for 1min with a decrease in annealing temperature to 1°C in each cycle; 26 cycles of 94°C for 30sec, 56°C for 30sec, 72°C for 1min; and once at 72°C for 5min to terminate the reaction. 2μl of the sample was loaded into 6% denaturing acrylamide-bisacrylamide gel (19:1) in 1× TBE buffer and electrophoresed at 1600volts for 120min. Then, the separated fragments were visualized with the silver staining.

2.2.6. Fluorescent in situ hybridization (FISH)

FISH was performed following the protocol routinely used in our laboratory and combined with other methods descried in references [14,15]. The insert DNA from the positive BAC clones or subclones were labelled with biotin or DIG using Nick Translation Mix (Roche) and hybridized to root-tip metaphase cells of *O. rufipogon* or other wild rice, cultivated rice germplasms. Hybridization signals were amplified and later visualized using fluorophore-conjugated avidin or antibodies. Chromosomes were counterstained with PI after hybridization. The two images of green FITC fluorescent signals and the red PI chromosome were captured separately in the fluorescent microscope with a cooled CCD camera and merged into single image with imaging software.

TABLE 2. PRIMERS USED IN DEVELOPING MOLECULAR MARKERS

Primer	Name	Sequence (5'- 3')
Anchored primer	r2-1	AGTCTCAGGGTGTTTCCTTG
	r2-2	TCTACACGGCGATGGTATTC
	r3-1	GGG AGCGTTTAG AGCGGT
	r3-2	GCTGTTTCCCATTTGTCT
Adaptor sequences	MPA-1	GACGATGAG TCCTGAG
	MPA-2	TACTCAGGACTCAT
Pre-amplification primer	MP-0	GATGAGTCCTGAGTAA
Selected-amplification primer	MP-CA	GATGAGTCCTGAGTAACA
	MP-GA	GATGAGTCCTGAGTAAGA
	MP-GAA	GATGAGTCCTGAGTAAGAA
	MP-GAC	GATGAGTCCTGAGTAAGAC
	MP-GAG	GATGAGTCCTGAGTAAGAG
	MP-GAT	GATGAGTCCTGAGTAAGAT

2.2.7. Linkage mapping and QTL analysis of cold tolerance

In order to map genes and QTL's for cold tolerance of Dongxiang wild rice, 114 SSR primers showed polymorphism between Dongxiang wild rice and cultivated rice (the sequences of primer result from Gramene database: http://www.gramene.org). The PCR reaction products were separated on polyacrylamide gels or 3.5% agarose. PCR profile: 95^{o}C 5min, 35 cycles of 94^{o}C for 40sec, 55^{o}C for 30sec, 72^{o}C for 1min, the final extension at 72^{o}C for 10min. The marker genotype of each SSR marker with polymorphism was identified for each BC2F1 plant by PCR.

In this study, we obtained 144 BC2F1 populations and their BC2F2, BC3F2 groups based of N11 as the recurrent parent. The cold tolerance of BC2F1 could be reflected by root conductivity, and the smaller its value is, the strongest of cold resistance is. Because, measuring conductivity need to clipping a number of root and BC2F1 groups need to plant into fields for the continuation of backcrossing or self-cross, the root conductivity of BC2F1 groups would not be directly measured by this way. Theoretically, BC2F1 groups consist of plant with or without Dongxiang wild rice cold-resistant genotypes. Each BC2F1 plant self-generated corresponding BC2F2, and the cold tolerance separated between strains. Therefore, using the root conductivity differences between BC2F2 strains can indirectly reflect BC2F1 genetypes. In that case, 50 BC2F2 seeds from each BC2F1 plant were randomly selected, and placed into light incubator. When they grow to the period of three-leaf heart, low-temperature was given at the conditions of light intensity of 25,000lx, 4±1^{o}C temperature and light 12h per day. After 48h, the roots of all plant in same line were immediately clipped and mixed. About 0.1g root cut into 1cm segments and placed in 100ml distilled water. And root conductivity value（us/cm） was measured by METTLER TOLEDO 326-type conductivity meter. With the value of distilled water as a blank control, each strain conductivity value=root cells exudate measured values - the value of blank control.

2.2.8. Principal component analysis of genetic relationship

Over sixty rice varieties were selected to check whether these specific markers can distinguish *indica* and *japonica*, including twenty-four *indica* varieties from the China, two from Thailand, one from the Philippines, one from India and three from the USA, as well as twenty-four *japonica* varieties from the China, one from Italy, one from the Philippines, one from the USA and one from Japan. All the banding patterns were transferred into 0 (absent) or 1 (present) data matrix on the basis of the electrophoretic pattern of each DNA sample. The data matrices constituted by the 0, 1 pattern were subjected to principal component analysis (PCA) using the programme MATLAB7.1.

3. RESULTS AND DISCUSSION

3.1. Construction of genomic BAC library and selection of BAC clones with repetitive sequence in wild rice

We have constructed about 5000 BAC (bacterial artificial chromosome) clones for *O. rufipogon.* The insert size of BAC clone ranges from 80 to 100Kb (Fig. 1A).

The genomic DNA of *O. rufipogon* **(**AA genome*)* and *O. officinalis* (CC genome) were used as probes to screen the BAC library for isolation of positive clones with AA genomic specific repetitive sequences. Over 50 candidate positive clones were identified from 3000 BAC clones (Fig. 1B,C). In order to eliminate some negative BAC clones, all candidate positive clones were dot-blot hybridized with labelled *O. rufipogon* and *O. officinalis* genomic DNA probes again. Lastly, 21 positive BAC clones were identified (Fig. 2).

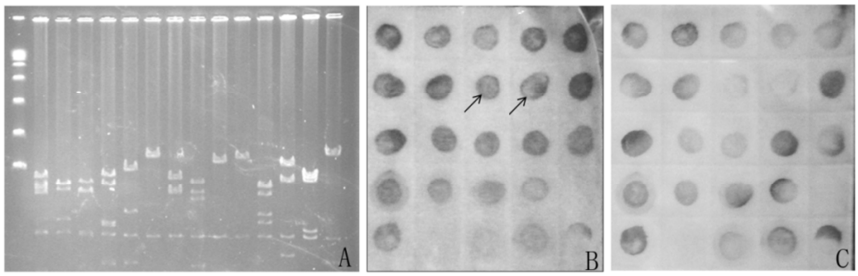

Fig. 1. BAC library of wild rice and screening results for positive clones. A: Insert fragment in BAC. B: AA genomic DNA (O. rufipogon) as probe. C: CC genomic DNA (O. officinalis) as probe. Arrows: positive BAC clones.

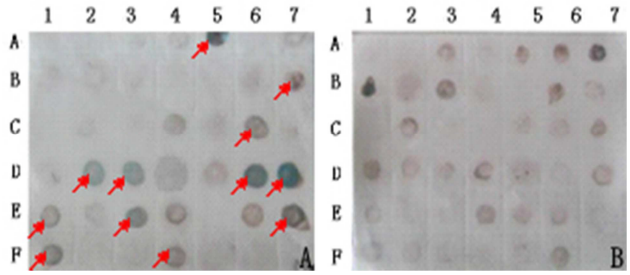

Fig. 2. The results of candidate positive clones by Dot Blot. A: AA genomic (O. rufipogon) DNA as probe. B: CC genomic (O. officinalis) DNA as probe.

3.2. Subcloning for positive BAC clones

Based on selected positive BAC clone with repetitive sequences, we selected 19 positive BAC clones to do subclone. The average 0.5-1.0Kb DNA fragment in length was inserted into vector pGATA. We picked up 20-72 subclones from each BAC clone. The DNA of each subclone was isolated and hybridized with genomic DNA of Dongxiang wild rice *(O. rufipogon)* and cultivated rice N11 as probe by dot-blot hybridization (Fig. 3). A lot of subclones showed strong signals hybridized with genomic DNA of Dongxiang wild rice as probe but weak signals hybridized with N11 as probe. We can confirm that these subclones contain specific *O. rufipogon* repetitive sequences in this way.

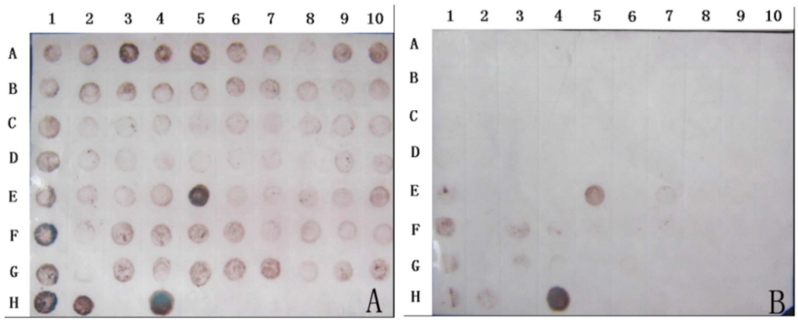

Fig. 3. Dot blot results showing subclones contain specific O. rufipogon repetitive sequences A: Genomic DNA of O. rufipogon as probe. B: Genomic DNA of O. sativa as probe; H-4: wild rice (O. rufipogon) genomic DNA as control.

3.3. Characterization of subclones thought DNA sequencing

To find the repetitive DNA sequences in Dongxiang wild rice, we sequenced 60 subclones and 31 sequences were deposited with Genbank (ID number. EF513613 - EF513615; EF527879 - EF527880; EU282824 - EU282838 and DQ861441 - DQ861452). Blast analysis method was used to find the possible function of these sequences. The sequences of DQ861441, DQ861442 and DQ861447 were partial sequences encoding some transposon protein. The sequence of DQ861450 related to acyltransferase and other clones belonged to rice retrotranposons *RIRE3*, *RIRE2* and *RIRE*8. These sequences were different from previously reported repetitive sequences: RC48 (AA), TrsA (AA), TrsB

(FF), TrsC (CC), G1043 (AA) [22,23]. And Blast analysis results also indicated that these repetitive sequences would be dispersed on most chromosomes and with a relationship to transposable element in AA genome. But their copy numbers are variable in the different chromosome. They provide favourable conditions for develop molecular marker system based of retrotransposon.

3.4. Southern hybridization for repetitive sequence

Southern hybridization was done with the selected repetitive DNA sequences, A8 and A15 as probes and the genomic DNA of five rice species representing AA, BBCC, CC, CCDD, EE genomes were digested with *Hind*III and *Bam*HI. Each clone produced a strong hybridization signal in AA genome. The weak or no hybridization signal in BBCC, CC, CCDD and EE genomes indicates that the sequences A8 and A15 were highly specific to AA genome, and very low in other genome (Fig. 4). The hybridization pattern of A8 and A15 indicates that these sequences have many copies and dispersion in the genome.

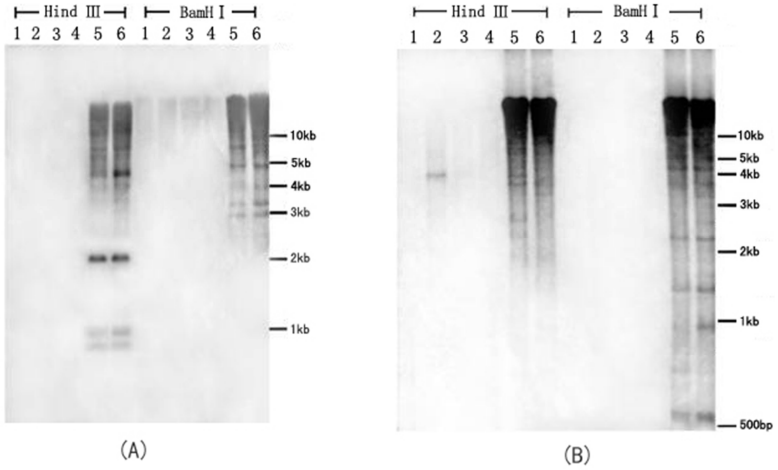

Fig. 4. Southern result of A8, A15 sequences hybridized to AA, BBCC, CC, CCDD, EE genomes A: A8 as probe; B: A15 as probe; 1: O.australiensis (EE）; 2: O.alta (CCDD）; 3: O.minuta (BBCC); 4:O.officinalis (CC); 5: O.sativa (AA); 6: O.rufipogon (AA); M: Marker.

3.5. Development of novel molecular markers based on isolated repetitive sequences

We used the isolated AA genome-specific repetitive DNA sequences from Dongxiang wild rice to develop the novel molecular marker by a modified TD protocol. One reaction of PCR amplification can generate multiple bands with abundant polymorphic profiles among the *O. sativa* accessions. The number of amplified fragments, ranged from 20 to 40 bands in size from 150 to 800bp depending on different primer combinations. In order to confirm the reproducibility of this marker system, four PCR reactions were done with 2 different thermocyclers and 2 different *Taq* polymerases. Most of the amplified bands were the same in these four reactions (Data not show). The amplified profiles were proved to be highly reproducible in rice.

We selected primer combinations to amplify specific band types between *indica* and *japonica* for molecular fingerprint analysis. A total of 22 *indica* and *japonica*-specific amplified profiles were found with seven primer combinations (Fig. 5). Based on the markers′ polymorphism information, principal component analysis (PCA) was done to show 60 cultivated rice varieties evolutional relationship. The 60 cultivated rice accessions clustered into two distinct groups, *indica* and *japonica* (Fig. 6). Along the first principal component, 30 *indica* varieties from different geographical regions were take up the first group, formed the *indica* group, second group occupied by 30 *japonica* varieties. But 3 *indica* varieties scattered near *japonica* group, they showed closer genetic relationships with the *japonica* group at the molecular level.

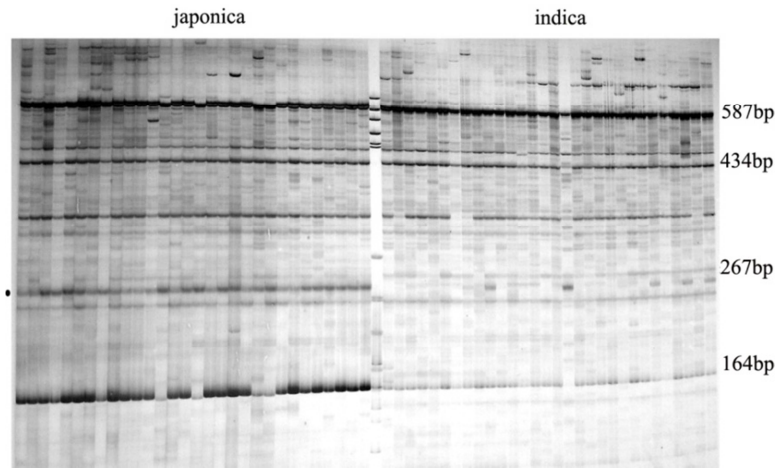

Fig. 5. Representation of amplified bands between indica and japonica by r2-1/MPCA primer combination in rice: japonica-specific marker.

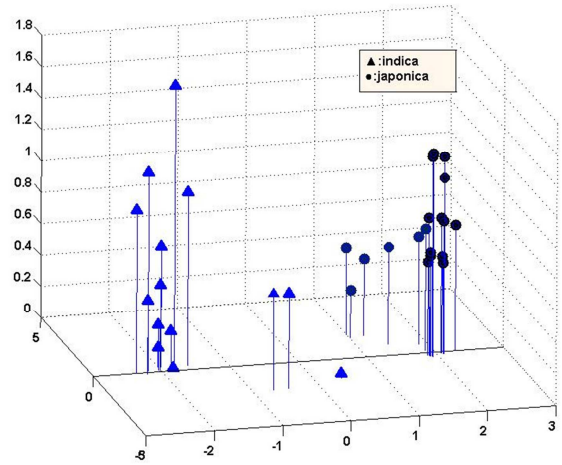

Fig. 6. The scatter plotting showing the genetic relationships among the indica and japonica rice varieties.

The origin and genetic differentiation of Asian cultivated rice is an important questions to be addressed for a wide range of scientists, including geneticists, evolutionary biologists, and rice breeders. The distribution of the *indica* group was more scattered than that of the *japonica* group, which indicated that the *indica* varieties had higher genetic diversity than the *japonica* varieties. Some insertion/deletions (InDels) of LTR exist in *indica* or *japonica* species. If anchored primer was paired with the flanking sequence of insertion/deletions site, polymorphic bands between *indica* and *japonica* can be produced when PCR was done. A longer amplification band could be produced in 'inserted species', and a shorter band produced in 'deleted species'. If anchored primer was paired with insertion/deletions site, an amplification band could be produced in one species existed no insertion/deletions, but no band produced in other species existed insertion/deletions. So, the main reason of producing subspecies-specific markers was LTR insertion/deletions (InDels) [24,25]. The retrotransposon-based insertion polymorphisms (RBIPs) provide evolutionary relationship information among different rice species. However, the degree of polymorphic was influenced by the age of retrotransposon. Vitte [26] designed 11 primers from the different retrotransposon to find RBIPs; the results showed the highest accuracy in distinguish *indica* and *japonica* varieties. Using the information of retrotransposons sequence to study the origin of rice (*japonica*) and (*indica*) domestication by a genomic palaeontology approach, the results indicated these two subspecies diverged from one another at least 200,000 years ago, and *indica* and *japonica* rice arose from two independent domestication events in Asia. Based on our study, the cloned fragments of *RIRE3* and *RIRE2* showed high copy in the AA genome, and various construct between cultivated and wild rice. These molecular

markers based on polymorphism of retrotransposon insert site should be very useful in the study of *indica-japonica* differentiation and the origin of *O. sativa*.

3.6. Fluorescence in situ hybridization (FISH) for BAC and repetitive sequences

Three positive BAC clones were selected from BAC library for FISH analysis. The hybridization signals could be found at the same site near the centromere on several different chromosomes, and also in the middle of chromosome arms (Fig. 7). However, the physical location of hybridization signals could not be clearly identified on a specific chromosome.

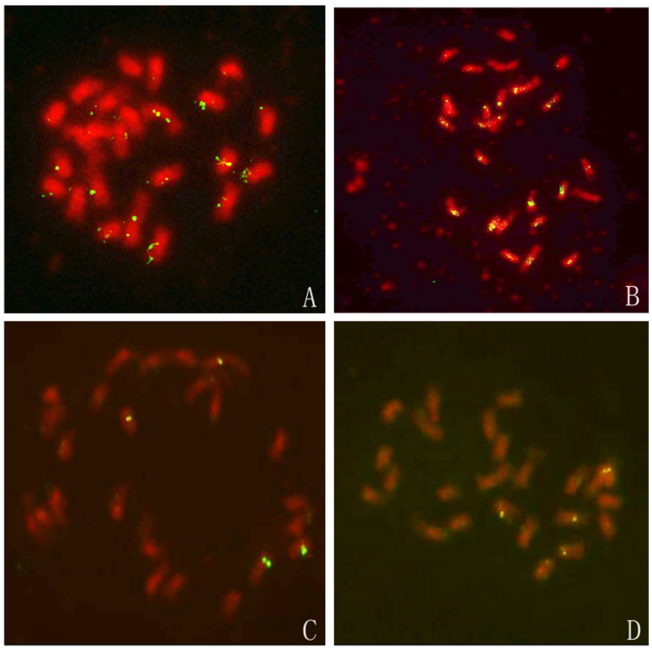

Fig. 7. FISH results of BAC clones and subclones with repetitive sequences on wild rice chromosomes A: BAC clones with H_1 sequence; B: BAC clones with H2 sequence; C: subclones with H_1 sequence; D: subclones with H2 sequence.

The results of FISH for two positive subclones showed that the number of chromosomes with hybridization signals was lower than that of BAC as probe. However, we could not identify which chromosomes had the hybridization signals. We identified a genome specific repetitive DNA sequence of AA genome (H2) by Southern analysis. Sequence analysis revealed that its repeat unit was about 615bp in size and distributed over many chromosomes of rice. The result of fluorescence in situ hybridization with the H_2 sequence indicated that hybridization signals could be found at almost the same site near the centromere and telomere on different chromosomes. This sequence should be used as a genome specific probe in research of rice evolution.

3.7. QTL mapping of cold tolerance gene in Dongxiang wild rice

When rice plants of parents have grown to 3-4 leaves stage in normal condition, one part of them has been treated with 5^{o}C low-temperature for 48hours and the others still been grown in normal condition. The results of root relative conductivity showed that there is no significant difference in Dingxiang wild rice between low-temperature treated and not, but very different in cultivated rice (N11). So, the root relative conductivity can be used as useful physiological index to identify phenotype of cold tolerance plants in marker analysis population. Three amplified profiles could be identified in BC2F1 groups, SSR amplified patterns same with the N11 recorded as ‘1’, and same with Dongxiang wild rice as recorded as ‘2’, missing or can not judge recorded as ‘0’. The total of 114 SSR primer pairs with polymorphism between the parents was used to screen BC2F1 (144 plants).

The cold tolerance QTLs were mapped using the composite interval mapping method [28], using the Mapmaker/EXP3.0 software to analysis the linkage. Reorganization values were converted into genetic distance (cM) by the Kosambi function, linkage was mapped and cold resistance genes detected using the Windows QTL Cartographer 2.5 software. The existence of a QTL was determined at the condition of LOD> 2.5, and QTL naming was followed the McCouch *et al.* [29]. There were three cold tolerance QTLs (*qRC-10-3,4,5*) on 10 chromosome of Dongxiang wild rice (Table 2). According to these results, we could find that the character of cold tolerance of Dongxiang wild rice was related to this physiological index. Our results showed that the root relative conductivity could be used as useful physiological index for cold tolerance research in rice.

TABLE 2. ASSOCIATION E OF QTLS WITH COLD TOLERANCE

QTL	Chro.	Region of Marker	LOD	Contr. %	Additive Effect
qRC-10-3	10	RM304-RM25570	2.6083	23.17	4.235
qRC-10-4	10	RM25570-RM171	3.1814	10.80	2.0396
qRC-10-5	10	RM1108-M25661	3.2471	9.76	1.8972

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GENOMIC CHARACTERIZATION OF THE CHILI PEPPERS (*CAPSICUM SOLANACEAE*) GERMPLASM BY CLASSICAL AND MOLECULAR CYTOGENETICS

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Abstract

Within the framework of the IAEA coordinated research project entitled 'Physical mapping technologies for the identification and characterization of mutated genes contributing to crop quality' we carried out genomic characterization of wild and cultivated samples of chilli peppers (genus *Capsicum*) by classical chromosome staining methods (AgNOR and fluorescent chromosome banding) and fluorescent in situ hybridization (FISH). For the first approach, fluorochromes with affinity for specific chromosome regions were used, i.e. chromomycin A3 (CMA) and diamidino-phenyl-indole (DAPI) which have preference for GC-rich and AT-rich regions, respectively. In addition, Ag-staining to detect active nucleolus organizing regions was applied. The heterochromatin could be characterized in respect to type, amount and distribution in the different accessions examined. On the other hand, the number and position of active NORs could be determined. Using FISH, different DNA probes were used in order to map specific sequences in the chromosomes, i.e. 45S and 5S rDNA, telomeric sequences and cloned restriction fragments of repetitive nature. As an example of the work done, we present the results obtained on a sample of *Capsicum annuum* var. *annum* (cultivar NMCA 10272), the most broadly exploited cultivar of chilli pepper. The results allowed us to characterize the *Capsicum* species and accessions and the possible evolutionary pathways for chilli peppers was deduced based on the available cytogenetic data. It is worth mentioning that the research work done under this CRP is part of work being done within an exsting network of chilli pepper research of this important plant group utilized by man and among one of the first cultivated plants in the history of humanity.

1. INTRODUCTION

Solanaceae is an economically important family because it includes several plants of interest for food, drug, stimulant and ornamental uses. On the other hand, some species are toxic or become aggressive weeds. Although the family is cosmopolitan, it has the major concentration of genera and species in South America [1]. In this sense, one highly important group is the New World genus *Capsicum* L. which comprises around 30 species. Five of them, *C. baccatum* L., *C. pubescens* Ruiz et Pav. and the members of the *C. annuum* L. complex (*C. annuum*, *C. frutescens* L. and *C. chinense* Jacq.), were domesticated by American natives and have been exploited world-wide since Columbus because of their fruits. The fruits, which have high nutritional content, specially in vitamins, and are constituents of the human diet, either the pungent forms as spice ('ajíes', 'chillies', 'hot peppers') or the sweet ones as vegetables ('sweet pepper'). Moreover, the genus has medical and ornamental applications [cf. 1-4].

One of the main disadvantages arising from cultivating plants is the resulting susceptibility to a multitude of diseases and pests due to the narrow genetic base from which most of the commercial cultivars are developed. Thus, plant breeders are forced to search for resistance in the existing gene pool and in the wild relatives of the crop species [5,6]. For success in breeding programmes in general, it is essential to achieve basic information on the genetic diversity of the available germplasm, and also on the genomic affinity between the possible donors of valuable alleles and the crop to be improved. Therefore, germplasm characterization is an important aspect for the conservation and utilization of plant genetic resources. In this respect, one of the main aspects to consider is the cytogenetic analysis.

The cytogenetics of *Capsicum* has received early attention for breeding purpose with reference to the domesticated taxa and their possible wild ancestors [3,7]. The success of interspecific crosses,

obviously related to the genomic homology of the parental species, could allow the genetic improvement of cultivars by introducing valuable alleles such as those responsible for pathogen resistance from the wild entities [5,6]. In this sense, a broad gene mapping and genome characterization programme in the genus by classical and molecular cytogenetics will be useful for hybridization and biotechnological approaches, including transformation.

Within the frame of the IAEA coordinated project entitled 'Physical mapping technologies for the identification and characterization of mutated genes contributing to crop quality' we carried out a genomic characterization of wild and cultivated samples of chilli peppers [8-12]. This work comprised a broad programme of classical and molecular studies on somatic chromosomes, i.e., chromosome banding (fluorescent and AgNOR staining) and DNA sequence (ribosomal RNA genes, i.e. 5S and 45S rDNA, telomeric sequences and restriction fragments of repetitive nature) mapping by fluorescence in situ hybridization (FISH), in order to evaluate the inter- and intraspecific cytogenetic variability. We expected to characterize species, varieties and cultivars, with the ultimate aim of enhancing our knowledge of the genome organization and evolution in *Capsicum*, with reference to the origin of the crop species, and attempted to obtain useful information for breeding purposes.

In this report, the cytogenetic characterization of a sample of the most important and widespread pepper crop, *C. annuum* var. *annuum*, a species possibly domesticated in southern highland Mexico [3,4], is presented as an example of the work done under the CRP.

2. MATERIALS AND METHODS

2.1. Plant material

The chilli pepper germplasm bank at the Multidisciplinary Institute of Plant Biology (IMBIV), National University of Cordoba, Argentina, comprises around 400 accessions, including cultivated and wild relatives from different localities. The studied sample presented in this paper is *Capsicum annuum* var. *annuum* cultivar NMCA 10272 from Mexico, was provided by Paul W. Bosland, New Mexico State University, USA, and cultivated at the greenhouse of the IMBIV.

2.2. Chromosome preparations

Root tips (5-10mm long) obtained following seed germination were pre-treated with a saturated solution of p-dichlorobenzene for 2h in the dark at room temperature, then, fixed in freshly prepared absolute ethanol/glacial acetic acid (3:1) and stored at -20ºC until preparation. Somatic chromosome spreads were prepared according to Schwarzacher *et al.* [13]. Root apices were macerated in an enzyme solution [1% (w/v) cellulase plus 10% (v/v) pectinase] at 37ºC for 40 min, and then squashed in 45% acetic acid. After removal of the coverslip with CO_2, slides were air dried, aged for 1-2days at room temperature, and then kept at -20ºC until use.

2.3. Chromosome banding methods

Silver impregnation to detect active nucleolar organizing regions (NORs) was performed according to the Ag-I procedure by Bloom and Goodpasture [14] with the modifications of Kodama *et al.* [15]. Fluorescent chromosome banding to reveal amount, distribution, and type of constitutive heterochromatin was done according to the triple staining method of Schweizer and Ambros [16] with the fluorochromes chromomycin A3 (GC-specific binding), distamycin A and 4'-6-diamidino-2-phenylindole (AT-specific binding) (CMA/DA/DAPI).

2.4. Fluorescent in situ hybridization (FISH)

The 45S rDNA and 5S rDNA repeated sequences were localized using the following DNA probes: R2, a 6.5-kb fragment of the 18S-5.8S-26S (45S) rDNA repeat unit from *Arabidopsis thaliana*, including internal transcribed spacers ITS1 and ITS2 and a short 5' segment of the intergenic region (IGR) [17], and pXV1, a 349 bp fragment of the 5S rRNA gene repeated unit from *Beta vulgaris*, including the

adjacent intergenic spacer [18]. The first probe was labelled with biotin-11-dUTP (Sigma, USA) and the second one with digoxigenin-11-dUTP (Roche, Germany), both by nick translation. The telomeric sequences were localized using an *Arabidopsis*-like telomeric probe amplified by PCR with the oligomer primers (5'-TTTAGGG-3')s and (5'-CCCTAAA-3')s according to Ijdo *et al.* [19] labelled with biotin-11-dUTP (Sigma). Labelling of the probes, pre-treatment of the preparations, chromosome and probe denaturation, conditions for the in situ hybridization, post-hybridization washing, blocking, indirect detection by fluorochrome conjugated antibodies (Dako, Denmark), i.e. anti-biotin conjugated to tetramethyl-rhodamine isothiocyanate (TRITC) and anti-digoxigenin conjugated to fluorescein isothiocyanate (FITC), and chromosome counterstaining with DAPI were performed according to Moscone *et al.* [20,21].

2.5. Microscopy and image acquisition

After the application of the different methods, metaphase chromosomes were observed and photographed, depending on the procedure, with transmitted light or epifluorescence in a Leica DMLB microscope equipped with the appropriate filter sets, a Leica DC250 digital camera, and the IM 1000 Leica image management system. Particularly, for epifluorescence red, green, and blue images were captured in black and white using appropriate filters for TRITC, CMA, FITC, and DAPI excitation, respectively. Digital images were pseudocoloured and combined using the IM 1000 Leica software package (Leica, Switzerland), and then, imported into Adobe Photoshop 7.0 for final processing.

2.6. Karyotype analysis

Five to ten metaphase plates per sample were used to make the karyotype characterization. The arm ratio [r=q (long arm length) / p (short arm length)] was used to classify the chromosomes according to Levan *et al.* [22] in m (metacentric, r=1.00-1.69), sm (submetacentric, r=1.70-2.99) and st (subtelocentric, r = 3.00 – 6.99). In Table 1 and Figs. 1-3 the chromosomes were numbered according to Moscone *et al*. [10,11]. The procedure for measurements of chromosomes and fluorescent heterochromatic bands, in order to build the respective idiograms, is described in Moscone *et al.* [11]. Karyotype variants below the species level were considered as 'cytotypes'.

TABLE 1. MEASUREMENTS OF THE CHROMOSOME COMPLEMENT IN *C. ANNUUM* VAR. *ANNUUM* CV. NMCA 10272

Pair	Length		r (q/p)	Type	Position of fhcb	Length of fhcb	
	Absolute (µm) X (sd)	Relative (% of HKL)	X (sd)			Absolute (µm) X (sd)	Relative (% of HKL)
1	7.10 (0.66)	10.40	1.06 (0.04)	m	q	0.09 (0.04)	0.14
2	6.19 (0.80)	9.07	1.18 (0.12)	m			
3	5.76 (0.71)	8.44	1.15 (0.08)	m			
4	5.74 (0.71)	8.41	1.17 (0.10)	m	p	0.05 (0.01)	0.07
5	5.71 (0.72)	8.37	1.15 (0.09)	m			
6	6.00 (0.49)	8.79	1.09 (0.05)	m	p^(77.35)	0.20 (0.06)	0.29
					q^(61.23)	0.05 (0.01)	0.07
7	5.53 (0.45)	8.10	1.13 (0.08)	m			
8	5.41 (0.68)	7.93	1.33 (0.14)	m	p^(88.12)	0.05 (0.03)	0.07
9	5.29 (0.54)	7.75	1.20 (0.10)	m	p	0.08 (0.02)	0.12
10	5.17 (0.44)	7.58	1.14 (0.07)	m			
11	5.48 (0.31)	8.04	1.85 (0.29)	sm-NOR	p	0.70 (0.01)	1.03
12	4.86 (0.62)	7.13	3.08 (0.31)	st-NOR	p	0.43 (0.08)	0.63

Abbreviations: *cv*, cultivar; *X*, mean value; *sd*, standard deviation; *HKL*, haploid karyotype length; *r*, braquial index; *q*, long arm; *p*, short arm; *m*, metacentric; *sm*, submetacentric; *st*, subtelocentric chromosome; *NOR*, nucleolus organizing region; *fhcb*, fluorescent heterochromatic band.
^ intercalary band (in brackets is indicated the distance to the centromere).

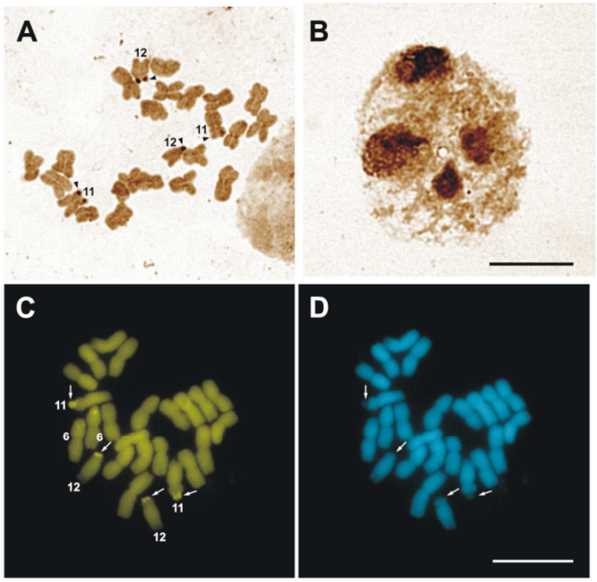

Fig. 1. Chromosome banding in Capsicum annuum var annuum cv. NMCA 10272 (2n = 24). A-B, Ag-NOR banding showing two chromosome pairs (Nos. 11 and 12) with active nucleolus organizing regions in metaphase pointed with arrowheads (A), and four Ag-stained nucleoli in an interphase nucleus (B). C-D, Fluorescent banding pattern displaying CMA+ (C) and DAPI- (D) heterochromatic bands; arrows indicate bands related to NORs. Scale bars represent 10 µm.

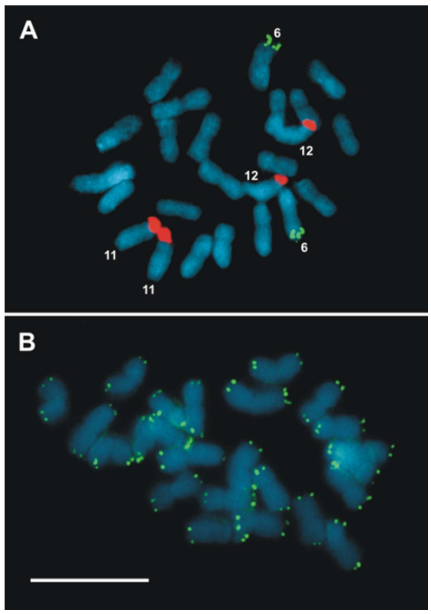

Fig. 2. Fluorescent in situ hybridisation (FISH) in Capsicum annuum var annuum cv. NMCA 10272 (2n = 24). A, Mapping of rRNA genes, 45S rDNA loci (two pairs) are displayed in red and 5S rDNA loci (one pair) in green. B, Mapping of telomeric sequences which are displayed in green. Scale bar represents 10 µm.

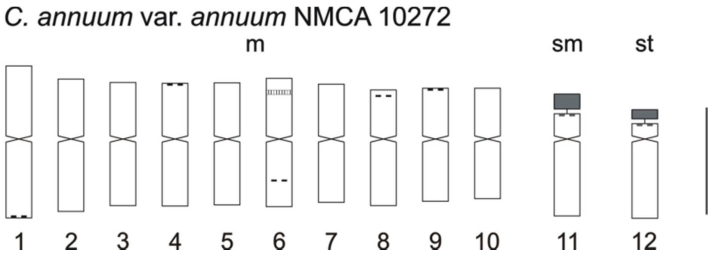

Fig. 3. Idiogram of Capsicum annuum var annuum cv. NMCA 10272 (2n = 24). All heterochromatic bands are CMA+/DAPI- , which include the rDNA loci. 45 S rDNA loci are shown in grey and the 5S rDNA locus in a hatched intercalary region. Scale bar represents 5 µm.

3. RESULTS AND DISCUSSION

Illustrations of chromosome banding, i.e., AgNOR staining, fluorescent CMA/DA/DAPI staining, and FISH experiments for localizing 5S and 18S, 5.8S, 26S (45S) rDNA and telomeric repeated sequences in *C. annuum* var. *annuum* cv. NMCA 10272, and the resulting idiogram are shown in Figs. 1-3. The measurements of the chromosomes from which the idiogram was built are given in Table 1.

The cultivar NMCA 10272 was, selected to show how the cytogenetic methodological approaches applied to chilli peppers is useful for chromosome identification. NMCA 10272 displayed 2n = 24, with a karyotype formula of 10m+1sm+1st chromosome pairs, where pairs nos. 11 (sm) and 12 (st) have active nucleolus organizing regions (NORs) on short arm. The total haploid karyotype length was 68.24µm. As it is typical in the genus, the fluorescent heterochromatic bands are CMA+/DAPI-, which indicates a GC-rich heterochromatin constitution. As usual in this species, the total heterochromatin amount of the cultivar was low (2.42%) and mostly placed in the terminal regions of the chromosomes.

The FISH patterns of the 45S ribosomal RNA gene family concerning number, position and size of loci differ between species, varieties and cultivars of *Capsicum* and resemble, although not completely, the corresponding specific fluorescent banding patterns after the triple staining with chromomycin, distamycin and DAPI, which shows CMA+/DAPI- bands [9,11,23]. This phenomenon, which was also observed in other plant groups (*Brassica* L.) [24], could be explained by possible cross-hybridization of the 45S probe with the CMA+/DAPI- (GC-rich) heterochromatic regions in the genus, as it is known that in most plant species ribosomal DNA is rather GC-rich [25]. It should be considered that the R2 and pTa71 probes used in our studies and by Park *et al.* [26], contain at least part of the intergenic 45S rDNA spacer (IGR), which carries repetitive elements and, thus, could be cross reactive [27]. In *Nicotiana* L., a satellite sequence with a GC-rich subrepeat, which is homologous to the 45S rDNA IGR and occurs outside of rDNA loci in species of section *Tomentosae* Goodsp was been reported. [28].

In the diploid complements of the taxa studied during this project, the number of 45S rDNA loci was variable, whilst invariably there was only one pair of 5S sites. Prior to this work, data on rRNA genes localization by FISH have been reported by other authors only in one accession each of the five cultivated *Capsicum* species, including *C. annuum*, *C. baccatum* and *C. pubescens* [26,29]. In *C. annuum* cultivar different from the one analysed in our study, only one pair of 45S rDNA loci was reported by non-fluorescent in situ hybridization methods [30].

Fluorescent chromosome banding patterns, AgNORs number and position and cytological mapping of rRNA gene clusters has revealed chromosome homologies between chilli pepper species [10,11,23] and allowed the speculation of species relationships and the possible evolutionary pathways. Generally, the information from our classical and molecular cytogenetic studies supports previous conclusions on systematic affinities and phylogenetic relationships in *Capsicum*, based on different methodological approaches [1,3,8,12,31]. It should be noted that the cytogenetic results obtained during this project allowed us to speculate a widely accepted evolutionary picture in the genus, where the x = 13 basic number appeared two times, once very early the evolution of the genus, and after that in a more advanced line. In addition, the observed intraspecific variation at cytological level helped in finding the possible original place of domestication for the cultivated pepper species [4], i.e. *C. annuum* var. *annuum* in Mexico and Peru, *C. chinense* and *C. frutescens* in Brazil, *C. baccatum* var. *pendulum* in Bolivia, and *C. pubescens* in Peru. Most of the cytogenetic information generated under this CRP was included in a related global project [32].

In the near future, we will attempt to localize in the chromosomes of the *Capsicum* species an available set of gene-derived markers (resistance gene analogues or RGAs), which are related to disease resistance genes of *Arachis* (Leguminosae). It should be noted that chilli peppers and peanut crops could be affected by similar diseases and pests, such as fungal leaf spots (*Cercospora*) and nematode root-knots (*Meloidogyne*), the resistance searched in the wild relatives [7]. Finally, the construction of integrated molecular linkage maps in members of the *C. annuum* complex [33-35]

open the prospect to attempt comparisons between the physical chromosomes identified cytogenetically and the pure genetic linkage groups, using chromosome-specific BAC clones based on genetically mapped restriction fragment length polymorphism (RFLP) markers [36,37].

4. CONCLUSIONS

The distribution and size variation of fluorescent heterochromatic bands, AgNORs and rDNA gene clusters, allowed us to distinguish wild and domesticated chilli peppers. Fluorescent banding, silver staining and rDNA FISH patterns were useful even with discriminate cultivars from the same species. This is significant, particularly, since most chromosomes in chilli pepper species are of similar in size and difficult to distinguish one from another. Thus, the methods used in study demonstrated to be very valuable for characterizing pepper germplasm.

FISH technique using ribosomal RNA gene (5S and 45S rDNA) probes combined in single experiments, together with fluorescent and AgNOR banding methods [32], brought out useful markers for chromosome identification and for further DNA sequence mapping by FISH, including genes contributing to crop quality in peppers.

Classical and molecular cytogenetics provided sufficient chromosome landmarks to postulate homologies between species. Valuable data to enhance the knowledge of the phylogeny, genome organization and evolution in *Capsicum* were obtained.

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INDUCTION AND STUDY OF TOMATO AND PEPPER MUTANTS WITH HIGH NUTRITIVE QUALITY

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Abstract

The aim of this project was to increase the levels of health-related compounds such as carotenoids in tomato and pepper, through induced mutagenesis integrating them with other economically important characters, and, to study the genomic changes in the mutants, for crop quality improvement. New genetic resources of tomato were developed by using mutant genes controlling high level of carotenes. The genetic potential to synthesize high lycopene and β carotene was evaluated in tomato lines carrying the mutant genes hp and ogc, as well as in progeny of *Solanum lycopersicum* with the wild tomato background. Molecular characterization using tomato lines originating from interspecific hybridizations between *S. lycopesicum* and either, *S. pimpinellifolium* or *S. chilense* showed that AFLP markers are useful for early identification of F1 hybrids. A relatively large scale of variation throughout the harvest dates was observed in lycopene content of the lines and hybrids studied but the genotypes investigated ranked almost in the same way despite of the variability in the pigment content. Several pepper mutants with increased β carotene levels in fruit, male sterility, lack of anthocyanins, determinate habit, altered fruit shape and changes in fruit position were developed from induced mutagenesis using physical and chemical mutagen. Using mutants with increased β carotene levels as parents F1 hybrids with a twofold increase in their β carotene content were selected. Molecular studies allowed us to establish a marker for orange fruit colour useful for MAS.

1. INTRODUCTION

Tomato and pepper are among the most important vegetable crops, consumed and provide human with nutrients, bioactive substances, ie antioxidants such as lycopene and β carotene. The nutritive value of horticultural crops is influenced by available cultivars and consumers and markets require new varieties with improved quality and high added food value [1]. Unidirectional breeding for important agricultural characters (including yield, resistances, etc.) in existing tomato varieties has resulted in the loss of some part of its biological value, including characteristics of the wild species. Genetic diversity is an essential result from crop breeding [2]. Phytonutrients should be improved by the breeders while selecting at the same time for yield, quality, and disease resistance, among others [3]. It is critical that plant breeders and geneticists ascertain if negative relationships exist between phytonutrient levels and other economically important traits, such as linkages between optimal phytonutrient level and characteristics such as inferior yield. Successfully breaking up these negative linkages may be crucial to the profitable commercialization of a phytonutrient-enriched crop. In this regard, genetists should also pay close attention to genetic variation not only of the targeted phytonutrients, but also of genetically and biochemically unrelated compounds occurring in crops that affect consumer acceptance and farmer preferences.

The content of the non-enzymatic strong antioxidants, the carotenoids, is among the most important criteria for fruit quality due to its health benefits [4]. Carotenoids play an important role in aging, vision, immune function, prevention or fighting off infections, bone growth, reproduction, cell division and differentiation [5]. Their deficiency is most often associated with protein/calorie malnutrition and affects over 120 million children worldwide [6]. The usual doses of beta carotene, one of the most valuable among carotenoids, from 15 to 50 mg/day, can prevent development of certain types of cancer and many severe chronic diseases such as cardiovascular diseases, respiratory, urinary, and intestinal tracts [7].

Bulgaria is traditionally a leader in the development of tomato and pepper lines, including varieties possessing high nutritive value [8]. Increasing the biodiversity has been difficult and it is a long process using conventional breeding. The advances in plant molecular biology, and the application of induced mutation technologies, useful tool to enhance our efforts, breeding, by enabling the generation and characterisation of a wealth of mutants which can be used in breeding programmes [9,10]. Until

recently, improvement programmes were based mainly on using natural sources of germplasm, cross breeding and heterosis effect of F1 hybrids [11]. Current advances in molecular biology have contributed to the growing interest in the use of induced mutations [12]. Developing appropriate science-based breeding strategies toward crop quality in vegetable crops would benefit breeding practice and genebanks, generating information to be used in selecting superior parental combinations and predicting progeny performance, accurate variety identification, control, registration and protection.

The aim of this project was to increase the levels of health-related compounds such as carotenoids in tomato and pepper, through induced mutagenesis, integrating them with other economically important characters, and, to study the genomic changes for crop quality improvement.

2. MATERIALS AND METHODS

Investigations were carried out between 2002 and 2008 at the Maritsa Vegetable Crops Research Institute, Plovdiv, Bulgaria.

2.1. Induced mutagenesis in tomato and sweet pepper

2.1.1. Tomato Germplasm

Studies were performed on the following tomato genotypes:

i) A tomato accession line 120D was used as a source of explants to develop new tomato genotypes using gamma rays irradiation. Line 120D originates from cross between a local tomato line *Solanum lycopersicum* L. and a high lycopene accession *S. lycopersicum var. humboldtii* (Dun.) Luckw (which is a synonym of *S. lycopersicum f. cerasiforme*). The lycopene content of line 120D reaches up to 90% to 95% of the total pigment content [3].

ii) We included progenies of wild tomato relatives (*S. cheesmanii, S. pimpinellifolium* and *S. chilense*), with high-value agronomic and quality traits crossed with *S. lycopersicum* lines, which had previously been characterized [15-17]. These lines were: XXIV-13, St-993, C-19, B-317, VK-1 and G-32.

iii) In order to get a more reliable baseline for comparative evaluation of the variation in fruit lycopene content, tomato genotypes developed by using genes controlling high level of lycopene: Ailsa Craig (AC) and the near isogenic lines (NILs) differing in genes hp (high pigment) and ogc (old gold-crimson) described by [18] and their F1 hybrids were included in the study.

2.1.2. In vitro mutation induction

Explants from sterile grown *in vitro* plants from seeds were used for callus induction and developing by the method of Tan et al. [19] for 4 weeks. At the end of the period callus was irradiated by 20 and 30Gy of γ rays ^{60}Co and transferred to regeneration agar medium – Murashige and Skoog macro- and micronutrients, vitamin B5, 2mg/l zeatine-ribozide and 30g/l sucrose (pH 5.8). Obtained plant-regenerants were micropropagated and 3 of each new regenerants were grown in soil for studying and seed formation. From changed forms seeds were gathered for the next generation. Segregation of valuable characters were estimated in M2 and confirmed in M3 generation. Morphological and biochemical traits of irradiated populations were characterized following a descriptor list derived from the IPBGR, for Lycopersicon (IPBGR, 1996) and changed forms were selected for molecular study.

2.1.3. Induced mutagenesis in sweet pepper

Studies were performed also on *Capsicum annuum* L. sweet pepper cultivars (cv.) and breeding lines (bl.) for fresh consumption. The pepper mutant germplasm used in the study are described below:

i) Pepper mutants developed using Ethyl methansulphonate (EMS) (0.5-0.7%) and gamma irradiation ^{60}Co (80-160Gy) of the cultivar (cv.) Hebar and identified by observation of

phenotypic alterations [20]. Anthocyanin-free plants were observed at the seedling stage of M2 plants and their phenotype has been further screened.

ii) An orange fruit mutant (Mof) developed from the treatment of dry seeds of red fruited (rf) local cultivar Pazardzhishka kapiya 794 with 120Gy of X rays [21] and a mutant cv. Oranzheva kapiya (WT1) developed by Daskalov and colleagues [22].

iii) An anthocyaninless mutant (Mal) which is early maturing, high yielding, with more attractive fruit shape and better flavour developed in cv. Albena (WT2) by Daskalov [23]. The mutant character orange fruit colour (of) were transferred into genotypes bearing the al mutation and a series of Mof,al recombinant inbred lines (RILs genotypes 32, 33, 34, 35, 36) were developed [24,25].

iv) Mutant Zlaten medal ms8 (bl.28) (WT3) possessing male sterility was obtained by the application of 120Gy Rö rays to the fertile (f) local cv. Zlaten medal 7 (WTf) [21]. The bl. 29 and 30 were obtained from 28ms8 crossed by lines free of anthocyanin with orange fruit colour (iii) by Daskalov and colleagues [22] combining different mutant characters - of, al, and in our study were assessed as fertile. The bl. 28ms8 and 30Ms,of,al were crossed to combine of, al and ms8. The type of segregation (fertile (f)(Ms): sterile (s)(ms8) was defined in 124 F2 plants. These plants were obtained from seeds produced from different isolated fruits and plants of the same cross. Pollen fertility or lack of pollen in anthers in the blossoming period was assayed in 4% acetocarmine and glycerol (1:1) and microsporogenesis by squashing anthers in 4% acetocarmine.

v) Near isogenic lines (NILs) differing in fruit colour (red and orange) were advanced to M12-15 The advanced pepper mutants M15 possessing orange fruit colour, and their respective WT lines were used in crosses with the corresponding WT. Mutant genes introduced into elite pepper Mof material were studied for their influence on β carotene content in mature fruit.

2.2. Methods

2.2.1. Biochemical and phenotypic analyses

To compare the genetic potential of (i) tomato genotypes with the mutant genes hp and ogc genes and others with a potential to synthesize high lycopene and/or β carotene, and (ii) wild type and mutant pepper lines to synthesize target carotenes and xanthophylls, biochemical analysis of the levels of some carotenoids was done.

The biochemical screening was based on column chromatographic absorption (CCA) according to [26] for routine analysis and high performance liquid chromatography (HPLC) method was developed for confirmation. HPLC for the analyses of tomato was published [27].

HPLC for the analysis of pepper:

Chemicals

Standards of lutein, zeaxanthin, β-cryptoxanthin (BCX), lycopene, α carotene and β carotene were purchased from CaroteNature (GmbH, Lupsingen Switzerland). HPLC grade solvents including acetonitrile, methanol and ethyl acetate were obtained from Merck (KGaA, Darmstadt, Germany).

Sample preparation

The pigments were extracted from a 2 g sample in duplicate, to which 0.2 g magnesium carbonate had been added, with 15 ml extraction solvent methanol:tetrahydrofuran (1:1, v/v) [28], containing 0.1% butyl hydroxytoluene (BHT).

Saponification

The residue from the extraction was saponified for 1 h with 10 ml 10% methanolic potassium hydroxide, containing 0.1% BHT in the dark at room temperature, according to [28].

Chromatographic separation

The HPLC analysis was performed on a Hewlett-Packard chromatograph system (series 1050), equipped with a quaternary pump, an UV-Vis detector (Hewlett-Packard, series 1050) and a column thermostat (Hewlett-Packard, series 1100). Data analyses were carried out using HP ChemStation software (Agilent Technologies). Separation was conducted on a RP Prevail C18, 5mm (150×4.6mm i.d., Alltech) column, coupled to Alltima C18 5 mm (20×4.6mm i.d., Alltech) guard column. The solvent system was composed according to [29] of acetonitrile:methanol (95:5, v/v) (eluent A) and acetonitrile:methanol:ethyl acetate (60:20:20, v/v/v) (eluent B). The mobile phase contained 0.1% BHT and 0.05% triethylamine (TEA). The modified gradient elution programme was performed as follows: initial conditions were 100% A for 5 min, followed by linear gradient to 100% B at 13 min, maintaining this proportion until the end of the run. The column was then returned to the initial conditions and equilibrated for 15 min. The flow rate was 1 ml/min. The column temperature was set at 28oC. Injection volume was 20 ml. The detection was done at the wavelength of 450 nm.

Identification and quantification

Identification of carotenoids was carried out by comparison of the HPLC retention times with corresponding 2 standards and co-chromatography with added standards. Additional identification was carried out comparing the spectral data obtained with photodiode array detector Waters 991 with reported values [30,31]. Carotenoids were quantified using external calibration method. To prepare the stock standard solutions 1 mg of each standard was dissolved in 10 ml chloroform, containing 0.1% BHT. The exact concentrations of the standards were determined spectrophotometrically using corresponding extinction coefficients: lutein-2550 at 445 nm in ethanol, zeaxanthin-2480 at 451 nm, lycopene-3450 at 472 nm, BCX-2460 at 451 nm, β carotene-2560 at 450 nm and β carotene-2800 at 444 nm in hexane [28]. Mix working standard solutions were prepared each day from individual stock solutions by evaporating aliquots with nitrogen and diluting with acetonitrile:methanol:ethyl acetate (60:20:20, v/v/v).

Method validation

The response of lutein, zeaxanthin, BCX, lycopene, α carotene and β carotene was linear (correlation coefficients 40.99) from 0.04 to 5 μg/ml ($r>0.999$).

The Limits of detection (LOD) defined as amount of the carotenoid, resulting in a peak height three times the baseline noise and limits of quantification (LOQ) set at 2.5 [32]. The following limits of detection were estimated using a signal to noise ratio of 3: 0.0100 μg/ml for lutein, 0.0090 μg/ml for zeaxanthin, 0.0171 μg/ml for lycopene, 0.0096 μg/ml for BCX, 0.0109 μg/ml for β carotene and 0.0117 μg/ml for ß carotene.

In order to determine recovery (%) of added analytes under an established extraction procedure, samples were spiked (n=3) with several concentrations of each carotenoid (0.07 to 0.5 μg/ml). The spiked samples were subjected to the entire process of extraction, saponification and HPLC analyses. The recoveries of carotenoids were calculated based on the ratio of the concentration obtained after HPLC to the concentration added in the beginning. The recoveries of the carotenoids were: 95% for lutein, 96% for zeaxanthin, 97% for lycopene, 102% for BCX, 104 % for ß carotene and 95% for ß carotene.

In order to check the precision of the method within-day and between-day repeatability of six fruit sample determinations within 1 day and the same sample determined 10 times over the following 2

days were assessed. The average short-term reproducibility (CV %) was 6.0%. The average long-term reproducibility was 7.4%.

Sampling for biochemical analyses

For each genotype ten individual samples were analyzed. The individual sample represented a composite sample consisting of all fruits of uniform size, colour and in botanical maturity, collected per individual plant. Three analytical samples were analysed from the composite sample, which comprised 50-200 g. Each sample was finally chopped up and homogenized, and 2 portions of 2 g were taken for duplicate analysis. All samples were analyzed raw on the same day they arrived at the laboratory using CCA method. The samples for the analysis by HPLC method were lyophilised.

The F1 progenies resulting from the M12-15.x WT crosses were evaluated for morphological character and β carotene content in the pepper fruits was determined in six F1 and the corresponding parents (WTs and Ms) using the CCA method.

Analysis of segregation in F2 plants by β carotene content in the fruit [33] was done using the cross obtained between one of the wild type and the mutant line using the CCA method.

Variation in lycopene and β carotene levels in tomato was evaluated depending on different harvest dates.

Photosynthetic pigments in leaves and fruits were analyzed two times during the botanical stage of the plant development. The pigments were extracted in acetone according to [34]. The amounts of the chlorophyll a, chlorophyll b and total carotenoids were determined by spectrophotometer VSU-2P. Dry matter content was determined by weight method [35].

The β carotene hydroxylase (CrtZ) activity was analyzed in WT and mutant pepper lines. To analyze the enzyme activity of β carotene hydroxylase, crude extracts of green and mature lyophilized fruits were used. The β carotene was used as standard which conversion into BCX was detected by spectrophotometer according to the change of the colour.

The bl. 28ms8 and 30Msof,al were crossed by us to combine of, al and ms8. The type of segregation (fertile (f)(Ms): sterile (s)(ms8) was defined in 124 F2 plants. These plants were obtained from seeds produced from different isolated fruits and plants of the same cross. Pollen fertility or lack of pollen in anthers in the blossoming period was assayed in 4% acetocarmine and glycerol (1:1) and microsporogenesis by squashing anthers in 4% acetocarmine.

2.2.2. Molecular characterisation

M3 and M5 mutant tomato lines with determinate habit (dm), low fertility (f) and male sterility (ms), selected following their morphological characteristics, were evaluated by ISSR and AFLP analyses to ascertain polymorphism between mutants and the wild types.

Genomic DNA was isolated from lyophilized leaf material (first true leaf) using a PhytoPure Kit (Amersham) and CTAB protocol of [36]. Inter Simple Sequence Repeats - Polymerase Chain Reaction (ISSR-PCR) method was performed, using a protocol of [35]. The amplified products were separated on 2% Agarose, high gel strength and a lambda DNA-Hind III digest was used as molecular weight standards.

Amplified Fragment Length Polymorphism (AFLP) was conducted on a genomic DNA according to the protocol of [33], using the restriction enzymes EcoRI, MseI; EcoRI-based end labelling with γ-ATP-P32; Selective amplification with EcoRI/MseI: I-ACT/CAT, II-ACA/CAT, III-AGG/CTT, IV-ACT/CAG, V-ACC/CAC.

Molecular characterization of the advanced pepper mutants with orange fruit colour and the corresponding wild type lines were performed by PCR technique with gene-specific primers. Gene-specific primers for the genes: Ggpps, Psy, Pds, Zds, Ze, Ccs, CrtL and CrtZ-2 related to β carotene biosynthesis and CrtZ designed based on available cDNA sequences in GeneBank (NCBI) and reported data [38], [39], [40], were used to screen the sample DNAs using the recommended PCR protocol by Amersham Pharmacia Biotech, Ready-To-Go PCR Beads.

2.2.3. Statistical analysis

The relationship among the mutant lines evaluated based on their morphological characteristics, biochemical assay and genotypic data generated from the molecular marker assays, were analyzed using the STATISTICA software 6. The morphological and biochemical data were subjected to frequency analysis and expressed as relative frequencies. A cluster analysis using Euclidian distances was used to determine genetic distances among mutant lines based on the AFLP and ISSR data.

3. RESULTS AND DISCUSSION

3.1. Induced mutagenesis in tomato

Characterisation of mutant tomato lines with new economically useful traits induced with gamma irradiation

The diversity of tomato fruit colour (red, green, pink, orange, yellow, whiteish) is mainly due to the composition and content of carotene, since fruits of various pigmentation types only a little differ in xanthophyll content. The main pigments in the majority of tomatoes are the carotenes: lycopene and ß carotene. In this study lycopene followed by ß carotene were the pigments detected in both the WT and mutant lines with insignificant traces of lutein in some cases.

High lycopene content was detected in some of the mutant plants studied by the chromatographic absorption (CCA) biochemical assay in the consecutive mutant generations in 2002-2004. The average lycopene content in fruit from the mutant plants was 39.751 mg lycopene/100 g dry matter. This value exceeded the average one of the initial line 120D that was 31.032 mg lycopene/100 g dry matter. For example the lycopene content in No1101-3 was 64.840 mg/100g dry matter, and 41.217 mg/100g dry matter in the mutant line No1101-1, that were 2 times and 1.3 times more, respectively, compared to the average lycopene value of the control 120D. This apparent increased lycopene quantity in some of the mutants was not confirmed in the next generations. The WT and the mutants differed statistically but in some of the cases the difference was in the opposite direction. A relatively large scale of variation throughout individual plants was observed in lycopene. For example, No1094-7 contained 17.369 mg lycopene/100g dry matter and was lower than the average value of the control 31.032 mg lycopene/100g dry matter and lower than the lowest quantity in the WT plant, 25.253 mg lycopene/100g dry matter. We conducted a selection for high lycopene within the different mutant plants in consecutive generations using the CCA for routine screening and HPLC for confirmation.

Evaluation of new tomato genotypes using genes controlling high level of lycopene and/or β carotene

The first steps of our study of carotene content in tomato were centred on evaluating the methodology of comparison, as well as the number of screening procedures necessary for determining individual plants or lines possessing genetic potential to synthesize high lycopene and β carotene content in tomato fruits. Investigations were carried out during two consecutive years under field conditions (Table 1).

TABLE 1. VARIATION IN FRUIT LYCOPENE CONTENT OF TOMATO GENOTYPES SAMPLED AT THREE HARVEST DATES

lycopene (mg % fresh matter) by absorption chromatography /Rank (R)							HPLC mg /100g dry matter)/R	
Genotype	July 25th	R	August 10th	R	August 25th	R	August 25th	R
G32 *hp*, *og*^{c}	8.6 ± 0.4	1	9.0 ± 0.3	1	10.9 ± 0.2	1	117.0 ± 0.2	2
A. Craig *hp*	6.7 ± 0.2	2	8.4 ± 0.3	2	8.9 ± 0.4	2	150.3 ± 0.4	1
A. Craig *og*^{c}	5.8 ± 0.2	4	7.4 ± 0.1	3	7.7 ± 0.3	4	92.8 ± 0.3	3
XXIV-13	4.8 ± 0.1	7	7.2 ± 0.2	4	7.8 ± 0.3	3	80.9 ± 0.2	4
AC *og*^{c} x AC *hp*	5.0 ± 0.3	6	6.9 ± 0.4	5	6.9 ± 0.1	6	75.4 ± 0.1	6
AC *hp* x AC *og*^{c}	5.6 ± 0.2	5	6.5 ± 0.2	6	7.2 ± 0.1	5	77.5 ± 0.1	5
A. Craig	4.7 ± 0.1	8	6.4 ± 0.4	7	6.8 ± 0.2	6	69.1 ± 0.2	8
VK 1	4.3 ± 0.1	9	4.5 ± 0.1	9	4.3 ± 0.1	9	51.3 ± 0.2	10
B 317	4.6 ± 0.2	8	5.0 ± 0.4	8	5.2 ± 0.3	7	54.1 ± 0.3	9
St 993	5.9 ± 0.3	3	6.4 ± 0.1	7	6.9 ± 0.2	6	70.4 ± 0.2	7
C 19	3.9 ± 0.1	10	4.2 ± 0.4	10	4.8 ± 0.1	8	50.4 ± 0.3	11

In all genotypes, fruit lycopene content increased from July 25th to August 10th to August 25th. Fruit lycopene content evaluated on August 25th exceeded that on July 25th by, on average, 25%. According to Helyes *et al.* [41] rainfall, temperature and light intensity during the period preceding the harvest date exercise a considerable effect on lycopene content. It was found that the genotypes possessing genetic potential to synthesize high lycopene and β carotene content might be assessed based on one only analysis. Fruits should be collected and analyzed at one harvest date. Evaluation of lines or individual plants possessing the highest lycopene and β carotene content within a number of genotypes analyzed for these characteristics on different harvest dates could be misleading. It is possible that environmental factors influenced the fruit content of the carotenes on different harvest dates. The second and if possible the third analysis might be helpful as they could provide additional information on the genotype capacity to synthesize lycopene and β carotene, as well as their varying limits throughout the harvest season (Table 2).

From our results, the genotypes could be divided into three groups based on their lycopene content: 1) genotypes that always have the highest lycopene content (G 32, Ailsa Craig hp and Ailsa Craig ogc), 2) genotypes always having low lycopene content (VK 1, B 317, C 19), and 3) genotypes with lower lycopene content than those from group 1, but significantly higher lycopene content than those from group 2 (XXIV 13, St 993, Ailsa Craig, Ailsa Craig ogc x Ailsa Craig hp). The same applied to the b carotene content although the variability in the content of this compound in some genotypes was relatively low. Regardless of the harvest date the highest content of b carotene was observed in Ailsa Craig hp and the lowest one – in Ailsa Craig and Ailsa Craig ogc.

TABLE 2. VARIATION IN FRUIT B carotene CONTENT OF TOMATO GENOTYPES SAMPLED AT THREE HARVEST DATES

β carotene (mg % fresh matter) by absorption chromatography / Rank (R)							HPLC mg/100 g dry matter/R by	
Genotype	July 25th	R	August 10th	R	August 25th	R	August 25th	R
A. Craig *hp*	3.32 ± 0.03	1	2.84 ± 0.05	1	2.66 ± 0.08	1	16.899± 0.2	1
G 32 *hp og*^{c}	1.74 ± 0.09	6	1.95 ± 0.06	2	1.92 ± 0.06	6	5.522 ± 0.4	6
XXIV -13	1.77 ± 0.06	5	1.94 ± 0.05	3	2.08 ± 0.07	2	5.834 ± 0.4	3
AC*og*^{c} x AC*hp*	2.18 ± 0.07	3	1.47 ± 0.09	9	1.56 ± 0.03	8	5.133 ± 0.1	9
AC*hp* x AC*og*^{c}	2.21 ± 0.04	2	1.51 ± 0.04	8	1.77 ± 0.02	7	3.860 ± 0.1	11
A. Craig	1.63 ± 0.01	9	1.53 ± 0.03	7	1.42 ± 0.05	10	5.500 ± 0.2	7
A. Craig *og*^{c}	1.37 ± 0.04	10	1.28 ± 0.02	11	1.32 ± 0.03	11	6.870 ± 0.3	2
VK 1	1.71 ± 0.1	8	1.89 ± 0.1	5	1.94 ± 0.02	5	4.943 ± 0.02	10
B 317	1.73 ± 0.2	7	1.86 ± 0.1	6	1.96 ± 0.02	4	5.134 ± 0.4	8
St 993	1.83 ± 0.07	4	1.92 ± 0.06	4	1.98 ± 0.05	3	5.622 ± 0.4	5
C 19	1.30 ± 0.04	11	1.38 ± 0.04	10	1.52 ± 0.03	9	5.770 ± 0.3	4

3.2. Induced mutagenesis in pepper

3.2.1. Induction of new mutations

In this study concentrations of EMS from 0.4% to 0.6% and doses of gamma irradiation from 60 to 140 Gy seemed to have a lower toxic effect on C. annuum L. lines. There was no correlation between the percentage of surviving plants and the concentrations of EMS. The percentage of the surviving plants decreased with higher doses of gamma-irradiation.

Phenotypes were screened in M2 plants and mutant characters selected as valuable were fixed in further generations. Selected M2-3 plants included absence of anthocyanins, those with erect fruit, erect fruit in a bunch and altered fruit shape. A mutant with determinate habit noted in M2, was confirmed in M3 and maintained. These were advanced to the M4 generations to confirm the traits and for further development of advanced mutant lines. Four lines with different additional morphological alterations were selected for a development of advanced mutant lines. The absence of anthocyanins observed in plants raised from M1 seeds was overcome in the subsequent generations. The mutant population from the tomato cv. Hebar treated with both EMS and gamma irradiation presented several morphological changes in the M4 generations (Fig. 1).

3.2.2. Cytological characterisation of mutation resulting in male-sterility

The cytological results (Fig. 2a,b,c) undertaken on the F2 segregating progeny (28×30) showed that the average ratio of fertile:sterile plants was 4.24:1, which is close to the expected 3:1 ratio. The expression of the mutant gene ms8 was stable and no pollen formation in the anthers of the studied sterile plants in both - the mother line 28 and the sterile F2 plants was observed, which is consistent with previous investigations [45]. The anthers were deformed and without pollen grains. Results of the meiotic analysis in pollen mother cells of sterile plants from line 28 revealed that lethality of the cells occurred after second telophase, by blocked cytokinesis and tetrad formation in the ms8ms8 plants was not observed.

Fig. 1. Induced pepper mutants in the M4 generations following EMS treatment and Co60 irradiation.

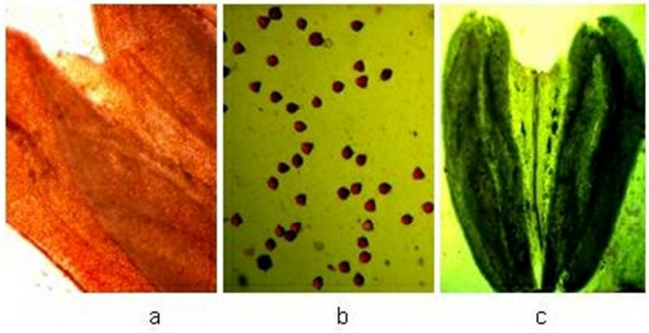

Fig. 2. Anthers of F2 plants: fertile (a) with pollen grains (b) and sterile (c).

3.2.3. Biochemical analysis of mutation resulting in orange fruit colour

Biochemical investigations were focused on the evaluation of carotenoid compound in sweet pepper and the assessment of their biological value. The levels of six carotenoids in green and mature fruits from mutant and wild type genotypes intended for fresh consumption were investigated by HPLC analyses. Increased β carotene contents in mutants were associated with a decrease in β-cryptoxanthin and zeaxanthin, compared with the wild type genotypes (Fig. 3). Mutants differed clearly from wild types by the α carotene accumulation and the corresponding lutein decrease. To explain the boosting of the health-related compounds β carotene and α carotene in sweet pepper fruits resulting from X rays irradiation, we suggested a suppressed enzyme activity due to a mutation in the gene encoding β carotene hydroxylase. The later is responsible for the hydroxylation of β-rings in both carotene pigments and their conversion into xanthophylls.

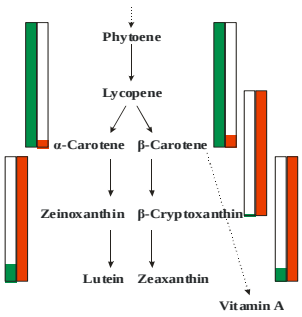

Fig. 3. Comparison between carotenoid levels in wild type and mutant pepper genotypes. Ms-left side; WTs-right side.

The biochemical data obtained in 2005 of the orange-fruited mutants demonstrated that the β carotene content was genotype dependant. Comparative analyses between the groups of Mof and WTrf revealed much higher accumulation of β carotene in the orange-fruited (of) lines. All Mof had higher β carotene content (12.57 to 19.58mg/100 g fresh matter (fm)) than the WTrf (bl. 28,31) which had red fruit (rf; 6.68 and 4.30 mg/100 g fresh matter (fm)). The β carotene levels in different F1 hybrids (MofxWTrf; MofxMof) were also greater than the WTrf. The highest concentrations were obtained when two parental Mof were hybridized. The β carotene content was the greatest in the F1 [35×17] hybrid (41.05 mg/100 g fm). The very high β carotene levels of the F1 hybrids were preserved in three consecutive years, despite of the variation in this trait. Higher β carotene levels in most of F1 hybrids were established combined with lower variation in this trait compared to the corresponding parents (Table 3).

TABLE 3. VARIATION IN β carotene CONTENT PER 100G FRESH FRUIT IN INDIVIDUAL PLANTS OF PEPPER GENOTYPES AND THE CORRESPONDING F_1 HYBRIDS

Genotype: Parents and F_1 hybrids			β carotene mg% fm		Error of the difference	
Line number		Fruit colour			$t_{experimental}$	VC%
P_1	28	red	6.68***	0.71	4.53 (F_1&P_1)	35.32
P_2	29	orange	12.57***	1.01	4.75 (P_2&P_1)	26.74
F_1	[28×29]	red	12.20	0.98	-0.26 (F_1&P_2)	25.60
P_1	28	red	6.68***	0.71	4.65 (F_1&P_1)	35.32
P_2	30	orange	15.84***	1.55	5.36 (P_2&P_1)	31.00
F_1	[28×30]	red	17.01	2.10	0.44 (F_1&P_2)	27.64
P_1	32	orange	12.80***	1.38	6.77 (F_1&P_1)	35.98
P_2	31	red	4.30***	0.45	-5.81 (P_2&P_1)	28.05
F_1	[32×31]	red	23.98***	0.89	19.62 (F_1&P_2)	10.54
P_1	35	orange	13.62**	1.07	3.26 (F_1&P_1)	24.92
P_2	31	red	4.30***	0.45	-7.98 (P_2&P_1)	28.05
F_1	[35×31]	red	27.87***	4.23	5.53 (F_1&P_2)	26.32
P_1	32	orange	12.80*	1.38	2.68 (F_1&P_1)	35.98
P_2	17	orange	19.58*	2.00	2.78 (P_2&P_1)	32.33
F_1	[32×17]	orange	18.89	1.79	-0.25 (F_1&P_2)	25.13
P_1	35	orange	13.62***	1.07	11.36 (F_1&P_1)	24.92
P_2	17	orange	19.58*	2.00	2.62 (P_2&P_1)	32.33
F_1	[35×17]	orange	41.05***	2.16	7.28 (F_1&P_2)	10.54

Df=18, tcritical, P5% (2.1), P1% (2.88), P0.1% (3.92); VC - variation coefficient %; - Average value; ±S - Error of

The F1 hybrids obtained from the lines 35, 31 and 30 could contribute to improved market performance due to the preference of red-fruited peppers. The presence of mutant gene in all Mof lines and hybrids demonstrated a greater potential for increased β carotene levels. Variability in the β carotene content of Mof with the same origin indicated that besides this mutation there are other genetically determined factors contributing to increase in this compound, reported for other pepper genotypes [26]. Despite the statistical significant difference (95%) between WTrf and Mof groups, the inheritance of the β carotene trait varied from partial dominance to over dominance in the same F1 hybrids. In each WTrf and Mof genotype a further selection towards the β carotene levels was conducted to decrease the variation of the target character (data not shown).

The quantity of chlorophylls, and total carotenoid content, was genotype dependent. Effects of mutation in chlorophylls and total carotenoids in fruit were detected. Chl-a contributed most to increasing of the total chlorophylls. Despite the increase in β carotene content, the rest of carotenoids contributed to a decrease in the total carotenoid content (data not shown).

Chromatographic absorption analyses of orange fruits from mutant genotypes and red fruits from wild type genotypes clearly revealed the higher accumulation of β carotene in the mutant lines. Generally, the β carotene levels in F1 generation originating from crosses between mutants and wild type lines were also elevated (Table 4). These results encouraged us to proceed further with crosses aiming to create new F1 hybrid pepper varieties, for fresh consumption, with boosted β carotene levels.

TABLE 4. PCR AMPLIFICATION WITH SPECIFIC PRIMERS FOR THE GENE *CRTZ* AND *CRTZ*-2

Gene/ fragment amplified	Primer from cDNA	Primer sequences	Results from amplified fragments Band length from
CrtZ-C/C	607-885	F GAGCTGAACGATATTTTTGCC	~620 bp in WT; absent in M
CrtZ2-C/C	586-864	R TAGGAACAAGCCATATGGGA	~760 bp in WT and ~760 bp in M
CrtZ-D/C	526-885	F AGATGGGCGCATAGAGCACTA	~700 bp in WT; absent in M
		R TAGGAACAAGCCATATGGGA	
CrtZ-D/D	526-866	F AGATGGGCGCATAGAGCACTA	~750 bp in WT; absent in M
		R ACCCCATCAAATTTGTCCGA	
CrtZ-E/E	20-866	F CGTACATGGCTGCTGAAATT	~1400 bp in WT; absent in M
		R ACCCCATCAAATTTGTCCGA	
CrtZ-C/D	607-866	F GAGCTGAACGATATTTTTGCC	~580 bp in WT; absent in M
		R ACCCCATCAAATTTGTCCGA	

The β carotene content depended on the genotype. The colour of the fruit was inherited independently from the β carotene levels. Crosses between wild type and mutant and among mutant lines themselves were performed to evaluate the fruit colour and β carotene content inheritance. Data for the parent and F1 hybrids corresponded to segregation by two genes with partially dominant character. The investigated mutant lines with orange fruit possessed steadily higher β carotene levels while the red-fruited wild-type lines showed lower β carotene levels.

On the base of the results we selected the genotypes to be used as donors for development of hybrid varieties with improved β carotene levels including the mutant lines 35, 31 and 30 which contributed to a greater extent the heterosic effect for increased β carotene content in there crosses.

Analysis of variance in pairs between parents and progenies confirmed differences in β carotene content due to the genetic potential of parents and/or to the effect of environmental factors. The quantitative characters like carotenoid content are variable due to the polygenic determination, strong effect of the environment and their interaction.

Significant variation in plastid pigment content in leaves was detected (from VC 10.54% to 35.98%). The quantity of leaf chlorophylls and total carotenoid content were genotype dependent. The results did not establish a relationship between mutation in the chlorophylls and carotenoids in leaves. However, we detected relation between chlorophylls and total carotenoids in fruits of the mutant. The Chl-a contributed to the increasing total chlorophylls in mutant lines compared to the wild type lines. Despite the increase in β carotene levels, the levels of the total carotenoids were low resulting in the decrease of the total carotenoids.

On the basis of the data for β carotene and total carotenoid levels, a hypothesis for a mutation that affected a gene encoding an enzyme responsible for hydroxylation of β carotene to β-cryptoxanthin was erected. Significantly less hydroxylase (e.g. CrtZ) enzyme activity in ripe fruit was noted in the Mof compared to the control WTrf (Fig. 4). This enzyme is responsible for the hydroxylation of β-rings and the conversion of carotenes into xanthophylls. Thus, data from analysis of enzyme activity support the hypothesis.

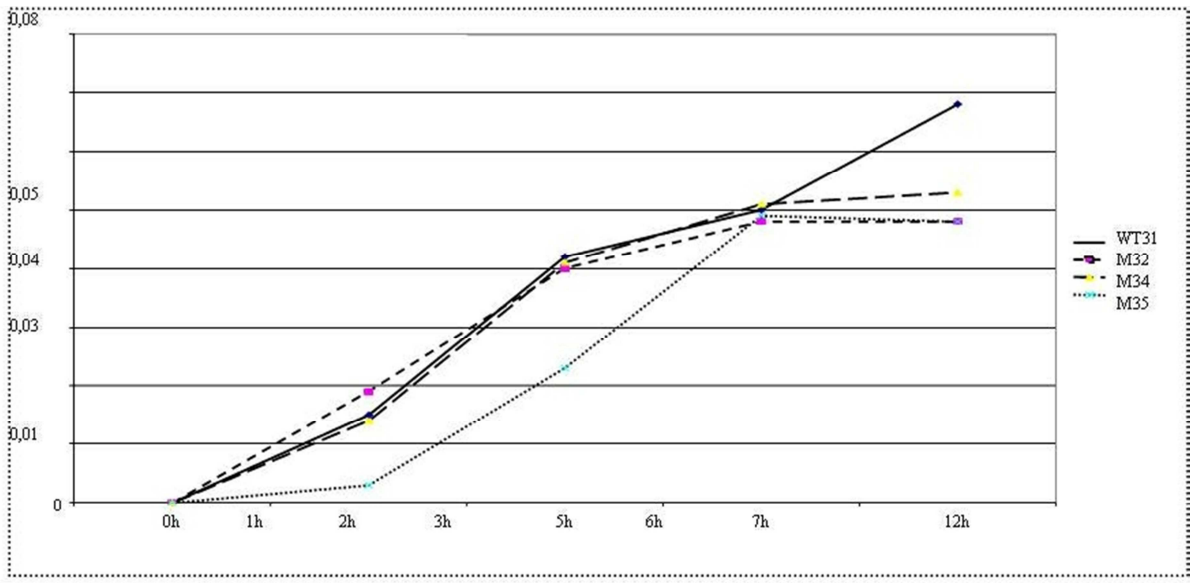

Fig. 4. CrtZ activity registered in pepper fruit of WTrf and Mof at botanical maturity.x - time of registration of the enzyme activity – 2, 5, 7, 12, 24 h after starting of the reaction; y - decrease of absorption values.

The β carotene hydroxylase activity was analyzed in green and mature fruits of pepper mutants and wild type lines. Smaller differences of the β carotene content were detected in the green pepper fruits. We noted a significant change in enzyme activity in the mutant genotype compared to the wild type control at a later stage of the fruit development.

Molecular studies have also supported the hypothesis built on the basis of the biochemical results. PCR reactions with different gene-specific primers were undertaken for two hydroxylase structural genes known to be involved in pepper biosynthetic pathway (Table 4).Both hydroxylases are expressed in pepper [39], and in tomato [40], and localized in the green tissue and flower [41]. A band

from CrtZ was amplified using primer combinations CrtZ-C, D, D/C and E in all WTrf but was absent in Mof. Fragments amplified from CrtZ2 in all WTrf and Mof showed monomorphic DNA profiles. This result was confirmed by a following cloning and sequencing of fragments isolated from CrtZ and CrtZ2 after amplification with the CrtZ-C/C.

Two DNA fragments were amplified and isolated from each WTrf corresponding to CrtZ2 and CrtZ genes and a unique fragment - from Mof corresponding to Crt2. A mutation occurring in the 3'-terminal region in the mutant was very probable. The noted primer combinations could be efficient as gene-marker for selection performing towards orange fruit colour in Mof.

Monomorphism was detected in the comparison between all WTs and Ms in electrophoretic patterns obtained by PCR in structural genes, encoding enzymes participating in the formation of the carotenoids in pepper. The amplified genes were: Ggpps design of primers for amplification was done using the information of the Accession No X80267, Psy - Acc. No X68017, Pds - Acc. No X68058, Zds - Acc. No X89897, Ze - Acc. No X91491, CrtL - Acc. No X86221, bCrtZ/BCH1/BCH2 - Acc. No Y09722, Ccs - Acc. No X77289.

4. CONCLUSIONS

New genetic resources of tomato and pepper were developed by inducing, using and combining mutant genes controlling high level of carotenes with other high value mutant genes.

A relatively large scale of variation throughout the individuals and harvest dates was observed in lycopene and β carotene of the tomato and pepper lines and hybrids.

Biochemical methods and procedures were developed as tools for evaluating the nutritive potential of the investigated crops.

Selective PCR, AFLP and ISSR markers with a direct application in the breeding of pepper and tomato lines and hybrids were revealed useful for marker assisted selection.

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FLUORESCENT IN SITU HYBRIDIZATION AS A GENETIC TECHNOLOGY TO ANALYZING CHROMOSOMAL ORGANIZATION OF ALIEN WHEAT RECOMBINANT LINES

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Abstract

Fluorescent in situ hybridization is a valuable method for physical mapping of DNA sequence to chromosomes and genomes and to analyzing their organization, diversity, evolution and function. Using genomic DNA the origin of chromatin in hybrids and alien introgression lines can be identified and followed through breeding programmes. We have applied this technology to study the chromosome composition of new recombinants and genomes derived from spontaneous and induced translocations in particular involving rye and the goat grass *Thinoyrum intermedium* that transfer disease and stress resistance to wheat. We have established flow diagrammes for easy identification of the alien chromosome material.

1. INTRODUCTION

Plant breeders have been remarkably successful in developing new varieties of most major crops, with a significant acceleration in the last 50 years [1,2]. Modern crop varieties deliver higher yields and better quality than their predecessors and this has been achieved despite the continuing emergence of new diseases and new races of diseases (biotic stress), changing cultivation practices, and movements in areas where crops are grown, with exposure to new environmental (abiotic) stresses. The potential yield increase and the resistances exclusively arise from improvements in the genetic constitution of the plants; a study of cereals in the UK shows that 90% of the recent increase in yield can be attributed to improved varieties [3].

It is important that the plant breeders continue with their success, meeting the challenges of continuing to increase yields, diseases adapting to current varieties, changing climates including water and salinity, and new market requirements for varieties suitable for storage and processing. A remarkably similar range of challenges face crops whether they are grown by subsistence farmers or smallholders, or large commercial operators, and whether in developing or industrialized countries. It is also essential that the successful genetic improvement strategies applied in the major crops are available widely for minor, niche and high-value species.

New genes, gene regulation and combinations of genes are needed to meet the new challenges faced by crops. Most crop species have a genetic bottleneck in their ancestry associated with a small number of genes critical to domestication see Ref. [2] Vaughan *et al.,* 2007 and 'Crop Domestication' Annals of Botany Special Issue 100/5; 2007), so the full range of variation present in the wild genepool is not available. It is also possible that intensive crossing, selection and improvement over recent decades has eliminated not only undesirable genetic alleles but also reduced the useful variation for generation of new varieties, although the evidence for this is equivocal: [4] found no reduction in variation in bread wheat and genetic diversity of wheat has remained at least constant for 70 years [5] while only some reduction in variation in durum wheat was reported by [6].

New gene alleles can be generated by induced mutation, and procedure that can be very beneficial in correcting well-defined weaknesses in existing varieties. The 2000 mutant varieties produced by direct use of induced mutations (IAEA Mutant Variety and Genetic Stocks Database, http://mvgs.iaea.org) all have important new characters but maintain other quality and agronomic attributes of their progenitor.

The genepool contained in wild species is also an important source for new variation to introduce new characters into the gene pool. This can be accessed by sexual crossing of cultivated and wild relatives of crops. Within the cereals, the Triticeae tribe in the grasses includes wheat, barley, rye and some forage grasses. The whole tribe can be intercrossed using appropriate techniques (including crossing intermediates or bridge-crosses, chromosome doubling and tissue culture rescue of embryos), and thus forms a single pool with enormous variation available at each genetic locus. To exploit the variation, characterization of the chromosomal constitution of the parents, design of crossing and backcrossing strategies, and analysis of chromosomes and chromosome segments that are transferred is essential. In this chapter, we show methods that allow detailed analysis of the introgressed alien (wild) chromatin into crop species, an approach which can accelerate and improve success rates in practical breeding.

A key method is in situ hybridization, allowing alien chromatin to be identified in chromosome preparations of alien-cross derived plants. Because of the evolutionary separation of the progenitor species, total genomic DNA, with a high content of genome specific repeats, can be used as probe for in situ hybridization to identify chromosomes and chromosome segments of different origin [7,8]. It has therefore become the method of choice when interspecific crosses and derived introgressed lines are analysed to reveal alien chromosomes and translocations [9-17].

For in situ hybridization, genomic DNA can be combined easily with cloned probes to give further information about the specificity of the chromosome involved in the introgression or detect rearrangements. In the Triticeae several repetitive probes are cloned and can be used for identification of chromosomes and often are specific for certain genomes [18-22]. Physical mapping of differentially labelled repetitive DNA sequences simultaneously in one experiment and in combination with total genomic DNA allows to detect the presence of introgressed chromosomes or chromosome segments from alien species in wheat lines, identifying the chromosomes involved and determining the nature and organization of any chromosome rearrangements [10,13-15,23-26].

In this report, we aim to show two examples of the analysis using in situ hybridization of wild wheat germplasm and the introgression of alien wheat chromosomes or chromosome segments in material with novel genetic variation which is of value to plant geneticists and breeders.

2. MATERIAL AND METHODS

2.1. Lines

The following wheat lines and varieties were used: *Triticum aestivum* hexaploid wheat ‘Chinese Spring’, and ‘Chinese Spring’ line carrying a 1DL.1RS translocation form rye ‘Imperial’ and variety ‘Beaver’ carrying a 1BL.1RS translocation.

Fixed triticale x wheat derivatives (RL lines) from CIMMYT material were selected after continuous selfing and growing in the remote regions of Trans- Himalayas.

Wheat breeding lines with *Wsm-1* from the materials evaluated in the study of Ref. [27] including the line subsequently named ‘*Mace*’ [28].

2.2. Chromosome and probe preparation

Seeds were germinated on Petri dishes in the dark and 1-2cm long emerging roots were fixed in alcohol:acetic acid 3:1 after synchronization with 24h ice treatment. Roots were digested with 3% (w/v) pectinase (Sigma, 450units ml^{-1}), 1.8% (w/v) cellulase (Calbiochem, 4000units g^{-1}) and 0.2% (w/v) cellulase (Onozuka RS, 5000units g^{-1}) and chromosome preparations made on glass slides by squashing in 45% and 60% acetic acid as described by [29].

The following probes were used:

pTa71 contains a 9 kb *Eco*RI fragment of the repeat unit of 25S-5.8S-18S rDNA isolated from *T. aestivum* [30] recloned in pUC19.

pSc119.2 or CS13 both contain a 120 bp tandem repeated DNA sequence isolated from *Secale cereale* [31, 20]

dpTa1 contains a tandem repeat with a monomeric length of 390 bp isolated from *T. aestivum* and subcloned by [32] and homologous to pAs1 [33] and the 340bp Afa-repeat sequences [21].

Total genomic DNA from rye 'Petkus' wheat 'Chinese Spring' and *Thinopyrum intermedium* 'Haymaker' was sheared, to 5-8kb pieces by autoclaving.

pTa71 was linearised with *Hind*III before labelling. CS13 and dpTa1 inserts were amplified by PCR using the universal M13 sequencing primers and the products of respective lengths were cut and cleaned out after running on 1.2% agarose gels. For labelling of cloned and gnomic DNA, biotin-16-dUTP and digoxigenin-16-dUTP (Roche Diagnostics) were incorporated in separate reactions using the Bioprime Biotin or CGH kits (Invitrogen) respectively.

2.3. In situ hybridization

DNA:DNA in situ hybridization followed the method described by [29] with minor modification. The probe mixture contained 50% (v/v) formamide, 20% (w/v) dextran sulphate, 2 x SSC, 25-100ng probe, 20 µg of salmon sperm DNA and 0.3% SDS (sodium dodecyl sulfate) as well as 0.12mM EDTA (ethylene-diamine-tetraacetic acid) and autoclaved total genomic DNA from wheat 'Chinese Spring' as blocking DNA at 4-20 x probe concentration. Probe and chromosomal DNA was denatured together on a Hybaid Omniblock for 6-10mins at 72-78°C and slowly cooled to the hybridization temperature of 37°C. Washes were carried out with 20% (v/v) formamide and 0.1 x SSC at 42°C at an equivalent to 85% stringency. Hybridization sites were detected with 2.0µg/ml streptavidin conjugated to Alexa594 (Molecular Probes) and 4µg/ml antidigoxigenin conjugated to FITC (flourescein isothiocyanate) (Roche Diagnostics). Chromosomes were counterstained with 0.2µg/ml DAPI (4′,6-diamidino-2-phenylindole) diluted in McIlvaines buffer pH7 and mounted in antifade solution (Citiflour). Preparations were analysed on a Zeiss epifluorescence microscope single band pass filters equipped with a CCD camera (Optronics, model S97790) and overlayed using Adobe Photoshop CS2 or 3.

3. RESULTS AND DISCUSSION

3.1. Wheat rye translocation lines

The rye (*Secale cereale* L.) genome has shown potential for improvement of bread wheat (*Triticum aestivum* L.), where wheat-rye substitutions and translocations have been and are frequently used in resistance breeding [34] and the 1BL.1RS wheat-rye translocation is present in the highest yielding cultivars currently grown in Europe and Canada [35,36]. However, the 1BL.1RS varieties have lost high molecular glutenins of 1BS that are responsible for good bread making quality and are hence used for feed and biscuit wheat only [37]. Several breeding programmes have hence tried to exchange either the 1AS or 1DS arm for the 1R arm as well as aiming to introgress other rye arms into wheat [38,34,17].

In order to analyse such lines, chromosome numbers should be established first using phase contrast, Feulgen or DAPI staining and genomic in situ hybridization using rye DNA will identify whether whole chromosomes, whole arms or segments have been transferred as addition, substitution, or translocation lines (See Fig. 1). 45S rDNA have been mapped to the large nucleolus organising regions (NORS) half way up the short arm of wheat chromosome 6B and 1B, 6B generally being

stronger than 1B [39] see Fig. 3B. Chromosome 5D carries a medium 45S rDNA site on the end of the short arm, while the small site at the end of the small arm of 1A is visible in most, but not all FISH experiments. Additionally, very small sites sub-telomerically on the long arm of the equal armed chromosome 7D and variable sites on other wheat chromosomes.

Combining genomic in situ hybridization with the 45S rDNA probe and counting major and minor sites, allows identification of the presence of 1RS and whether it is substituting the 1BS, 1AS or 1DS arm (Fig. 2). In Fig. 3A, a root tip metaphase of a 1B.1R translocation line is shown. The genomic rye DNA has labelled the two rye 1RS arms that are characterized by a large 45S rDNA site. Further 45S sites are on chromosome 6B, 5D 1A and 7D, while chromosome arm 1B is missing. In the 1DL.1RS translocation line (Fig. 3A) six major 45rDNA sites are visible on 1R, 6B and 1B.

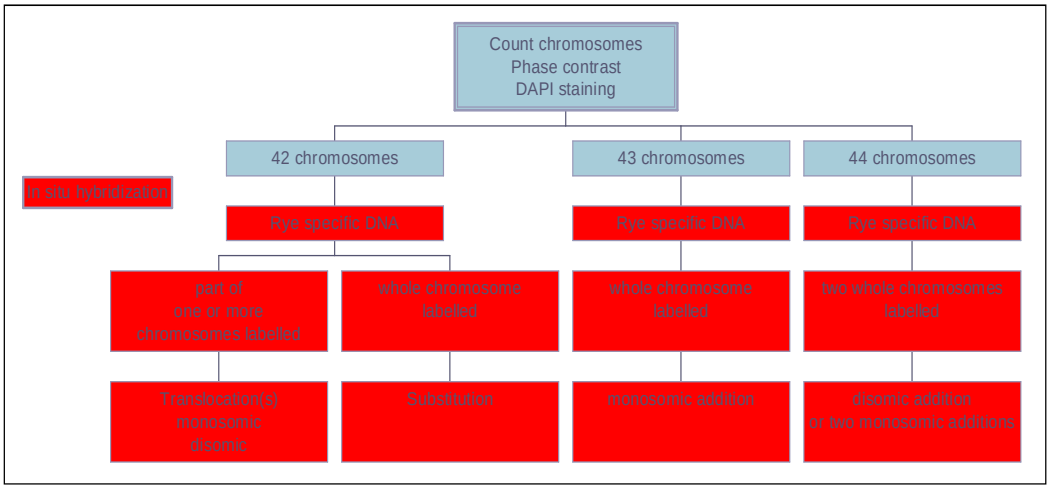

Fig. 1. Flow diagram to determining the presence of rye-wheat additions, substitutions or translocations.

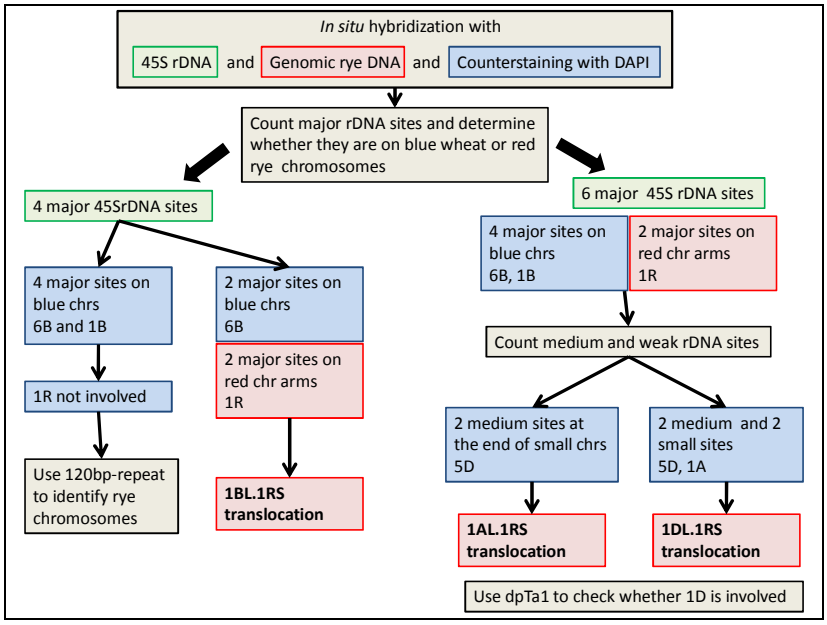

Fig. 2. Flow diagram for the identifying rye and wheat chromosomes involved in translocations. Fluorescent in situ *hybridization is assumed with green detection of the 45S rDNA and red detection of rye chromatin while the wheat chromosomes are blue with DAPI (see Fig.3A and B).*

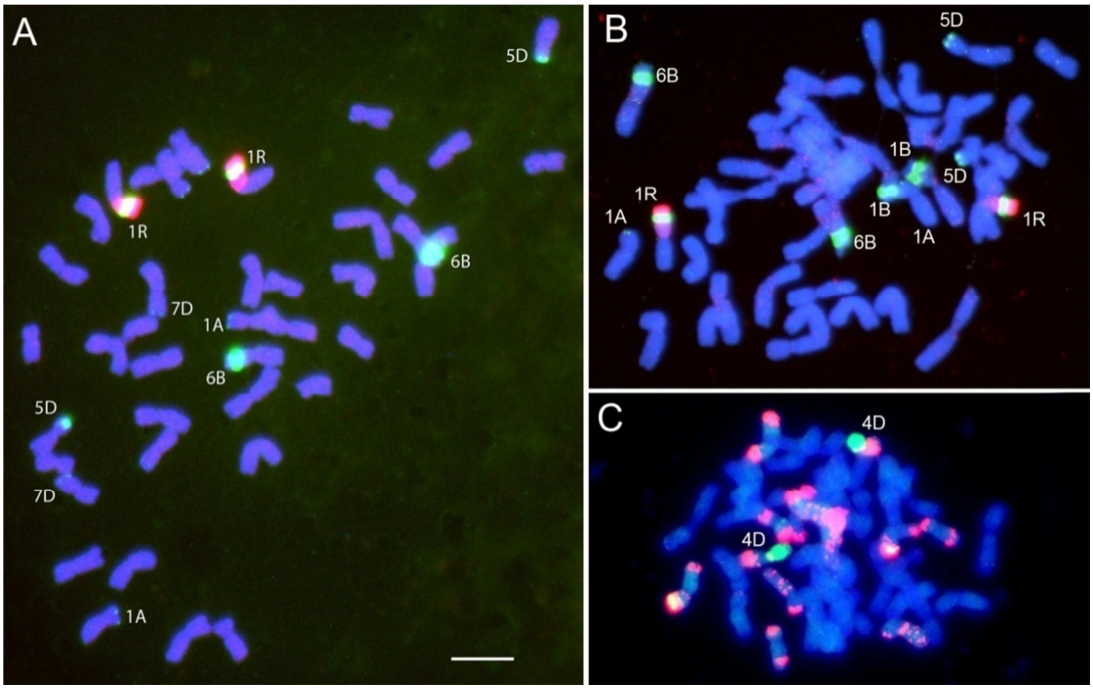

Fig. 3. Root tip metaphases of alien-to-wheat chromosomal translocation lines after FISH with genomic DNA from rye (shown in red; A: 1BL.1RS 'Beaver' and B: 1DL.1RS) and Thinopyrum intermedium (green in C; line giving rise to 'Mace'). Wheat chromosomes are shown in blue with DAPI fluorescence and repetitive probes are used to identify chromosomes. The 45S rDNA (green in A and B) identifies the major NOR sites on chromosomes 1R, 6B, 1B as well as minor sites on chromosomes 5D, A and 7D). In C, the Th. intermedium chromosome arm is translocated to chromosome 4D identified by the D-genomic specific sequences dpTa1. Bar equals 10µm.

In an example of a project to examine derivatives of wheat-rye breeding programme, sixteen spring-type bread wheat lines derived through wheat-rye introgression following hexaploid triticale x wheat hybridization were used. The programme involved further reshuffling of the genes by making crosses amongst the reconstituted lines involving various north-west Himalayan landraces, and these lines were screened for the detection and characterization of the rye chromatin substitutions/translocations by fluorescent in situ hybridization. Genomic in situ hybridization with rye genomic DNA detected the presence of one arm of a rye chromosome translocated into the 10 reconstituted wheat lines out of 16, and total number of the somatic chromosomes was unchanged. The presence of this part of the chromosome was further confirmed as short arm of 1R chromosome of rye through fluorescent in situ hybridization (FISH) using the 45S rDNA probe recognizing the NOR region on the sub-terminal end of this translocated chromosome's arm similar to the variety 'Beaver' shown in Fig. 3A. Additional karyotyping and was carried out using the B genome specific 120pb repeat sequence (not shown) and confirmed the involvement of the 1BL arm in the rye translocation. The remaining six lines were observed to be exhibiting neither any substitution/addition/translocation nor deletion of any wheat chromosome.

3.2. Wheat-Thinopyrum lines

Wheat Streak Mosaic Virus (WSMV) is an important disease, limiting winter wheat production in the US. The disease is seed born and spread via curl mite (WCM) *Aceria tosichella Kiefer* [40]. There is no known effective WSMV resistance within the genetic pool of wheat, and if present is non-effective above 18°C [41]. The Triticeae species *Thinopyrum intermedium* (2n=6x=42) has a vast reservoir of useful agronomic traits and shows good resistance to WSMV. It has been used to increase the genetic diversity in wheat. *Th.intermedium has* the resistant gene Wsm1, which limit infection and WCM colonization [16,42,27]. Hybrids and back crosses were developed with the aim to transfer WSM resistance gene, Wsm1 to wheat from *T. intermedium* [27], and these authors obtained six populations, each comprised of resistant and susceptible lines. Breeding lines with potential WSMV resistance can

be screened and selected at seedling stage without difficult pathological tests, and if analysed by PCR using the primers named STSJ15L &STSJ15R [43] giving a 420bp product, a marker linked to resistance. A new cultivar, Mace, incorporating WSMV resistance from the wild species has been released [28]. Genomic in situ hybridization with total genomic DNA from *T. intermedium* (Fig. 3C) is able to identify the introgresses translocated chromosome segment as a 4D.4Ag chromosome.

Currently, this programme is being extended to identify breakpoints and for further characterization of a family of wheat-intermediate wheatgrass (*Triticum aestivum – Thinopyrum intermedium*) recombinant lines spanning the wheat streak mosaic virus resistance locus; methods including both SSR markers and FISH are being applied.

4. CONCLUSIONS

The examples above show how molecular cytogenetics with genomic DNA and cloned DNA probes can be used to identify chromosome introgression in wheat breeding material. These crosses are valuable to increase the range of variation present in bread wheat, in some situations bringing in gene alleles that are entirely absent from current varieties. The cytogenetics is also valuable to identify ploidy of lines (in parents and in the hybrids themselves), to check for aneuploidy, and to track marker chromosomes through breeding programmes.

Parallel approaches are valuable in other crops, and relatively little background information is required about the material, at least compared to the extensive marker development or recombinant DNA libraries needed is some programmes. Thus, molecular cytogenetics and wide hybridization can be applied to minor crops, and enables more directed germplasm exploitation and improvement to be associated with breeding programmes.

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CHARACTERIZATION OF ALUMINUM TOLERANCE IN RYE

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Abstract

Large insert libraries, cosmid, yeast artificial chromosomes (YACs), bacteriophage P1, bacterial artificial chromosomes (BACs), and P1-derived artificial chromosomes (PACs), have proven to be valuable tools for gene cloning, physical mapping, and comparative genomics. Of all the large insert libraries, BAC libraries are the most widely used, because of their ease of creation, large insert size, and stability. This report describes a simplified method for plant BAC library construction, which involves isolation and partial digestion of intact nuclei, selection of appropriate size of DNA via pulsed-field gel (PFG) electrophoresis, elution of DNA from agarose gels, ligation of DNA into the BAC vector, electroporation of the ligation mix into Escherichia coli cells and estimation of insert sizes. The whole process takes 1-3 months depending on the genome size and coverage required. We used this approach to produce a BAC library from different rye (*Secale cereale* L.) for utilization in our attempts to clone the gene complex controlling aluminum tolerance.

1. INTRODUCTION

Characterization and manipulation of Aluminum (Al) tolerance genes for cereal improvement offers a solution to the Al toxicity problem involving crop cultivation on acid soils. On a world scale, acid soils comprise approximately 40% of all arable land or about 2.5 billion hectares of land potentially useful for agricultural production. By exploiting the rice/wheat/barley/rye syntenic relationship, the potential for genetically and physically locating Al tolerance gene complexes of interest, and most importantly for map-based cloning of gene complexes controlling Al tolerance in rye, can be explored. Rye was selected for analysis because it has long been ranked as the most Al tolerant of all the cereals, rye>>rice>maize≈sorghum≈wheat>>barley.

2. CHARACTERIZATION OF ALUMINUM TOLERANCE

A large 1100 line rye F2 mapping population was developed that was segregating for Al tolerance. Attempts at high resolution mapping the rye Al tolerance gene and cloning an Al tolerance gene from rye were initiated utilizing several different kinds of PCR-based molecular markers (RFLP-both genomic and cDNA, SSR, AFLP, EST), from wheat, barley, and rice, and rye. Two PCR-based markers flanking the Al tolerance gene were derived from a rice BAC clone that had been fully sequenced and characterized. In order to obtain a very tight linkage to the Al tolerance gene required for initiating map-based cloning of the gene, several additional PCR-based markers were developed from the 32 rice coded sequences located on the rice BAC, and were also mapped to the rye Al tolerance region. Two of the rice-derived markers flanked the Al tolerance locus at a distance of 0.05 cM, while two others co-segregated with it. The rice/rye micro-colinearity worked well in delineating and mapping the Al tolerance gene region in rye. A rye 400 bp fragment from the middle of a suspected Al tolerance candidate gene present on the rice BAC clone was identified using rice-derived primers. However, at this level of rye/rice microsynteny, the rye/rice relationship broke down and we could no longer develop primers from the rice sequence flanking regions that would work in rye. Apparently, because of evolutionary divergence, the genic and flanking region sequence differences existing between rice and rye were such that we have been unable to use any PCR-based chromosome walking techniques to clone the full-length candidate gene in rye using existing rye/rice microsynteny. The evolutionary divergence of rice and rye, in this specific region has been significantly changed (deletions, insertions, translocations, mutations, etc.).

Currently, the rice-derived PCR-based markers are being used in marker-assisted-selection (MAS) programs designed to transfer rye Al gene(s) into hexaploid wheat via wheat/rye hybridizations

followed by backcrossing to wheat and selecting for the presence of a wheat plant phenotype and presence of the markers flanking the rye Al tolerance region.

Since our approach of map-based cloning using a form of PCR walking proved not to be useful in sequencing any rye candidate gene, the creation of a rye BAC library appeared to be the most promising approach to break down the large complex diploid rye genome into more manageable pieces that could be sequenced and utilization for gene cloning in rye and to identify other gene complexes or genomic regions responsible for specific value-added traits of interest. In addition to gene characterization and positional cloning, the insert DNA could also be used to create BAC-contig maps of specific genomic regions by various approaches including BAC fingerprinting [1], and physical mapping [2] [3-6]. The insert DNA from the BAC library could also be used to evaluate the differences between physical and genetic distances within regions of a genome [7], in map saturating of molecular markers and microsatellite development [8], to compare related species [9], to analyze the fractions of complex genomes [10], and to characterize genome architecture [11]. We are sure that there are several additional uses of large insert DNA that have not been mentioned.

The insert size found in most BAC libraries varies and ranges from averaging less than 100 kb to 150 kb, and can be up to as large as 350 kb [12]. However, BAC libraries offer many advantages including ease of construction and manipulation [13], lower amounts of instability, generally contain fewer rearrangements in the repetitive DNA [14], contain fewer chimeric clones [15].

So far, no BAC library had been constructed for diploid rye, which is the last of the large cereal genomes lacking a BAC library, thus resulting in a phylogenetic gap in evolutionary comparisons of the Triticeae and greatly limiting our ability to study and manipulate the various Triticeae genomes for cereal improvement. In addition, as stated earlier, the lack of a rye BAC library also creates difficulties in positional cloning of value-added genes (i.e. abiotic stress, disease, drought tolerance, etc.) of interest for use in wheat and rye improvement, and in large scale physical mapping of gene-rich regions in rye for utilization in studying the syntenic relationship between wheat, rye, and rice, to develop physical maps, and for studying genome structure.

The isolation and characterization of agronomically important value-added trait genes from rye has been hampered by the massive size and complexity of the rye genome. Our previous attempts at chromosome walking and positional cloning in rye for example; [16] failed probably due to the presence of an extremely large diploid genome containing a massive amount more than 80% [17] of highly repeated sequences. At the present time we do not know how much the presence of large amounts of repeated sequences within rye BACs will hinder the characterization of BAC contigs and chromosome walking in other regions of value-added traits. The presence, within BACs, of large amounts of repeated sequences can reduce our ability to characterize and utilize BACs. These problems are already beginning to make their presence known in sequencing rye BACs (Gustafson, unpublished data). Regardless, the rye BAC library will definitely provide a direct source of material for cloning desirable value-added traits, such as acid and saline soil tolerance, disease resistance, drought tolerance, high lysine, winter hardiness, etc., for use in the improvement of other cereals. Granted that the grass ‘model’ species, rice, has been sequenced and is a very powerful tool, but recent work has shown that the colinear relationship between rice and other cereals only works so far using map-based cloning approaches [18]. Many rye value-added traits will only be obtained and characterized using a BAC library as a source of gene complexes.

Therefore, our primary objective was to construct a rye BAC library for conducting studies in evaluating rye as a source for the map-based positional cloning of various abiotic genes and gene complexes for utilization in gene characterization and for cereal improvement. Bu-Jun Shi at the Australian Centre for Plant Functional Genomics (ACPFG), Adelaide, South Australia, constructed the rye BAC library utilizing pIndigo BAC-5 (Epicentre) as a vector. Genomic DNA fragments from the spring white-seeded, daylength insensitive diploid rye cultivar ‘Blanco’ were used as inserts. The library constructed contains approximately 6x genome equivalents with an average insert size of 130kb. The rye BAC library is composed of 963 384-well plates containing approximately 369,792 clones picked using the ACPFG, Adelaide, using Bio-Rad VersArray CPAS Robot. The insert size is

slightly larger than the average observed in many other BAC libraries. The construction of the rye BAC library required considerable effort as the number of clones in the rye library equal approximately 7.8 rice BAC libraries.

The ACPFG in Adelaide used a very important second PFGE separation, which increased the concentration of more desired higher molecular weight fragments and eliminated most of the small fragments. But the two-step selection for larger size appeared to decrease the transformation efficiency, thus resulting in a massive increase in the amount of work required to pick large numbers of big sequences. The more sequences that were picked per plate, the smaller in size were the sequences, thus the ACPFG had to make compromise between the number of transformations per picking plate and the size of the fragments. Chalhoub *et al.* [19] described in detail several improvements that would increase the size and quality of clones, which should be considered when creating any new BAC libraries.

Using filters containing only 4,608 clones, we established that only a very small portion of the total library (0.98 %) was comprised of chloroplast DNA. The number of clones containing mitochondrial DNA was also estimated and again only a very small portion of the library (0.02 %) was contaminated. When the average insert sizes (130 kb), the percentage of clones containing chloroplast and mitochondrial DNA (0.98% and 0.02%, respectively), the rye genome size of 8.110 Gb [20], the number of clones being 369,792, and the number of empty clones was taken into account, the 'Blanco' rye BAC library was found to agree with our conclusion that the library represents approximately 6× genome equivalents.

We are currently in the process of fully sequencing three rye BAC clones that have been shown to contain an aluminum tolerance gene-rich region.

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ABBREVIATIONS

AFLP	Amplified fragment length polymorphisms
BAC	Bacterial artificial chromosomes
Comet Assay	A cytological test also known as single cell gel electrophoresis (SCGE), called comet assay based on th structure of the detected product
DArT	Diversity arrays technology
DNA	Deoxyribonucleic acid
EMS	Ethyl methanesulfonate
EST	Expressed sequence tag
FISH	Flourescent in situ hybridization
GISH	Genomic in situ hybridization
HVI	High-volume instruments
IRAP	Inter-retrotransposon Amplified Polymorphism
ISSR	Inter-simple sequence repeat
LINEs	Long interspersed nuclear elements
LTR	Long-terminal repeat
MAS	Marker assisted slection
MNU	N-nitroso-N-methylourea (synonym n-methyl-n-nitrosourea, old acronym NMU)
NIRS	Near infrared reflectance spectroscopy system
PCR	Polymerase chain reaction
PRINS	Primed in situ labeling
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic dnas
rDNA	Recombinant DNA
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
SCAR,	Sequence amplified characterized region
SINEs	Short interspersed nuclear elements
SNP	Single nucleotide polymorphisms
SSR	Simple sequence repeats
TILLING	Targeting Induced Local Lesions in Genomes
TUNEL Assay	Terminal Transferase dutp Nick End Labeling

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