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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 4 (4): 479-482, 2001

Use of Molecular and Morphological Markers as a Quality Control in Plant Tissue Culture

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Abstract: The concept of utilizing markers in plant breeding as an indirect method of selecting desirable recombinant genotypes is well established. Until relatively recently, the markers employed were morphological characters which are limited in number and often agronomically undesirable. As a means of increasing the number of markers, different strategies have been developed based on existing natural variation present in plant genomes. The significance of this variation is that a large number of genetic markers can be assembled in a single cross. Further more, these markers are inherited in a Mendelian and hence, predictable manner. The availability of such markers provides new opportunities to improve the speed and precision of gene transfer in crop improvement. The suitability of different markers to be used to characterize the tissue culture plants has been reviewed in this paper. As the micropropagation industry is expanding somaclonal variation is becoming problems for the commercial propagator of a genotype. There is a severe need to have an effective reliable fast screening system to assure the genetic basis of the plants regenerated through tissue culture as a quality control measure.

Key words: Molecular marker, somaclonal variation, polymorphism micropropagation

Introduction

The fact that *in vitro* culture can cause genetic variation in regenerated plants aroused much interest and discussion and this type has been frequently reviewed (Evans and Bravo, 1986;). Larkin and Scowcroft (1981) detailed various sources of variation in regenerated plants. They considered that plants derived from any form of cell and tissue culture should be called somaclones and genetic variation produced in these clones should be called somaclonal variation.

It may of course be that different processes are at work in different species or that a number of processes are operating simultaneously in the one culture. Muller *et al.* (1990) reported that most of somaclonal variations are directly or indirectly related to alteration in the state of DNA methylation. Other mechanisms observed in regenerated plants by many workers are, chromosomal aberration i.e. changes in chromosome numbers (Orton, 1983), chromosome rearrangements, translocations, reciprocal translocation and ring chromosomes (non-homologous translocations) (Shepard, 1982). Published work also suggests that chromosome deletions, additions, inversion, and crossing-over also occurs in regenerated plants. (Grunewaldt and Dunemann, 1991). Cryptic changes can result not only in the loss of genes and their functions but also the expression of genes, which have been silent. For example a rearrangement may delete or otherwise switch off a dominant allele allowing the recessive allele to affect the phenotype. In eukaryotes evidence suggests that certain unstable mutants may be explained by transposable elements. The excision and reinsertion of the genetic element can directly affect the expression of the neighboring structural gene. Weill and Reynaud (1980) referred to the high mutability and consequent adaptiveness in somatic tissue as 'somatic Darwinism'. Somatic genes rearrangements, amplification and depletion have also been suggested to be involved in somaclonal variation. (Nagl, 1979).

Morphological Markers

Morphological assays generally require neither sophisticated equipment nor preparatory procedures. So their prime advantages are simplicity and lack of expense to score even from preserved specimens e.g. herbarium sheets and in maize (*Zea mays* L.). The main disadvantage of this approach is that possession of a normal phenotype is no guarantee that cryptic changes have not occurred. In addition, many of these changes may be recessive and consequently when in heterozygous forms, do not appear until plants have been selfed and progeny examined (Brown *et al.*, 1993).

Chromosome numbers and cytomorphological traits have also served as genetic markers, especially in polyploid crop complexes, where these have been important tools for elucidating systematic and evolution (Simmonds, 1976). Chromosome morphological features include size, centromere position, mitotic configurations, and occurrence of satellites which are observable following staining (Dyer, 1979). All these karyotype features have contributed critical data to plant systematic and evolutionary studies. Karyotypic/cytogenetic observations require specialized equipment (microscopes) but preparative protocols are otherwise relatively simple and inexpensive (Dyer, 1979). Correctly interpreting certain cytomorphological features does require considerable training, experience, and advanced knowledge of cytogenetics.

Proteins: Protein and isozyme variants (Weeden and Wendel, 1989) that migrate at different rates under electrophoresis have been the most widely employed molecular genetic markers during the last quarter century. Isozymes are generally fractionated by starch gel electrophoresis whereas, proteins are generally analysed via Polyacrylamide gels (PAGE) in sodium-dodecyle sulphate SDS (Cooke, 1984).

Isozymes: During the last 20 years isozymes revealed through starch gel electrophoresis (SGE) have been the genetic markers most frequently employed in many fruit species. These were used for the identification of cultivars as well as characterisation of somaclonal variation. (Damiano *et al.*, 1995). They are generally but not always governed by single Mendelian genes. They can be assayed from a wide variety of organ and tissues, and analytical procedures are not exceptionally complicated (Weeden and Wendel, 1989). In studies of genetic diversity and divergence, isozymes with

similar enzymatic activity and electrophoretic migration rates are presumed to be homologous although this assumption cannot be validated without amino acid sequencing.

Genetic Markers

The greater utility of molecular markers arises from six inherent properties that distinguish them from morphological markers (Powell *et al.*,1994).

1 The phenotype of most morphological markers can only be determined at the whole plant level, whereas molecular loci can be assayed at the whole plant, tissue, and cellular levels.

2 Allele frequency tends to be much higher at molecular loci compared with morphological markers.

3 In addition, morphological mutants tend to be associated with undesirable phenotypic effects.

4 Alleles at morphological loci interact in a dominant-recessive manner that limits the identification of heterozygous genotypes.

5 Molecular loci exhibit a co-dominant mode of inheritance that allows the genotypic identification of individuals in a segregating population.

6 Fewer epistatic or pleiotropic effects are observed with molecular markers than with morphological markers. Hence a large number of polymorphic markers can be generated and monitored in a single cross.

RFLP: Various DNA polymorphism are the most highly heritable of all genetic markers. In plants, they can be assayed from three distinct genomes, i.e. nuclear, chloroplast, and mitochondria which may each evolve according to different modes and tempos (Wolfe *et al.*, 1987). The emergence and general accessibility of molecular biological techniques has allowed the relatively extensive variation, which occurs in the DNA sequence of a given organism to be exploited. The standard method by which this is achieved relies on the ability of certain bacterial enzymes, restriction endonucleases, to recognise and cleave specific DNA sequences within the extremely long DNA molecules, which comprise a plant's genome. Cleavage results in the generation of a set of restriction fragments of differing lengths which reflects restriction site changes within a given individual. After electrophoretic separation of the fragments according to their length, and transfer to a solid membrane. Identification of restriction fragments is achieved by southern DNA:DNA hybridisation with a radioactivity labelled cloned DNA probe and visualised by exposure of the hybridisation membranes to photographic film. The variation, which occurs, has been termed restriction fragment length polymorphism (RFLPs). Since the mid-1980s, RFLPs have been used extensively for the construction of genetic linkage maps, and RFLPs linked to many desirable characters have been identified. RFLPs are also useful for other applications including cultivar identification, evaluating germplasm resources, identifying distantly related parents for inclusion in a breeding programs and for phylogenetic studies.

RFLPs are superior genetic markers, because they are firstly ubiquitous throughout the plant tissue and throughout the plant genomes coding and non-coding sequences and secondly highly heritable, relatively highly polymorphic, and co-dominantly inherited. (Helentntjaris and Burr, 1989).

A major drawback of RFLPs is that their application is technically difficult and in the majority of laboratories the detection methods rely on the use of short-lived radioisotopes. At present, RFLP analysis is relatively slow and labors intensive. It may involve expensive and sometimes radioactive/toxic reagents. (Helennljaris and Burr, 1989). In many cases, these features may inhibit the routine application of RFLPs in plant breeding.

AFLP: The Amplified Fragment Length Polymorphism (AFLP) technique is based on the amplification of subsets of genomic restriction fragments using the polymerase chain reaction (PCR). DNA is cut with a restriction enzyme, and double stranded (ds) adapters are ligated to the ends of the DNA-fragments to generate template DNA for amplification. Thus, the sequence of the adapters and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments from a total genomic digest. Selective nucleotides are added to the 3' ends of the primers that therefore can recognise only a subset of the restriction fragments. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified.

Two restriction enzymes, a rare cutter and a frequent cutter generate the restriction fragments for amplification. The AFLP procedure results in the predominant amplification of those restriction fragments that have a rare cutter site on one end and a frequent cutter site on the other end. Fingerprints are produced without prior sequence knowledge using a limited set of generic primers. Typically 50-100 restriction fragments are co-amplified and detected in each AFLP reaction. This technique is therefore extremely powerful for the detection of DNA fragments, and hence for the identification of DNA polymorphism. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer sets. The AFLP technique is robust and reliable because stringent reaction conditions are used for primer annealing: the reliability of RFLP techniques is combined with the power of PCR techniques and of gel analyses of amplified fragments.

The AFLPs like RFLPs are highly heritable, relatively highly polymorphic, apparently selectively neutral, and can be isolated from virtually every type of plant tissue. The AFLP fragments are generally inherited in a simple dominant Mendelian fashion, whereas fragment absence is recessive. In this respect AFLP markers are inferior to co-dominant genetic markers and with expensive DNA polymerase enzymes at a relatively high cost. Nevertheless, AFLP analysis may be amenable to automation, requires very little tissue for analysis, and does not require blotting, probing, probe maintenance in bacteria, or other expensive steps associated with RFLP analysis (Smith and Smith, 1992).

RAPD-PCR: Considerable progress has been made in the application of DNA-based methodologies for the identification of phylogenetic relationships in many crop species. Exciting new opportunities for improving techniques of identification and diagnosis were created by the introduction of the polymerase chain reaction (PCR). However, the advent of PCR has brought the potential of a DNA-based marker system one step closer to being routinely and reliably applied. PCR technology hinges on the availability of DNA polymerase (Taq polymerase) from the thermophilic bacterium *Thermus aquaticus* that retains activity even after prolonged incubation

at temperatures, which denature double stranded DNA templates. Defined segments of minute quantities of target DNA can be specifically amplified by supplying Taq polymerase, excess nucleotides and oligonucleotide primers (which are exactly complementary to sequences flanking a target sequence), and repeating a thermal cycle which denatures the DNA, allows the primers to anneal to their complementary sequence and finally activates the DNA polymerase. Taq polymerase will copy the single stranded target DNA unidirectionally from the annealed primers. Any sequence up to a size of approximately 4000 nucleotides, which is flanked by two primer binding sites, can be amplified exponentially by repeating the thermal cycle up to 45 times. The specificity of amplification is determined by the nucleotides of the individual primers. After amplification sufficient product is produced to be visualised directly after electrophoresis by ethidium bromide staining and illumination by UV light.

One of the most important developments occurred in 1990 when a variation of PCR was developed independently by two different laboratories (William *et al.*, 1990;}. The technique concerns the analysis of Random Amplified Polymorphic DNA (RAPDs) and provides a novel and effective method for distinguishing organisms according to the banding patterns of their DNA as well as providing a new means of obtaining genetic markers. RAPD is a modification of PCR technique, which requires no prior knowledge of nucleotide sequence and is becoming increasingly attractive as a DNA based marker system. The approach is based on the probability that in the genome of the organism under study, a given single nucleotide sequence will occur in inverse orientation within a distance that is amplifiable by PCR. The primers used are generally only 10 nucleotides in length with their sequence determined arbitrarily. Differences in the sequences amplified from related individuals are caused by either mutation in the primer binding sites or by DNA rearrangements. Differences detected using this technique have been called random amplified polymorphic DNA markers or RAPDs.

The technique is fast, technically easy, and requires few materials. One of the main advantages of RAPD analysis, in contrast to many other PCR-based protocols, is that there is no requirement for a prior knowledge of the molecular biology of the organisms under study; the technology is not dependent upon predetermined nucleic acid sequence data. Traditional PCR-based techniques depends on the availability of DNA or RNA sequence information in order to design primers. Such sequence information is lacking for most organisms and, therefore, systematic studies have tended to be biased towards limited numbers of well-suited genes. In these cases it has been possible to construct primers based on relatively highly conserved regions of the genes in question from other species. The unique feature of RAPD analysis is that a single primer of arbitrary nucleotide sequence is added to the PCR. At low stringency, the primer will anneal to a number of complementary sequences within the template DNA. When the single primer binds to the genomic DNA at two sites on opposite strands of the DNA template which are within an amplifiable distance of each other, discrete DNA fragments will be produced through thermocyclic amplification. The multiple products of different sizes, which are produced during the reaction from a single genomic DNA template, may be resolved by gel electrophoresis. The presence of an amplification product indicates complete or partial nucleotide sequence homology between the genomic DNA and the oligonucleotides primers, at each end of the amplified product. Different primers, and the choice is practically unlimited, will initiate the amplification of different parts of the genome. Many markers can readily be identified as a variety of taxonomic levels and in comparison with DNA sequencing, the effort and cost are modest so that many individuals can be assayed. Ideally these arbitrary primers yield at least several but not too many marker bands, that generally are inherited as dominant. Arbitrary primers methods are most useful when analysing closely related germplasm (Smith, 1992).

Which of the polymorphism analysis techniques should one chose the answer depends first of all on the nature of the project and needs dictated by the specific application, and also on the facilities resources and skill that are available. It is suggested that one should go for morphological markers then biochemical markers if it is possible because both morphological and biochemical markers represent only those genes or a part of genome which is func1tional. As most of the DNA is junk or simple repetitive and DNA markers sampled all the genomes randomly there for the variation present may not necessarily express itself in morphology of plant or it may not be affected economical traits. Secondly The DNA markers are very very expensive.

Polymorphism obtained in the progenies of tissue culture plants in comparison to the control mother explant source could be correlated with the apparent morphological changes. Although this is very time consuming but once it is done then this will give an ideal markers system. This can be used commercially in a routine testing for quality control in large-scale micropropagation system. These makers will further enhance our ability to understand the insight effect of the different variable on the production of somaclonal variation in plants regenerated from tissue culture.

If the objective is to characterize (Fingerprint) genomes or to perform a study of genetic diversity in a population then methods such as AFLP and RAPDs are appropriate. AFLP also allows for the extremely rapid identifications of new markers (Primarily dominant), either spread throughout the whole genome or confined to specific genetic regions. RAPDs technology provides a very easy entry into mapping even for the worker with no experience in molecular methods. RAPD analysis is frequently chosen by those beginning to study a normal genetic system, but having minimal amount of DNA or other resource at their disposal. Although the reproducibility of RAPDs patterns between laboratories remains a concerns, appropriate precautions and care with assay will eliminate most problems. The dominant nature of RAPDs may also limit the utility of RAPDs for some mapping needs. RFLP technology offers codominent markers, and is easy and convent if existing collection of RFLP probes are available. Concerns regarding the use of radioactive materials can be avoided if non radioactive detection system are used such as chemiluminescence. RFLP analysis is also fastest way to place cloned gene or other genomic fragment on an existing map.

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