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 rate of gene transfer and further 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 has been much interested and discussed and this type has been frequently reviewed (Evans and Bravo, 1988). Larkin and Scoccroft (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. (1950) 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. (Grunevaldt and Dunemann, 1981). 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. Well 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. (Nagi, 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, 1978). 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-dodecyl 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., 1996). 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. **Allele frequency** tends to be much higher at molecular loci compared with morphological markers.

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

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

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

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 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 polymorphisms (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.

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.

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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 1980 when a variation of PCR was developed independently by two different laboratories (William et al., 1980). 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 genic 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 depend 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 conserved 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 choose 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 functional. As most of the DNA is junk or simple repetitive and DNA markers sampled all the genomes randomly. The question presents 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 variables 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 concern, 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 codominant markers, and is easy and convenient 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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