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Molecular Markers for Genetic Fidelity Assay of Tissue Cultured Crops

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Tissue culture techniques are being widely used for the production of large-scale disease-free planting material aseptically on artificial media within a lesser span of time and space. This technique has gained tremendous popularity in recent days for the commercial production of several clonally propagated plants such as banana, bamboo, pointed guard, seed potato, strawberry, pineapple, papaya etc. Even in seed-grown plants, this technology has application in overcoming the barriers like dormancy, sexual compatibility, germination etc. But the major drawback of tissue culture raised plantlets is that they show somaclonal variation, i.e. alteration in the genetic constitution of the plant leading to the disappearance of true-to-type nature and resulting variants may not be desirable. In such case, the genetic fidelity test is mandatory to confirm the true-to-type genetic makeup of newly derived plantlets. Several strategies have been used to ascertain the genetic fidelity of the *in vitro* raised progenies comprising morpho-physiological, biochemical and cytological approaches. These approaches are mainly based on phenotypic characters, which can be affected by the *in-vitro* manipulation, environment and types of plant tissue; hence it is not easy to differentiate clonal fidelity with a high probability. In contrast, for a similar purpose, the DNA- based molecular markers are versatile tools with broader applicability in various fields of applied biology. These molecular markers have the edge over aforementioned

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traditional non-DNA based markers concerning environmental interaction, developmental stage, tissue, time and cost. The present paper contains different techniques of genetic fidelity assay to confirm the true-to-type genetic constitution in tissue culture raised crops.

Keywords: DNA marker; genetic fidelity; somaclonal variation; tissue culture.

1. INTRODUCTION

Tissue culture is the recent technique for the multiplication and regeneration of the crops [1]. It is one of the important techniques of vegetative propagation which can be accomplished in a shorter time and space under a contamination-free condition [2]. Although the tissue cultured crop shows similarity in genetic constitution, few crops of the clonal population may undergo genetic or environmentally influenced changes. It has come to be known that cell or tissue cultures which undergo genetic changes are also expressed at morphological, molecular levels. The reason for genetic changes may be polyploidy, aneuploidy, structural changes in the chromosome, gene amplification and mutations. Larkin and Scowkraft (1981) gave the general term “somaclonal variation" to the resulting variations of plant derived from tissue cultures. Somaclonal variation in regenerated plants is generated during *in vitro* culture stage due to artificial manipulation. Crops such as Banana strawberry, papaya, grapes, pineapple, tomato, cucumber, watermelon, rhododendron etc., are generally propagated through tissue culture methods. But instances of somaclonal variations have been reported in horticultural crops such as banana where the occurrence of off-types from tissue-cultured plantlets ranged from 6 to 38% in Cavendish cultivars [3]; however, it is expected as high as 90% [4]. This phenomenon is the major obstacle in the path of crops which are mainly propagated through tissue culture. Here it becomes essential to examine the fidelity or genetic uniformity of the micropropagated plantlets to confirm being true-to-the-type and rejection of variants to avoid variations of any kind, which is induced may multiply very fast and lead to loss of the parental characteristics of the parent genotypes [5].

2. IMPORTANCE OF GENETIC FIDELITY IN TISSUE CULTURE

The prime objective of tissue culture is to obtain true to type plants to maintain the germplasm of the desired trait. But during tissue culture, there is a chance of genetic aberration which is commonly known as 'somaclonal variations'. Thus, the plantlets obtained from micropropagation must be screened for its genetic fidelity [6]. Genetic fidelity is the maintenance of the same genetic constitution of a particular clone through its lifespan [7]. The occurrence of deleterious genetic defects arising variation in the regenerants can limit the broader utility of tissue culture systems [8]. Propagation through tissue culture and preservation of elite genotypes, selected for the desired trait, require a high degree of similarity in genetic constitution. Somaclonal variation is a bottleneck for both *in vitro* cloning as well as a germplasm preservation method. Therefore, it very essential to confirm the uniformity of the genetic constitution of tissue culture raised plants before initiation of tissue culture.

3. SOMACLONAL VARIATIONS IN TISSUE CULTURED CROPS

Tissue culture techniques are common practices for the production of large-scale disease-free planting material aseptically on artificial media within a lesser span of time and space. This technique has gained tremendous popularity in recent days for the commercial production of several clonally propagated plants such as banana, bamboo, pointed guard, seed potato, strawberry, pineapple, papaya etc. Even in seed grown plants, this technology has application in overcoming barriers like dormancy, sexual compatibility, germination etc. But the major drawback of tissue culture raised plantlets is that they show somaclonal variation i.e. alteration in the genetic constitution of the plant leading to the disappearance of true-to-type nature and resulting variants may not be desirable. alterations are associated *vitro* propagation, which may have phenotypic consequences, and are collectively called somaclonal variation [9]. Now a day various techniques are available for the detection and characterisation of somaclonal variants. Among them, genetic markers are one of the important tool, which includes-differences in morphological traits, for the determination of numerical and structural variation in the

chromosomes, biochemical and molecular DNA markers.

4. TYPES OF GENETIC MARKERS USED IN THE GENETIC FIDELITY ASSAY

A genetic marker is one of the tools of genetics to detect variation and similarity. Genetic markers have been classified into two categories: classical markers and DNA/molecular markers. Morphological, cytological and biochemical markers are classical markers which are the result of expression of genes. On the other hand, molecular/DNA markers are based on the variation in the sequence of genotypes. Each kind of Genetic marker is being dealt here in detail.

4.1 Morphological Marker

This is one of the oldest and classic methods to detect variation and fidelity. It is easily noticeable methods and may also be applied to detect variants resulting from tissue cultured plants. Morphological characters like the variation in plant height, canopy structure, leaf morphology, pigmentation abnormality etc. may be employed as morphological markers [10]. But the number of morphological markers are limited, developmentally regulated and often environmentally affected. This feature of morphological markers fails to give much information about the genetic composition of a plant [11].

4.2 Cytological Marker

Cytological markers include numerical and structural changes in chromosome and quantitative changes in the RNA/DNA. This qualitative and quantitative feature has been also exploited to study the genetic fidelity of horticultural crops [12]. The cytological analysis may be done by conventionally stained, chromosomal aberration using light microscopy, oil immersion or other complex microscopy techniques and this been done by several workers [13]. This technique has severe limitations such as time-consuming and often cumbersome particularly when chromosome number is high or difficult to observe due to their small size [8]. Presently, the problem of counting and examining chromosomes has been overcome by precise and sensitive, advanced technology, flow cytometry [14], which involves preparation of aqueous suspensions of intact nuclei whose DNA is stained using a DNA fluorochrome, followed by suspending them in a stream of fluid and passing them by an electronic detection apparatus. The absence of any morphological and cytological variations among *in vitro* raised plants does not mean that (epi) genetic differences among them do not exist. Moreover, the most probable changes may result from point mutations, small deletions or alterations in methylation patterns; therefore, this technique has limitation too and can be furnished by recent marker system [15].

4.3 Protein Marker

Morphological variation results from the expression of genes. Temporal and spatial expression of genes into enzyme (protein) influences the quality and quantity of chemical substances in the cell has been recognised as a technique to determine the genetic variation among all protein marker, if any, appears in vitro-derived plants. Isozymes are protein markers, which differ in amino acid sequence but catalyse the same chemical reaction. The technique is based on the principle that allelic variation exists from many different proteins. Therefore, the isozymes from two different alleles of the same gene would not cover to the same distance in a polyacrylamide gel due to the difference in their electrophoretic mobility. As a result, the discriminating isozymes is an of polymorphic loci be identified and genetically characterised in an organism [16]. Therefore, Lassner and Orton [17] proposed the use of isozyme markers to study somaclonal variation. Since then, proteins and isozymes such as peroxidase, malate dehydrogenase and superoxide dismutase have been extensively used to study variation in different horticultural crops [18]. This technique is useful for detecting differences among individuals hence changes in isoenzyme patterns could reflect gene expression, or even gene changes [19]. Protein and enzyme polymorphism is the basis of detection of a somaclonal variation. The limitation of this technique is that salt soluble protein or enzyme cannot be exploited by Gel Electrophoresis. Also, the sequence of protein shows more redundancy than that of DNA. Mandal et al. [20] also reported the salt soluble peptide as molecular markers for varietal identification of banana cultivars. Therefore, this technique is not considered as a promising tool to detect variants, and it has been replaced by other more sensitive and precise alternative techniques like molecular markers.

Table 1. Merit and demerits of different markers system

S.No.	Marker	Merits	Demerits
1	Morphological marker	Noticeable from the naked eye, observing characters like plant height, canopy structure, leaf morphology, pigmentation abnormality etc.	The number of these markers is limited, developmentally regulated and often affected by environmental factors. These limitations do not reveal the true changes in the genetic composition of a plant
2	Cytological marker	Easily done by conventional methods of microscopy and by more recent tools such as flow cytometry.	The absence of cytological variations among tissue culture raised plants does not necessarily mean (epi) genetic difference. Moreover, the most probable changes may result from point mutations, small insertion/deletion or alterations in methylation patterns, which are not revealed through the cytological marker
3	Protein Marker	Useful for detecting differences among individuals hence changes in isoenzyme patterns could reflect gene expression or even gene changes	It is restricted to isoenzyme and isoprotein. It becomes cumbersome when isoenzyme or proteins are salt soluble proteins.
4	DNA marker	Based on the sequence of the genome and. not regulated by developmental factors	Commonly based on Thermo-cycler (PCR machine) and very sensitive

4.4 DNA Markers

Morphological, cytological and protein markers have been used over the years in the classification and identification of plants. These markers have certain limitations as they could be influenced by environmental and developmental effects. Therefore, DNA markers have emerged as an important tool to assess genetic fidelity in tissue culture grown long-term cultures of economically important fruit plants [21]. There is a different type of DNA markers which are used in the genetic fidelity of tissue cultured crops. But the markers which show a low level of polymorphism encouraged rely more on DNA markers such as RAPD, ISSR, AFLP and SSRs [22]. These markers are PCR based marker, and comparative account of all markers are dealt in Table 2.

Table 2. Comparative account of the development of different DNA marker

Technical features	SSR	RAPD	AFLP	ISSR
Genomic abundance	Medium	Very high	Very high	Medium
Part of Genome surveyed	Whole genome	Whole genome	Whole genome	Whole genome
Required DNA	Low	Low	Medium	Low
Inheritance	Co-dominant	Dominant	Dominant	Dominant
Type of polymorphism	Change in length of repeats	Insertion/Deletion	Insertion/Deletion	Insertion/Deletion
Detection of alleles	Yes	No	No	No
Automation	High	Medium	Medium	Medium
Type of Primers	Specific repeat DNA sequence	Random 10 bp primer	Specific sequence	Specific repeat DNA sequence
Cloning/Sequencing	Yes	No	No	No
Radioactive detection	No	No	No/Yes	No
Proprietary Right Status	Some are licensed	Licensed	Licensed	No
Level of Polymorphism	High	High	Very High	High

4.4.1 Random Amplified Polymorphic DNA (RAPD)

This marker is developed by the amplification of DNA fragments by polymerase chain reactions (PCR) using random primers. This marker system has the ability to produce multiple bands using a single primer. Therefore, a relatively small number of primers can be used to generate a very large number of DNA fragments from different location of the genome. These multiple loci may be evaluated very quickly [23]. These markers have been used by different workers for genetic fidelity assay too. The usefulness of RAPD markers for genetic analysis of tissue cultured plants of *Populus deltoids* Marsh has been reported [24]. RAPD markers have also been used to assess genetic stability among somatic embryos in spruce species. Somaclonal variations in tissue date palm plants have also been detected using RAPD [25]. RAPD finger-printing diagnostics for genetic integrity of enhanced auxiliary branching derived plants of 10 forest tree species have also been studied by Rani et al. [26]. Similarly, RAPD analysis has been employed to study genetic uniformity of micropropagated *Pusa Urvashi* plantlets, a newly released grape cultivar [27]. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers have been employed to assess the genetic stability of *Spilanthes acmella* (L.) Murr., plants multiplied through auxiliary bud multiplication with up to 20 *in vitro* subcultures [28] and shown fidelity of true to type. Despite the wide use of RAPD marker in genetic fidelity analysis, lack of reproducibility and being dominant are two main disadvantages.

4.4.2 Amplified Fragment Length Polymorphism (AFLP) marker

A recent approach by Vos et al. (1995) known as Amplified Fragment Length Polymorphism (AFLP), is a technique which is also used to detect genetic fidelity of most of the tissue cultured crops. This technique is based on the variation of the amplified fragment of DNA by PCR after restriction digestion of genomic DNA. This technique can be used for DNAs of any origin or complexity. The fingerprints are produced, with the help of primer generated from the known sequence of the adaptor. The number of fragments detected in a single reaction can be adjusted by the addition of arbitrary base in the earlier primer. AFLP technique is more reliable as stringent primer annealing is required in the process of its development. This technique thus shows an ingenious combination of RFLP and PCR techniques and is extremely useful in the detection of polymorphism between closely related genotypes and genetic fidelity testing. AFLP consist of unique properties as it has reliability like RFLP and efficiency like RAPD (Vos et al., 1995).

4.4.3 Simple Sequence Repeats (SSR) marker

Simple sequence repeats (SSRs), is also known as microsatellite, short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs). It is the smallest class of simple repetitive DNA sequences. Allelic variation in the SSR marker is the results of variable numbers of tandem repeat units within the microsatellite region of the chromosome. The repeated sequence is often simple, two, three or four base. Dinucleotide repeat (CA)n, refers to the total number of repeats that ranges between 10 and 100 is a common example of SSR. Microsatellite loci are more common in some organisms than in others, and screening may produce few useful loci in some species [29]. The efficiency of development of SSR marker lies on the abundance of repeats in the target species and the ease with which these repeats can be developed into informative markers. Microsatellites are highly sensitive markers for monitoring genetic variation that may signal potential deleterious mutations during tissue culture because they reflect a relatively high rate of mutation and the corresponding level of genetic variability [30]. Genetic fidelity testing using SSR Marker assay has also confirmed trueness to type of micropropagated coconut (*Cocos nucifera* L.) plantlets derived from unfertilized ovaries [31].

4.4.4 Inter-Simple Sequence Repeat (ISSR) marker

Inter-simple sequence repeat (ISSR) marker technique is a PCR-based method. It involves amplification of DNA segment an amplifiable distance two identical microsatellite repeat. In this case, these repeats remain oriented in different directions. Microsatellites, usually 16 to 25 bp long, are used as primers in a single primer PCR reaction genomic loci to amplify mainly the inter-SSR sequences of different sizes. Di-nucleotide, tri-nucleotide, tetranucleotide or penta-nucleotide SSR repeats may be utilised as primers. The primer used can be either unanchored [32] or more usually anchored at 3' or 5' and with 1 to 4 degenerate

bases extended into the flanking sequences [33]. The ISSR technique shows most of the advantages of AFLP analysis with the universality of RAPD. ISSRs have high reproducibility rather than RAPD (10 mers) probably due to the use of longer primers (16- to 25-mers) permitting subsequent use of high annealing temperature (45 to 60ºC) leading to higher stringency [34]. A large number of micropropagated plantlets of banana that were developed from auxiliary explants over a 10 years period have been their genetic stability. of genetic stability in long-term micro propagated shoots of banana using RAPD and ISSR has been carried out by Lakshmanan et al. [35]. Inter-simple sequence repeat (ISSRs) have been used as an efficient and effective tool for clonal fidelity and genome mapping in various crops [36].

4.5 Potential of Marker System and Genetic Fidelity Testing of Tissue Cultured Crop

Each molecular marker has some advantage and disadvantage. Therefore, no single molecular markers can fulfil all the requirements needed by researchers. Among the various kind of markers used in the genetic fidelity assay, DNA-based markers are more attractive means for examining genetic fidelity of tissue cultured crops as these markers are more informative and not influenced by environment also. RAPD suffer from a lack of reproducibility [37]. Also, RAPDs are dominant diallelic markers; hence it can't differentiate between homozygote and heterozygote of a diploid organism. Therefore, dominant markers, including amplified fragment length polymorphisms (AFLPs), are not entirely informative enough for examining somaclonal variation although it is more reproducible. The sensitivity, reproducibility, co-dominance and strong discriminatory power of microsatellite DNA/SSR (simple sequence repeat) markers [34] make them particularly suitable for detecting somaclonal variation, but their application in the study of somaclonal variation has been rather quite limited [38]. A technique which gives higher resolution and elimination of faint bands are thus ideal for determining genetic fidelity. The reliability and efficiency of markers in the detection of large genomic manipulation depends on the type of marker used in the process. The variations due to genetic or epigenetic factors are very likely to be reflected in the banding profiles developed by employing different marker systems [39]. This variation is quite common in the case of ISSR maker, probably due to the high melting temperature in case of ISSR primers which does permits not only more stringent annealing conditions but also more specific and reproducible amplification consequently. This is evident from the work of Devarumath et al. [40] who revealed that ISSR fingerprints detected more polymorphic loci than RAPD fingerprints. Therefore, now a day ISSR markers are being used for the genetic fidelity assay [41].

5. FUTURE PERSPECTIVE OF GENETIC MARKER

An attractive alternative to RAPD is the ISSR marker system, which requires no prior genomic information and produces a large number of DNA bands that are more reproducible than RAPD patterns. AFLPs and SSRs marker may also result in useful markers for detecting somaclonal variation, but are more demanding technically or require prior sequence information. SSR marker has been designed only to check repetitive sequences such as SSR (Vazquez, 2001). A combination of several techniques might be most suitable for the evaluation of the genetic stability of regenerated plants [42,43]. For situations where a higher level of marker specificity is required such as cultivar identification or marker-assisted selection, in that case, SSRs, Sequence characterised amplified region (SCARs), or Single nucleotide polymorphism (SNPs) may be the choice approach [44].

6. CONCLUSION

Molecular markers have the edge over aforementioned traditional non-DNA based markers concerning environmental interaction, developmental stage, tissue, time and cost. The present paper contains different techniques of genetic fidelity assay to confirm the true-to-type genetic constitution in tissue culture raised crops. Despite the availability of various makers, to the date, no single technique can guarantee the identification of a single random mutation/ point mutation in the genome. This is essential in the identification of random mutation and somaclonal variation [45]. RAPD fingerprinting has become popular technique for detecting somaclonal variation and genetic stability. However, RAPD analysis suffers from various limitations, particularly the lack of reproducibility of results.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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