



Somaclonal Variation and Methods Used for Its Detection

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V. M. Ranghoo-Sanmukhiya

Abstract

During plant tissue culture, the genetic stability of in vitro plantlets is often compromised and the underlying factors include the plant species or varieties involved, mode of regeneration used, genotype and ploidy level, composition of the growth medium, duration of the callus phase, and total time in culture. During tissue culture, conversion of the explant into in vitro tissue in fact imposes stress to the plant cells, which undergo a genomic shock and require restructuring of their genomes. An array of morphological, biochemical, and molecular methods has been used to assess somaclonal variation in different plant species and variable efficiency has been observed. The array of methods including molecular markers used for ensuring genetic stability of regenerated cultures in different plants is reviewed in this chapter.

Keywords

Somaclonal variation · Tissue culture · Molecular markers

1.1 Introduction

Tissue culture experiments involve growing plant tissues under a set of specific laboratory conditions different from their normal growth conditions in nature in an aim to eventually propagate and conserve them or for ultimate genetic transformation. When the explant is placed in vitro, a specific tissue culture protocol has to be optimized or designed for each species used. Moreover, adjustments in physiology, anatomy, and metabolism have to be made for them to be able to grow and

V. M. Ranghoo-Sanmukhiya (✉)
Faculty of Agriculture, University of Mauritius, Réduit, Moka, Mauritius
e-mail: m.sanmukhiya@uom.ac.mu

multiply in culture. During tissue culture, any plant cell type or organ including embryos, spores, roots, leaves, and protoplast are used as explants. There is normally a cycle of dedifferentiation of tissues from the explant referred to as (callus production) under conditions set by the researcher followed by proliferation for a number of generations (callus proliferation) culminating in the regeneration of the plants (Larkin and Scowcroft 1981). Regeneration only involves repeated mitotic divisions, which ideally should yield genetically uniform plants (Larkin 1998). The “somaclone” is the regenerated plant obtained following tissue culture, while “calliclones” and “protoclones” refer to regenerated plants obtained from stems and protoplast (Chawla 2000).

During tissue culture, conservation of the genetic integrity of the mother plant is a desirable component, but regeneration by tissue culture has been reported to yield plants which exhibit unexpected and undesired phenotypic anomalies and variation (Karp 1994). Conditions inherent *in vitro* include regimented media components, low light levels, and more importantly high humidity which eventually cause several physiological and developmental aberrations of the regenerated plantlets. In fact, somaclonal variation refers to culture-induced anomalies or variation, which eventually becomes genetic and is inherited by the clonal progenies (Chawla 2000; Us-Camas et al. 2014). There are other variations induced in culture which are only temporary and reverse back to the normal state when faced with other culture conditions. The temporary changes which are nonheritable and not reversible are in fact caused by epigenetic and physiological factors, otherwise heritable and permanent changes represent “*de novo*” genomic variation (Kaepler et al. 2000). Initially, somaclonal variants were classified as artifacts caused by overexposure to phytohormones and epigenetic events (Chawla 2000; Isah 2015). In fact, genetic variation observed following regeneration is a result of aberration of genome expression which is due to the formation of chromosome mosaics and spontaneous mutation. Moreover, somaclonal variation can be induced by a directed approach through *in vitro* selection and can be minimized as well without any *in vitro* selection (Brar and Jain 1998; Chawla 2000). The genetic basis of somaclonal variation, its uses in agriculture, associated disadvantages and methods of detection are discussed.

1.2 Uses of Somaclonal Variation in Agriculture

In the current era, we need to secure food availability faced with the impending crisis of global warming and declining arable soil. Somatic embryogenesis can be exploited to produce plants with certain variable characteristics as compared to the starting material and without using transgenics. Under tissue culture conditions, the plant will do what it is programmed to do but variation will result by using epigenetic regulation and hence inducing variation. In addition, the number of embryos produced can be increased by making use of specific chemicals, e.g., 5-Aza for plants which are recalcitrant for SE and hence increase production. Somaclonal variation can be considered as an advantage as it allows creation of new genotypes which have been exploited in agriculture for the following:

1.2.1 Generation of New Agronomic Variants with Favorable Traits

Somaclonal variation has given rise to new breeding lines with variants of important agronomic traits. Many variants in different crops have been generated using somaclonal variation which can further be integrated in breeding programs for introgression of important agronomic traits in different lines. Somaclonal variants were selected for: high oil yield in *Cymbopogon winterianus* (Dey et al. 2015), higher flowering and fruiting ability in strawberry (Biswas et al. 2009), high sucrose yielding sugarcane varieties (Rastogi et al. 2015), finger millet of higher seed and biomass yield (Baer et al. 2007), non-browning potato varieties (Arihara et al. 1995; Thieme and Griess 2005).

1.2.2 Generation of Disease Resistance Varieties

Disease resistance for different crop varieties has been obtained through induced somaclonal variation. Somaclonal variants from different crops with increase disease resistance include sugarcane which were resistant to eyespot and red rot disease (Rastogi et al. 2015), wheat resistant to white blotch disease (Arun et al. 2003), and *Fusarium* wilt-resistant banana (Asif and Othman 2005).

1.2.3 Generation of Biochemical Variants with Abiotic Resistance

Biochemical variants can be generated and these can be used in plant metabolic pathway studies. Somaclonal variants in wheat which exhibit frost and freezing tolerance have been obtained and these have been directly related to the production of proline (Dorffling and Melz 1997). Somaclonal variation has also successfully yielded plants which exhibit salt and herbicide resistance in various crop species, e.g., drought and salt resistance in sugarcane (Rastogi et al. 2015) as well as drought tolerance rice (Adkins et al. 1995).

1.3 Disadvantages of Somaclonal Variation

1. Somaclonal variation generates uncontrollable and unpredictable variation which are of no agricultural use. Most changes are cultivar independent.
2. Genetic changes during regeneration occur at variable frequencies and are not stable and nonheritable.
3. Somaclonal variants are not novel genotypes and have frequently to be checked for novelty.
4. Generation of somaclonal variants is limited only to those plants which have the power to multiply in culture and regenerate into whole plants.
5. Somaclones have reduced growth rate, reduced fertility as well as low-performance rate. They sometimes lose the power of regenerating.

1.4 Causes and Genetic Basis of Somaclonal Variation

Conditions in tissue culture which induce mutations include wounding, removal of the cell wall, sterilization which includes exposure to high concentration of chemicals including sterilants, sugars, and plant growth regulators in the nutritive media. Genetic alteration of the regenerants in culture is inevitable and increases with prolongations (Duncan 1996; Us-camas et al. 2014). The in vitro environment is stressful and hampers proper morphogenesis of tissues and this is due to erratic gene expression which results in disrupted hormonal synthesis and signaling. In addition, oxidative stress has been reported in plant tissue culture which normally results in elevated reactive oxygen species production (Isah 2015). During regeneration of plants using tissue culture, there is normally differentiation and redifferentiation of cells leading to qualitative and quantitative genomic changes. Somaclonal variation can be traced back to changes which have occurred at the genome level and include:

1. Single gene mutation: Somaclonal variants in tomato have been linked to single gene mutation inside the genome of crop species including wheat, maize, rice (Edallo et al. 1981), and tomato (Evans and Sharp 1983).
2. Activation of transposable elements: Transposable elements have been linked with somaclonal variation and were first reported to cause colored spots in *Nicotiana tabacum* species (Lorz and Scowcroft 1983). Activation of the transposable elements has been observed in in vitro maize and tobacco tissues (Peschke and Phillips 1991). In vitro genetic rearrangements (Hirochika et al. 1996; Sato et al. 2011) as well as insertion and excision of transposable elements have also been observed (Gupta 1998; Sato et al. 2011).
3. Karyotype changes: Variant plants with altered chromosome numbers inducing ploidy level changes namely polyploidy which includes chromosomal abnormality have been observed (Hang and Bregitzer 1993; Mujib et al. 2007; Leva et al. 2012).
4. Changes in chromosome structure: Changes in chromosome structure which include chromosome breakage and rearrangement, centric and acentric fragments, abnormal arrangement of chromosomes, ring chromosomes, and formation of micronuclei (Kaepler et al. 1998; Alvarez et al. 2010).
5. Plastid genetic change: Plastid genetic changes including deletion, addition, and intramolecular recombinations have occurred in somaclones (Cassells and Curry 2001; Bartoszewski et al. 2007).
6. Methylation: Methylation in single-copy sequences, methylation of the genome as well as histone modifications have been reported in tissue culture (Kaepler et al. 2000; Linacero et al. 2011; Miguel and Marum 2011).

1.5 Methods Used for Assessing Somaclonal Variation

1.5.1 Morphological Assessment of Somaclonal Variation

Morphological assessment of somaclonal variation has been traditionally used to detect “variants” been referred to as “off types” regenerated plantlets. Characters most often used for the assessment include plant stature, leaf morphology, and pigmentation. Examples of somaclonal variants include banana dwarf off-types (Rodrigues et al. 1998) or excessive vegetative growth in palms (Zaid and Al Kaabi 2003). Morphological assessment for somaclonal variation may not be reliable since the number of characters used may be limited and more importantly be artifacts and caused by the impeding stress inherent of the tissue culture conditions (Cloutier and Landry 1994). Moreover, genomic changes caused during tissue culture but these may not be reflected in the phenotype (Bairu et al. 2011).

1.5.2 Biochemicals Used to Assess Somaclonal Variation

Responses of explants to certain chemicals such as gibberellic acid have been used for the assessment of somaclonal variation (Phinney 1985; Sandoval et al. 1995; Graebe 2003). Gibberellic acid is well known for its regulation of growth and development and somaclonal variants differ from normal plantlets in having disturbed gibberellic acid metabolism and endogenous level allowing somaclone identification (Bairu et al. 2011). Normal banana plants have been found to be gibberellic acid responsive and hence developed significantly greater leaf sheaths as compared to dwarf somaclonal variants which were nonresponsive (Damasco et al. 1996). Synthesis of chlorophyll and carotenoids has been used to differentiate somaclones with normal plantlets (Mujib et al. 2007; Wang et al. 2007).

1.5.3 Protein Markers Used to Assess Somaclonal Variation

Isozymes are different molecular forms of proteins which are used to actively control biochemical processes inside cells (Kunert et al. 2003). Isozymes fingerprints are normally generated and can be used as a basis for finding differences between genomes of parental plants and regenerated individuals. Enzymes commonly measured include esterase (EST), endopeptidase, alcohol dehydrogenase, peroxidase, polyphenol oxidase amongst others (Kunert et al. 2003). Isozyme analysis has advantages such as facilities of usage and low cost. Isozymes assays, however, have limited polymorphisms which are highly influenced by environmental conditions (Kunert et al. 2003). Somaclonal variation has been assayed using isozymes in palms (Salman et al. 1988; Saker et al. 2000), soybean (Amberger et al. 1992), citrus (Carini and De Pasquale 2003), and potato (Afrasiab and Iqbal 2012).

1.5.4 Cytogenetic Analysis for the Determination of Somaclonal Variation

Chromosomal abnormalities can occur in tissue culture plantlets and could be the causal factors of somaclonal variations (D'Amato 1985; Kaepler et al. 2000). Presence of an abnormal number of chromosomes, aneuploidy, has been reported during callus induction and cell suspension phases of citrus and oil palm (Hao and Deng 2002; Giorgetti et al. 2011). Chromosomal alterations due to non-separation of chromatids during mitosis and meiosis can lead to the production of chromosomal abnormalities and segmental or complete aneuploidy (D'Amato 1985; Holland and Cleveland 2009; Kaepler and Phillips 1993; Kaepler et al. 2000). Variations in chromosome number among cells in cultures can be detected using fluorescence in situ hybridization, flow cytometry, and microscopic examination. Flow cytometry is a technique which allows the calculation of the DNA content analysis through the use of a fluorochrome specific to the DNA found in whole cells, protoplast, nuclei, or chromosomes. Using flow cytometry, diploids and tetraploids were recovered from calli cells of *Arapidopsis* which had cytogenetic abnormalities such as chromosomal translocations, deletions, and duplications (Orzechowska et al. 2013). Flow cytometry has allowed detection of chromosomal abnormalities during somaclonal variation in many plants including eucalyptus (Pinto et al. 2004), cotton (Jin et al. 2008), papaya (Abreu et al. 2014), and *Viola uglinosa* (Slazak et al. 2015).

1.5.5 Detection of Somaclonal Variation Using RFLP

The restriction fragment length polymorphism (RFLP) is one technique which is used for genome analysis and it involves digestion of genomic DNA using enzymes restriction endonucleases, followed by separation of the digested products on agarose gel for the generation of DNA profiles. Variations in the number and size of the bands will occur in the DNA profiles of the true to type regenerated plantlet as compared to the somaclonal variant. Variations in the band number and sizes observed inside the genome can be explained by insertion, deletion, or single nucleotide polymorphisms (Agarwal et al. 2008; Bairu et al. 2011). RFLP markers have been used to screen for somaclonal variation in rice (Müller et al. 1990), maize (Brown et al. 1991) sugar beet (Levall et al. 1994), *Hypericum perforatum* (Halušková and Čellárová 1997), and oil palm (Jaligot et al. 2002).

1.5.6 Detection of Somaclonal Variation Using Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) is a PCR-based technique which relies on the selective amplification of fragments by using primers of a random nature. These primers are around ten nucleotides in length which have the ability to bind to complementary sites within the genome followed by amplification of the

sites delimited. RAPD represents one among the most popular markers used for the assessment of somaclonal variation due to low input cost and low technology required. Presence of certain bands in the parental strains and absence in regenerated plantlets indicates genomic rearrangement during tissue culture and account for somaclonal variation. Common bands which are found in the somaclones originate from mutation inherited from the mother embryogenic callus tissues. Variations in the genome level caused by somaclonal variation include nucleotide substitution events such as transition and transversion (Ngezahayo et al. 2007). RAPDs have been however associated with low reproducibility, reliability, and transferability among different labs. RAPDs have actually been used in the assessment of somaclonal variation in sugarcane (Taylor et al. 1995; Tawar et al. 2008), banana (Trujillo and Garcia 1996; Tang 2005; Abdellatif et al. 2012), orchids (Chen et al. 1998), rice (Yang et al. 1999), maize (Osipova et al. 2001), tomato (Soniya et al. 2001), family of Zingiberaceae (Islam et al. 2004; Wondyifraw and Wannakrairoj 2004; Mohanty et al. 2011), and *Cymbopogon* (Dey et al. 2015).

1.5.7 Detection of Somaclonal Variation Using Simple Sequence Repeat (SSR)

Simple sequence repeat (SSR) also referred to as microsatellite markers consist of short DNA sequence motifs of few base pairs which are repeated tandemly inside the eukaryotic genome. Each genome varies in having a characteristic number of repeats and these can be amplified by using the polymerase chain reaction (PCR) using special primers. Different somaclones will have different numbers of these repeats units which are exhibited with high levels of polymorphism following amplification (Levinson and Gutman 1987; Coggins and O'Prey 1989). SSRs are highly reproducible and codominant which make them ideal, rapid, simple genotyping assays. SSRs are very versatile in asserting genetic variation and are highly used in marker assisted breeding. Moreover, SSR markers have also been used to screen for somaclonal variants in cotton populous (Rajora and Rahman 2003), bananas (Hautea et al. 2004; Ray et al. 2006), rice (Khai and Lang 2005), pine (Marum et al. 2009), sorghum (Zhang et al. 2010), and grapevine (Nookaraju and Agrawal 2012).

1.5.8 Detection of Somaclonal Variation Using the Inter-Simple Sequence Repeat (ISSR) Markers

Inter-simple sequence repeat markers represent the DNA fragments which are found between simple sequence repeats which are oppositely oriented. The ISSRs can be amplified by using primers which are complementary to the microsatellite regions. These inter-simple sequence repeats can be amplified by using the PCR technique which makes use of primers specially designed to amplify part of the microsatellite DNA core sequences as well as selectively amplifying the adjacent regions. The targeted region inside the genomes is found between two closely spaced SSRs which

may also be oppositely oriented. Following amplification around 20–50 fragments will be amplified from multiple loci containing the SSR motifs but they will be polymorphic in their sizes. The ISSR technique has been successfully used on several occasions for the genome screening of somaclones of mulberry (Vijayan and Chatterjee 2003), tea (Thomas et al. 2006), and gerbera (Bhatia et al. 2009).

1.5.9 Detection of Somaclonal Variation Using the AFLP Technique

Molecular markers mentioned above targeted highly repetitive sequences inside the genome and genotyping relies on the variation in the number of repeat counts. However, since multiple targets are being exponentially amplified at the same time, DNA profiles obtained may not be reproducible and be variable due to the quality of DNA and other reaction conditions.

The AFLP method involves both restriction enzymes digestion with PCR amplification of fragments obtained and allows generation of fragment length polymorphisms. The AFLP technique relies on the use of primers which are complementary to known adapter sequences to selectively amplify restriction-digested fragments and DNA profiles obtained are more reproducible. The AFLP technique is a more suitable and sensitive marker system with higher reproducibility (Meudt and Clarke 2007). The AFLP technique has been recognized as the ideal marker system for assessing somaclonal variation in *Arabidopsis thaliana* (Polanco and Ruiz 2002), *Coffea arabica* (Sanchez-Teyer et al. 2003), bean (Rosales-Serna et al. 2005), *Asparagus officinalis* (Pontaroli and Camadro 2005), pea (Smýkal et al. 2007), grapevine (Schellenbaum et al. 2008), and *Feesia hybrida* (Gao et al. 2010).

1.5.10 Detection of DNA Methylation Events Using the Methylation-Sensitive Amplification Polymorphism (MSAP)

DNA methylation of the genome is normally associated with several inheritance processes such as transcriptional silencing, genetic imprinting, and genomic stability. Methyl groups are added to DNA cytosine to form 5-methyl cytosine (He et al. 2011) during the methylation process without any change occurring in the DNA sequence but results in an increase in the compression level of chromatin affecting gene expression eventually as access to transcription by RNA Polymerase II is debarred. The tissue culture environment induces abiotic stress during regeneration and is responsible for global genome-wide methylation. The long callus generation phase allows for the methylation event and induces variation during plant regeneration. In cassava, genome-wide methylation changes have been observed during meristem micropropagation using genome sequencing (Kitimu et al. 2015). DNA methylation events appear to be exclusive to repetitive DNA more specifically to transposons (Lippman et al. 2004). Decrease in the level of methylation may be due to gene activation during cell reprogramming to facilitate plant regenerations.

Changes in methylation have been reported to be the cause of somaclonal variation in many crops (Gonzalez et al. 2013). Epigenetic changes to DNA of plant tissues in culture appear to allow the reactivation of multiple transposon classes. Insertions of the retrotransposon family Tos and that of the class II TE family (nDiaZ) have been reported to increase with tissue culture period (Huang et al. 2009a). Transcriptional activity of the MERE1 retrotransposon family (associated with a decrease in cytosine methylation) has also been found to increase in two accessions of *Medicago truncatula* and has led to 40% of novel insertions mostly within coding sequences (Rakocevic et al. 2009). In grapevines, an increase in TE insertions has also been noted (Lizamore 2013).

The methylation-sensitive amplification polymorphism (MSAP) technique is used for the evaluation of the total methylation level of a genome. The MSAP is actually a modification of the AFLP (Vos et al. 1995) technique in which methylation-sensitive restriction endonucleases such as isoschizomers HpaII and Msp I to digest the DNA. These enzymes have different sensitivity to methylated cytosines and generate digestion patterns which allow identification of the methylated DNA (Yaish et al. 2014). The MSAP technique has efficiently been used to detect somaclonal variants in different plants including coffee and oil palm (Matthes et al. 2001; Bobadilla Landey et al. 2013; Francischini et al. 2017).

1.5.11 Detection of Somaclonal Variation Using Transposon-Based Marker Systems

Transposable elements represent DNA fragments which have the ability to make copies of themselves and can insert into new loci of the genome during transposition. When transposable elements insert themselves within genes there will be a hindrance to normal gene function (Makarevitch et al. 2015) and gene expression will be affected (Hollister and Gaut 2009; Lisch 2009). Induction of transposable elements has been observed in rice (Huang et al. 2009b) and maize (Makarevitch et al. 2015). Retrotransposons were also identified in tobacco and rice and have been reported to be involved with induced mutations in tissue culture (Hirochika 1993; Hirochika et al. 1996).

With the advent of genome sequencing projects, transposable elements have been recognized as being dynamic and abundant for the development of markers namely sequence-specific amplified polymorphism (SSAP), inter-retrotransposon-amplified polymorphism (IRAP), and retrotransposon-based insertion polymorphisms (RBIP) (Fig. 1.1). The sequence-specific amplified polymorphism (SSAP) marker which made use of transposon-specific sequence within an AFLP reaction was developed by Waugh et al. (1997). The SSAP technique has been found to be more specific than SSR-based markers in different agricultural crops namely oats, grapevine, tomato, sweet potato, and pea (Berenyi et al. 2002; Labra et al. 2004; Tam et al. 2005; Lizamore 2013). IRAP markers make use of the long terminal repeat primers (singly or in pairs) to amplify different retrotransposons distributed inside the genome of the somaclones, hence, amplifying regions of the genome which are found between

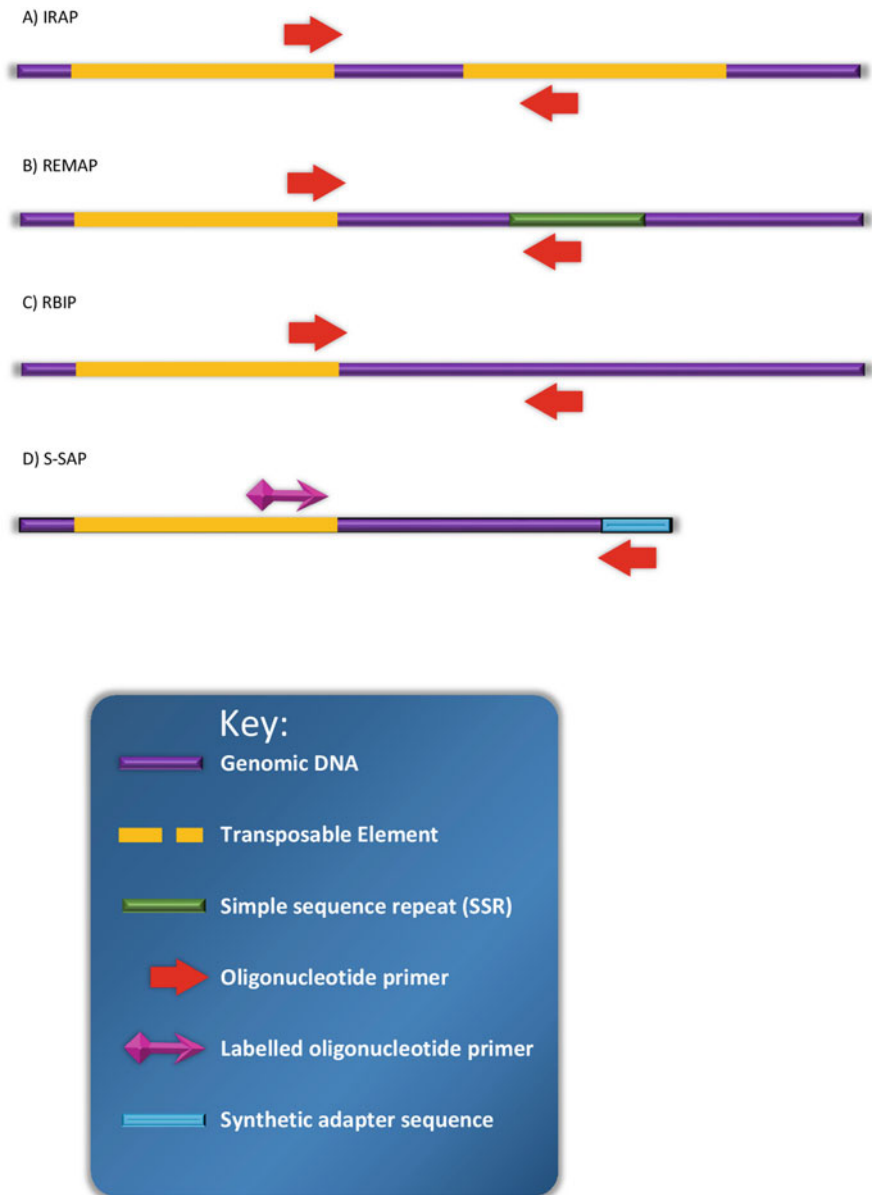


Fig. 1.1 Shows the different types of markers involving transposons discussed above

retrotransposons insertion sites (Kalendar et al. 1999; Kalendar and Schulman 2006). IRAP markers have been used to ensure genetic fidelity in banana, barley, wheat, and *Diospyros* (Muhammad and Othman 2005; Guo et al. 2006; Li et al. 2007; Carvalho et al. 2010; Campbell et al. 2011). Retrotransposon-based insertion

polymorphisms (REMAP) use primers specifically targeting transposon insertion sites and aim at testing presence or absence of insertions hence amplifying regions between microsatellites and adjacent retrotransposons (Carvalho et al. 2010; Lizamore 2013). REMAP aims at amplifying regions between microsatellites and adjacent retrotransposons (Lizamore 2013). All markers are represented in Fig. 1.1.

1.5.12 Next-Generation Sequencing (NGS) for the Determination of Somaclonal Variation

Regeneration of plants by tissue culture is stressful and can lead to both genetic and epigenetic changes which lead to phenotypic polymorphism. NGS-based analyses have been carried out in *Arabidopsis* (Jiang et al. 2011) and rice (Sabot et al. 2011; Miyao et al. 2012) to confirm the extent of genomic change which occurs during tissue culture for induction of somaclonal variation. An elevated genome-wide DNA sequence mutation rate was observed in *Arabidopsis* regenerated plantlets with base substitution as major genomic change but with no detectable change in transposable element reactivation (Jiang et al. 2011). In rice, single nucleotide polymorphism (SNPs), nucleotide changes (including insertions and deletions) as well as transpositions were observed (Sabot et al. 2011; Miyao et al. 2012; Zhang et al. 2014). In wheat, however, transposable elements were found to be the most likely factors causing somaclonal difference at the genome level (Bara Anek et al. 2016).

1.5.13 MicroRNA Involvement in Tissue Culture

MicroRNA or miRNA, first discovered in *Caenorhabditis elegans*, has strong gene regulatory activity. These miRNAs are noncoding, approximately 19–24 nucleotides in length which have been recognized to play crucial regulatory roles in many biological processes and pathways (Carrington and Ambros 2003). MiRNAs have been reported to be highly involved in plant defense especially in stress responses, plant development, hormonal signaling, and seed germination. Recently miRNAs have been reported to be involved during in vitro culture of plants (Miguel and Marum 2011; Rodriguez-Eneiquez et al. 2011). Somaclonal variation is thought to be caused by both genetic and epigenetic factors which relate to transposable element transposition and small RNA-directed methylation (Smulders and De Klerk 2011; Neelakandan and Wang 2012).

Differential microRNA production (namely Mir169a and miR390) has been recorded in strawberry plantlets regenerated by tissue culture and these have been correlated with existing differences with the phenotype (Li et al. 2012). Regulatory roles of miRNAs have been highlighted during somatic embryogenesis of many plants (Nodine and Bartel 2010; Su et al. 2015; Zhang et al. 2015a, b) as well as their involvement in gene regulation of epigenetic processes. However, to date, no direct relationship has been found between somaclonal variation and microRNA. Further studies are required to characterize the miRNA expressed during somaclonal

variation from different plants so that diagnostic miRNA associated with this phenomenon can be compiled and their possible use as biomarkers facilitated.

1.6 Conclusion

Regenerating plants by tissue culture may be aimed at maintaining the genetic integrity and the production of true to types but at times somaclonal variation may be desirable to increase the gene pool of a population with a narrow genetic base. Various methods for the detection of somaclonal variation have been reviewed and methods for assessing variant genomes at the molecular level allow for early and easy selection during a breeding experiment. These methods may be used in isolation but preferably in concertation to ascertain genetic fidelity.

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