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# TRANSPOSABLE ELEMENTS AND THE DETECTION OF SOMACLONAL VARIATION IN PLANT TISSUE CULTURE: A REVIEW

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## ABSTRACT

Plant tissue culture uses the genetic potential, or totipotency, of plants to regenerate and give rise to a whole plant. Tissue culture-derived plants are expected to have identical genetic material to the parent, and thus can serve as an effective tool for controlled, mass clonal propagation. However, somaclonal variation has been observed in plant tissue culture due to either point mutations, transposition activity of mobile genetic elements, chromosomal rearrangements, or ploidy level changes, causing genetic instability. Tissue culture-induced mutations associated with transposable element activities have been reported from many plant studies, related to stress conditions during tissue culture such as wounding, exposure to hormones and/or specific compounds in the growth media, and genomic shocks from cytological changes. Transposable elements are repetitive DNA fragments with the ability to transpose from one region to another within a genome. Mutations that may occur during plant tissue culture suggest that the plantlets have to be regularly monitored. Methyl-sensitive transposon display (MSTD) is an advanced, efficient DNA fingerprinting technique that can simultaneously detect genetic variation, changes in transposable element insertion sites and the status of cytosine methylation of DNA in plant genomes. The main principles and notes for application of MSTD such as design of element-specific primers, identification of transposable element sequences in plant genomes, selection of the isochizomer enzymes used, and different classes of banding pattern shown are briefly discussed based on our preliminary work with *Nicotiana benthamiana* (Tnt1 retroelement) and *Musa acuminata* (Copia-33 Mad-I retroelement) examples.

**Key words**: Methyl-sensitive transposon display (MSTD), somaclonal variation, transposable elements

## INTRODUCTION

Plant tissue culture, also known as plant cell, sterile, axenic or *in vitro* culture, is a technique of growing plant cells, tissues or organ in an artificial gel or liquid media supplemented with nutrients, vitamins and plant growth regulators under controlled and sterile conditions (Singh and Kumar, 2009). Plant cells possess sufficient genetic potential to be able to regenerate and give rise to a whole plant (totipotency) – making plant tissue culture an important method in plant biotechnology studies as well as having commercial applications (Thorpe, 2007). Originally proposed by Gottlieb Haberlandt in 1902, single cells were predicted to regenerate into a complete and functional plant (Krikorian and Berquam, 1969), this hypothesis of totipotency was first proved using tobacco cells (Vasil and Hildebrand, 1965) and later using tobacco protoplasts to regenerate a new plant (Takebe *et al.,* 1971).

Plants grown via tissue culture are expected to have identical genetic material to the parent and thus, keep their intrinsic characteristics. This method of establishing genetically identical clones from an organism's tissue, capable of generating into a complete plant is known as clonal propagation or micropropagation (Vizel *et al.,* 2010). Micro-propagation has been extensively used for the multiplication and growth of many plants in horticulture and agriculture, ranging from wild species such as orchids, ferns, carnivorous plants,

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medicinal plants, woody plants, and cultivated crops (Fay, 1992; Debnath *et al.,* 2006; Gatica-Arias *et al.,* 2008; Dam *et al.*, 2010; Vergara-Galicia *et al.,* 2010; Paek *et al.,* 2011). Main advantages associated with micropropagation include the large number of homogenous and genetically stable plants that can be produced under sterile condition, relative ease of controlling the environmental and nutritional conditions and rapid production of new varieties (Dobránzski and Texeira de Silva, 2010). Two commonly used methods in micropropagation include organogenesis and somatic embryogenesis (Rout *et al.,* 2006). These techniques share similar main aims of enhancing the rate of multiplication of regenerated plants and the production of disease free plants (Eka *et al.,* 2005).

**Mutation leads to phenotypic, genetic variation and genome instability**

Plant tissue culture can be seen as an effective tool for the large scale, controlled production of plant material and represent an option for mass clonal propagation (Chandrika and Rai, 2009). Even though plant tissue culture provides many advantages, one major drawback associated with it is mutations that can cause phenotypic and genetic changes i.e. somaclonal variation. Mutations can cause phenotypic alteration, genetic variation and genome instability among the regenerated plants from a single donor clone. Possible mechanisms that may cause a mutation to occur during the tissue culture process are under the controls of genetic and epigenetic systems where it can affect the genetic and genome stability of the plant (Temel *et al.,* 2008). Somaclonal variation may arise due to point mutations, the activation of mobile genetic elements, chromosomal rearrangements, or ploidy level changes (Jaligot *et al.,* 2000). Any genetic changes induced by tissue culture condition will probably produce a plant with unique heritable characteristics (Soniya *et al*., 2001). Even though the resulting mutant phenotypes of the plants (e.g. alteration in leaf shape, dwarfing, and other changes in growth habit) are unique compared to the mother plant, they are not normally deemed useful in micropropagation or for crop improvement (Evans, 1989).

Mutations are induced by specific components of culture medium where the unnatural condition and environment, and hormone supplemented in the media can generate spontaneous and heritable genetic changes due to a "shocking" experience of isolation. Rates of genetic instability (mutations) are dependent on the type and quantity of the plant growth regulators, such as auxin, cytokinin and abscisic acid in the media (Michiba *et al.,* 2001), the duration of the regenerated plant in the culture (Fras and Maluszynska, 2004) and the degree of endopolyploidy of the explants (Nontaswatsri and Fukai, 2005). Typically, high number of subcultures, exposure to high concentration of plant growth regulators and long term culture are factors that can result in mutations (Marum *et al.,* 2009). Epigenetic changes are not due to sequence modification and chromosomal aberration (Akimoto *et al.,* 2007). Tsaftaris *et al.* (2005) defined epigenetic changes as "all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself". This aspect commonly occurred when the plants are under stress conditions – in tissue culture environments such as prolonged culture time and exposure to plant growth regulators (Vázquez and Linacero, 2010). One interesting source of genomic instability is linked to the activation of transposable elements that might be responsible for tissue-culture induced mutation (Momose *et al.,* 2010; Karajol and Naik, 2011; Bui and Grandbastien, 2012).

**Activation of transposable elements during plant tissue culture**

Mobile genetic elements or transposable elements (TEs) are repetitive DNA fragments that have the ability to move or transpose from one area to another area within a genome (Lisch, 2009). Transposable elements contribute to the size of plant genomes; for example, in certain species of grass, they may comprise of 50% up to 80% of the genomes (Meyers *et al.,* 2001; Piegu *et al.,* 2006; Dooner and Weil, 2007; Macas *et al.,* 2007). Shirasu *et al.* (2000) reported that about 40% to 70% of the total DNA content of crop plants may be made up of repetitive elements, and it was reported that a correlation exists between the evolution of LTR retro-transposons and genome size (Zedek *et al.,* 2010). Transposable elements may greatly impact host genomes by causing mutations when they transpose into or near a gene (Chen *et al.,* 2005). They can alter expression of a nearby gene by operating as promoters, silencers, enhancers, or act as targets of epigenetic modification and other alternative splicing events (Kazazian, 2004; Bui and Grandbastien, 2012). They can also serve as locations for homologous recombination, resulting in chromosomal rearrangements such as deletions, inversions, duplications or translocations due to high, repetitive copies of integrated elements (populations of the same family of elements) existing in the genome (Devos *et al.,* 2002; Kolomietz *et al.,* 2002; Bailey *et al.,* 2003; Sen *et al.,* 2006).

Generally divided into two classes based mainly on their transposition intermediates, retrotransposons (also known as Class I elements) are elements which move via an RNA intermediate while DNA transposons (or Class II elements) directly transpose in the form of DNA (Feschotte *et*

*al.,* 2002; Todorovska, 2007). Retrotransposons represent a substantial percentage of eukaryotic genomes as they transpose by a "copy and paste" mechanism catalyzed by reverse transcriptase and endonuclease (EN) domains of a polyprotein encoded by themselves or by other retrotransposons (Collier and Largaespada, 2007). Based on their structure, retrotransposons can be further divided into two major types – elements that are flanked by long terminals repeats (LTRs) and non-LTR retrotransposon. Non-LTR retrotransposons are divided into long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) (Feschotte *et al.,* 2002; Karajol and Naik, 2011). LINEs are autonomous retrotransposons that encode the enzymatic machinery required for their propagation (Ostertag and Kazazian, 2001) while SINEs are non-autonomous and require the enzymatic machinery of LINE elements for retrotransposition (Lenoir *et al.,* 2001; Ostertag *et al.,* 2003).

In general, DNA transposons move only as DNA from one genomic area to another and can be deleted precisely though at a relatively low frequency (Huang *et al.,* 2008). Mobility of these elements is performed by a "cut-and-paste" mechanism using an element encoded transposase (TPase) (Arkhipova and Meselson, 2005). However, more recent work interestingly revealed that DNA transposons may be further divided into three major classes which consist of (i) "cut-and-paste" DNA transposons, (ii) rolling-circle DNA transposons or Helitrons, which were found to use a replicative transposition mode and (iii) self-synthesizing DNA transposon or Polintons. DNA transposons are characterized by a superfamily-specific transposase core which differs between superfamilies (Kapitonov and Jurka, 2008). DNA transposons are also distinguished by terminal inverted repeats (TIRs) at their extremities and similar to retroelements, both classes of elements form target site duplication (TSDs) during insertion (Wicker *et al.,* 2007). Upon transposition, the TIRs are identified by the DNA-binding domains of TPases and this element usually excises from one location and reinserts into elsewhere in the genome (Chandler and Mahillon, 2002). Both class I and II transposable elements have been found to contain autonomous and non-autonomous elements, whereby autonomous elements have open reading frames (ORFs) that encode the products required for the whole process of transposition. Non-autonomous elements require the presence of an active autonomous element in order to move since they have no significant coding capacity (Lisch, 2009).

During the process of plant tissue culture, transposable elements have been found to be a significant source of mutations and their activities can be influenced (silenced) by some epigenetic mechanisms due to changes in the environment and culture condition (Gaut and Ross-Ibarra, 2008). Tissue culture stress is related to wounding during the isolation of explants, exposure to hormones and/or specific compounds in the growth media during *in vitro* cell culture or tissue culture. All these factors may activate transposable elements and result in changes in their copy numbers (Melayah *et al*., 2001; Kubis *et al*., 2003; Bui and Grandbastien, 2012). Due to the (potential) highly mutagenic impact of TE activities, it is known that host plants have evolved different epigenetic mechanisms to regulate element activity. These mechanisms include transcriptional silencing via DNA methylation, histone modification and alterations in chromatin packing and condensation, as well as post-transcriptional silencing of elements by the RNAi pathway, and the more recently described transposon - derived small interfering (si) RNAs and RNA directed DNA methylation (RDRM) and histone modification to suppress transpositional activity (Martienssen and Colot, 2001; Lippman and Martienssen, 2004; Matzke and Birchler, 2005; Slotkin and Martienssen, 2007; Ito 2012, 2013).

In plants, DNA (CpG) methylation have been reported to normally repress transpositional activity of diverse transposons, and studies comparing transcriptional and transpositional activities of elements (Evade, ONSEN), using methylation and siRNAs mutants also demonstrated regulation of transposable elements via RNAi-mediated chromatin modifications (Mirouze *et al.*, 2009; Ito *et al.,* 2011). Therefore, normal epigenetic program and re-patterning that may not be established properly in regenerated plants could lead to activation of transposable elements due to hypomethylation in tissue culture conditions (Kaeppler *et al.,* 2000). However, it is interesting to note that exceptions have been detected, whereby CpG methylation instead elevated transpositional activity of certain elements (Sleeping Beauty, Frog Prince and Minos) of the Tc1/mariner superfamily (Yusa *et al*., 2004; Ikeda *et al.,* 2007; Jursch *et al*., 2013). It was also proposed that chromosome breakages that sometimes occurred in tissue culture induce genomic shock which in turn activates the transposable elements, though the main cause of chromosome breakage in plant tissue culture is still unclear but may be related to changes in DNA methylation pattern resulting in chromosome bridges and breakage (Kaeppler *et al.,* 2000).

Tissue culture-induced mutations associated with transposable element activities have been reported in many plant species - various studies have reported that *in vitro* tissue culture can activate transposable elements such Ac in maize (Brettell and Dennis, 1991), Tto1 in *Nicotiana tabacum*

(Hirochika, 1993), Tos17 (Miyao *et al.,* 2003) and Lullaby (Picault *et al*., 2009) in *Oryza sativa*, Tnt1 in *Medicago truncatula* (d'Erfuth *et al.,* 2003), and Rtsp-1 in *Ipomea batatas* (Tahara *et al.,* 2004). Hirochika *et al.* (1996) originally showed that Tos retrotranposons were inactive in normal plants but increased in transcription during tissue culture. Kikuchi *et al.* (2003) observed the activation of MITEs (*mPing)* during anther culture of rice, which led to transpositions into new gene locations. Interestingly, Huang *et al*. (2009) reported transposition of *nDaiZ9* was activated in most of the samples upon treatment with a DNA methylation inhibitor (5-azaC). Another study suggested that prolonged tissue culture is needed for more transposition of *Tos17* in rice tissue culture leading to more and severe gene mutations (Kuan *et al.*, 2010). Kour *et al.* (2009) showed that transposable elements had been activated in callus regenerated plants bearing the B chromosome of *Plantago lagopus*, and deduced that activation of transposable elements was highly related to the condition in tissue culture since there were no changes in the B chromosome of the mother plant. Recently, microarrays and next generation sequencing (NGS) analysis detected genome-wide activities of transposable elements in tissue culture and suspension cells of *Arabidopsis thaliana* (Tanurdzic *et al*., 2008) and tissue culture derived plants of *Oryza sativa* (Picault *et al.,* 2009; Sabot *et al*., 2011; Miyao *et al.,* 2012).

**An advanced molecular marker technique to detect genomic instability: Methyl-Sensitive Transposon Display (MSTD)**

Mutations that may occur during plant tissue culture suggest that the plantlets have to be regularly monitored. Various molecular marker techniques are available for use to detect somaclonal variation. Most of the molecular marker techniques involve amplifications of genomic DNA with short random or specific primers. For example, tissue-culture induced mutations of different plant tissue cultures have been screened using Random Amplified Polymorphic DNAs (RAPD) (Eshraghi *et al.,* 2005; Rasheed *et al.,* 2005; Qin *et al.,* 2007; Venkatachalam and Sreedhar 2007; Sianipar *et al.,* 2008; Elmeer *et al.,* 2009), Amplified Fragment Length Polymorphism (AFLP) (Pontaroli and Camadro, 2005; Puente *et al.,* 2008; Chuang *et al.,* 2009), microsatellite markers or simple sequence repeats (SSR) (Morgante *et al.,* 2002; Lopes *et al.,* 2006; Burg *et al.,* 2007), Sequence-Specific Amplified Polymorphism (SSAP) (Venturi *et al.,* 2006; Du *et al.,* 2009; Wegscheider *et al.,* 2009) and Methyl-Sensitive Amplified Polymorphism (MSAP) (Peraza-Echeverria *et al.,* 2001; Jaligot *et al.,* 2004; Lu *et al.,* 2008). Recently, another modified advanced method called Methyl-Sensitive Transposon Display or MSTD (Figure 1a), was shown to be useful for detecting genetic variation, changes in transposable element banding pattern and DNA methylation all in one technique (Parisod *et al.,* 2009).

Methyl-sensitive transposon display (MSTD) is actually a combination method of MSAP (Reyna-Lopez *et al.*, 1997) and Sequence Specific Amplification Polymorphism (SSAP), which is highly similar to Transposon Display (Casa *et al.,* 2000; Syed and Flavell, 2007). Both MSAP and MSTD are efficient DNA fingerprinting methods to detect the conditions of large scale cytosine methylation (status) in plant genomes, and differences in the banding patterns rapidly reveal and identify changes in methylation between the genomes of the sampled plants. In addition, MSTD is also able to provide information on epigenetic (methylation status) of adjacent regions and population dynamics (copy numbers) of transposable elements (TEs) insertions (Zerjal *et al.,* 2009). The MSTD technique relies on primer(s) anchored at TE extremities to specifically amplify digested genomic DNA of the insertion sites of TEs and therefore, this technique can be used to comparatively evaluate the copy number of TEs. An amplified fragment will consist of sequences of part of the element, and the genomic sequence adjacent to the border of the element - and this varies in length according to the location of the nearest restriction site on the genomic DNA (de Setta *et al.,* 2007; Parisod *et al.,* 2014). Results from MSTD are also useful to potentially provide information on the impact of TE insertions on the genome since certain elements such as retrotransposons are widely distributed throughout the genome (Miyao *et al.,* 2003) and can give insights to improve our understanding, predict and possibly track genomic evolution due to transposition events (Mandal *et al.,* 2006). Parisod *et al*. (2009) performed a study in *Spartina* using three elements, namely *Ins2*, *Cassandra* and *Wis-like,* which were mostly shared by both parents and hybrid plants. Their results found that CpG methylation and major structural changes have occurred near the TE insertion sites and thus caused alterations in the plant genome following inter-specific hybridization and/or genome duplication.

At present, a limited number of studies have been performed to detect genomic instability that may have occurred during plant tissue culture of Malaysian crops. Perhaps one main hindrance is the design of element-specific primers, which requires existing DNA sequence information of well-characterized elements. Nowadays, however, many genomics studies using the application of high-

throughput sequencing technologies have been performed resulting in abundant information on genomic sequences for many crop genomes (for example, see Plant Genomes Central at National Center for Biotechnology Information (NCBI) for current list of plant genomic projects, @ http://www.ncbi.nlm.nih.gov/genomes/PLANTS/PlantList.html). Available full genome sequence information (datasets) have even been exploited to develop a method that can predict the transposon insertion display banding pattern of a given transposable element primer (Le and Bureau, 2004) so designed primers can be tested virtually before being applied in MSTD. In addition, there is an existing site hosting the Plant Repeat Databases (http://plantrepeats.plantbiology.msu.edu/about.html), created to assist in the compilation and identification of repeat sequences in plant genomes whereby repetitive DNA sequences of selected plant genera are regularly queried and compiled from GenBank and other published records (Ouyang and Buell, 2004).

Our own preliminary work testing MSTD on the model plant *Nicotiana benthamiana* and a crop plant, banana (*Musa acuminata*) showed that it was relatively easy to adopt the MSTD protocol. The primer used for *Nicotiana* was based on the well-studied tobacco retroelement Tnt1, which is one of the few active retrotransposons in plants (Melayah *et al*., 2001). Transposition of this element has been shown previously to be activated during protoplast culture in tobacco and tissue culture in the heterologous hosts *Arabidopsis thaliana* and *Medicago truncatula* during the early steps of the *in vitro* transformation-regeneration process (Grandbastien *et al*., 1998; d'Erfurth *et al.,* 2003). Sequences used to generate a retroelement primer for *Musa acuminata* were directly obtained from NCBI whereby a quick search revealed the availability of a large Bacterial Artificial Chromosome (BAC) clone sequence (AC226035). The program Repeat Masker, Genetic Information Research Institute (GIRI) (Kohany *et al.,* 2006) identified several transposable elements of which one was named Copia-33 Mad-I, similar to *copia* elements from *Malus domestica.* The primer used was then designed using Primer3 Input version 0.4.0 (Rozen and Skaletsky, 2000), positioned facing outwards on the long terminal repeat (5' LTR) sequence of the Copia-33 Mad-I retrotransposon element.

Our test application of MSTD on *Nicotiana* (normal and mutant phenotype "*grassy*") and banana tissue cultured plantlets (normal and mutant phenotype "*cabbage-like*") with the Tnt1 and Copia-33 Mad-I primers respectively produced results as shown in Figure 1(b) and 1(c). Results demonstrated banding pattern differences between the normal and mutant plantlets in both the *Nicotiana* and banana samples (Azman, 2011). Polymorphic bands can be seen between normal and mutant plantlets, as well as between the samples digested by *MspI* and *HpaII*, by the disappearance and appearance of bands, indicating differences in DNA methylation pattern occurring near the retroelement insertion sites or element activity, in both normal as well as mutated plants. In addition, the MSTD gel results also demonstrated that the genome of banana contained many copies of Copia-33 Mad-I, suggesting that this retrotransposon might be present in high copy number and thus possibly play a role in the evolution of the banana genome.

A notable step in the MSTD technique which could influence the success of the analysis is selection of the isochizomer enzymes used for detection of methylation status. Isochizomers *MspI* and *HpaII* have been widely used for methyl-sensitive displays since these are frequent cutters compared to other isochizomer enzymes such as *MseI* and *Taq1* (Takata *et al.,* 2007). Both *MspI* and *HpaII* recognize the same tetranucleotide sequence of 5'-CCGG-3' but have different sensitivity to methylation (Krauss *et al.,* 2009). *HpaII* activity is blocked when either internal or external cytosine is fully methylated (both strands) while *MspI* is only blocked when the external cytosine is fully methylated or in a rarer case, when the sequence is hemi-methylated (Xiong *et al.,* 1999). Different levels of methylation of the internal cytosine will result in different cutting by the restriction enzymes thus generating uneven PCR bands between two digests (Zerjal *et al.,* 2009). Hence, the quality of extracted genomic DNA (gDNA) used for the digestion process is an important aspect to ensure the success of the protocol. Significant contamination occurring in the gDNA samples may affect the ability of the restriction enzyme to digest the gDNA as the presence of proteins, polysaccharides and/or polyphenols may interfere with restriction enzyme activity during the digestion process, as well as *Taq polymerase* activity during PCR amplification (Angeles *et al.,* 2005).

The MSTD analysis can provide a powerful technique to investigate genomic changes around transposable elements that occurred during the plant tissue culture process. Three classes of bands may be identified from the polyacrylamide gel electrophoresis (PAGE) results – the first class represent un-methylated bands, present in both *MspI*- and *HpaII*- digested gDNAs; a second class with presence of bands in gDNA digested with *MspI* but missing in *HpaII* digested gDNA, representing methylated sequences and a rare third class of bands present in *HpaII* digested gDNA but missing from

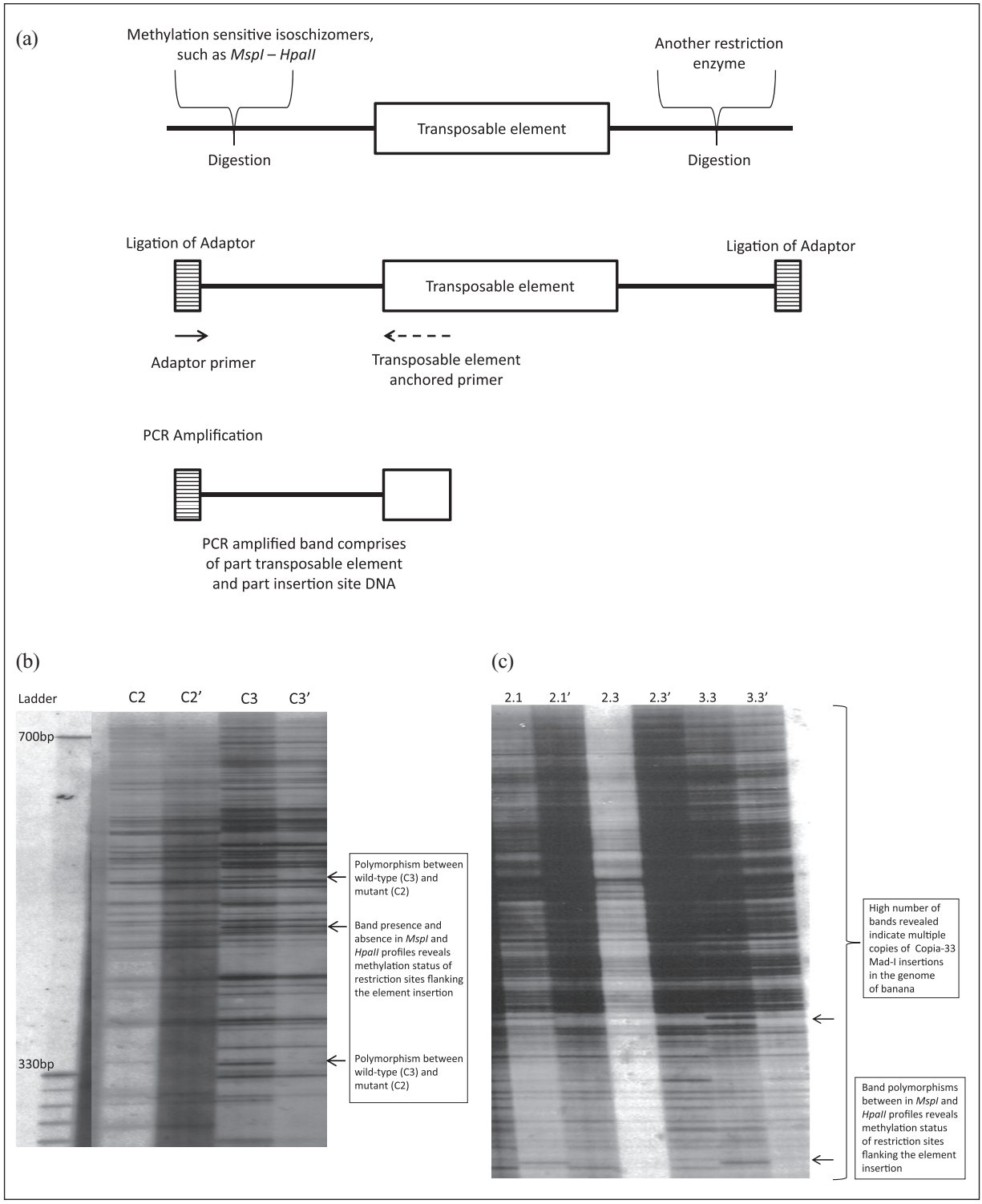


**Fig. 1.** (a) Schematic representation depicting the principle of methyl-sensitive transposon display (MSTD) adapted from Parisod *et al*. (2014). Digestion of genomic DNA with methylation sensitive isoschizomers (such as *MspI* and *HpaII*) is followed by digestion with a frequent cutter such as *EcoRI*, and then adaptors are ligated to the DNA fragments. PCR amplification using a specific transposable element anchored primer with the complementary adaptor primer will generate bands that are separated by polyacrylamide gel electrophoresis. (b) An example of MSTD profiles generated by using the restriction enzymes *MspI* (C2 and C3) and *HpaII* (C2' and C3') of tissue culture-derived samples of normal (C3) and mutant "*grassy*" (C2) *Nicotiana benthamiana.* (c) Preliminary example of MSTD profiles generated by using the restriction enzymes *MspI* (2.1, 2.3 and 3.3) and *HpaII* (2.1', 2.3' and 3.3') of tissue culture-derived samples of normal (2.1) and mutant "cabbage" (2.3 and 3.3) *Musa acuminata*.

the *MspI* digested gDNA – these bands might indicate hemi-methylation of the external cytosine (Zerjal *et al*., 2009; Parisod *et al.,* 2014). Presence of a specific band from a selected sample may correspond to either a transposition event or de-methylation of the area near a TE insertion. However, CpCpG methylation on both DNA strands prevents the enzymes from digesting the DNA, therefore, this technique does not perform well in regions of the genome that are heavily methylated. An absence of a band may signify a change in TE insertion or increased levels of methylation and this should be recognized during interpretation of the MSTD profiles (Parisod *et al.,* 2014). Protocol adjustments and further explanations to aid the interpretation of the banding patterns produced by the MSAP method, which is similar to MSTD, is available for samples that display difficult, complex patterns (Fulneček and Kovařík, 2014).

## CONCLUSION

The occurrence of mutations during plant tissue culture can be a problem as it leads to plant genomic instability. As a result, the plantlet will possess somaclonal variation and may display different phenotypes from the mother plant. Studies which can offer insights into the causes of mutations are important in order to better understand the types of mutation that may happen during the *in vitro* process so that improvement on the procedure can be done to reduce the number of mutants. Transposable elements constitute a large fraction of repetitive regions in plant genomes and its mobilization affect the evolution of current genomes. Tissue culture conditions have been observed in many plant studies to induce transposable element activities, making elements a source of mutations during tissue culture. An advanced molecular marker technique such as MSTD has not only been proven efficient for the detection of genomic changes in mutant plants and able to detect the complexity of DNA methylation changes during plant development, it also offers an opportunity to study the effect(s) of transposition of TEs by examining insertions potentially associated with specific phenotypic changes of the plant. As a start, this can be done as unique or specific MSTD bands can be isolated, cloned and sequenced in order to discover the affected DNA regions in the host plant. Further genetic studies can then be performed to follow the inheritance (segregation) of the specific marker with the phenotype to confirm the genotype-phenotype association.

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