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Analysis of genetic and epigenetic variation in *in vitro* propagated potato somatic hybrid by AFLP and MSAP marker

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Abstract

Background: Genetic and epigenetic changes (DNA methylation) were examined in the tissue-culture propagated interspecific potato somatic hybrids between dihaploid *Solanum tuberosum* and *S. pinnatisectum*. Amplified fragment length polymorphism (AFLP) and methylation-sensitive amplified polymorphism (MSAP) were applied to detect the genetic and epigenetic changes, respectively in the somatic hybrids mother plants (1st cycle) and their regenerants (30th cycles sub-cultured).
Results: To detect genetic changes, eight AFLP primer combinations yielded a total of 329 scorable bands of which 49 bands were polymorphic in both mother plants and regenerants. None of the scorable bands were observed in term of loss of original band of mother plant or gain of novel band in their regenerants. AFLP profiles and their cluster analysis based on the Jaccard's similarity coefficient revealed 100% genetic similarity among the mother plant and their regenerants. On the other hand, to analyze epigenetic changes, eight MSAP primer pair combinations detected a few DNA methylation patterns in the mother plants (0 to 3.4%) and their regenerants (3.2 to 8.5%). Out of total 2320 MSAP sites in the mother plants, 2287 (98.6%) unmethylated, 21 (0.9%) fully methylated and 12 (0.5%) hemi-methylated, and out of total 2494 MSAP sites in their regenerants, 2357 (94.5%) unmethylated, 79 (3.1%) fully methylated and 58 (2.3%) hemi-methylated sites were amplified.
Conclusion: The study concluded that no genetic variations were observed among the somatic hybrids mother plants and their regenerants by eight AFLP markers. However, minimum epigenetic variations among the samples were detected ranged from 0 to 3.4% (mother plants) and 3.2 to 8.5% (regenerants) during the tissue culture process.

Keywords: AFLP; DNA methylation; *in vitro* propagation; MSAP; potato; somatic hybrids.

INTRODUCTION

Plant tissue culture is recognized as one of the valuable components of biotechnology methods because of its potential to rapid multiplication of true-to-type genotypes. In potato, *in vitro* clonal propagation is used to produce micro or mini tubers for healthy seed stocks identical to mother plant. However, *in vitro* cultures pose a problem of genetic stability caused by genetic and epigenetic changes (somaclonal variations) in regenerants. So in the clonal regeneration, one of the most crucial concerns of curators is to retain genetic stability of *in vitro* propagating material (Zilberman and Henikoff, 2007). It was proposed that apart from genetic changes, epigenetic modifications may play an important role in plant growth and development. Moreover, term epigenetic refers to a mechanism that controls gene expression without altering DNA sequence and leads to genetic modifications by DNA methylation, histone and chromatin changes. Studies show that changes in DNA methylation are quite stable and are frequently transmitted during meiosis and mitosis (Smulders and De Klerk, 2011).

Until now, the best known epigenetic process is DNA methylation, partly because it has been the easiest to study with existing technology and plays a key role in regulating gene expression. In general, any method capable of displaying polymorphism of digested DNA fragments can be used to detect DNA methylation. Detection of DNA methylation may depends on the application of restriction enzymes such as isoschizomers. Isoschizomers share the same recognition sites but show differential sensitivity to DNA methylation. Polymorphic DNA fragments can be generated after digestion of methylated genomic DNA with isoschizomers (Chen, 2007). Among various molecular markers, amplified fragment length polymorphisms (AFLP) and methylation-sensitive amplified polymorphisms (MSAP) are still a reliable and relatively cheap alternative. The MSAP method, a modification of the AFLP technique, can detect DNA methylation patterns by restriction digestion of DNA with the isoschizomers such as *Hpa*II and *Msp*I. The isoschizomers recognize the same tetranucleotide sequence (5'-3') CCGG but have different sensitivities to the cytosines methylation. The enzyme *Hpa*II cuts when external cytosines is hemi-methylated (single DNA strand methylated), whereas the enzyme *Msp*I cleaves when internal cytosines is fully-methylated (both DNA strands methylated). On the other hand, for a given DNA sample, two major methylation sites namely i) full methylation of internal cytosine and ii) hemi-methylation of external cytosine can only be distinguished using isochimeres *Hpa*II and *Msp*I. They cannot distinguish between unmethylated and fully methylated cytosines or hemi-methylated internal cytosines. Thus, the methylation percentages obtained by MSAP should be lower than the total absolute values existing at CCGG sites (McClelland et al. 1994). In spite of this limitation, the MSAP method has been successfully applied in a wide range of studies where alterations in cytosine methylation were detected in various crop species (Bednarek et al. 2007). The AFLP and the MSAP markers have been used to detect significant genetic and epigenetic changes, respectively in a number of crop species for example potato (Joyce and Cassells, 2002; Dann and Wilson, 2011), *Solanum aculeatissimum* (Ghimire et al. 2012), *Triticum aestivum* (Meng et al. 2012), *Gardenia jasminoides* (Wu et al. 2012), *Ungernia victoris* (Bublyk et al. 2012), *Capparis spinosa* (Carra et al. 2012), *Phaseolus* ssp. (Abid et al. 2011), *Nicotiana tabacum* (Yang et al. 2011), *Freesia hybrida* (Gao et al. 2010), *Ocotea catharinensis* (Hanai et al. 2010), *Cymbidium* (Chen et al. 2009), *Vitis* spp. (Baránek et al. 2010), *Brassica oleracea* (Salmon et al. 2008), *Hordeum brevisubulatum* (Li et al. 2007) and *Humulus lupulus* (Peredo et al. 2006). The aim of this study was to detect genetic and epigenetic variations in *in vitro* propagated somatic hybrids mother plants and their regenerants using AFLP and MSAP molecular markers.

MATERIALS AND METHODS

Plant material and culture conditions

In the present study, *in vitro* propagated interspecific potato somatic hybrids between dihaploid *Solanum tuberosum* and *S. pinnatisectum* namely P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, P12 and P13 were used (Sarkar et al. 2011). Somatic hybrids mother plant (1^{st} cycle: original mother plant regenerated from one callus in the previous study of Sarkar et al. 2011) and 30^{th} cycles sub-cultured somatic hybrids regenerants (here after called regenerants) were used to detect the genetic and epigenetic changes. Tissue culture plants were maintained in the Cell and Molecular Biology Laboratory, Division of Crop Improvement, Central Potato Research Institute, Shimla, India. *In vitro* plantlets were multiplied by sub-culturing leafy node(s) (1-2) on MS (Murashige and Skoog, 1962) medium (pH 5.8) supplemented with sucrose (20 g l^{-1}) and solidified with gelrite (2 g l^{-1}). Cultures were grown at 20ºC under a 16-hrs photoperiod (light intensity 50-60 µmol m^{-2} s^{-1}). Triplicate *in vitro* plantlets of the each mother plant and regenerant were used for DNA analyses.

DNA isolation

Plant DNA was isolated from 100 mg leaves collected from fresh *in vitro* plants using the GenElute Plant Genomic DNA MiniPrep Kit (Sigma-Aldrich, St. Louis, USA). *In vitro* plantlets (in triplicates) were pooled together for the DNA isolation separately of the mother plants and the regenerants. DNA quality and quantity were determined with NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), and quality was also assessed on 0.8% (w/v) agarose gel. The isolated DNA was used for various molecular analyses in the present study.

Amplified fragment length polymorphism (AFLP) analysis

To detect genetic changes, AFLP analysis was demonstrated in the somatic hybrids mother plant and their regenerants. Independently isolated DNA of the each genotype was used for analysis. Adapters, primers sequences and methods of AFLP procedures were followed as described by Dann and Wilson (2011) according to the basic protocol of the enzyme combination *Eco*RI + *Mse*I (Vos et al. 1995). List of AFLP adapters and primers (pre-selective and selective primer pairs combinations) sequences are listed in Table 1. Genomic DNA (1000 ng) was restricted by 10 U of each enzyme *Eco*RI and *Mse*I (New England Biolabs, Ipswitch, USA) in total of 50 µL reaction mix by incubation at 37ºC for overnight. Restricted DNA fragments were ligated with adapters with 10 U of T4 DNA ligase enzyme at 16ºC for overnight containing 1 x T4 ligase buffer (NEB), 1 µM *Eco*R-adpaters, 5 µM *Mse*-adapters and made up to 60 µL with sterile distilled water. Reactions were diluted to 1:5 with sterile distilled water and stored at -20ºC. Adapter mixes were prepared by adding equimolar amounts of both adapters and heating to 95ºC for 5 min and slowly cooled to room temperature. Pre-selective PCRs were prepared using AmpliTaq Gold® PCR Master Mix (Applied Biosystems, California, USA) that includes AmpliTaq Gold DNA Polymerase (0.05 U/µL) for automated Hot Start PCR (polymerase chain reaction), 1 x Gold PCR Buffer (30 mM Tris/HCl, pH 8.05, 100 mM KCl), 400 µM each dNTP, 5 mM $MgCl_2$, 0.5 µM EcoR-T primer, 0.5 µM Mse-C primer and made up to 20 µL with sterile distilled water. The polymerase chain reaction (PCR) was performed in a Mastercycler Gradient (Eppendorf, Hamburg, Germany) following the reaction conditions: 95ºC for 15 min followed by 30 cycles of 94ºC for 30 sec, 56ºC for 30 sec, 72ºC for 2 min and extension at 72ºC for 7 min. All PCRs were visualised in 1.5% agarose gel electrophoresis with ethidium bromide stain in 1 x TBE buffer. The reaction mixture was diluted 1:20 with sterile distilled water for selective amplification and stored at -20ºC. Selective PCRs were prepared using same AmpliTaq Gold® PCR Master Mix (ABI) including selective primer pairs combinations of 0.5 µmol *Eco*RI and 0.5 µmol *Mse*I. Selective amplification was performed by the touchdown PCR conditions in a Mastercycler Gradient (Eppendorf) as follows: 95ºC for 15 min, 13 cycles at 94ºC for 30 sec, 65 to 56ºC for 30 sec (with subsequent reduction by 0.7ºC per cycle) and 72ºC for 2 min; and another 23 cycles of PCR amplification were used following the touchdown program. The denaturing step was done at 94ºC for 30 sec, annealing at 56ºC for 30 sec, extension at 72ºC for 2 min; and a final extension at 72ºC for 10 min. Final selective amplification products were denatured at 95ºC for 5 min and then AFLP fragments were analyzed on '3500 Genetic Analyzer' (ABI).

Methylation-sensitive amplified polymorphism (MSAP) analysis

To detect epigenetic changes among the mother plants and their regenerants, MSAP analysis was carried out as above like AFLP methods following adapters and primer sequences described by Chen et al. (2009). List of MSAP adapters and primers (pre-selective and selective primer pairs combinations) sequences are listed in Table 1. Two sets of restriction digestion reactions were carried out independently each at a concentration of 10 U μl^{-1} by mixing *Eco*RI with two isoschizomers, *Hpa*II and *Msp*I (*Eco*RI + *Hpa*II; and *Eco*RI + *Msp*I) separately for mother plants and their regenerants. In the first reaction, ~1000 ng DNA of the samples was digested at 37ºC overnight with *Eco*RI + *Hpa*II in 50 µl reaction volume. In the second reaction, 1000 ng DNA of the same samples was digested with *Eco*RI + *Msp*I under the same reaction conditions. Subsequently adopters ligation, pre-selective amplification and selective amplification were followed as described in AFLP analysis.

Scoring and data analysis

A data matrix was constructed on the basis of presence (1) or absence (0) of bands of the amplified DNA fragments. Missing data were scored as '9'. All reactions were repeated at least twice, and only distinct, reproducible, polymorphic and well-resolved bands across all runs were considered for analysis. In the fragment analyses, peak intensity (≥ 100) and band size (≥ 100 bp) were considered for analyses which were scorable. DNA fragments of low visual intensity, which could not be readily distinguished as present or absent, were considered to be ambiguous markers and were not scored. Genetic diversity analysis was performed with the program NTSYS-PC 2.21 (Rohlf, 2006). A similarity matrix was calculated by Jaccard's coefficient and the dendrogram was generated using unweighted pair-group method (UPGMA) clustering method. Fragment analysis of AFLP and MSAP data was performed using GeneMapper® Software Version 4.1 (ABI). A 500-bp 'GS 500 ROX' standard was used to estimate the molecular size of the fragments.

Table 1. Adapters and primer (per-selective and selective) pair sequences used for AFLP and MSAP analyses of potato somatic hybrids mother plant and their regenerants.

AFLP primers	*EcoR* I (5'-3') Sequence	*Mse*I (5'-3') Sequence
Adapter-1	CTCGTAGACTGCGTACC	GACGATGAGTCCTGAG
Adapter-2	AATTGGTACGCAGTCTAC	TACTCAGGACTCATC
Pre-amplification primers	GACTGCGTACCAATTCT (*Eco*R-T)	GATGAGTCCTGAGTAAC (*Mse*-C)
Selective amplification primers	GACTGCGTACCAATTCAA(E11)	GATGAGTCCTGAGTAACAC (M1)
	GACTGCGTACCAATTCAG (E13)	GATGAGTCCTGAGTAACTG (M2)
	GACTGCGTACCAATTCAAC(E32)	GATGAGTCCTGAGTAACAC (M1)
	GACTGCGTACCAATTCACA(E35)	GATGAGTCCTGAGTAACTG (M2)
	GACTGCGTACCAATTCACC(E36)	GATGAGTCCTGAGTAACAC (M1)
	GACTGCGTACCAATTCACG(E37)	GATGAGTCCTGAGTAACTG (M2)
	GACTGCGTACCAATTCACT(E38)	GATGAGTCCTGAGTAACAC (M1)
	GACTGCGTACCAATTCAGC(E40)	GATGAGTCCTGAGTAACTG (M2)
MSAP primers	*EcoR* I (E) (5'-3')	*Hpa*II/*Msp*I (H/M) (5'-3')
Adapter-1	GACGATGAGTCTAGAA	CTCGTAGACTGCGTACC
Adapter-2	CGTTCTAGACTCATC	AATTGGTACGCAGTC
Pre-amplification primers	GACTGCGTACCAATTC(E00)	GATGAGTCTAGAACGG(H/M00)
Selective amplification primers	GACTGCGTACCAATTCAA(E11)	GATGAGTCTAGAACGGTA(H/M23)
	GACTGCGTACCAATTCAG (E13)	GATGAGTCTAGAACGGTC(H/M24)
	GACTGCGTACCAATTCAAC(E32)	GATGAGTCTAGAACGGTAA(H/M79)
	GACTGCGTACCAATTCACA(E35)	GATGAGTCTAGAACGGTAG(H/M81)
	GACTGCGTACCAATTCACC(E36)	GATGAGTCTAGAACGGTAT(H/M82)
	GACTGCGTACCAATTCACG(E37)	GATGAGTCTAGAACGGTCA(H/M83)
	GACTGCGTACCAATTCACT(E38)	GATGAGTCTAGAACGGTGT(H/M90)
	GACTGCGTACCAATTCAGC(E40)	GATGAGTCTAGAACGGTTC(H/M92)

RESULTS

AFLP analysis

To detect genetic changes, eight AFLP selective primer pair combinations generated a total of 329 scorable bands including 23 to 61 bands per primer, ranging from 101 to 492 bp in size, of which 49 (14.8%) bands were polymorphic (Table 2). The number of bands were varied from 23 (E38 + M1) to 61 (E11 + M1), with an average of 41.12 bands per primer and 6.1 polymorphic bands. In the study, AFLP profiles resulted into complete genetic similarity and no variations were observed among the mother plants and their corresponding regenerants. Moreover, none of the loss of original bands of the mother plants or gain of novel bands in the regenerants were observed during AFLP analysis. The highest bands count was observed in primer combinations E11 + M1 (61) followed by E36 + M1 (56), and the lowest in E38 + M1 (23) followed by E32 + M1 (26). Cluster analysis based on the Jaccard's

similarity coefficient revealed 100% genetic similarity among the mother plants and their regenerants. To illustrate, AFLP profile using the primer pair combination E11 + M1 shows complete similarity among the mother plant (P7) and its regenerants (Figure 1).

Table 2. AFLP bands amplified by the eight primer pairs, and variable bands in somatic hybrids regenerants relative to the mother plants.

Primer pair combinations	Scorable band size range (bp)	No. of total band scored	No. frequency of polymorphic band	Loss of original band	Gain of novel band	No. singleton
E11 + M1	102-488	61	8 (13.1%)	0	0	0
E13 + M2	104-456	50	6 (12.0%)	0	0	0
E32 + M1	100-363	26	4 (15.3%)	0	0	0
E35 + M2	101-466	42	4 (9.5%)	0	0	0
E36 + M1	103-492	56	7 (12.5%)	0	0	0
E37 + M2	110-456	38	8 (21.0%)	0	0	0
E38 + M1	105-365	23	5 (21.7%)	0	0	0
E40 + M2	106-465	33	7 (21.2%)	0	0	0
Total	101-492	329	49 (14.8%)	0	0	0

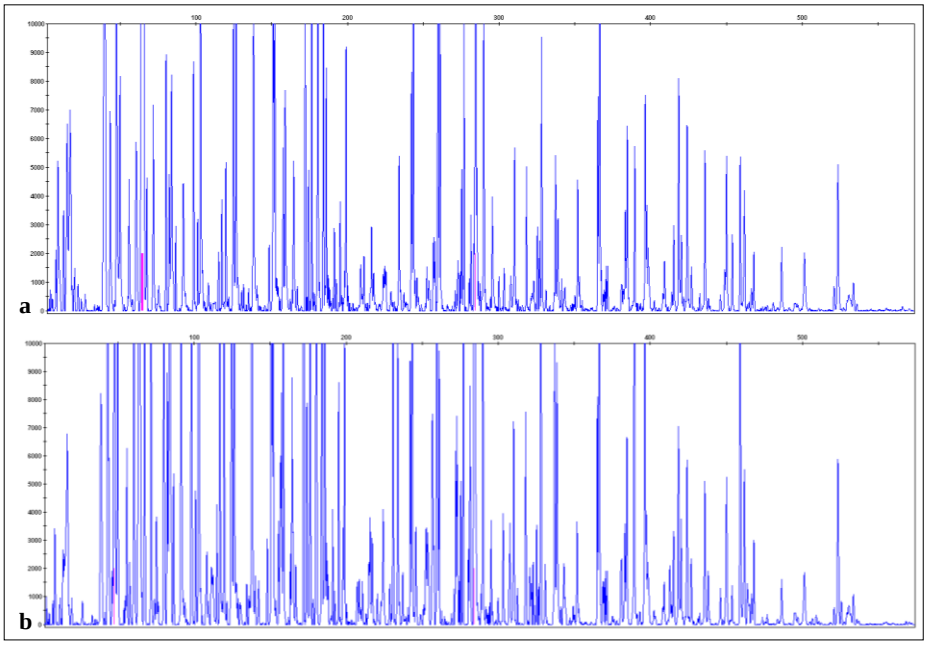

Fig. 1 AFLP profiles of the primer pairs E11 + M1 showing genetic similarity of the somatic hybrid mother plant P7 (a) and its regenerant (b).

MSAP analysis

Eight MSAP selective primer pairs combinations yielded in total 144 to 235 and 153 to 253 clear and reproducible bands in the mother plants and regenerants, respectively (Table 3). The numbers of total, non-methylated, hemi-methylated and fully-methylated CCGG sites were calculated based on the MSAP profiles. In the mother plants, out of total 2320 MSAP sites, 2287 (98.6%) unmethylated, 21 (0.9%) fully methylated and 12 (0.5%) hemi-methylated sites were amplified. Total methylation level in the mother plants was 1.4% (varied between 0.0 to 3.4%), which was comprised of 0.9% full-methylation at the internal cytosines (varied between 0.0 to 2%) and 0.5% hemi-methylation at the external cytosines (varied between 0.0 to 1.9%). In particular to the mother plants, the highest total methylation sites (hemi- + fully-methylated) were 5 (P1 and P12), followed by 4 (P2 and P6) and the lowest 0 (P4, P7 and P8).

Whereas, in the regenerants, a total of 2494 MSAP sites, 2357 (94.5%) unmethylated, 79 (3.1%) fully methylated and 58 (2.3%) hemi-methylated sites were amplified. Compared to the mother plant, regenerants showed both kinds of detectable cytosine methylation levels, *i.e*., full methylation of the internal cytosines and hemi-methylation of the external cytosines, at the CCGG sites. Among the regenerants, total methylation level was 5.4% (varied between 3.2 to 8.5%), which was comprised of both full methylation of the internal cytosines (3.1%) (ranged between 0.9 to 5.2%) and hemi-methylation of the external cytosines (2.3%) (ranged between 0.9 to 3.3%) showed higher values compared to the mother plants. Among the regenerants, the highest total methylation sites were 13 (P1 and P7), followed by 12 (P2, P3, P5, P6, P8 and P13) and the lowest 7 (P9). Cluster analysis based on Jaccard's similarity coefficient of MSAP profiles of primers combination of both enzymes (*EcoR*I + *Hpa*II/*EcoR*I + *Msp*I) showing genetic distinctness among mother plants and their regenerants is shown in Figure 2.

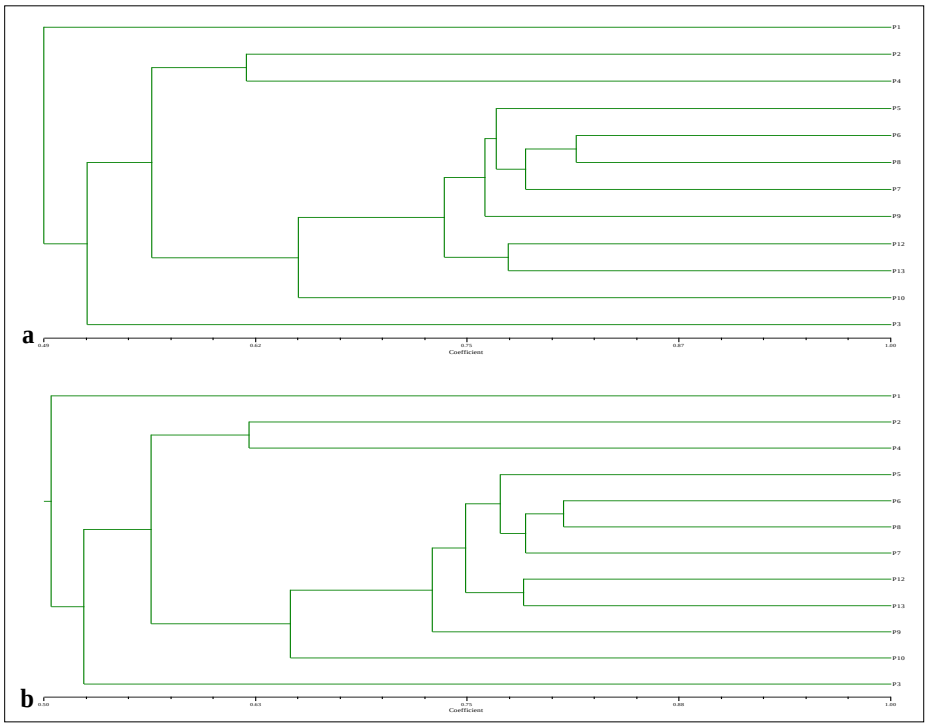

Fig. 2 Cluster analysis based on Jaccard's similarity coefficient of MSAP profiles of both primer combinations (*EcoR*I + *Hpa*II/*EcoR*I + *Msp*I) showing genetic distinctness among the somatic hybrids mother plants (a) and their regenerants (b).

DISCUSSION

Genetic and epigenetic changes has been reported in the literature and observed frequently in plant tissue culture, nevertheless, the underlying mechanism remains largely unknown. Recently, there has been an increased interest in understanding the role of DNA methylation in controlling gene expression in plant. The MSAP technique has been used in various studies on cytosine methylation in plants genome, and has proven to be a highly efficient and powerful tool for investigating methylation patterns in many crop species as mentioned in the introduction.

In the present study, genetic changes were investigated by eight AFLP markers that revealed complete genetic similarity among the mother plants and their regenerants. The somatic hybrid mother plant and regenerants had been independently sub-cultured by nodal cuttings in tissue culture for the last three years. Though, only 8 AFLP primers combinations were used in the study, similar number of primer combinations (7 nos.) was also used earlier to test the genetic stability in potato (Zarghami et al. 2008). There are a number of findings in the literature which reports on detection of genetic stability of mother

plants and their regenerants using molecular markers, for example *Solanum* species (Aversano et al. 2009) and *Lilium orientalis* (Liu and Yang, 2012) by inter simple sequence repeat (ISSR) markers. Zarghami et al. (2008) investigated genetic stability in potato cultivars using seven AFLP primer combinations and resulted 97 and 100% genetic similarity in the cv. Agria and Marphona plantlets stored under cryopreservation and non-cryopreservation conditions. However, Dann and Wilson (2011) detected genetic differences ranged from 8.75 to 15.63% in long-term nodal tissue culture potato clones compared to our study where no genetic differences in the somatic hybrids mother plants and their regenerants. These small changes may be due to the variation on tissue culture procedures, plant types and molecular analyses system except minor peaks/fragments which were not scorable in AFLP and MSAP markers. Variations (genetic and epigenetic) in potato microplant morphology *in vitro* and DNA methylation were also studied by Joyce and Cassells (2002).

Table 3. Alteration in cytosine DNA methylation level in somatic hybrids regenerants relative to the mother plants, based on MSAP analysis using eight primer pairs.

Sample	Total sites (bands)	Unmethylated CCGG sites	Methylated CCGG sites		
			Fully methylated sites (internal cytosines)	Hemi-methylated sites (external cytosines)	Total methylation
Somatic hybrid mother plant (1st cycle of original plants)					
P1	144	139 (96.5%)	3 (2.0%)	2 (1.3%)	5 (3.4%)
P2	170	166 (97.6%)	2 (1.1%)	2 (1.1%)	4 (2.3%)
P3	150	148 (98.6%)	2 (1.3%)	0	2 (1.3%)
P4	169	169 (100%)	0	0	0
P5	210	208 (99.0%)	1 (0.4%)	1 (0.4%)	2 (0.9%)
P6	235	231 (98.2%)	3 (1.2%)	1 (0.4%)	4 (1.7%)
P7	211	211 (100%)	0	0	0
P8	222	222 (100%)	0	0	0
P9	209	206 (98.5%)	2 (0.9%)	1 (0.4%)	3 (1.4%)
P10	183	181 (98.9%)	2 (1.0%)	0	2 (1.0%)
P12	207	202 (97.5%)	4 (1.9%)	1 (0.4%)	5 (2.4%)
P13	210	204 (97.1%)	2 (0.9%)	4 (1.9%)	6 (2.8%)
Total	2320	2287 (98.6%)	21 (0.9%)	12 (0.5%)	33 (1.4%)
Somatic hybrid regenerants (30th cycles sub-cultured plants)					
P1	153	140 (91.5%)	8 (5.2%)	5 (3.3%)	13 (8.5%)
P2	176	164 (93.2%)	7 (4.0%)	5 (2.8%)	12 (6.8%)
P3	164	152 (92.7%)	7 (4.3%)	5 (3.0%)	12 (7.3%)
P4	185	174 (94.1%)	6 (3.2%)	5 (2.7%)	11 (5.9%)
P5	231	219 (94.8%)	7 (3.0%)	5 (2.2%)	12 (5.2%)
P6	253	241 (95.3%)	7 (2.8%)	5 (2.0%)	12 (4.8%)
P7	229	216 (94.3%)	8 (3.5%)	5 (2.2%)	13 (5.7%)
P8	237	225 (94.9%)	5 (2.1%)	7 (3.0%)	12 (5.1%)
P9	222	215 (96.8%)	2 (0.9%)	5 (2.3%)	7 (3.2%)
P10	198	188 (94.9%)	5 (2.5%)	5 (2.5%)	10 (5.1%)
P12	224	213 (95.1%)	9 (4.0%)	2 (0.9%)	11 (4.9%)
P13	222	210 (94.6%)	8 (3.6%)	4 (1.8%)	12 (5.4%)
Total	2494	2357 (94.5%)	79 (3.1%)	58 (2.3%)	137 (5.4%)

To uncover epigenetic changes, MSAP markers were demonstrated among mother plants and regenerants. A detection method of methylated DNA was followed by an addition of methylation-sensitive restriction digestion of genomic DNA prior to PCR. MSAP has been proved to be a robust method for detecting genome-wide cytosine methylation alterations in both level and pattern in plant and animal genomes (Zilberman and Henikoff, 2007). In the present study, total alteration in cytosine methylation level in the regenerants was higher (3.2 to 8.5%) than their corresponding mother plants (0.0 to 3.4%). However, Dann and Wilson (2011) observed higher epigenetic (12.56-26.13%) variations among regenerants of potato derived from long-term nodal tissue culture. Several findings on DNA methylation levels associated with tissue culture have been reported by MSAP analysis in crop plants. In higher plant, total cytosine methylation level varied in different plant species such as from 11.1 to 26.7% in *Cymbidium hybridium* (Chen et al. 2009), 23.5 to 27% in barley (Li et al. 2007), 8.1 to 9.2% in *Freesia hybrida* (Gao et al. 2010).

Our study indicated long-term nodal tissue culture induced epigenetic variations in the potato somatic hybrids regenerants. It is well known that changes in DNA methylation level is accompanied by growth and developmental stages of plant (Joyce and Cassells, 2002). These changes are also accompanied by changes in gene transcription controlling methylation process. DNA methylation is generally recognized to suppress gene expression as regulatory factors, homozygosity/heterozygosity of methylated DNA may be involved in inbreeding depression/heterosis (Nakamura and Hosaka, 2010). A considerable change in the methylation pattern is critical during embryogenesis process and gene expression such as in *Phaseolus* interspecific hybrids and could be involved in the disruption of the regulation or maintenance of the embryogenesis (Abid et al. 2011). This provides further insight into the molecular mechanisms involved epigenetic variations in the somatic hybrids regeneration by more molecular markers. Further experiments are needed to elucidate the causal relationships between alterations in DNA methylation and genetic changes at sequence levels at different developmental stages in the somatic hybrids mother plants and regenerants. Extensive sequencing of the methylation-sensitive fragments and their gene expression analyses may be a valuable strategy to examine genomic regions most affected by genetic and epigenetic changes. Nevertheless, chromatin-immuno precipitation techniques by microarray technology and next generation sequencing technology may also reveal underlying mechanism of genetic and epigenetic control.

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