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ORIGINAL ARTICLES

In Vitro Micropropagation of *Freesia hybrida* and the Assessment of Genetic and Epigenetic Stability in Regenerated Plantlets

Xiang Gao · Dan Yang · Donghui Cao · Man Ao · Xin Sui · Qinmei Wang · J. N. Kimatu · Li Wang

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Abstract *Freesia hybrida* is an important worldwide cut flower, especially in America and Europe. For efficient regeneration of this flower from young inflorescence and rachillae in tetraploid, we developed a simple in vitro micropropagation protocol. Explants of *Freesia hybrida* can regenerate plantlets through somatic embryogenesis via two kinds of pathways, that is, directly from the epidermal cells or indirectly from an embryonic callus, depending on the exogenous plant growth regulators (PGRs) used in the culture media. In direct embryogenesis, when the explants were cultured on Murashige and Skoog (MS) medium supplemented with 11.43 μM indole acetic acid (IAA) and 4.44 μM 6-benzylaminopurine (6-BA), the induction rate was 84% for young inflorescence and 100% for rachillae. After the multishoots were subcultured on the rooting MS medium containing 1.08 μM α-naphthalene acetic acid (NAA), the rooting rate was close to 100%. In indirect embryogenesis, embryonic calluses were formed when the culture medium contained 22.22 μM 6-BA and 4.52 μM 2,4-dichlorophenoxy acetic acid (2,4-D), and the induction rate was 92.4% for young inflorescence and 100% for rachillae. After the embryonic calluses were transferred to the medium supplemented with 11.43 μM IAA and 13.33 μM 6-BA, they could develop into plantlets with roots. In assessing the two regeneration pathways in terms of genetic and epigenetic fidelity of the regenerants, two kinds of molecular markers [amplified fragment length polymorphism (AFLP) and methylation-sensitive amplified polymorphism (MSAP)] were employed. The AFLP analysis used 20 primer pairs that yielded 916 scorable bands among the donor plant and 11 regenerants from direct embryogenesis, of which 8 (0.87%) were polymorphic. The regenerants from indirect embryogenesis had 1075 clear bands of which 3 (0.27%) were polymorphic scorable bands from 18 primer pairs. Moreover, the variant band patterns included two types, that is, loss-of-original and gain-of-novel bands. MSAP analysis revealed that tissue culturing of the flower induced DNA cytosine methylation alterations in both CG and CNG levels and patterns compared with the donor plant. The variation rate was 1.1 and 1.3% for the direct and indirect embryogenesis pathways, respectively. The findings show that tissue culture of flowering plants is a form of stress which can induce some heritable epigenetic variations and should be considered in future long-term genotype preservation programs of *Freesia hybrida*.

Keywords *Freesia hybrida* · Somatic embryogenesis · DNA methylation · Somaclonal variation · Genetic and epigenetic stability

X. Gao · D. Yang · M. Ao · X. Sui · Q. Wang · L. Wang (✉)
Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China
e-mail: wangli@nenu.edu.cn

X. Gao
e-mail: gaoxiang424@yahoo.com.cn

D. Cao · J. N. Kimatu
Key Laboratory of Molecular Epigenetics of MOE, Institute of Genetics and Cytology, Northeast Normal University, Changchun 130024, China

Introduction

The *Freesia* species has been grown widely as a cut flower and its popularity is increasing. Current market research shows that over 110 million *Freesia* stems are sold in the UK each year (http://www.flowers.org.uk/public/flower_search_by_name.php). Studies show that it originated from

the southern region of Africa and was first imported to Europe at the end of the 19th century. Now it has become one of the most popular flowers in the world. Somatic embryogenesis has been induced in the tissue culture of diploid *Freesia refracta* either directly from the epidermal cells of young leaves and inflorescences or indirectly via intervening callus (Wang and others 1990). However, tissue culture in the tetraploid *Freesia hybrida*, which accounts for the largest proportion of the *Freesia* cut flowers in the tissue culture market, has not been reported so far.

Knowledge of the optimal conditions favoring differentiation under in vitro conditions is an important step in the application of in vitro manipulation techniques in higher plants. Successful plant regeneration depends on factors such as the genotype, explant type, age of the donor plants, the number of subcultures (Jain 1998; Veilleux and Johnson 1998), and the composition of medium, especially plant growth regulators (PGRs). Two major PGRs are auxin and cytokinin (Skoog and Miller 1957).

In vitro clonal propagation techniques provide the ability to efficiently multiply and maintain large numbers of elite genotypes. However, tissue culture-induced phenotypic and genotypic variations, collectively termed "somaclonal variation," are commonly observed in plants and are at least partly due to in vitro-induced stress (Larkin and Scowcroft 1981; Evans and others 1984). The molecular basis of somaclonal variation is not precisely known; however, both genetic and epigenetic mechanisms are thought to play a role. Somaclonal variation may arise as a result of point mutations, rearrangements in nuclear or organellar DNA (Phillips and others 1994), the activation of mobile elements (Kidwell and Lisch 1997; Kazazian 2004; Liu and others 2004), ploidy (Sunderland 1977; Bayliss 1980; Creissen and Karp 1985), or epigenetic changes (Jaligot and others 2000; Kumar and Mathur 2004; Gostimsky and others 2005; Kuznetsova and others 2005) causing deviations from a desired phenotype quality or standard. In principle, somaclonal variation is defined primarily as epigenetic change that alters gene expression patterns without changes in the DNA sequence (Russo and others 1996; Kaeppler and others 2000). One chief contributor implicated in epigenetics is DNA methylation. In higher plants, cytosine is primarily methylated in a CG dinucleotide context and CNG sites (Gruenbaum and others 1981) and, less abundantly, in CNN sequences (Tariq and Paszkowski 2004).

In this study, the amplified fragment length polymorphism (AFLP) technique was used to investigate genetic changes induced by tissue culture. The AFLP method is based on the principle of selectively amplifying a subset of restriction fragments from a complex mixture of DNA fragments obtained after digestion of genomic DNA with restriction endonuclease (Vos and others 1995). To determine the extent of DNA methylation changes and the presence of methylated CCGG sites, the methylation-sensitive amplified polymorphism (MSAP) technique (Xiong and others 1999) was used; it is a modification of the AFLP method that makes use of the differential sensitivity of a pair of isoschizomers, *Msp*I and *Hpa*II, to cytosine methylation. The objectives of this study were (1) to develop a highly efficient and relatively stable tissue culture protocol for routine plant regeneration in the tetraploid *Freesia hybrida* and (2) to assess the occurrence and extent of genetic and epigenetic instabilities induced by tissue culture using AFLP and MSAP molecular markers so as to be incorporated in future long-term genotype preservation programs of *Freesia hybrida*.

Materials and Methods

Tissue Culture

Two kinds of *Freesia* cultivars were used for tissue culture, red *Freesia hybrida* were for direct embryogenesis and yellow *Freesia hybrida* were for indirect embryogenesis. Young inflorescences with rachillae were obtained from greenhouse-grown plants and were surface sterilized with 70% ethanol followed by immersion in 0.1% mercuric chloride for about 4 min with frequent agitation, and then rinsed five times successively using sterile distilled water. Before being cultivated onto the solid MS medium, the inflorescences and rachillae were dissected and cut into 2-3-mm-thick segments by removal of surrounding leaves. The medium was supplemented with different concentrations of plant growth regulators (PGRs), 2.5% sucrose, and 0.6% agar, and the pH was adjusted to 5.8. All cultures were incubated in growth chambers at 24 ± 3°C, with a 12-h photoperiod and a photon flux density of 1600 $\mu E\ m^{-2}\ s^{-1}$. Subculture was under taken every 5 weeks.

Plantlets regenerated from both direct embryogenesis medium without inducing roots by NAA and indirect embryogenesis medium were used for AFLP and MSAP analysis.

AFLP and MSAP Analysis

Genomic DNA was extracted from expanded leaves of the donor plants and regenerated plants using a modified cetyl trimethyl ammonium bromide (CTAB) method. A standard AFLP protocol with minor modifications was followed (Wang and others 2005). One preselective and 23 selective primer combinations were used (Table 1). MSAP was performed using *Hpa*II and *Msp*I, a pair of isoschizomers that recognize the same restriction site (5′-CCGG) but have

Table 1 Preselective and selective primers used in AFLP and MASP

Preselective primers	
H/M + 0	ATCATGAGTCCTGCTCGG
E + A	GACTGCGTACCAATTCA
M + C	GATGAGTCCTGAGTA
Selective primers	
*Eco*RI + 3 primers	
E-A	GACTGCGTACCAATTCAAC
E-B	GACTGCGTACCAATTCAAG
E-C	GACTGCGTACCAATTCACA
E-D	GACTGCGTACCAATTCACT
E-E	GACTGCGTACCAATTCACC
E–F	GACTGCGTACCAATTCACG
E–G	GACTGCGTACCAATTCAGC
10 *Hpa*II/*MspI* + 3 primers	
H/M1	ATCATGAGTCCTGCTCGGTCT
H/M2	ATCATGAGTCCTGCTCGGTCG
H/M3	ATCATGAGTCCTGTCGGTCC
H/M4	ATCATGAGTCCTGCTCGGTTC
H/M5	ATCATGAGTCCTGCTCGGTTG
H/M6	ATCATGAGTCCTGCTCGGTTA
H/M7	ATCATGAGTCCTGCTCGGTGA
H/M8	ATCATGAGTCCTGCTCGGTGT
H/M9	ATCATGAGTCCTGCTCGGTGC
H/M10	ATCATGAGTCCTGCTCGGTAC
9 *Mse*I + primers	
M1	GATGAGTCCTAGGTA ACA A
M2	GATGAGTCCTAGGTA ACA C
M3	GATGAGTCCTAGGTA ACA G
M4	GATGAGTCCTAG GTA ACAT
M5	GATGAGTCCTAGGTA ACT A
M6	GATGAGTCCTAGGTA ACTC
M7	GATGAGTCCTAGGTA ACTG
M8	GATGAGTCC TAG GTA ACTT
M9	GATGAGTCCTAGGTA ACCA

different sensitivity to methylation of the cytosines. Specifically, *Hpa*II will not cut if either of the cytosines is fully methylated (double-strand); in contrast, *Msp*I will not cut only if the external cytosine is fully or hemimethylated (McClelland and others 1994). Thus, for a given DNA sample, two major methylation states at the CCGG sites, (1) full methylation of the internal C, which are bands absent from *Hpa*II but present in the corresponding *Msp*I digest, that is, pattern H/M = −/+, and (2) hemimethylation of the external C, which are bands present in *Hpa*II but absent from the corresponding *Msp*I digest, that is, pattern H/M = +/−, will be readily recognized in the MSAP profiles (Reyna-Lopez and others 1997; Cervera and others 2002). Nonetheless, full methylation of the external cytosine or both cytosines indigested by both *Hpa*II and *Msp*I are invisible; thus, the level of cytosine is lower than reality. One pair of preselective and 18 pairs of selective primers were used (Table 1). The restriction enzymes *Eco*RI, *Mse*I, *Hpa*II, and *Msp*I and T4 ligase were purchased from New England Biolabs Inc. (Ipswich, MA; http://www.neb.com). The amplification products of AFLP or MSAP were resolved by denaturing polyacrylamide gel electrophoresis (5% acrylamide) and visualized by silver staining. Only clear and completely reproducible bands were scored.

Data Analysis

The AFLP or MSAP bands were scored as a binary character for absence (0) or presence (1). To rule out confounding effects of nucleotide sequence change at the CCGG sites in the computed MSAP data, the method described by Cervera and others (2002) was used.

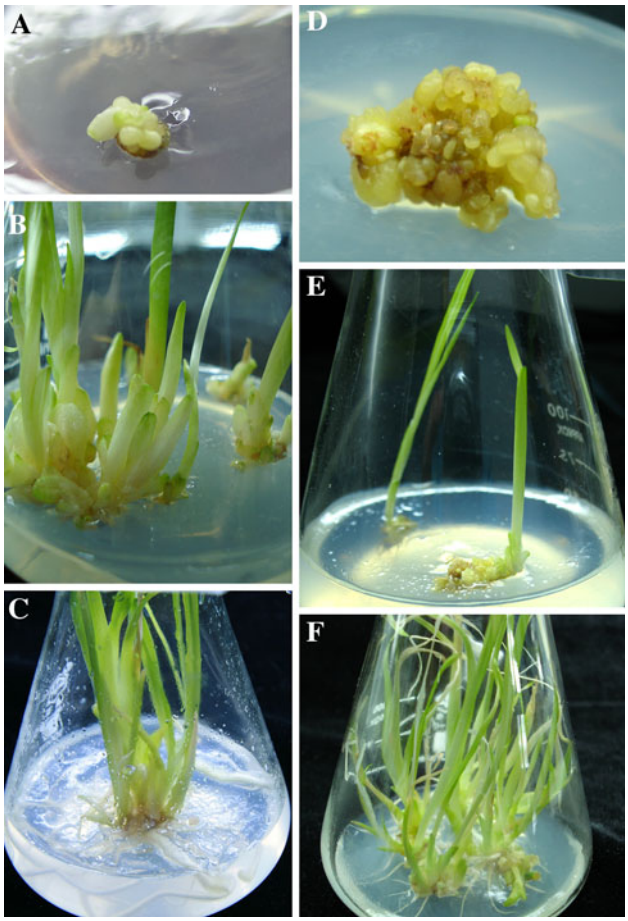

Fig. 1 In vitro culture of *Freesia hybrida* via direct and indirect somatic embryogenesis pathways. **a** Embroyoids. **b** Multishoots. **c** Plantlets with roots. **d** Embryonic callus. **e** Single shoot. **f** Plantlets with roots

Results

In Vitro Micropropagation of *Freesia hybrida*

Explants of *Freesia hybrida* can regenerate plantlets through somatic embryogenesis via two kinds of pathways, that is, directly from the epidermal cells or indirectly from an embryonic callus stage, depending on the exogenous PGRs used in the culture media. We found that segments of young inflorescences and rachillae were responsive for embryoids then developing into multishoots and embryonic calluses. During the direct somatic embryogenesis pathway, globular embryoids (Fig. 1a) emerged at the periphery of explants after 7-10 days in culture on the MS medium containing IAA and 6-BA combinations at different concentrations. After 50 days, multishoots appeared (Fig. 1b). Plantlets with roots (Fig. 1c) were regenerated 30 days after transferring the multishoots onto the MS basal medium containing only 1.08 μM NAA; the rooting rate was close to 100%. When the same explants were cultivated on the medium supplemented with 6-BA and 2,4-D combinations, big masses of pale yellow embryonic calli (Fig. 1d) were induced after 25-30 days in culture; they developed into single or multiple shoots (Fig. 1e) about 30 days after subculturing on the differentiation medium containing 11.43 μM IAA and 13.33 μM 6-BA. Multiple plantlets (Fig. 1f) bearing roots were obtained after one more month in culture.

Table 2 shows that different types and concentrations of PRG combinations may induce different regeneration pathways and the induction rate was also dissimilar. By testing a series of embryoids and callogenic media, we found that three IAA and 6-BA combinations resulted in direct embryogenesis. Indeed, when the IAA:6-BA value was 11.43:4.44 μM, the induction rate was the highest: 84% for young inflorescences and 100% for young rachillae. As for indirect embryogenesis, MS medium supplemented with 6-BA and 2,4-D was found to be more suitable, with the highest induction rate of 92.4% for young inflorescences and 100% for young rachillae. Obviously, the use of young rachis as explants was more efficient than inflorescences in causing induction.

Table 3 Scorable AFLP bands amplified by each of 20 selected primer pairs, and variable bands of 11 regenerated plants relative to the donor plant in *Freesia hybrida* regenerated from the direct somatic embryogenesis pathway

Primer combinations	No. total band scored	No. and frequency of polymorphic band	Loss of original band	Novel band	No. singleton
E-A/M1	35	0	0	0	0
E-A/M2	37	0	0	0	0
E-A/M3	51	1 (2%)	0	1	0
E-A/M5	50	0	0	0	0
E-A/M6	34	2 (5.9%)	0	2	2
E-A/M7	40	1 (2.5%)	0	1	0
E-C/M2	63	0	0	0	0
E-C/M3	61	0	0	0	0
E-C/M4	70	0	0	0	0
E-C/M5	41	0	0	0	0
E-C/M6	30	0	0	0	0
E-C/M7	34	2 (5.9%)	0	2	0
E-C/M8	41	0	0	0	0
E-D/M1	45	0	0	0	0
E-D/M2	48	0	0	0	0
E-D/M3	57	0	0	0	0
E-D/M4	58	0	0	0	0
E-D/M8	54	1 (1.9%)	0	1	1
E-D/M9	22	0	0	0	0
E-E/M8	45	1 (2.2%)	0	1	0
Total	916	8 (0.87%)	0	8	3

Table 2 Patterns of somatic embryogenesis in explants of young inflorescences and rachides of *Freesia hybrida* under MS medium and hormone conditions

Hormones and concentrations (mg l^{-1})			Pattern of induction	Number of explants		Number of explants induced successfully		Induction rate (%)	
IAA	6-BA	2,4-D		YI	YR	YI	YR	YI	YR
2	4	–	D	53	11	35	8	66	73
2	3	–	D	51	11	24	8	47	73
2	1	–	D	44	11	37	11	84	100
–	2.5	0.5	I	50	11	30	10	60	91
–	5	0.5	I	46	10	31	10	67.5	100
–	5	1	I	53	10	49	10	92.4	100
–	10	1	I	49	9	29	8	59.2	89

D direct embryogenesis, *I* indirect embryogenesis, *YI* young inflorescence, *YR* young rachis

Assessment of Genetic and Epigenetic Instabilities in Regenerants of *Freesia hybrida* by Two Different Types of Molecular Markers

AFLP Analysis

Twenty-three primer pairs for selective amplification were selected among 45 combinations (9 *Mse*I + 3 primers combined with 5 *Eco*RI + 3 primers, see Table 1) for AFLP analysis.

Regenerants from Direct Embryogenesis Twenty primer pairs yielded 916 scorable bands when the donor plant and 11 regenerants from direct embryogenesis were analyzed (Table 3), of which 8 (0.87%) were polymorphic. The average number of bands generated by each primer pair was 45.08 (range = 22-70), with an average of 0.4 polymorphic bands (range = 0–2). The polymorphic bands included only gain-of-novel bands (Fig. 2) in the regenerants, and three of the eight novel bands were singletons, that is, variant band(s) present or absent in a single regenerant for a given primer pair.

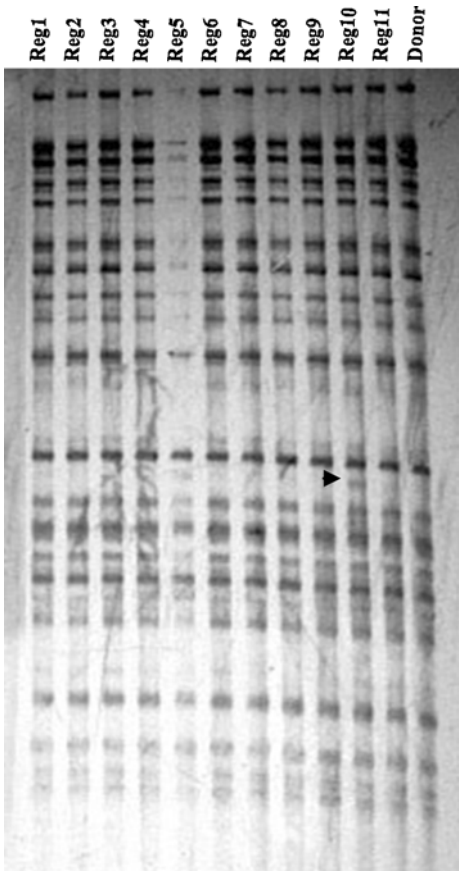

Fig. 2 An example of AFLP profiles of the donor plant and 11 regenerants (Reg 1–11) in *Freesia hybrida* by primer pair *Eco*RI + AAC and *Mse*I + CAG. The type of variant band, that is, gain-of-novel band, is labeled by an arrowhead

Table 4 Scorable AFLP bands amplified by each of 20 selected primer pairs, and variable bands of 11 regenerated plants relative to the donor plant in *Freesia hybrida* regenerated from the indirect somatic embryogenesis pathway

Primer combinations	No. total band scored	No. and frequency of polymorphic band	Loss-of-original band	Novel band	No. singleton
E-A/M1	43	0	0	0	0
E-A/M5	45	2 (4.4%)	0	2	2
E-A/M6	54	0	0	0	0
E-A/M7	50	0	0	0	0
E-C/M2	77	1 (1.3%)	1	0	0
E-C/M3	71	0	0	0	0
E-C/M4	64	0	0	0	0
E-C/M5	59	0	0	0	0
E-C/M6	53	0	0	0	0
E-C/M7	74	0	0	0	0
E-C/M8	83	0	0	0	0
E-D/M1	38	0	0	0	0
E-D/M2	54	0	0	0	0
E-D/M3	63	0	0	0	0
E-D/M4	65	0	0	0	0
E-D/M5	71	0	0	0	0
E-D/M6	55	0	0	0	0
E-D/M7	56	0	0	0	0
Total	1075	3 (0.27%)	1	2	2

Regenerants from Indirect Embryogenesis When regenerants from indirect embryogenesis were analyzed, 1075 with 3 (0.27%) polymorphic scorable bands were obtained using 18 primer pairs (Table 4), and the average number of bands was 59.72 (range = 38-83). Contrasting with the results from direct embryogenesis, the variant band patterns included two types: (1) loss-of-original bands seen in Fig. 3 and (2) gain-of-novel bands, of which two were singletons. Furthermore, we noticed that the variant bands existed only in a few of the regenerants.

MSAP Analysis

Based on the same criteria for AFLP primer selections, 18 selective amplification primer pairs for MSAP were chosen among 70 combinations (10 *Hpa*II/*Msp*I + 3 primers combined with 7 *Eco*RI + 3 primers; Table 1).

Regenerants from Direct Embryogenesis By using 12 pairs of selected *Eco*RI + *Hpa*II/*Msp*I primer combinations, 541-544 clear and reproducible bands were amplified in DNA from the donor plant and 15 regenerants, of which 11 plant samples were used as previously described in the AFLP Analysis section. By tabulating the number of bands

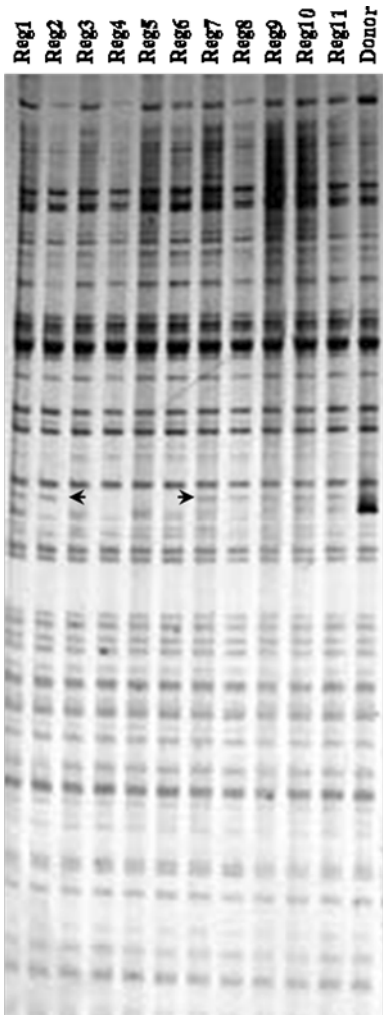

Fig. 3 An example of AFLP profiles of the donor plant and 11 regenerants (Reg 1–11) in *Freesia hybrida* by primer pair *Eco*R-I + ACA and *Mse*I + CAC. The type of variant band, that is, loss-of-novel band, is labeled by an arrow

representing the various types of MSAP pattern, the levels of CG, CNG, and simultaneous CG/CNG methylation were calculated (Table 5). The levels of all three kinds of methylation in the 15 regenerated plantlets tended to be quite similar to the methylation level of the donor plant, though with a little reduction. We also examined the MSAP patterns in a locus-specific manner. Table 6 shows that (i) both hypo- and hypermethylation occurred in regenerated plants, and the total DNA methylation variation rate was 1.1%. (ii) The level of hypomethylation was higher than hypermethylation (0.73 vs. 0.37%). (iii) As for hypomethylation, the number of variant bands for both CNG and CG hypomethylation (3) was greater than for only CG hypomethylation alone (1).

Regenerants from Indirect Embryogenesis Eighteen pairs of selected *Eco*RI + *Hpa*II/*Msp*I primer combinations were used for MSAP analysis. A total of 689 scorable bands were amplified from the same set of plant samples used for AFLP analysis. The number of nonmethylated, CG, CNG, and simultaneous CG/CNG methylation sites was calculated based on MSAP profiles (Table 7). The donor plant had a total methylation level of 9.8%, comprising 6.1, 2.9, and 0.8% at CG, CNG, and simultaneous CG/CNG methylation sites, respectively. Compared with the donor plant, 11 regenerated plantlets showed a similar total methylation level although it was lower (except Reg 10); the lowest was 9.2%.

MSAP profiling also allowed comparison of the cytosine methylation patterns in a locus-specific manner. By using the donor plant as a control, all scorable MSAP loci in the studied samples were classified into four major groups: CG, CNG, CG/CNG hypomethylation, and CG/CNG hypermethylation (Fig. 4). As seen in Table 8, the number of CG, CNG, CG/CNG hypomethylation, and CG/CNG hypermethylation variant bands was 2, 2, 4, and 1, respectively. Obviously, the hypomethylation level was higher than hypermethylation level, and the global methylation variation rate was 1.3%.

Discussion

Culture systems aimed at inducing embryogenesis have been developed for many angiosperm and gymnosperm species (Brown and others 1995; Dunstan and others 1995; Krishnaraj and Vasil 1995; Thorpe and Stasolla 2001). To date, the only conclusive evidence on this subject is that a cell culture has to be grown in a medium containing a high concentration of auxin to become embryogenic. Due to its characteristics as an important cut flower in the world, especially in Europe and America, and the apparent potential held by modern biotechnology for further improvement, establishing an efficient, stable, and simple in vitro micropropagation protocol in *Freesia hybrida* is of interest. This study demonstrates the feasibility of regenerating plantlets from isolated young inflorescences and rachillae via both direct and indirect somatic embryogenesis. Conceivable advantages of using young inflorescences and rachillae as explants may include high totipotency of its constituent primordial cells, easier decontamination, and lower incidence of virus infection in the regenerants.

Plant growth regulators can affect the rate of somaclonal variation both directly (Stover 1987) and indirectly by increasing the multiplication rate and inducing adventitious shoots (Damasco and others 1998; Bairu and others 2006). We observed that the concentrations of 2,4-D played a critical role in inducing embryonic calli and in maintaining regeneration potential during subcultures in *Freesia hybrida*. In addition, IAA and 6-BA were both suitable for direct

Table 5 Cytosine DNA methylation level in 15 regenerated plants (Reg 1-15) relative to the donor plant, based on MSAP analysis using 12 primer pairs in *Freesia hybrida* regenerated from the direct somatic embryogenesis pathway

Sample	Total band	Unmethylated CCGG sites	Methylated CCGG sites			Total
			CG	CNG	CG and CNG	
Donor	541	496 (91.7%)	27 (5.0%)	14 (2.6%)	4 (0.7%)	45 (8.3%)
Reg 1	542	498 (91.9%)	27 (5.0%)	14 (2.6%)	3 (0.5%)	44 (8.1%)
Reg 2	542	498 (91.9%)	27 (5.0%)	14 (2.6%)	3 (0.5%)	44 (8.1%)
Reg 3	542	498 (91.9%)	27 (5.0%)	14 (2.6%)	3 (0.5%)	44 (8.1%)
Reg 4	542	498 (91.9%)	27 (5.0%)	14 (2.6%)	3 (0.5%)	44 (8.1%)
Reg 5	542	496 (91.5%)	29 (5.0%)	14 (2.6%)	3 (0.5%)	46 (8.5%)
Reg 6	542	498 (91.9%)	27 (5.0%)	14 (2.6%)	3 (0.5%)	44 (8.1%)
Reg 7	542	498 (91.9%)	27 (5.0%)	14 (2.6%)	3 (0.5%)	44 (8.1%)
Reg 8	544	500 (91.9%)	27 (5.0%)	16 (2.9%)	1 (0.2%)	44 (8.1%)
Reg 9	543	499 (91.9%)	27 (5.0%)	15 (2.8%)	2 (0.3%)	44 (8.1%)
Reg 10	542	498 (91.9%)	27 (5.0%)	14 (2.6%)	3 (0.5%)	44 (8.1%)
Reg 11	543	499 (91.9%)	27 (5.0%)	15 (2.8%)	2 (0.3%)	44 (8.1%)
Reg 12	544	500 (91.9%)	27 (5.0%)	16 (2.9%)	1 (0.2%)	44 (8.1%)
Reg 13	542	498 (91.9%)	27 (5.0%)	14 (2.6%)	3 (0.5%)	44 (8.1%)
Reg 14	542	498 (91.9%)	27 (5.0%)	14 (2.6%)	3 (0.5%)	44 (8.1%)
Reg 15	542	498 (91.9%)	27 (5.0%)	14 (2.6%)	3 (0.5%)	44 (8.1%)

embryogenesis and transferring indirect embryogenesis to the direct pathway. Similar studies were done in *E. pulcherrima* (Osternack and others 1999), *Dendrathema grandiflora* (May and Trigiano 1991), and in several other cereal crops, including barley and maize (Bregitzer and others 1995; Bohorova and others 1995; Carvalho and others 1997).

We detected both genetic and epigenetic changes in randomly chosen regenerants of *Freesia hybrida*, although the possibility that such changes were due to preexisting heterozygosity or natural mutations in the explants was minimized by using explants taken from a single donor plant (Meins 1983). Therefore, it can be concluded that all scored changes were more likely to have resulted from genetic and epigenetic instabilities induced by the tissue culture stress process. Similar research has been done in callus and regenerants of maize (Brown 1989; Brown and others 1991; Kaeppler and Phillips 1993b), in progeny of regenerated rice plants (Brown and others 1990; Muller and others 1990), and in a continuously proliferating, dedifferentiated cell suspension culture of *Arabidopsis* (Tanurdzic and others 2008). Usually AFLP is more likely to find band losses, but minor losses were detected in our research; thus, there is the very likely possibility that this *Freesia* is tetraploid. In mammals, DNA methylation is normally confined to one of the alleles related to imprinting and X-chromosome inactivation (Feil 2006). When four alleles are present in *Freesia*, most losses of bands may not be visible as other copies of the alleles are still present.

Somaclonal variation has been documented to occur frequently in plant tissue culture but the underlying mechanism remains largely obscure (Kaeppler and others 2000; Xu and others 2004; Peredo and others 2006). The heritability of epigenetic changes can be thought of as a precursor for genetic change, especially when stress conditions are maintained. Furthermore, the epigenetic mechanisms play a fundamental role in somaclonal variation in that they may induce a broad range of genomic mutations, from single-point mutations to chromosome breakage and polyploidy (Kaeppler and others 2000). Hence, epigenetic variation is deemed to be more frequent than genetic variation. Indeed, this was the observed scenario in this study of the in vitro micropropagation protocol of *Freesia hybrida* in which we found methylation polymorphism to be greater than DNA polymorphism. For example, during indirect embryogenesis, the level of methylation variation was 1.3% versus 0.27% in DNA polymorphism. The same results were obtained in rice (Ashikawa 2001), *Arabidopsis thaliana* (Cervera and others 2002), cotton (Keyte and others 2006), and maize (Kaeppler and Phillips 1993b). On the other hand, there are several studies indicating that there are intimate correlations between frequencies of genetic changes and DNA methylation alterations (Guo and others 2006, 2007; Li and others 2007). This suggests that the genetic and epigenetic instabilities occurring under tissue culture conditions may share a common mutagenic mechanism.

The induction of somatic embryogenesis consists of the termination of a current gene expression pattern in the explant's tissue and its replacement with an embryogenic gene expression program. It has been proposed that PGRs play a central role in mediating the signal transduction

Table 6 Alteration in cytosine DNA methylation pattern in regenerated plants (Reg 1-15) relative to the donor plant, based on MSAP analysis using 12 primer pairs in *Freesia hybrida* regenerated from the direct somatic embryogenesis pathway

Pattern	Donor				Regenerated plants															
					No. of sites and frequencies (%)															
	H	M	H	M	Reg 1	Reg 2	Reg 3	Reg 4	Reg 5	Reg 6	Reg 7	Reg 8	Reg 9	Reg 10	Reg 11	Reg 12	Reg 13	Reg 14	Reg 15	Total
Both hypo	−	−	+	−	0	0	0	0	0	0	0	2 (0.37)	1 (0.18)	0	1 (0.18)	1 (0.18)	0	0	0	4 (0.73)
	−	−	+	+	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	1 (0.18)	
CNG hypo	−	−	−	+	0	0	0	0	0	0	0	0	0	0	0	1 (0.18)	0	0	0	
CG hyper	+	+	−	+	0	0	0	0	2 (0.37)	0	0	0	0	0	0	0	0	0	0	2 (0.37)
Total																				1.1

Table 7 Cytosine DNA methylation level in 11 regenerated plants (Reg 1-11) relative to the donor plant, based on MSAP analysis using 12 primer pairs in *Freesia hybrida* regenerated from the indirect somatic embryogenesis pathway

Sample	Total band	Unmethylated CCGG sites	Methylated CCGG sites			Total
			CG	CNG	CG and CNG	
Donor	684	617 (90.2%)	42 (6.1%)	20 (2.9%)	5 (0.8%)	67 (9.8%)
Reg 1	684	618 (90.4%)	41 (5.9%)	20 (2.9%)	5 (0.8%)	66 (9.6%)
Reg 2	684	619 (90.5%)	40 (5.8%)	20 (2.9%)	5 (0.8%)	65 (9.5%)
Reg 3	686	623 (90.8%)	40 (5.9%)	20 (2.9%)	3 (0.4%)	63 (9.2%)
Reg 4	686	621 (90.5%)	41 (6.0%)	21 (3.1%)	3 (0.4%)	65 (9.5%)
Reg 5	686	620 (90.4%)	41 (6.0%)	22 (3.2%)	3 (0.4%)	66 (9.6%)
Reg 6	686	620 (90.4%)	42 (6.1%)	22 (3.2%)	2 (0.3%)	66 (9.6%)
Reg 7	684	618 (90.4%)	41 (5.9%)	21 (3.1%)	4 (0.6%)	66 (9.6%)
Reg 8	686	624 (90.7%)	41 (6.0%)	20 (2.9%)	3 (0.4%)	64 (9.3%)
Reg 9	684	618 (90.4%)	41 (5.9%)	20 (2.9%)	5 (0.8%)	66 (9.6%)
Reg 10	683	616 (90.2%)	41 (6.0%)	20 (2.9%)	6 (0.9%)	67 (9.8%)
Reg 11	684	618 (90.4%)	41 (5.9%)	20 (2.9%)	5 (0.8%)	66 (9.6%)

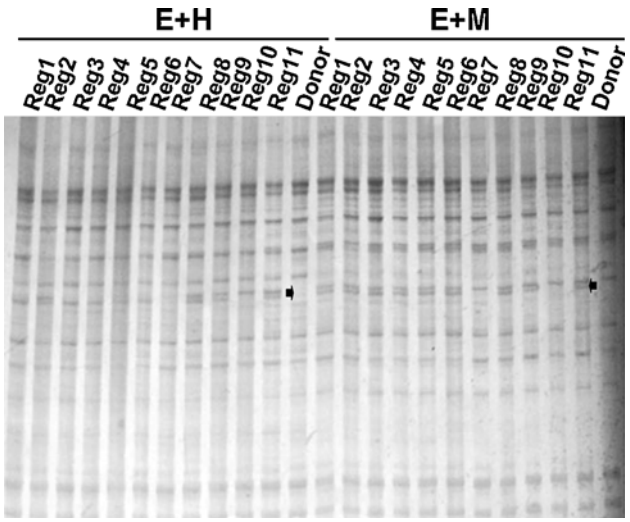

Fig. 4 An example of MSAP profiles of the donor plant and 11 selected regenerants (Reg 1-11) in *Freesia hybrida* by primer combination *Eco*RI + ACG/*Hpa*II(*Msp*I) + TTA. E + H, the combination of *Eco*RI and *Hpa*II; E + M, the combination of *Eco*RI and *Msp*I. Some typical methylation and changing methylation patterns, as detailed in the text, are marked by arrowheads

cascade leading to the reprogramming of gene expression (Arnold and others 2002). One possible mechanism for regulation of current gene expression is DNA methylation, which is influenced by auxins (LoSchiavo and others 1989; Chakrabarty and others 2003). Similar methylation alterations have also been observed in rose somatic embryos (Xu and others 2004), potato microplants (Joyce and Cassells 2002), germinating pepper seeds (Portis and others 2004), micropropagated banana (Peraza-Echeverria and others 2001; Baurens and others 2003), and oil palm (Jaligot and others 2000). Some alterations were reported to have occurred upon exposure to in vitro conditions (Phillips and others 1994) and some by wounding (Kaeppler and Phillips 1993a). The alteration in cytosine methylation level in *Freesia hybrida* occurred in regenerants, with a general trend of decreased methylation in the second plantlet regenerating pathway. Similar findings of a general decrease in methylation levels associated with tissue culture have been reported previously in several plants, including maize (Kaeppler and Philipps 1993b), soybean (Quemada and others 1987), oil palm (Jaligot and others 2000; Matthes and others 2001), and barley (Li and others 2007), although increased methylation was also reported in some cases (Cecchini and others 1992; Smulders and others 1995; Parra and others 2001; Xu and others 2004). Recent studies by Tanurdzic and others (2008) have shown that stress due to cell culture causes epigenetic changes in which the euchromatin becomes hypermethylated while some hypomethylation takes place in the heterochromatin indicating a search for a new genomic stability after the induced stress.

Table 8 Alteration in cytosine DNA methylation pattern in regenerated plants (Reg 1-11) relative to the donor plant, based on MSAP analysis using 18 primer pairs in *Freesia hybrida* regenerated from the indirect somatic embryogenesis pathway

Pattern	Donor				Regenerated plants											
					No. of sites and frequences (%)											
	H	M	H	M	Reg 1	Reg 2	Reg 3	Reg 4	Reg 5	Reg 6	Reg 7	Reg 8	Reg 9	Reg 10	Reg 11	Total
Both hypo	−	−	+	−	0	0	1 (0.15)	1 (0.15)	2 (0.29)	2 (0.29)	1 (0.15)	0	0	0	0	8 (1.16)
	−	−	+	+	1 (0.15)	1 (0.15)	2 (0.29)	2 (0.29)	1 (0.15)	1 (0.15)	0	3 (0.44)	1 (0.15)	0	1 (0.15)	
CNG hypo	−	−	−	+	0	0	0	0	0	1 (0.15)	0	0	0	0	0	
	+	−	+	+	0	0	1 (0.15)	0	0	0	0	0	0	0	0	
CG hypo	−	+	+	+	1 (0.15)	2 (0.29)	2 (0.29)	1 (0.15)	1 (0.15)	1 (0.15)	1 (0.15)	1 (0.15)	1 (0.15)	1(0.15)	1 (0.15)	
Both hyper	+	+	−	−	1 (0.15)	1 (0.15)	1 (0.15)	1 (0.15)	1 (0.15)	1 (0.15)	1 (0.15)	1 (0.15)	1 (0.15)	1(0.15)	1 (0.15)	1 (0.14)
Total																1.3

In general, the levels of cytosine methylation polymorphism and DNA polymorphism were very low in this study: 1.1 and 0.87% and 1.3 and 0.27% for direct and indirect embryogenesis, respectively. Hence, it can be concluded that the mode of regeneration does not have a significant effect on the balance between sequence and methylation state changes induced by a tissue culture process. This finding contradicts the commonly held belief that plants regenerated without a passage through a callus phase are less prone to somaclonal variation than those emerging after a period in callus (Thomas and others 1982; Young and others 1999).

In conclusion, to the best of our knowledge this is the first report on tetraploid *Freesia hybrida* in vitro micropropagation that comprises two efficient and simple protocols via direct and indirect somatic embryogenesis pathways. Furthermore, AFLP and MSAP analysis showed that regenerants were true-to-type of their donor plants with respect to genetic and epigenetic fidelity (Li and others 2006).

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