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Application of AFLPs to characterize somaclonal variation in anther-derived grapevines

C. F. Popescu[1)], A. Falk[2)] and K. Glimelius[2)]

[1)] Research Station for Viticulture Stefanesti-Arges, Romania
[2)]Genetic Centre, Uppsala, Sweden

Summary

The AFLP (amplified fragment length polymorphism) technique was used to characterize genetic variation in grapevine (*Vitis vinifera* L.) regenerated from anther culture. 12 plants obtained by direct embryogenesis from cv. Valerien, and twelve plants obtained by indirect embryogenesis from cv. Mission were evaluated by AFLP. For each genotype the results were analyzed in comparison to control, represented by one field-grown plant (anther-donor).

In contrast to cv. Mission, where no difference in polymorph pattern was observed in the DNA restricted with *Pst*I/*Mse*I, in cv. Valerien we found bands with different distribution among anther-derived plants and between them and the mother plant.

When the DNA samples were digested with *Eco*RI in combination with methylation sensitive restriction enzymes *Msp*I or *Hpa*II, the AFLP products showed a higher polymorphism in cv. Mission with respect to the number of specific bands in correlation with the age of culture at the moment of regeneration from anthers. Direct embryogenesis from anther culture of cv. Valerien was associated with genetic variation induced in a very early stage of *in vitro* culture, while the indirect somatic embryogenesis, specific for cv. Mission and/or long-term culture was accompanied by changes in the methylation status. There is some evidence that all the analyzed grapevine somaclones regenerated from *in vitro*-cultured anthers are genetically distinct from the original cultivars.

K e y w o r d s : *Vitis,* AFLPs, anther culture, direct and indirect embryogenesis.

Introduction

Investigation of natural and induced genetic diversity and of genetic relatedness among cultivars is essential for evaluation of the available grapevine germplasm, and for increasing the efficiency of breeding programs. In the last two decades, plant tissue culture proved to be a potential source of genetic variation and, therefore, a possible mean for selecting new valuable genotypes among the regenerated somaclones exhibiting stable modification of a trait.

Although regeneration from various somatic tissues was shown to be possible for grapevine by employing a variety of *in vitro* methods, such as meristem culture (Alleweldt 1987; Mullins 1990; Torregrosa 1995; Torregrosa *et al.* 2001), somatic embryogenesis (Gray and Meredith 1992; Popescu 1999; Martinelli and Gribaudo 2001), protoplast regeneration (Papadakis *et al.* 2001), only little information was provided about phenotype variation and genetic variability in regenerated somaclones, presumably due to the high degree of morphological uniformity during the juvenile phase (Bouquet and Davis 1989; Fallot *et al.* 1989). Moreover, the few investigations on these aspects did not focus on the detection of genetic changes in regenerated grapevines, but only on relatedness to developmental competence of cells in dedifferentiated calli and established cultures (Schneider *et al.* 1996). On the basis of ampelographical criteria it has been concluded that *in vitro*-regenerated somaclones are very similar, but this has not been proved at the genetic level.

Molecular techniques, which can accurately detect any modification at the DNA level, are used increasingly to characterize grapevine germplasm collections, since there is general agreement that morphological markers are far from being as informative as molecular markers (Crespan and Milani 2001). In recent years, because the use of PCR- based molecular tools allows detection of DNA polymorphism at random or specific *loci* in genomic DNA, molecular markers were applied as objective tools for genotype identification (Striem *et al.* 1994; Thomas *et al.* 1994; Bowers and Meredith 1997), for establishing the progenitors of cultivars and their pedigree (Sefc *et al.* 1998; Verdison *et al.* 1999; Vidal *et al.* 1999), or for reconstruction of the events which led to certain cultivars (Regner *et al.* 1996; Dettweiler *et al.* 2000). Among molecular techniques, AFLP has been proved to be one of the most efficient tools for cultivar identification in germplasm collection (Cervera *et al.* 1998), in phylogenetic studies (Sensi *et al.* 1996), or to establish the geographic origin of grapevine cultivars (Labra *et al.* 1999).

In the present paper, we studied genetic variation and/or true-to-type for grapevines regenerated from cultured anthers, by using the AFLP technique. Results on the influence of direct or indirect embryogenesis on genetic variation in regenerated plants are also presented.

Material and Methods

P l a n t m a t e r i a l : Field-grown Mission and Valerien vines, representing the donors (controls) and potted plants regenerated by anther culture from single donor plants were used. For Valerien the 12 analyzed plants were obtained by direct embryogenesis after 4 months of culture, while for Mission the 12 anther-derived plants were regenerated by

Correspondence to: Dr. C. Popescu, Research Station for Viticulture, Stefanesti, 0343, Arges, Romania. Fax: +40-248-266808.

indirect embryogenesis after 6, 9, 12, or 15 months of culture. The protocol used for somatic embryogenesis was previously described by POPESCU (2001).

Shoot apex cultures, from both controls and somaclones, were initiated on Murashige-Skoog medium (MURASHIGE and SKOOG 1962) supplemented with 0.5 mg l^{-1} benzyladenine (BA), 0.5 mg l^{-1} indolyl-3-acetic acid (IAA), 2 % sucrose, and solidified with 3 % gelrite. The cultures were maintained in a growth room at 24 °C at a 16 h photoperiod. The micropropagated plants were subsequently maintained *in vitro* by periodical transfer to the same medium in order to obtain enough material for DNA extraction from each plant and in consecutive subcultures. DNA samples were used to assess genetic variation (if any) in the anther-regenerated plants, and to test the genetic fidelity during their micropropagation.

DNA extraction: Genomic DNA from *in vitro*-multiplicated plants was isolated according to a modified CTAB (cetyl-trimethyl-ammonium bromide) method (LODHI *et al*. 1994). DNA concentration was quantified by measuring absorption at 260 and 280 nm; its quality was estimated after staining with ethidium bromide and electrophoresis in agarose gel (0.8 %).

AFLP assay: AFLP analysis was performed according to VOS *et al.* (1995), with a few modifications. Genomic DNA (1 µl per sample) was digested with a mixture of restriction enzymes (*Pst*I with *Mse*I and *Eco*RI with *Msp*I or *Hpa*II) and incubated at 37 °C for 4 h. After 1 h of digestion the restricted fragments were ligated with specific double-strand adapters (10 pmol μl^{-1}) in order to provide known sequences for amplification and the incubation was further continued at 37 °C for 3 h. Template DNA (0.75 µl) together with the preamplification mixture [8 pmol μl^{-1} of specific AFLP primers, 2 mM of each dNTP, 1 U μl^{-1} *Taq* DNA and 10x PCR buffer] was amplified in 30 cycles of denaturation (30 s at 94 °C), annealing (30 s at 60 °C) and extension (60 s at 70 °C). The reaction mixture was diluted 6 times with double distilled water and 4 µl were used for selective amplification with the same two primers used in the first amplification but containing three more selective nucleotides. One of the primers E10 or P10 (8 pmol per µl) was endlabelled with γ-^{32}P-ATP. The selective amplification was carried out using one labelled primer and one of the primers (M32 or M33) complementary to *Mse*I adapter. The PCR conditions for the second amplification were those recommended by VOS *et al.* (1995). For analysis of DNA amplification products, 4 µl PCR amplified mixture was added to an equal volume of loading buffer (94 % formamide, 10 mM EDTA, 0.5 mg·ml^{-1} bromophenol blue and 0.5 mg·ml^{-1} xylene cyanol) denatured for 5 min at 90 °C and loaded onto a 5 % polyacrylamide gel. After electrophoresis in TBE buffer for 3 or 4 h at 100 W, the fingerprint patterns were visualized on an X-ray film.

Data analysis: The AFLP bands were scored as present or absent. The percentage of polymorphic bands was calculated for each plant dividing the specific bands (SB) of its restricted DNA in comparison to each other DNA sample by the total number of bands obtained with one primer (32 or 33).

For each pair of genotype the coefficient of genetic similarity (GS) was calculated. According to DICE (1945) the GS between pairs of somaclones and between control and somaclones, respectively, represents the value of the following equation: 2Nab/(Na+Nb), where Nab is the number of shared bands (either present or absent), while Na and Nb represent the total number of bands detected in plant a and b, respectively. The degree of homology is reflected in values ranging between 1 (identity of the two genotypes) and 0 (no link between the two genotypes).

Results

AFLP analysis: The first attempt to detect genetic variation in anther-derived grapevines, was carried out with DNA isolated from both donor and anther regenerated plants and restricted with *Pst*I and *Mse*I. Although the gels showed many common bands, cv. Valerien showed different distribution of DNA fragments among anther-derived plants, and also between these and the donor plant. The number of clearly amplified bands per sample varied between 101 and 117, depending on primer. As shown in Tab. 1, the percentage of specific bands was higher with primer 32 in comparison with primer 33. All the variants were polymorphic among one another and the control, the variations ranging between 4.8 % and 26.0 % with primer 33, and between 9.2 % and 23.7 % with primer 32. The number of polymorphic bands was correlated with the GS coefficient among somaclones and control, and revealed averages of 0.77 (primer 32) and 0.84 (primer 33), respectively (Tab. 2). Individual values, calculated from pairs of genotypes, ranged from 0.71 (control-somaclone 8) to 0.98 (somaclones 11-12) with primer 32, and from 0.69 (somaclones 7-8) to 1.0 with primer 33.

The results obtained with the two different primers indicated that the highest number of polymorphic bands and the lowest coefficients of GS were scored in DNA samples extracted from plants 1, 4, 7, and 8. A similar degree of variability (SB and GS) between somaclones and among these and the control were revealed with DNA extracted after 3 and 6 months of culture on multiplication media.

On the contrary, with cv. Mission no difference in the polymorphic pattern was observed among plants regenerated from different calli, or in comparison with the control plant with any of the primers used.

Only 4 plants belonging to cv. Valerien, showing the highest polymorphism (somaclones 1, 4, 7, and 8), and 4 plants belonging to cv. Mission (somaclones VI, IX, XII, XV) regenerated from anthers after different periods of culture (6, 9, 12, or 15 months), were selected for further analysis. When the DNA extracted from these plants was digested with *Eco*RI in combination with methylation sensitive restriction enzymes *Msp*I or *Hpa*II, the AFLP products showed that the degree of variability was higher in cv. Mission with a total number of 149 specific bands, in comparison to only 116 specific bands for cv. Valerien. Another distinctive feature of DNA pattern in cv. Mission was that the anther-derived plants obtained after 6 and 9 months of culture exhibited the highest number of specific bands.

The two methylation-sensitive enzymes used in this experiment, *Msp*I and *Hpa*II, recognize the same restriction

Table 1

Polymorphic bands (%) detected with two primers for 12 tested plants regenerated from anther culture in cv. Valerien, among one another and the control

Plant No.	Control	Primer 32 1	2	3	4	5	6	7	8	9	10	11	12
1	**24.8**	x											
2	22.2	11.1	x										
3	21.4	11.1	2.5	x									
4	**32.5**	21.4	19.6	20.5	x								
5	17.9	14.5	5.9	5.1	22.2	x							
6	20.5	10.2	6.8	5.9	17.1	7.7	x						
7	**23.9**	10.2	10.5	7.7	16.2	11.1	3.4	x					
8	**25.6**	28.2	22.2	23.9	29.0	17.9	22.2	25.6	x				
9	19.5	10.2	8.5	7.7	16.2	9.4	6.8	6.8	23.1	x			
10	20.5	0.8	2.6	18.8	15.9	6.8	10.5	10.2	21.3	8.5	x		
11	20.5	8.5	7.7	5.9	17.9	5.9	5.1	5.1	22.2	3.4	7.7	x	
12	19.6	9.4	8.5	6.8	18.8	5.1	5.9	5.9	23.1	4.3	8.5	0.8	x
Avg.	**22.4**	**13.4**	10.7	11.4	**20.6**	11.8	10.2	**11.4**	**23.7**	10.4	11.0	9.2	9.7

Plant No.	Control	Primer 33 1	2	3	4	5	6	7	8	9	10	11	12
1	**22.8**	x											
2	25.7	3.0	x										
3	25.7	3.0	0.9	x									
4	**28.7**	6.9	2.0	2.9	x								
5	24.7	2.9	0.9	0.9	2.9	x							
6	25.7	2.9	0.9	0.9	2.9	0.9	x						
7	**26.7**	1.9	0.9	0.9	2.9	1.9	1.9	x					
8	**33.6**	20.8	16.8	1.8	18.8	17.8	16.8	20.8	x				
9	25.7	4.9	3.0	3.0	2.9	0.9	0.9	4.9	16.8	x			
10	25.7	4.0	5.0	2.0	4.9	1.9	1.9	4.9	16.8	0.9	x		
11	23.7	4.0	5.0	2.0	4.9	1.9	1.9	1.9	19.8	1.9	0.9	x	
12	23.7	4.0	5.0	2.0	4.9	1.9	1.9	1.9	19.8	1.9	0.9	0.9	x
Avg.	26.0	**6.7**	5.7	5.2	**7.1**	4.8	4.8	**5.9**	**19.6**	5.6	5.8	5.7	5.7

site (CCGG), but differ in their ability to digest DNA, depending on the methylation status of the recognition sequence. Thus, *Hpa*II does not cleave the restriction site that contains one or both methylated cytosine, while *Msp*I recognizes the site with internal cytosine methylated in position C4 or C5. According to these properties, the number of DNA fragments were the same (no methylation), or higher (a specific methylation) in the case of digestion with *Eco*RI in combination with *Msp*I in comparison to digestion with *Eco*RI and *Hpa*II.

The AFLP profiles with primers 32 and 33 revealed differences among control and somaclones for both cultivars (Figure). When analyzing the distribution of DNA fragments, three situations are distinguished (shown by arrows):

- the bands are present in both cases of digestion (*Eco*RI + *Msp*I and *Eco*RI + *Hpa*II) = the recognition site does not contain methyl group (arrow 1),

- the restriction fragment is present in DNA digested with *Eco*RI + *Msp*I and absent in the case of digestion with *Eco*RI + *Hpa*II = the restriction site is methylated to internal cytosine that is recognized by *Msp*I, but not by *Hpa*II (arrow 2),

- lack of bands = methylation takes place in a position that blocks the cleavage of both enzymes, meaning to external C (arrow 3).

Analyzing the disposition of bands in gel, it was clear that the two genotypes presented specific reactions. In cv. Valerien, beside the three types of restricted fragments discussed above, specific bands were noticed in plants regenerated from anthers, but not in the control plant (arrow 4). Also, selected plants 4 and 8 presented specific fragments that were not found in DNA from the control and from the other two plants (arrow 5).

In the DNA samples of cv. Mission, the AFLP patterns showed a specific distribution of restriction bands depending on the moment of regeneration from anther culture or the length of calli phase. In comparison to DNA from the donor plant, the samples from anther-derived plants were distin-

Table 2

Coefficients of genetic similarity determined on pairs of anther-derived plants (somaclones 1, 2,…12) and donor plant (control) in cv. Valerien, according to DICE (1945)

Plant No.	Control	Primer 32 1	2	3	4	5	6	7	8	9	10	11	12
1	**0.74**	x											
2	0.78	0.89	x										
3	0.76	0.91	0.97	x									
4	**0.73**	0.86	0.88	0.85	x								
5	0.79	0.95	0.93	0.97	0.93	x							
6	0.81	0.87	0.96	0.97	0.90	0.98	x						
7	**0.80**	0.92	0.92	0.94	0.81	0.91	0.93	x					
8	**0.71**	0.71	0.78	0.76	0.74	0.75	0.78	0.77	x				
9	0.81	0.96	0.93	0.95	0.91	0.99	0.89	0.92	0.77	x			
10	0.77	0.89	1.0	0.97	0.88	0.95	0.96	0.85	0.79	0.93	x		
11	0.81	0.95	0.93	0.96	0.89	0.93	0.94	0.94	0.77	0.96	0.94	x	
12	0.81	0.93	0.92	0.95	0.87	0.91	0.97	0.93	0.77	0.95	0.93	0.98	x
Avg.	0.77	**0.88**	0.91	0.91	**0.85**	0.91	0.91	**0.89**	**0.76**	0.91	0.91	0.92	0.91

Plant No.	Control	Primer 33 1	2	3	4	5	6	7	8	9	10	11	12
1	**0.86**	x											
2	0..85	0.94	x										
3	0.85	0.94	1.0	x									
4	**0.82**	0.92	0.92	0.92	x								
5	0.84	0.94	0.96	0.96	0.94	x							
6	0.84	0.94	0.96	0.96	0.94	1.0	x						
7	**0.85**	0.98	0.98	0.96	0.92	0.93	0.93	x					
8	**0.72**	0.73	0.71	0.72	0.73	0.76	0.76	0.69	x				
9	0.84	0.93	0.96	0.95	0.94	1.0	1.0	0.93	0.80	x			
10	0.86	0.94	0.94	0.96	0.89	0.92	0.93	0.94	0.72	0.95	x		
11	0.86	0.94	0.95	0.96	0.89	0.92	0.93	0.94	0.72	0.95	1.0	x	
12	0.86	0.94	0.95	0.96	0.89	0.92	0.93	0.94	0.72	0.95	1.0	1.0	x
Avg.	0.84	**0.92**	0.93	0.93	**0.89**	0.92	0.93	**0.91**	**0.73**	0.93	0.92	0.92	0.92

guished by the presence of specific restriction fragments, which were different for the plants regenerated within the first 9 months (arrow 6), and for those regenerated after a longer period of culture (arrow 7).

Discussion

A common and at the same time disappointing characteristic of *in vitro*- regenerated grapevines is the uniformity of ampelographic traits in field-grown plants during the first two years and, as a consequence, the impossibility to select new clones in an early stage of development by using morphological markers.

In this study, somaclonal variation among anther-derived grapevines could be distinguished at the molecular level by using the AFLP technique. Since the two genotypes exhibited different potentialities for regeneration from this type of explant, the genetic variability was expressed in a specific manner. This method proved to be efficient to detect uniformity and/or variability in plants regenerated by both direct and indirect embryogenesis, and to confirm our previous hypotheses on genetic variation induced during developmental process *in vitro* (POPESCU 1996).

Using two primers and different types of restriction enzymes, it was possible to reveal the amplitude of gene polymorphism in grapevine plants regenerated from anther-derived callus. Moreover, the AFLP technique allowed us to demonstrate that the amplitude of genetic variation in regenerated plants depends on the genotype and the way of regeneration.

DNA digested with *Pst*I and *Mse*I yields a large number of polymorphic bands per primer combination, being highly efficient for detection of genetic variation, but only in cv. Valerien. The differences between the mother-plant and the anther-derived somaclones, quantified by the percentage of specific bands and the coefficient of genetic similarity, reflected the diversity of the new regenerated plants as a result either of their somatic origin from different anthers (or anther tissues), or mutation events induced during

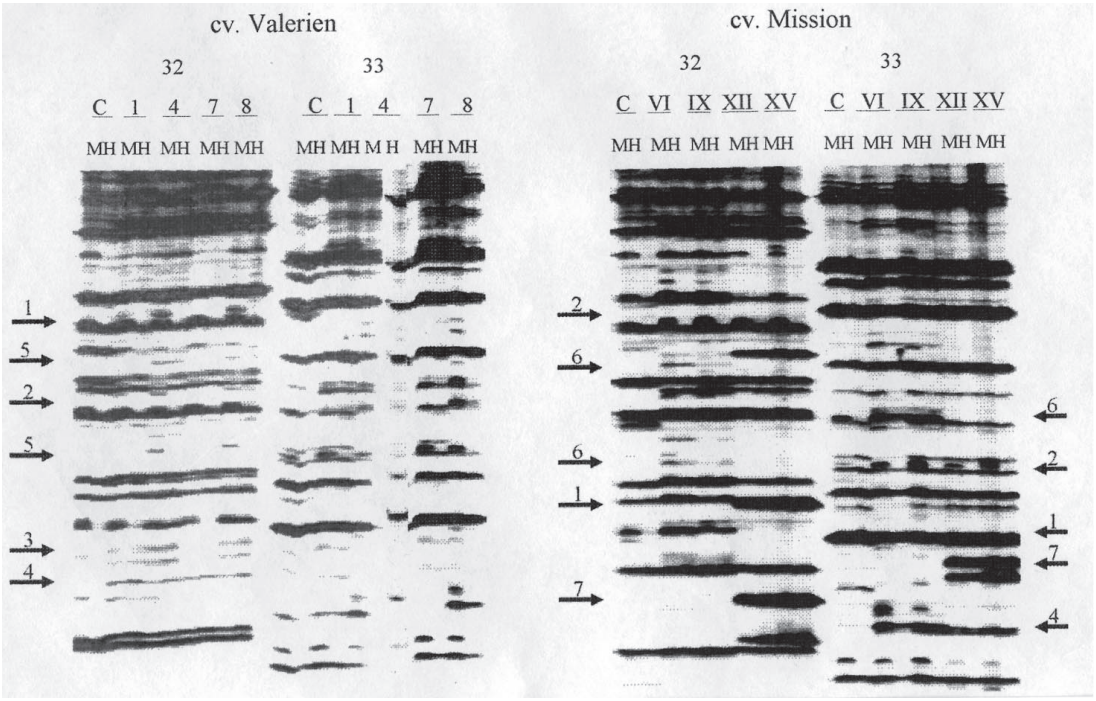

Figure: Detail of an autoradiogram showing the segregation of AFLP markers in cvs Valerien and Mission with primers 32 and 33. Plant samples: control (C) and plants no. 1, 4, 7 and 8 belonging to cv. Valerien, respectively control (C) and plants regenerated after 6, 9, 12 and 15 months belonging to cv. Mission. For each plant the first lane corresponds to DNA sample restricted with EcoRI and MspI (M) while the second lane corresponds to a DNA restricted with EcoRI and HpaII (H). Segregating AFLP markers are indicated by arrows.

in vitro regeneration. These differences between control and the regenerated plants could also be linked with the embryogenic type of regeneration (all the somaclones) which is generally considered to be proned to mutation (Gupta and Varshney 1999), involving specific genetic factors for cell division or *de novo* differentiation and a long period of culture. Since it was emphasized that in AFLP analysis GS values between 0.70 and 0.90 are indicating different varieties (Cervera *et al.* 1998), our data with cv. Valerien seem to provide evidence that all the analyzed grapevine somaclones regenerated from *in vitro*-cultured anthers are genetically distinct from the original cultivar. The most significant differences were found in somaclones 1, 4, 7, and 8, which were shown to be also distinctly different from the other anther-derived somaclones regenerated from the same cultivar by direct embryogenesis.

The DNA samples extracted from *in vitro*-multiplied plants after another 3 and 6 months, showed the same amplitude of polymorphism expressed by the number of amplified fragments different from the control. This underlines that the detected genetic variation resulted from genetic changes in the original plant material (anther-derived plants) and was not induced during *in vitro* multiplication (starting from apices of the acclimated plants).

We were surprised that the same two primers failed to generate polymorphism among Mission somaclones and that there was no difference to the control, although the plants were regenerated by indirect embryogenesis after long and different periods of culture (6, 9, 12 and 15 months after anther culture initiation). The main conclusion from these first results was that direct embryogenesis from anther culture of cv. Valerien was clearly accompanied with genetic variation induced in a very early stage of *in vitro* culture, while indirect embryogenesis from anthers of cv. Mission was associated with genetic uniformity.

The high number of bands obtained by using *Pst*I and *Mse*I for digestion and the lack of variability in DNA samples from the cv. Mission were the main reasons to take into account the possibility to obtain a lower number of restriction fragments, and presumable methylation changes during *in vitro* regeneration. The AFLP pattern with the distribution of restriction fragments gives a real image of this complex process and opens prospects to screen somaclones for genetic variation. The higher number of detectable and specific bands obtained with both primers and enzymes in comparison to control, revealed the increase of the methylation status in anther-derived plants. Our results agree with those of Benerjee *et al.* (1998) who investigated DNA variation in regenerated somaclones of rice; they suggested that during the *in vitro* regeneration process the rearrangement of DNA sequences either creates more restriction sites or changes the degree of methylation.

The added value of AFLP technology using the two methylation sensitive enzymes (*Mse*I and *Hpa*II) in combination with an insensitive one (*Eco*RI) enables the possibility to establish the relation between the way of regeneration and the amplitude of genetic variability, as well as the degree of methylation and the moment of regeneration. Our results support the idea that in cv. Valerien direct embryogenesis from anther culture is accompanied by rearrangement of genetic material, while in Mission indirect somatic embryogenesis and/or long-term culture are accompanied by changes in the methylation status. In this study AFLP proved to be very efficient to distinguish grapevine

somaclones obtained by anther culture and to identify the specific bands for the evolution of the somatic embryogenesis process. This information further provides important knowledge for understanding and controlling the process of regeneration and induced genetic variation.

Methylation and demethylation is a common process for *in vitro*-regenerated plants and seems to be related with gene fluidity and/or their activity during somatic embryogenesis (Phillips *et al*. 1990; Harding *et al.* 1996). Forthcoming studies will have to verify the stability of detected methylation changes in plants regenerated from callus culture and their influence on phenotypic traits after transfer to field conditions.

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