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BRIEF COMMUNICATION

AFLP analysis of somaclonal variations in *Eucalyptus globulus*

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Abstract

DNA variations of forty-eight *Eucalyptus globulus* plants, regenerated by successive culture from seven different explants were assessed by AFLP analysis using 18 primer combinations. At least one variation showed 66.7 % of the analyzed plants, and the numbers of polymorphic bands per plant ranged from 1 to 22. The proportion of polymorphic fragments did not correlate with the numbers of the regenerated plants. However, the more times of successive culture were done the more of polymorphic bands were found within the groups. On average, between 97.39 and 99.88 % of all fragments were shared within the same group. *AMOVA* analysis showed 39.33 % of the variation was found among the accessions that originated from different calli while 60.67 % was from same calli.

Additional key words: blue gum, polymorphic bands, successive cultures.

Micropropagation of *Eucalyptus globulus* Labill. has been successful, and the tissue culture-derived plants has been used in afforestation widely (Li *et al.* 2000). But little information is available on the somaclonal variation presented in regenerated plants. Somaclonal variation is of practical interest due to its potential uses in plant breeding. However, it is a problem, if clonal *in vitro* propagation is the main goal. Molecular marker techniques such as randomly amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) have been employed to detect the genetic fidelity of micropropagated plants (*e.g.* Feyissa *et al.* 2007, Joshi and Dhawan 2007). AFLP has been recognized as a more reliable and efficient DNA marker system, compared with RFLP, RAPD, ISSR or microsatellites (Pejic *et al.* 1998, Zhang *et al.* 2006). AFLP markers have been used extensively for studying genomic variations in different plant species because of their high reproducibility and multiplex ratio (Erschadi *et al.* 2000, De Riek *et al.* 2001, Mukherjee *et al.* 2003/4, Saker *et al.* 2006, Zhang *et al.* 2006). AFLP was able to detect variability within culture lines of pecan somatic embryos and within pecan trees from the same culture line (Vendrame *et al.* 1999, 2000). However, no somaclonal variation was detected, using AFLP technique, among embryogenic cell lines of various oak species (Wilhelm 2000). There are still no reports about somaclonal variation of *Eucalyptus globulus*. The aim of the present study was to evaluate the applicability of AFLP markers to assess genetic variability in regenerated plants of *Eucalyptus globulus* obtained by successive cultures.

A total number of 48 *Eucalyptus globulus* Labill. plants, which were regenerated by organogenesis from 7 calli, were analyzed. The calli were obtained from bud explants excised from 7 different seedlings. The groups were named A, B, C, D, E, F and G (Table 1), and they included seven (from A01 to A07), five (from B08 to B12), five (from C13 to C17), ten (from D18 to D27), five (from E28 to E32), nine (from F33 to F41), and seven (from G41 to G48) regenerated plants, respectively. Groups A and B consisted of regenerated plants of 6th, C, D and E of 8th, F and G of 10th successive culture. Tissue culture was performed as described by Li *et al.* (2000). For initial multiple shoot induction, the explants were cultured on Murashige and Skoog medium supplemented with 2.0 mg dm^{-3} benzyladenine (BA) + 0.5 mg dm^{-3}

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Abbreviations: AFLP - amplified fragment length polymorphism; ISSR - inter-simple sequence repeat; RAPD - random amplified DNA polymorphism; RFLP - restriction fragment length polymorphism; UPGMA - unweighted pair group method with arithmetic.
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indole-3-butyric acid (IBA) + 0.1 mg dm^{-3} α-naphthaleneacetic acid (NAA) + 20 g dm^{-3} sugar. Small microshoots grown on subculture medium were transferred to MS media supplemented with 2.0 or 2.5 mg dm^{-3} BA + 0.5 mg dm^{-3} IBA + 0.1 mg dm^{-3} NAA + 20 g dm^{-3} sugar for elongation. The elongated shoots of 3 - 4 cm length were subsequently transferred to half strength liquid MS medium supplemented with 0.5 mg dm^{-3} IBA + 0.05 mg dm^{-3} NAA + 15 g dm^{-3} sugar for rooting.

Leaves of regenerated plants were excised, frozen in liquid nitrogen, and ground to a very fine powder. DNA used for PCR detection was isolated according to Zhang *et al*. (2007). The AFLP analysis was performed following the manufacturer's protocol (*Life Technologies*, Merelbeke, Belgium). The DNA was digested simultaneously with restriction enzymes *Eco*RI and *Mse*I. Restricted genomic DNA fragments were ligated to *Eco*RI and *Mse*I adapters. The following preamplification cycle was performed 20 times: 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 60 s. The selective amplifications were performed using the primer pairs listed in Table 2. Primers within set *Eco*RI all included the sequence 5'-GAC TGC GTA CCA ATT C; primers of the *Mse*I set had the sequence 5'-GAT GAG TCC TGA GTA A. A touch-down cycle was performed: 1 cycle of 60 s at 94 °C, 60 s at 65 °C and 90 s at 72 °C; followed by 9 cycles of 1 °C lower annealing temperature each cycle, and 23 cycles of 30 s at 94 °C, 30 s at 56 °C and 60 s at 72 °C. The pre- and the selective amplifications were performed in a *2400 Perkin-Elmer* (Norwalk, USA) thermocycler. An equal volume (0.002 cm^3) of loading dye (95 % v/v formamide and 0.08 % m/v bromophenol blue, 20 mM EDTA) was added to each sample, which was then denatured at 95 °C for 3 min and placed on ice for 2 min before loading. Amplification products were analyzed by electrophoresis in a 6.5 % polyacrylamide gel. A 30- to 330-bp molecular mass ladder (*Invitrogen*, Carlsbad, USA) was used for each gel. The electrophoresis parameters were set to 1500 V, 40.0 mA, 40.0 W, 50 °C and the run time was set to 2.0 h. Separated AFLP products were visualized using silver staining as described in the *Promega* (Heidelberg, Germany) silver staining kit and gel images were analysed. Each accession was scored 1 for presence or 0 for absence of each band. Only bright, clearly distinguishable bands were used in the genetic analysis. A genetic distance matrix was obtained according to Link *et al.* (1995), and used to construct a UPGMA tree. A bootstrap analysis was performed using 1000 replicates (Felsenstein 1985). The above analyses were performed using the *TREECON* software (Van de Peer and Watcher 1994). *AMOVA* analysis was performed with the software package *Arlequin* (Schneider *et al.* 2000).

A total of 843 fragments were detected by AFLP among the 48 regenerated plants. Eighteen selective primers were screened against all 48 accessions. Five primer pairs were not included in the final analysis because either the amplification profile was consistently too faint to score accurately (E-ACT/M-CTG and E-ATG/M-CTC) or only monomorphic amplification products were produced (E-AAG/M-CCG, E-AAG/M-CCT, E-ATT/M-CCA). The thirteen informative primer pairs used in the final analysis are listed in Table 2. The average number of fragments per plant per primer pair was 64.85. From any given primer pairs, amplified DNA fragments ranging from approximately 50 to 330 bp were observed. Polymorphic fragments were distributed across the entire size range. Three (E-ATT + M-CCT) to 21 polymorphic bands (E-AAA + M-CGA) of variable lengths generated by a primer pair were detected, and the range of polymorphic rate was from 4.05 % (E-ATT + M-CCT) to 33.87 % (E-AAA +M-CGA) (Table 2). All the regenerated plants analyzed shared 622 (73.78 %) fragments. Another 170 (20.17 %) detected fragments showed variability among plants of the same group. The remaining 51 (6.05 %) fragments were not present in all the regenerated plants from one or several groups indicating variability between groups, due to differences among genotypes of maternal plants. In this study, seven

Table 1. The number of variable accessions and number of polymorphic bands in groups of different number of successive cultures.

Group	Generation	Total accessions	Unvariable accessions	Variable accessions	Polymorphic bands
A	6^{th}	7	3	4	22
B	6^{th}	5	2	3	15
C	8^{th}	5	2	3	27
D	8^{th}	10	3	7	57
E	8^{th}	5	2	3	24
F	10^{th}	9	2	7	76
G	10^{th}	7	2	5	58

Table 2. The number of bands and degree of polymorphism revealed by AFLP primer combinations.

Primer combinations	Total bands	Polymorphic bands	Polymorphic rate [%]
E-AAA + M-CGA	62	21	33.87
E-AAG + M-CGG	51	5	9.80
E-ATG + M-CCA	85	14	16.47
E-ACA + M-CTC	69	11	15.94
E-ATT + M-CCT	74	3	4.05
E-ACC + M-CCA	67	19	28.36
E-ACA + M-CTC	61	16	26.23
E-ACG + M-CCA	75	8	10.67
E-ACA + M-CCA	57	13	22.81
E-AAC + M-CCC	54	7	12.96
E-AAG + M-CTG	48	4	8.33
E-ACT + M-CCA	59	12	20.34
E-ACA + M-CCA	81	17	20.99
Total	843	150	
Average	64.85	11.54	17.79

different plants were used to obtain calli. However, we did not analyze the maternal plants. We thought that their AFLP pattern should be the same as in the regenerants without variable bands within the group.

The distribution of the variability was not consistent, and a few regenerated plants accumulated a large number of polymorphic bands, whereas some plants without any polymorphic bands. Some polymorphic bands appeared in the different groups, while some just appear in one special group, even just in one special plant. Results revealed that 66.67 % of the analyzed plants showed at least one polymorphic band, and the number of polymorphic bands per regenerated plant ranged from one up to 22, compared within groups. The proportion of polymorphic fragments did not correlate with the number of the regenerated plants. However, the more times of successive culture were done to the accessions, the more of polymorphic bands were accumulated within the groups. In the accessions obtained by 6th successive culture, group A and B, the average number of polymorphic bands was 3.08; in the ones of 8th, group C, D and E, the number was 5.40; in the ones of 10th, group F

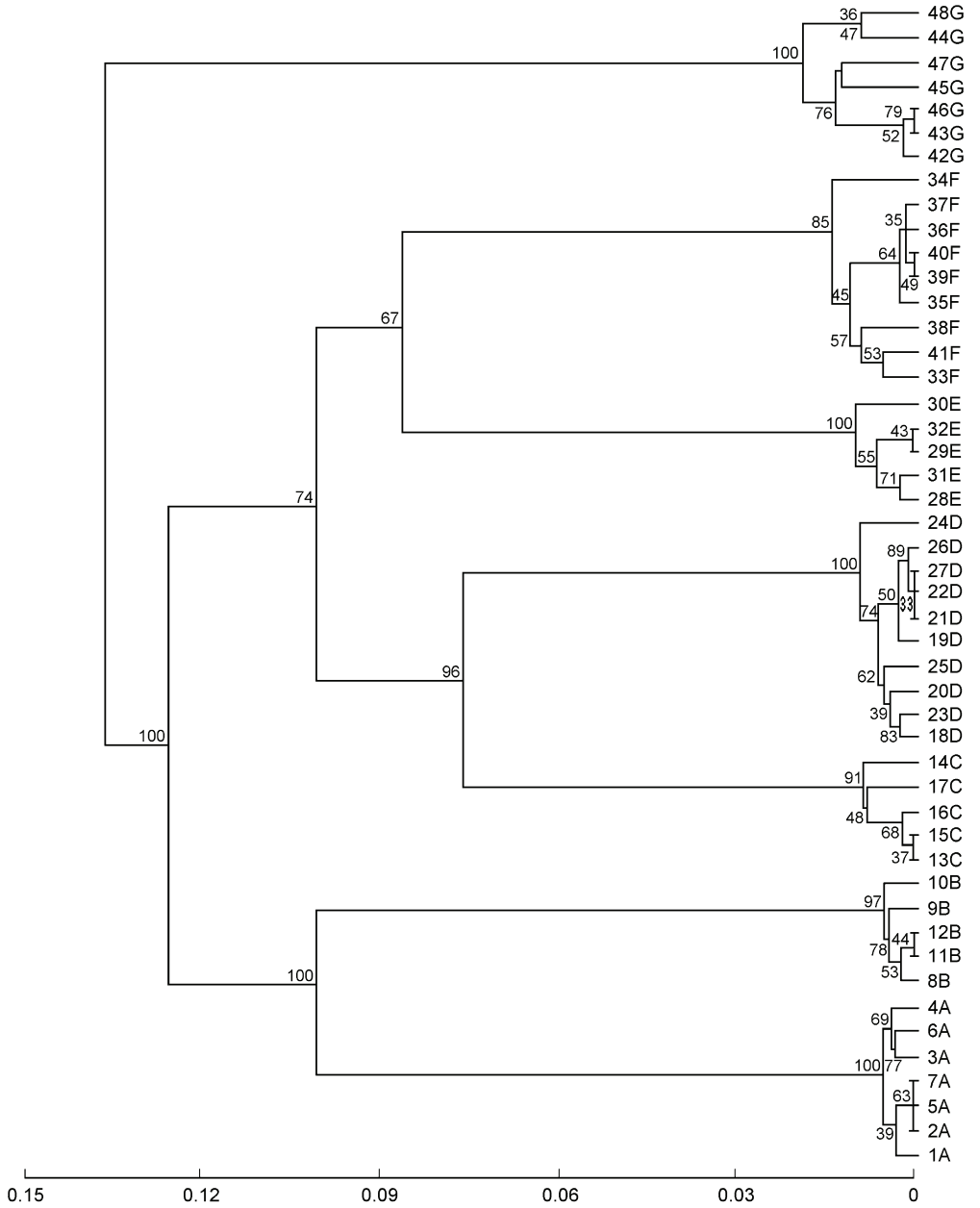

Fig. 1. Dendrogram obtained using UPGMA cluster analysis for the 48 analyzed regenerated plants. Plants obtained from the same explant are grouped showing the seven groups of regenerated plants studied: A (1 - 7), B (8 - 12), C (13 - 17), D (18 - 27), E (28 - 32), F (33 - 41), and G (42 - 48).

and G, the number was 8.38 (Table 1). The proportion of variable accessions negatively correlated with the number of successive culture. The proportion of accessions without any polymorphic bands was 41.67 % (5/12) among those obtained by 6th successive culture, 35 % (7/20) among those of 8th culture and 25 % (4/16) among those of 10th culture (Table 1).

A pairwise matrix of percent agreement between all the analyzed plants was obtained (data not showed), and between 97.39 and 99.88 % of all fragments were shared within the same group. The relationships among the plants are shown by the dendrogram (Fig. 1). The accessions were grouped into 7 major clusters. Regenerated plants from same calli clustered together, showing a close relationship. The more variable plants that accumulate several polymorphic bands were clearly distinguished for each group. Such polymorphic band was not related to any detectable phenotypic differences. The bootstrap values ranged from 33 to 100 % (Fig. 1).

When the genetic variation of the accessions was partitioned by *AMOVA*, 39.33 % of the variation was found among the accessions that originated from different calli while 60.67 % was found among those originated from same calli ($P < 0.001$).

The genetic variability induced by *in vitro* conditions (somaclonal variation) is not limited to any particular group of plants. Now AFLP analysis has revealed as a reliable and efficient method for identification of somaclonal variation in *Eucalyptus globulus* regenerants. In our study, 32 (66.7 %) of the 48 analyzed regenerated plants showed at least one polymorphic AFLP marker, compared with plants obtained from the same callus. The AFLP technique can be applied to quality-control system of tissue culture seedlings of *Eucalyptus globulus*.

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