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BRIEF COMMUNICATION

Using morphological diagnosis and molecular markers to assess the clonal fidelity of micropropagated *Echinacea purpurea* regenerants

S.J. CHUANG[1], C.L. CHEN[2], J.J. CHEN[1] and J.M. SUNG[3]*

Taiwan Seed Improvement and Propagation Station, Taichung County 42642, Taiwan[1]
Department of Agronomy, National Chung Hsing University, Taichung 40227, Taiwan[2]
Department of Food Science and Nutrition, Hung Kuang University, Taichung County 43302, Taiwan[3]

Abstract

Both morphological characteristics and amplified fragment length polymorphism (AFLP) markers were used to validate the genetic fidelity of 1 080 field-grown *Echinacea purpurea* plants regenerated from leaf explants of donor T5-9. Morphological diagnosis revealed that 1 067 out of 1 080 regenerants were normal, while 13 regenerants were aberrant. AFLP analysis was further performed to assess DNA variations among donor, 43 sampled normal regenerants and all 13 aberrant regenerants. Seven primer combinations generated 471 fragments among donor and normal regenerants, of which 9 fragments were polymorphic. The same primer pairs generated 484 fragments for aberrant regenerants, of which 417 fragments were polymorphic. UPGMA clustering indicated that 42 normal regenerants and donor fell into same cluster at similarity scale of > 0.99, while all 13 aberrant regenerants and one morphologically normal regenerant comprised the other clusters. AFLP analysis indicated that these 14 regenerants are off-types.

Additional key word: abberant regenerants, dendrogram, polymorphic fragments, somaclonal variation, UPGMA.

Echinacea purpurea has been commercially cultivated in North America, Europe and Australia where its propagation is predominantly done by seeds (Seidler-Lozykowska and Dabrowska 2003, Kreft 2005). However, the germination of *E. purpurea* seeds is rather low and inconsistent, and requires pre-sowing seed treatment to assure a successful field standing (Chiu *et al*. 2006, Abbasi *et al*. 2007). Moreover, *E. purpurea* is a natural cross-pollinator and maintain higher heterozygosity as compared to self-pollinators (Li 1998, Van Gaal *et al*. 1998). Therefore, it is very difficult to produce *E. purpurea* stock seeds with good uniformity in agronomic and phytochemical traits (Chen *et al*. 2008). Growing genetically similar plantlets with desirable traits propagated through *in vitro* regeneration techniques provides an alternative in commercial cultivation of *E. purpurea* (Abbasi *et al*. 2007, Jones *et al*. 2007). Nevertheless, *in vitro* propagation is often associated with the incidences of somaclonal variation (Jain 2001, Thomas *et al*. 2006). This somaclonal variation usually results in off-type reducing the commercial value of plants (Oh *et al*. 2007). Many methods (morphological, biochemical and molecular) are available to assess somaclonal variation (Vázquez 2001). Morphological diagnosis is relatively simple, but it requires laborious field experiments and it is time-consuming (Nwauzoma *et al*. 2002, Pitman *et al*. 2002, Piagnani *et al*. 2008). Additionally, morphological characteristics are frequently affected by the developmental stage and environment (Hussain *et al*. 2008). Molecular markers, such as amplified fragment length polymorphism (AFLP) (Chuang *et al*. 2009), randomly amplified polymorphic DNA (RAPD) (Nayak *et al*. 2003, Yang *et al*. 2008) or inter simple sequence repeat (ISSR) (Chandrika *et al*. 2008), provide an efficient way for identifying genetic uniformity of the micropropagated plantlets since these markers are not affected by environmental factors and present reliable and reproducible results (Agarwal *et al*. 2008).

In the present work, both morphological characteristics and AFLP markers were used to assess the genetic fidelity of field-grown *E. purpurea* plants directly regenerated from leaf explants. *Echinacea purpurea* (L.) Moench clone T5-9 selected from a consecutive mass selection program was used as donor (Chen *et al*. 2009). A total

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Abbreviations: AFLP - amplified fragment length polymorphism; UPGMA - unweighted pair group method with arithmetic mean.
* Corresponding author; fax: (+886) 4 35082021, e-mail: sungjm@sunrise.hk.edu.tw

of 1 080 *E. purpurea* plantlets were regenerated from leaf explants using the technique detailed by Chuang *et al.* (2009). The produced regenerants were hardened in the greenhouse for 3 to 4 weeks, and then grown in the experiment farm of National Chung Hsing University for field evaluation using the practices detailed by Chen *et al*. (2008). The morphological traits including branching, flowering patterns of flower heads and the colour of stem in each of regenerants were recorded during flowering stage. Any changes in these traits were considered as off-type (aberrant).

Genomic DNA was extracted from young leaves of field-grown normal and aberrant plants. AFLP assay was performed according to Vos *et al*. (1995) with some modifications (Chuang *et al.* 2009). AFLP fragments were examined using *LI-COR* saga generation 2 software and scored for presence (1) or absence (0), and then entered into a binary matrix representing the AFLP profile of each sample. The genetic similarity was estimated according to Jaccard's similarity coefficient (Jaccard 1908). The binary data were subjected to UPGMA (un-weighted pair group method with arithmetic mean) analysis using *NTSYS* software (Rohlf 1997). Similarity based relationship were presented in the form of the dendrogram.

The disorganized state of callus phase is widely assumed to be responsible for the higher rate of resultant somaclonal variation (Vázquez 2001). Therefore, only the primary regenerants directly derived from shoot organogenesis of leaf explants were used in the present study. Field-examination indicated that 1067 out of 1080 (98.8 %) *E. purpurea* primary regenerants (around 40 cm in plant height) propagated from donor clone T5-9 had erect and branched stems, with or without light green spots on the surface of stout stem. These regenerants generally produced 10 to 15 purplish flower heads per plant. These morphological traits agree with the field-grown T5-9 donor plant (data not present). However, a total of 13 T5-9 regenerants (1.2 %) showed several different morphological characteristics in comparison with normal T5-9 regenerants. These aberrant regenerants had the erect stem but without branching and produced only one flower head. Some of these regenerants also showed light red spots on stem and leaf surface.

For AFLP analysis, a total of 64 primer pairs were initially screened and finally 7 were chosen for the present study. Across all 43 normal regenerants and donor (N1) receiving PCR-amplification through 7 pre-selected primer pairs, a total of 471 fragments were observed (Table 1). Among these, 4 primer pairs produced no polymorphic fragments, while the other three primer pairs produced 2, 3 and 4 polymorphic fragments (Table 1). On the other hand, a total of 484 fragments, with 417 polymorphic fragments, were detected in 13 aberrant regenerants subjected to PCR-amplification using the same 7 primer pairs (Table 1). The polymorphic banding patterns of these primer pairs averaged from 61 to 100 %.

Extremely high Jaccard's similarity coefficients, ranged from 0.988 to 1.000, were observed among the sampled 43 normal regenerants and donor plants (data not shown). Forty-two out of 43 sampled normal regenerants and mother donor plant shared Jaccard's similarity coefficients of 0.991 to 1.000. Only 1 regenerant (N13)

Table 1. AFLP produced DNA fragments in normal and aberrant regenerants using various primer pairs (number of total/ polymorphic fragments.

Primer pair	Normal plants	Aberrant plants	Total
E-ACG/M-CAA	58/0	65/60	123/60
E-AGC/M-CAA	65/4	67/67	132/71
E-ACG/M-CAG	44/0	44/27	88/27
E-AAC/M-CAG	78/0	78/43	156/43
E-ACG/M-CAT	87/3	91/83	178/86
E-AGG/M-CAT	84/2	84/84	168/86
E-AAG/M-CAG	55/0	55/53	110/53

Table 2. Jaccard's similarity coefficients based on AFLP profiles of 13 aberrant (A1-A13), mother donor (N1) and normal regenerant (N2) of *Echinacea purpurea* clone T5-9 regenerants. The N1 and N2 were added to serve as comparisons.

	N1	N2	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
N2	1.000													
A1	0.963	0.963												
A2	0.985	0.985	0.965											
A3	0.990	0.990	0.973	0.988										
A4	0.985	0.985	0.969	0.992	0.992									
A5	0.985	0.985	0.969	0.988	0.992	0.992								
A6	0.973	0.973	0.961	0.980	0.979	0.979	0.980							
A7	0.425	0.425	0.430	0.423	0.425	0.426	0.423	0.419						
A8	0.848	0.848	0.836	0.850	0.851	0.850	0.850	0.841	0.494					
A9	0.786	0.786	0.781	0.784	0.792	0.784	0.784	0.775	0.472	0.825				
A10	0.930	0.930	0.914	0.932	0.936	0.936	0.936	0.927	0.431	0.810	0.756			
A11	0.489	0.489	0.495	0.490	0.492	0.487	0.487	0.484	0.495	0.492	0.520	0.497		
A12	0.466	0.466	0.466	0.470	0.473	0.470	0.467	0.467	0.508	0.474	0.505	0.483	0.593	
A13	0.312	0.312	0.315	0.310	0.318	0.310	0.315	0.305	0.353	0.339	0.343	0.320	0.391	0.393

showed similarity coefficients of 0.988 with other samples. Thus, these 42 primary regenerants are considered as true-to-type of T5-9 mother clone. On the other hand, the 13 aberrant regenerants identified through morphological traits showed greater variations in Jaccard's similarity coefficients, which ranged from 0.305 to 0.992, suggesting that they were rather distinct from each other (Table 2). These aberrant regenerants also showed Jaccard's similarity coefficients of 0.312 to 0.990 with donor N1 and representative of normal regenerants N2 (Table 2).

A dendrogram for all the samples was also constructed based on the UPGMA analysis using Jaccard's coefficients of similarity (Fig. 1). Genetic similarity between these normal and aberrant samples was 0.31. The dendrogram consisted of three major clusters. Cluster I comprised of 2 sub-groups. Sub-group Ia included donor and 42 sampled normal regenerants. However, N13 formed sub-group Ib at the similarity coefficient scale of 0.99. Thus, N13 regenerant was distinct, as it diverged from other normal regenerants in the dendrogram. Cluster II included 4 aberrant regnerants with 2 sub-groups. In cluster III, the remaining 9 aberrant regenerants formed 8 separate groups. It is evident from cluster analysis that all the 13 aberrant regenerants and N13 are separated from donor and 42 normal regenerants by using cutting point of 0.99 (Fig. 1), which clearly indicates that these somaclones are off-types.

In large-scale micropropagation system, using AFLP analysis as a quality control may be limited by the large number of individual regenerants that can to be processed. One possible approach to overcome this limitation is to analyze one or several bulked samples, rather than individual regenerants. Using bulked DNA samples can reduce the characterization effort considerably (Fu *et al*. 2003). However, drawbacks of the analysis with bulked DNA samples are the potential non-detection of alleles present at low frequencies as well as the loss of information concerning the amount of heterozygosity within samples (Reif *et al*. 2005). Using AFLP markers to detect DNA polymorphism in bulk samples have been reported in several plant species (Kölliker *et al*. 2001, Papa *et al*. 2007). Nevertheless, to our knowledge, there is no such a study focusing on micropropagated regenerants by using bulked DNA samples is conducted. In this regard, using bulked DNA based AFLP to detect genetic fidelity in *E. purpurea* somaclones should be conducted in the future.

In conclusion, our results re-confirm that tissue culture-induced somaclonal variations occur in *E. purpurea* primary regenerants derived from shoot organogenesis of leaf explants (Chuang *et al*. 2009). Both morphological and AFLP markers can be used to detect the somaclonal variation in *E. purpurea* regenerants. However, in rare case, morphological diagnosis fails to detect off-type. Thus, AFLP appears to be capable of characterizing somaclonal variation with greater precision and less effort than morphological diagnosis in micropropagated *E. purpurea* regenerants.

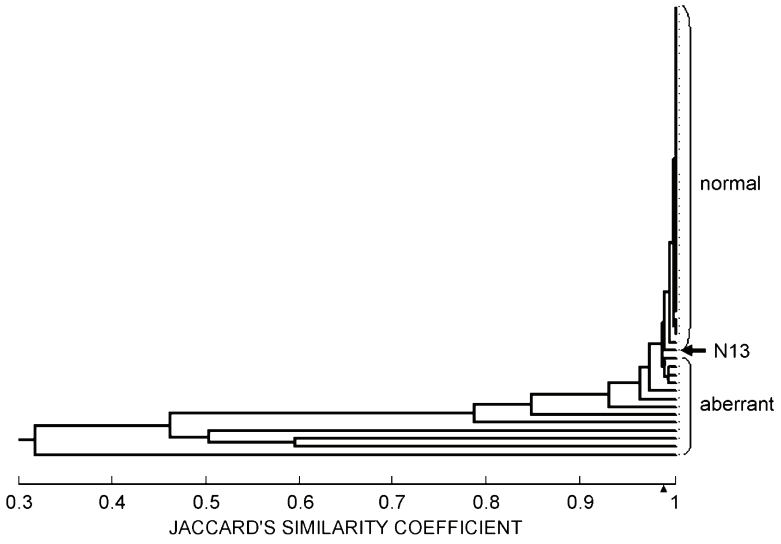

Fig. 1. Dendrograms constructed by UPGMA cluster analysis based on Jaccard's similarity coefficients of 7 selected primers combinations of AFLP markers on normal and aberrant *Echinacea purpurea* clone T5-9 regenerants. N13 (*arrow*) was the only morphologically normal regenerant diverged from other normal regenerants. *Triangle* indicates the similarity scale of 0.99.

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