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BRIEF COMMUNICATION

Determination of genetic stability of long-term micropropagated plantlets of *Platanus acerifolia* using ISSR markers

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Abstract

Inter-simple sequence repeat (ISSR) markers were used to assess the genetic stability of long-term micropropagated plantlets of London plane tree (*Platanus acerifolia* Willd.). Twenty micropropagated plantlets were chosen from a clonal collection of shoots that originated from a single mother shoot. This clonal collection had been maintained under *in vitro* culture conditions for at least 8 years, as achieved by axillary branch multiplication. Out of 38 ISSR primers screened, 16 primers were found to produce clear reproducible bands resulting in a total of 103 distinct bands with an average of 6.44 scorable bands per primer. Of these 103 bands, 86 were monomorphic across all 20 of the plants tested and 17 showed polymorphisms (16.5 % polymorphism). Based on the ISSR band data, similarity indices between the plantlets ranged from 0.92 to 1.00. These similarity indices were used to construct an UPGMA dendrogram and demonstrated that all 20 micropropagated plants grouped together in one major cluster with a similarity level of 91 %. A total of 1771 scorable bands were obtained from the full combination of primers and plantlets and only 51 (2.88 %) were polymorphic across the plantlets which indicates that this micropropagated line of *P. acerifolia* is genetically stable.

Additional key words: axillary branch multiplication, dendrogram, London plane tree, molecular markers, somaclonal variation.

London plane tree (*Platanus acerifolia* Willd.) belongs to the family *Platanaceae* and is a hybrid of *P. orientalis* × *P. occidentalis*. It has gained the acronym of "the king of street trees", reflecting its extensive use within temperate and subtropical urban plantings. A high-efficiency plant regeneration system for *P. acerifolia* has been reported previously (Liu *et al*. 2002, Liu and Bao 2003) and also a method for the reproducible *Agrobacterium*-mediated genetic transformation of this species has been developed (Li *et al*. 2007). Thus, genetic manipulation is a viable option for London plane tree.

It is clear for several plant species that the outcome of *in vitro* manipulation is highly genotype-dependent (Toshihikoi *et al*. 1998, Opabode 2006), and a suitable genotype for the efficient *Agrobacterium*-mediated genetic transformation of *P. acerifolia* has been isolated and identified. Thus, in order to preserve such a useful genotype it is imperative to multiply the line and micropropagation offers the only practical solution in the case of species with such a long inter-generation period. However, maintaining the genetic stability of the line is also very important and it is this point that we seek to address in this report.

The scaling up of any micropropagation methodology carries the risk of inducing genetic variability, namely somaclonal variation amongst sub-clones of one parental line (Larkin and Scowcroft 1981). Somaclonal variation within micropropagated plants has been demonstrated using morphological, cytological, physiological, biochemical and molecular traits in several species (Martins *et al*. 2004, Joshi and Dhawan 2007). Various DNA marker techniques, including random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and inter-simple sequence repeats (ISSR), have been developed and used to assess genetic stability within micropropagated plant

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Abbreviations: AFLP - amplified fragment length polymorphism; ISSR - inter-simple sequence repeat; RAPD - random amplified polymorphic DNA; SSR - simple sequence repeat.

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lines (Gupta and Varshney 1999, Devarumath *et al*. 2002, Carvalho *et al*. 2004, Feyissa *et al*. 2007). ISSR analysis involves the use of single simple sequence repeat (SSR) motifs in order to prime the polymerase chain reaction (PCR) and thereby amplify regions between adjacent, but inversely oriented, microsatellites (Zietkiewicz *et al*. 1994). ISSR markers have been successfully applied in the analysis of genetic fidelity within lines of cauliflower (Leroy *et al*. 2000), almond (Martins *et al*. 2004), banana (Venkatachalam *et al*. 2007) and *Swertia chirayita* (Joshi and Dhawan 2007). Moreover, ISSR markers offer other advantages in the detection of somaclonal variation notably, a high degree of sensitivity, reproducibility and the dominant representa- tion of polymorphic genetic alleles. Here, we report the use of ISSR markers in order to assess genetic fidelity within *in vitro* propagated plants of London plane tree (*P. acerifolia*). For this study, we utilized the plants that had been clonally propagated *in vitro* for a period of at least 8 years. To our knowledge, this is the first report of the analysis of DNA sequence-variation in micropropa- gated plants of *P. acerifolia*.

Plantlets had previously been regenerated from the leaves of a single *in vitro*-grown mother seedling of *Platanus acerifolia* Willd. (Liu and Bao 2003) and one of these plantlets, identified as clone P1-2, formed the basis of the clonal population used in this study. An *in vitro* micropropagation protocol *via* axillary branch multiplication has previously been standardized for *P. acerifolia* (Liu *et al*. 2002). Using this technique, a single shoot from clone P1-2 was excised and transferred to micropropagation medium (MM), composed of Murashige and Skoog (1962; MS) basic medium supplemented with 0.3 mg dm^{-3} 6-benzyladenine, 0.05 mg dm^{-3} α-naphthalene acetic acid, 30 g dm^{-3} sucrose and solidified using 7 g dm^{-3} agar (Liu and Bao 2003). Subsequently, the micropropagated shoots were separated and transferred to fresh MM every two months and incubated at 25 ± 2 °C under a 14-h photoperiod and a photosynthetic photon flux density (PPFD) of 50 µmol m^{-2} s^{-1} provided by 40 W cool white fluorescent tubes. A group of 20 micropropagated plants were selected indiscriminately from a batch of micropropagated shoots that originally derived from a single shoot of clone P1-2 and had been sub-cultured *in vitro* every two months for a period of > 8 years. These 20 plantlets were subjected to ISSR marker analysis. In addition, a control group was analysed, comprising two micropropagated plants that originated from a common mother shoot but had been subsequently sub-cultured for a period of only 2 years.

Total DNA was extracted from fresh leaves of the *in vitro*-propagated plantlets according to the method of Li *et al.* (2007). RNA was removed by treating the extract with RNase-A (*Fermentas*, Vilnius, Lithuania) for 30 - 60 min at 37 °C. The integrity and quantity of the DNA preparations were determined spectrophotometrically and visually (by ethidium bromide staining on 0.8 % agrose gels) and the DNA samples were diluted to a final concentration of 10 mg dm^{-3} before use in ISSR analysis.

A total of 38 primers were screened for effectiveness within inter-simple sequence repeat (ISSR) reactions, using 2 different DNA samples isolated independently from the micropropagated plantlets of clone P1-2 of *P. acerifolia*. Out of these 38 primers, only 16 primers (Table 1) gave reproducible and clearly identifiable bands and these, therefore, were used in further PCR analyses.

Table 1. List of ISSR primers used for detecting the genetic stability in micropropagated plants of *Platanus acerifolia* (Y = T or C, R = G or A)

Primer code	Primer sequence ($5^{/}$-$3^{/}$)	Number of monomorphic bands	Number of polymorphic bands	Total number	Size range [kb]
IS12	$(AG)_8YG$	7	-	7	0.6 -1.5
IS13	$(AG)_8YT$	9	-	9	0.6 -1.8
IS14	$(AG)_8YA$	7	-	7	0.5 -1.6
IS20	$(GA)_8YG$	9	-	9	0.25-1.9
IS23	$(GA)_8YT$	6	-	6	0.25-1.0
IS24	$(GT)_8YC$	3	4	7	0.5 -1.8
IS25	$(GT)_8YG$	6	3	9	0.5 -1.8
IS28	$(GT)_8RA$	7	-	7	0.75-1.8
IS30	$(GT)_8RG$	5	-	5	0.5 -1.8
IS32	$(AC)_8RG$	3	-	3	0.8 -1.5
IS34	$(AC)_8RA$	3	-	3	0.7 -1.0
IS36	$(AC)_8YG$	4	4	8	0.4 -1.2
IS38	$(AC)_8YA$	5	-	5	0.75-1.5
IS43	$(GTG)_5$	3	-	3	0.9 -1.6
IS44	$(GAC)_5$	5	4	9	0.3 -1.2
IS46	$(GGGGT)_3$	4	2	6	1.0 -1.9
Total		86	17	103	

Optimised PCR conditions, established for the 16 ISSR primers, were used for DNA amplification reactions. DNA amplification was performed in a 0.02 cm^3 final volume reaction mixture containing 10 ng template DNA, 1 U *Taq* DNA polymerase (*Fermentas*), 0.2 mM dNTP and 0.5 µM primer within 1× reaction buffer [75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, and 0.01 % *Tween 20*]. Amplification was carried out in a *PTC-100™ Programmable Thermal Controller* (*Bio-Rad*, Hercules, USA) according to the following conditions: an initial step of 94 °C for 4 min followed by four cycles of 94 °C for 30 s, 58 - 54 °C (a stepwise reduction of 1 °C for each cycle) for 45 s, and 72 °C for 90 s. In the subsequent 36 cycles, the annealing temperature was 53 °C and the reaction was finally terminated by 8 min at 72 °C.

Amplified products were separated on a 2.0 % (m/v) agarose gel (*Biowest*, Nuaillé, France) in 1× TAE buffer, run for 3 h at 60 mA. The gels were visualized under UV radiation after staining with ethidium bromide (0.001 %) and analyzed using a *KODAK Gel Logic2200 Imaging System* (*Carestreamhealth*, New Haven, USA).

Only distinct, reproducible and well-resolved fragments ranging from 250 bp to 2000 bp were considered in the analysis. These bands were scored either as present (1) or absent (0) for each of the ISSR markers within the 20 micropropagated plants. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered

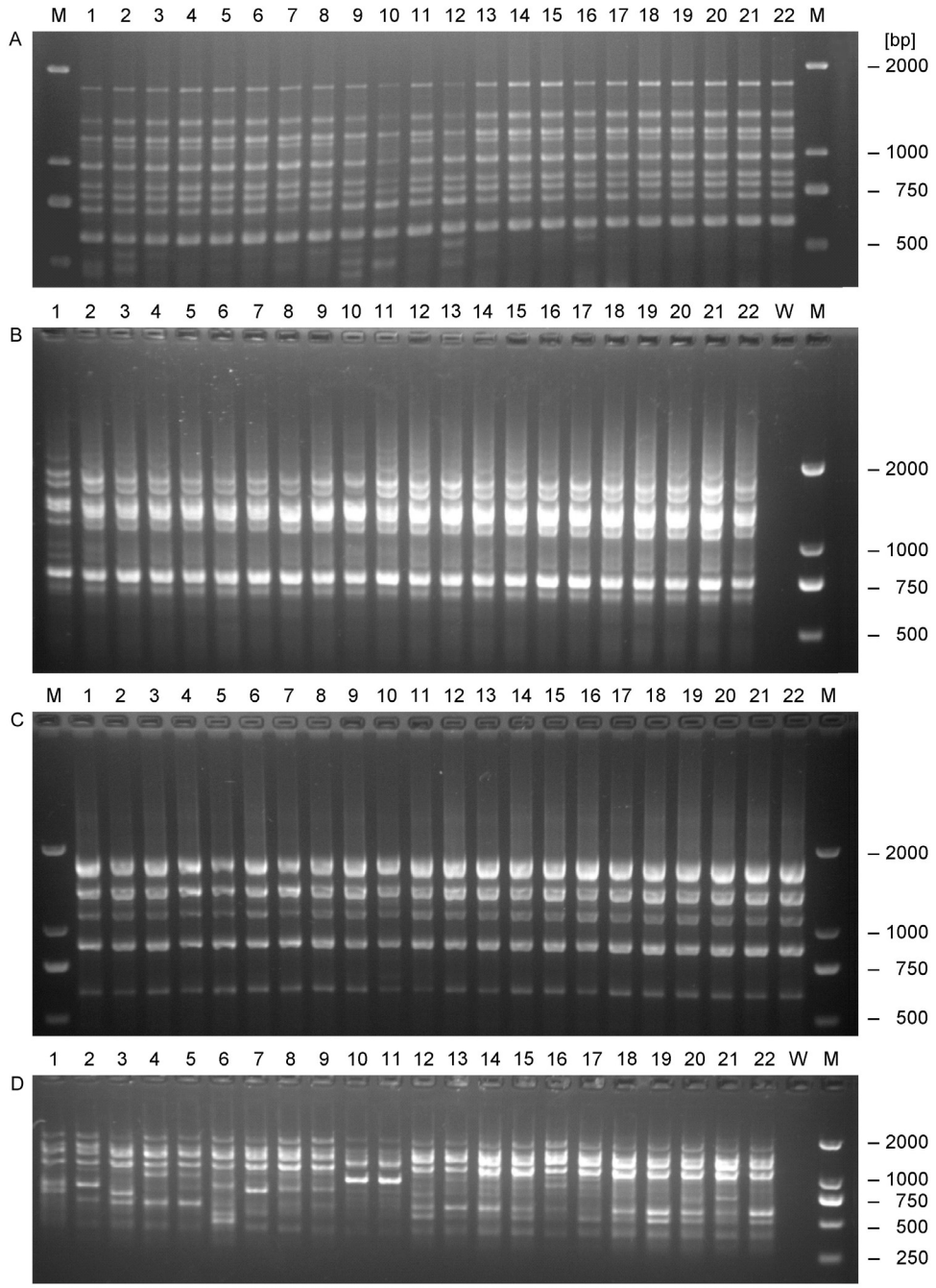

Fig. 1. Electrophoretic gel separation of the amplification products by primers IS13 (*A*), IS28 (*B*), IS30 (*C*) and IS46 (*D*) (see Table 1 for primer details). *Lanes* 1 to 20, respectively, represent the 20 micropropagated plantlets of *P. acerifolia* obtained following an *in vitro* culture period of ≥ 8 years; *lanes* 21 and 22 represent micropropagated plantlets obtained following a 2 years *in vitro* culture period, M shows the DL2000 molecular size markers (*BioRule*) and W shows a control reaction of purified water.

ambiguous markers and were not scored. The size of the amplification products was estimated using a *DL2000* molecular size marker (*BioRule*, Guangzhou, China). *NTSYSpc Version 2.10e* was used to perform the distance matrix and clustering analysis of the complete data set. Genetic similarities amongst different individuals were measured by the Jaccard's similarity coefficient (Jaccard 1908) using the *SIMQUAL* module. Similarity coefficients were used to construct a dendrogram using the unweighted pair group method with arithmetic average (UPGMA) and the sequential, hierarchical and nested clustering (SHAN) routine in *NTSYS* software.

A total of 38 primers were tested in ISSR reactions with the genomic DNA from clone P1-2 of *P. acerifolia.* Of these, 16 primers were found to yield distinct and reproducible amplification products and, therefore, were

used in the further analysis of the 20 long-term micropropagated P1-2 plantlets. These 16 primers yielded a total of 103 bands with an average of 6.44 scorable bands per primer. Primers IS13, IS20, IS25 and IS44 amplified the largest number of 9 bands, while primers IS32, IS34 and IS43 amplified the lowest number of 3 bands (Table 1). All of the scored amplified products ranged in size from 250 to 2000 bp. The majority (103 bands) aligned with a molecular mobility size between 1.0 - 1.9 kb (Fig. 1*D*). In addition, 13 bands aligned between 300 to 500 bp, 19 bands aligned with a ladder size of 1.6 to 2.0 kb and 6 bands were >1.8 kb.

The primers IS13, 28 and 30 produced 9, 7 and 5 monomorphic bands, respectively, across the 20 micropropagated plantlets (Fig. 1*A*,*B*,*C*). Primer IS46 produced 4 monomorphic bands and 2 polymorphic bands (33.3 % polymorphism) (Fig. 1*D*). The primers IS24 and IS25 revealed, respectively, 4 and 3 polymorphic bands (57.1 and 33.3 % polymorphism) (Table 1). Thus, from the total of 103 major products that could be clearly scored for presence/ absence, 86 bands were monomorphic across the 20 micropropagated plantlets. By contrast 17 bands, as collectively generated by the primers IS24, IS25, IS36, IS44 and IS46 (Table 1), were found to be polymorphic across the 20 sub-cloned plantlets (16.50 % polymorphism).

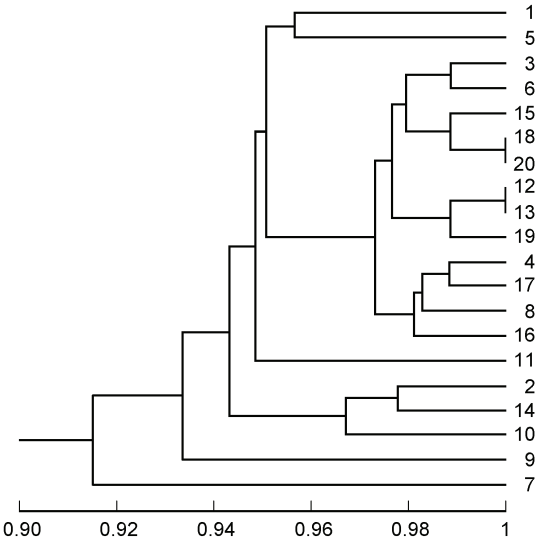

Fig. 2. UPGMA dendrogram of the genetic relationship of 20 long-term micropropagated plants of *Platanus acerifolia* constructed using similarity coefficients based on ISSR marker analysis.

Cluster analysis was performed on the basis of similarity coefficients generated from the ISSR data of the 103 scored bands. Similarity indices ranged from 0.92 to 1.00 among the 20 micropropagated plants (Fig. 2). According to this ISSR analysis, all 20 micropropagated plants could be grouped together in a single cluster with a 91% level of similarity. In view of the 8 year period of *in vitro* propagation, this indicated a very low rate of polymorphism. Only when assessed at the more stringent similarity level of 95 % could 4 distinct clusters be distinguished (Fig. 2).

A total of 1771 scored bands was detected in this study, comprising the 86 monomorphic bands found in each of the 20 plantlets (a total of 1720 bands) and a total of 51 polymorphic bands. Therefore, polymorphic bands comprised 2.88 % of the total scored genetic loci.

According to ISSR analysis, the group of 20 (non-preferentially chosen) micropropagated plantlets of *P. acerifolia* showed the high level of genetic similarity ranged from 91 to 100 %, and polymorphic bands formed just 2.88 % of the total bands scored. This very low percentage of genetic variation (polymorphism), despite more than 8 years of *in vitro* culture, demonstrates the genetic stability of *P. acerifolia* under these particular conditions of micropropagation. A similar conclusion was reached by a study on *Phoenix dactylifera*, where 2.6 % genetic variation was detected (Saker *et al*. 2006). These results indicate that the axillary branch micropropagation shows a high level of reliability for the multiplication of true-to-type materials.

The level of genetic instability that was detected may be attributed to naturally occurring variation or to the accumulation of mutation during the *in vitro* culture period. Although somaclonal variation may provide a useful source of novel variability (Vuylsteke 1998), it is more typically viewed as an undesirable side-effect of *in vitro* propagation (Larkin and Scowcroft 1981) and somaclonal variants may exhibit off-type phenotypes (Smith 1988, Bindiya and Kanwar 2003). The source of the explants used and the method of regeneration (organogenesis versus embryogenesis) may influence the rate of somaclonal variation. After axillary branch multiplication, somatic embryogenesis is generally considered to be the next best method of generating genetically stable clones (Leroy *et al*. 2000). By comparison, organogenic differentiation is highly prone to somatic variation (Vasil 1987, Pontaroli and Camadro 2005). These differences are probably attributable to that the greater resistance of organized meristems to genetic change than unorganized callus is found (Shenoy and Vasil 1992). The findings reported here for *P. acerifolia* support such ideas.

Extensive researches have been carried out into the mechanisms of somaclonal variation and these have been found to involve chromosomal rearrangements, point mutation, gene replication and deletion, DNA methylation or transposon activation (Kaeppler and Phillips 1993, Phillips *et al*. 1994, Joshi and Dhawan 2007). Genetic variation induced by such genetic and epigenetic mechanisms can be reflected in the profiles of molecular genetic markers. Both RAPD and ISSR markers have been often used for the detection of somaclonal variation within the micropropagated clones of several species (Rani and Raina 2000, Devarumath *et al*. 2002, Sahijram *et al*. 2003, Martins *et al*. 2004, Souframanien and Gopalakrishna 2004). However, ISSR analysis was frequently found to reveal a higher level of polymorphism than shown by RAPD markers (Devarumath *et al*. 2002, Souframanien and Gopalakrishna 2004) and, therefore, appears to offer a

sensitive method of detecting somaclonal variations among *in vitro* propagated plants.

This is the first report of ISSR markers used to assess genetic variation amongst micropropagated plants of *P. acerifolia*. The results showed a high similarity indices between all of the tested plantlets and detected just 2.88 % polymorphism of total genetic loci. Such a low level of somaclonal variation was deemed as the genetic stability of the micropropagated line (Saker *et al*. 2006). The use of the ISSR molecular marker system to successfully assess genetic variations within the *in vitro* materials of a woody species provides a substantially more rapid and more effective system than confirmation of adult phenotypic characteristics. Furthermore, these results demonstrate that the axillary branch multiplication for the micropropagation of *P. acerifolia* can be carried out for a considerable length of time (at least 8 years) with only a low risk of genetic instability. Such clonal plant materials are therefore suitable as the basis of an effective programe of *Agrobacterium*-mediated genetic transformation.

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