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Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh

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Abstract. RAPD markers were used to assess genetic fidelity of 23 micropropagated plants of a single clone (L34) of *Populus deltoides*. Eleven arbitrary 10-base primers were successfully used to amplify DNA from *in vivo* and *in vitro* material. Of these, 5 distinguished a total of 13 polymorphisms common across 6 micropropagated plants. Apart from these 6 plants, the amplification products were monomorphic across all the micropropagated plants, the mother plant and 4 additional field-grown control plants. Our results show that RAPD markers can be used to gain rapid and precise information about genetic similarities or dissimilarities in micropropagation systems that might not be so easily evident from other commonly used techniques.

Introduction

One of the most crucial concerns in *in vitro* propagation is to retain genomic integrity of the micropropagated plants vis-a-vis mother plant(s) so that the advantages (high yield, uniform quality of commercial product, shorter rotation period, etc.) in the use of elite genotype(s) over natural seedlings are maintained. Numerous reports indicate that somaclonal variation in micropropagation derived plants is not uncommon (see D'Amato 1978, Skirvin 1978, Earle and Demarly 1982). The documentation of divergent genetic changes (see Larkin 1987) associated with somaclonal variation is, however, a difficult task.

Several strategies that have been used to assess the genetic integrity of *in vitro* raised plants have, more often than not, been met with limited success. The detection of off-types among micropropagated plants, especially tree taxa, by phenotypic identification based on morphological traits requires extensive observations of plants until maturity, and in many cases it lacks definition and objectivity. Karyotypic analysis of metaphase chromosomes has been used to determine rearrangements and/or numerical variation in the chromosomes in somaclones by many workers (see Bhojwani et al. 1986). Such analysis as a means of determining change(s) has severe limitations in, for example, most of the economically important and successfully *in vitro* propagated hardwood tree species (Brown and Sommer 1982), wherein the chromosomes are small in size and/or the number is high due to polyploidy. The genetic integrity of the micropropagated plants could also be evaluated by isozyme electrophoresis. The major disadvantages of this technique lie in the limited number of informative markers and its proneness to environmental and developmental variation. Besides these limitations, the above approaches are not fully suitable for detecting DNA sequence polymorphism, if any, in the *in vitro* raised plants. Restriction endonuclease digestion of genomic DNA followed by hybridization with several probes, on the other hand, reveals restriction fragment length polymorphisms (RFLPs) at the DNA sequence level. This technique has more recently been used for molecular characterisation of tissue culture derived plants (Shimron-Abarbanell and Breiman 1991, Chowdhury and Vasil 1993, Vallés et al. 1993, Shirzadegan et al. 1991, Shenoy and Vasil 1992, Cloutier and Landry 1994). However, the technical complexity of performing RFLP analysis, relatively high cost, and the widespread use of radioisotopes in the detection method are some of the disadvantages for its routine application in micropropagation systems. Random amplified polymorphic DNA (RAPD) analysis can circumvent some of the problems associated with RFLP analysis. The RAPD technique developed by Williams et

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al. (1990) and Welsh and McClelland (1990) has the advantages of being technically simple, quick to perform, and requires only a small amount of DNA. A single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template, such as the plant genome.

In this paper, we evaluate the potential of using the technique of RAPD analysis for rapid appraisals in a micropropagation system, while contributing new and useful evidence toward genomic stability and/or variability in *Populus deltoides* plants produced through enhanced axillary branching cultures.

Material and methods

Plant material. Among the 6000 seedlings raised from open pollinated seeds of G48 x G3 (both obtained from Australia) *P. deltoides* clones, L34 has been recognized as one of the 7 most promising clones screened among the seedlings (Chaturvedi and Rawat 1994). This asexually (conventional rooted cuttings) propagated clone is now widely planted in North India.

The standard protocol for raising *P. deltoides* clone L34 micropropagated plants through axillary branching method was developed and tested at National Botanical Research Institute, Lucknow (Chaturvedi, unpublished). The suitable protocol thus developed was further tested, and subsequently adopted by Tata Energy Research Institute (TERI) for large scale multiplication at the Tissue Culture Pilot Plant (TCPP) facility at Gual Pahari in Northern India (Anonymous 1992, Pandey and Chaturvedi 1993, Dhawan personal communication). For the present study, a total of 23 micropropagated plants was randomly selected from a particular batch of about 500 plants maintained initially in the potting mix for about 4 months before being transferred to field conditions. These ~500 plants were derived from explants of a single mother plant. Briefly, the methodology of raising these plants was as follows: Freshly excised explants (nodal cuttings) from multiple positions of the single 7-year old tree of the clone L34, growing in natural environment, were surface sterilized and inoculated on MS liquid medium (Murashige and Skoog 1962) supplemented with 0.25 mg/l IAA + 0.2 mg/l BAP to initiate enhanced axillary branching. Microcuttings of nodal sections from the shoots that developed from the axillary buds or the miniature shoots were subcultured on the same medium + 0.8% Agar. After 6 passages on the multiplication medium, the propagules were transferred to hormone free MS medium for rooting. Well-rooted plantlets were hardened before transfer to potting mix, then transferred to the field conditions.

DNA extraction and PCR amplification. DNA was extracted from fresh leaves as described by Saghai-Maroof et al. (1984). For each micropropagated plant sampled in the survey, DNA was separately extracted approximately 3 and 6 months after their transfer to potting mix and field conditions, respectively. DNA was also extracted separately (leaves excised from multiple positions and bulked) from each of the five 5-to-7-year-old clone L34 trees, including the one used as the explant source in the present study. DNA concentration was measured by a Gilford Spectrophotometer at a wavelength of 260 nm.

Eleven arbitrary 10-base primers (10 mers) (Operon Technologies Inc., Alameda, California) were used for polymerase chain reaction (PCR) based on the protocol of Williams et al. (1990) with minor modifications. Amplifications were done in 25 μl of reaction mixture containing 2.5 μl of 10x assay buffer (Stratagene), 2.0 μl of 1.25 mM dNTPs (Pharmacia), 15 ng of the primer, 3 units of amplitaq DNA polymerase (Stratagene) and 50 ng of genomic DNA. The reaction mix was overlaid with 30 μl of mineral oil (Sigma). DNA amplification was performed in a Perkin Elmer Cetus DNA thermal cycler programmed for 45 cycles as follows: 1st cycle of 3.5 min at 92°C, 1 min at 35°C, 2 min at 72°C; followed by 44 cycles, each of 1 min at 92°C, 1 min at 35°C, 2 min at 72°C followed by one final extension cycle of 15 min at 72°C. The amplification products were size-separated by gel electrophoresis in 1.4% agarose (Pharmacia) gels with 0.5 x TBE and stained with ethidium bromide. All the reactions were repeated at least twice, and only the consistently reproducible bands were considered.

Results and Discussion

Of the 11 primers tested, 6 (OPA 02, OPA 12, OPA 13, OPA 14, OPC 18, OPF 20) produced amplification products that were monomorphic across all the micropropagated plants (Fig. 1c). The size of the 35 monomorphic bands produced by these primers ranged from 468-bp in OPA 02 to 1474-bp in OPF 20.

Thirteen out of the 39 bands produced by the remaining 5 primers (OPC 07, OPA 10, OPA 05, OPA 08, OPC 19) were scored as polymorphic within the micropropagated plants. These 13 markers were monomorphic among 17 plants, and polymorphic when the remaining 6 (lane 10, 13, 19, 20, 22, 23) plants were considered in the analysis (Fig. 1a,b). Interestingly, the marker profiles among these 6 plants were identical with all 5 primers. The differences between the groups of 17 and 6 plants lie in the presence of new, or absence of, specific amplification products as shown in the profiles of primers OPA 10 and OPC 19 (Fig. 1a,b). The other 3 primers (OPC 07, OPA 05, OPA 08) also revealed a similar kind of polymorphism. Primer OPA 08 produced 5 bands common to all 23 micropropagated plants, while in the 6 plants two additional amplification signals of 924 and 878-bp were observed in the profiles. Primers OPC 07 and OPA 05 produced 10 monomorphic bands each in the 17 plants. In comparison, the corresponding amplification products at 3 (723, 615, 485-bp) and 2 (1062, 864-bp) sites, respectively, were found to be missing in the profiles of the 6 plants. The number of amplification products generated by the 5 primers

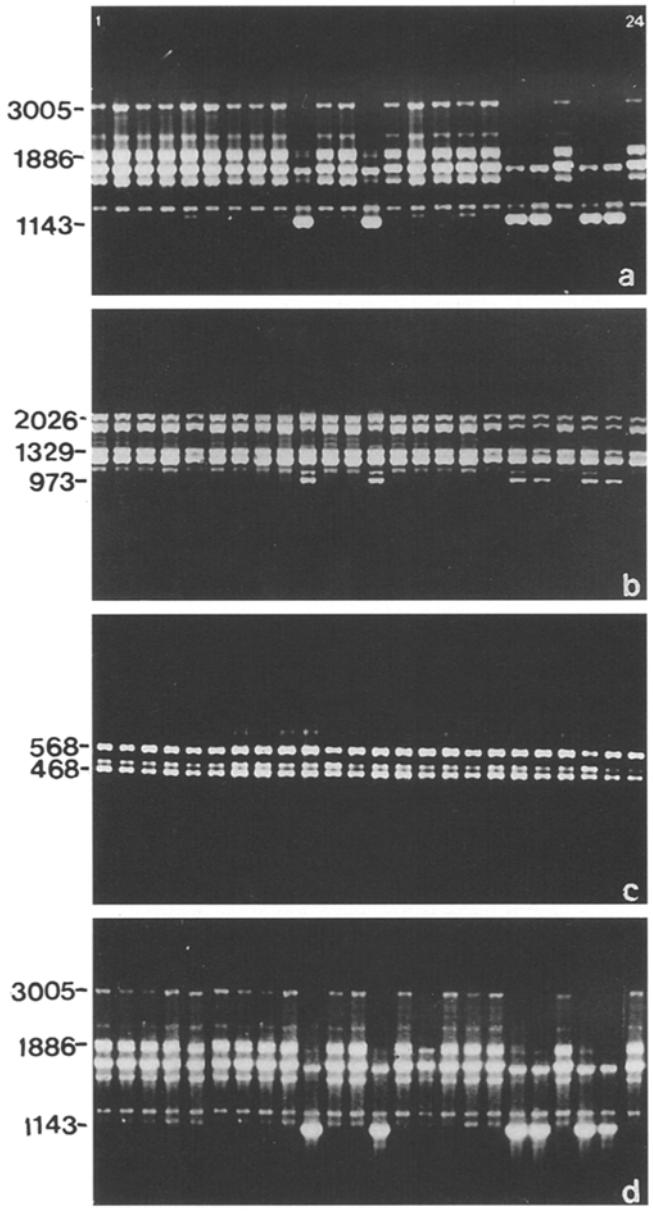

Fig.1. Gel electrophoresis of RAPD fragments obtained with primers OPA 10 (a, d), OPC 19 (b), OPA 02 (c). Lane 1 shows RAPD bands from the field-grown mother tree. Lanes 2-24 show RAPD products from micropropagated plants. DNA extracted separately for each micropropagated plant about 3 and 6 months after their transfer to potting mix and field conditions served as the template for generation of RAPDs in a-c, and d, respectively. Note the polymorphic fragments in 6 lanes (10, 13, 19, 20, 22, 23) in a, b and d. a and d indicate the occurrence of common bands between corresponding micropropagated plants using primer OPA 10. The size of the fragments is indicated on the left.

ranged from 7 in OPA 08 to 10 in OPC 07 with a size range of 254-bp in OPC 07 to 3005-bp in OPA 10. The polymorphic amplification products that were each present in the 6 plants versus the 17 other plants were verified (Fig. 1d) using new DNA extractions (from corresponding field transferred plants), with the same 5 primers, to establish that these polymorphisms were not artifacts of the DNA extraction.

Barring the change in amplification products in the 6 plants detailed above, all 11 primers tested revealed the same number and size of the bands between the single control and micropropagated plants (Fig. 1a-c). A survey with 5 primers (OPC 07, OPA 10, OPA 05, OPA 08, OPC 19) revealed monomorphism of the amplified DNA fragments among 5 naturally growing trees of clone L34. The present finding is in agreement with Castiglione et al. (1993) who found no RAPD fingerprint variation when different plants of the same clone of *Populus* species, including *P. deltoides,* were assayed independently.

Among the various methods (see Pierik 1987) developed to micropropagate plants, enhanced axillary branching culture has become the most important propagation method. This method is especially advantageous because it is simple and the propagation rate is relatively high (Pierik 1991). More importantly, it is generally considered to be an *in vitro* culture system with low risk of genetic instability (Pierik 1991, Schoofs 1992), because the organised meristems are generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions (Shenoy and Vasil 1992). Notwithstanding this consideration, however, there are numerous reports on the incidence of somaclonal variation among micropropagated plants (Maynard et al. 1991, Moore et al. 1992, Wakasa 1979, Schoofs 1992, Swartz 1990, Martinelli 1992, Grenan 1992, Leach 1979, D'Amato 1978, Skirvin 1978). For example, reports have indicated the occurrence of somaclonal variation in micropropagated bananas and plantains (see Schoofs 1992) raised through meristem culture. Various kinds of leaf chlorosis coupled with multiple apexing and dwarfing in strawberry (Martinelli 1992), more jagged and pubescent leaves in grapevine (Grenan 1992), spininess and albino strips in pineapple (Moore et al. 1992), and less shoot growth and curved stems (Leach 1979) in *Pinus taeda* were some of the common off-type features observed to occur in micropropagated plants.

The present study provides the first information on the molecular basis of polymorphism detected as RAPD markers in micropropagated plants of *P. deltoides.* The polymorphism in amplification products which represents one allele per locus could result from changes in either the sequence of the primer binding site (e.g. point mutations) or changes which alter the size or prevent the successful amplification of a target DNA (e.g. insertions, deletions, inversions). In our study, products of 254 to 3005-bp were amplified by 11 random primers tested. Thirteen amplified products

exhibited polymorphism and 61 products were shared among all the plants. The polymorphism was, however, due to occurrence of variation in only 6 plants. Taken by themselves the 6 plants also showed complete homology within the group. It is apparent, therefore, that there is a kind of discrete RAPD polymorphism detected in the present material. The occurrence of exactly the same polymorphism pattern in 13 different amplification products from 5 different primers indicates with near certainty that we are looking at a single somatic mutation event that occurred early and was then carried forward. From the practical application standpoint, the prevalence of 6 off-type plants (morphologically indistinguishable from the remaining 17) out of 23 presently investigated is a matter of great concern in any micropropagation system where clonal uniformity is required. Variation could have been induced by the *in vitro* process, or by added biochemicals and stresses (Swartz 1990), and therefore, understanding the event(s) which gave rise to such variation needs more study. Moreover, the detection of morphologically indistinghuishable off-types by RAPD markers in the present study warrants additional testing of tissue culture propagated plants at the molecular level.

In conclusion, our results demonstrate that the RAPD technique can be successfully applied to determine the genetic fidelity of micropropagated plants of poplar.

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