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Detection and fingerprinting of narrow-leaf mutants in micro-propagated hybrid poplar (*Populus sieboldii* x *P grandidentata*) using random amplified polymorphic DNA

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ABSTRACT

Two narrow-leaf phenotypes (A and B) were identified among a population of hybrid poplar (*Populus sieboldii* x *P grandidentata*) regenerated through in vitro culture. The two variants appeared alike in them in appearance but differed significantly in appearance and size from the donor plant. Morphological studies revealed that phenotypic variants had higher number of branches and much smaller, narrower and thicker leaves than the parent plant. To elucidate the basis of these phenotypic variations random amplified polymorphic deoxyribonucleic acid (RAPD) analysis was performed on variants as well as on the normal plants. Among the 120 decamer primers of Operon Technology, Calif, only primers OPG 12, OPQ 04, OPF 20, and OPT 02 differentiated normal plants from the variants. Only one primer OPB 04 differentiated phenotype A from the normal plants and phenotype B with an additional band at 350 bp. Out of 386 reproducible markers from 120 primers obtained only 4 correlated with narrow-leaf phenotypes.

Keywords: Hybrid poplar, in vitro culture, narrow-leaf phenotypes, RAPD, somaclonal variation

INTRODUCTION

Poplars are one of the fastest growing hardwoods in the world. The tissues of *Populus* exhibit a high degree of plasticity and are, therefore, one of the most widely studied genera in forest tree tissue culture. Since the earlier studies of Wolter (1968) and Winton (1968, 1971) several species and hybrids of poplar have been propagated through tissue culture technique (Chun 1993, Confalonieri et al 2003). In vitro cloning of plants from cells and tissues sometime leads to a wide range of variation among regenerants a phenomenon known as somaclonal variation (Larkin and Scowcroft 1981). Somaclonal variation which is unwanted in clonal propagation is

a rich and novel source of genetic variation for plant breeding. The source and expression of this variation is quite diverse but the occurrence of stable gene mutations has been proven in many species and several new varieties and clones have been obtained by this way in some plant species. Somaclones with phenotypic variation can be easily screened during in vitro regeneration. In this paper the molecular basis of polymorphism detected as RAPD markers in two narrow-leaf regenerants of hybrid poplar are reported.

MATERIAL AND METHODS

Plant material

Among a population of hybrid poplar derived from crossing between *Populus sieboldii* and *P grandidentata* a few promising clones were selected for further testing and utilization. In vitro platelet regeneration protocols for these clones have been achieved through callus cultures (Sato 1974, Ishii et al 1991). During the process of developing protocols for multiplying these clones in vitro 18 regenerants from clone number OP100 showing a significant variation in leaf morphology and branching habit were identified and isolated for further multiplication and testing (Ishii et al 1991). Of these 18 phenotypic variants only two variants could be maintained and multiplied in vitro. These variants were about one-fourth in leaf size of the original plant (Fig 1). The branching frequency of the variants was almost double to the normal plant (Fig 2). Eight regenerants each from the two phenotypic variant (A and B) and one normal plant line were randomly studied for their genetic fingerprints to find out the basis of phenotypic variations in the two variants.

For each sample DNA was extracted from about 150 mg leaves. The protocol of DNA extraction was essentially the cetyltrimethylammonium bromide (CTAB) method (Murry and Thompson 1980) as modified by Shiraishi and Watanabe (1995). Extracted DNA was

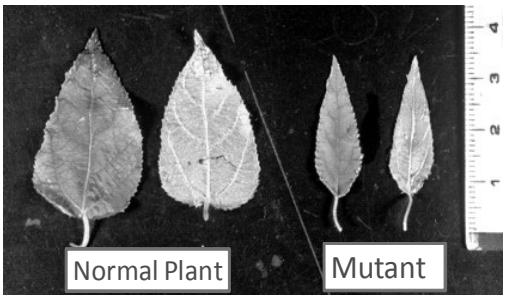

Fig l. Leaves of a normal and a mutant plant of hybrid poplar (*Populus sieboldii* x *P grandidentata*)

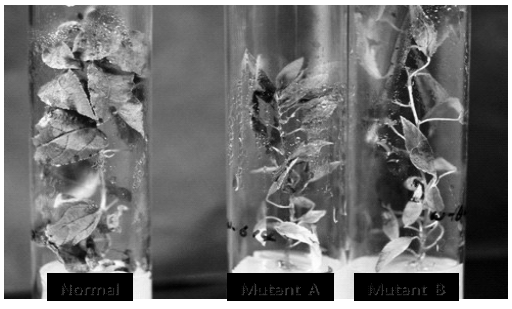

Fig 2. Normal and mutant plants (A and B) growing in vitro DNA extraction and polymerase chain reaction (PCR) amplification

then purified with ELU-QUIK™ DNA purification kit (Schleicher and Schuell). DNA concentration was measured by a Beckman DU-600 Spectrophotometer using Warburg-Christian Concentration Assay. Purified DNA was used as template DNA for RAPD analysis.

A total of 120 arbitrary 10-mer primers (Operon Technology, Calif) were used in RAPD analysis of micropropagated hybrid poplar. RAPD reactions were performed in 20 µl volume containing 10 ng of template DNA, 2.0 µl of 10 x Stoffel buffer (100 mM Tris-HCl, 100 mM KC1, pH 8.3), 0.20 mM of each dNTP, 0.25 mM of RAPD primer, 3.0 mM of $MgCl_2$ and 1 unit of AmpliTaq DNA polymerase Stoffel Fragment (Perkin-Elmer cetus). For DNA amplification the reactions were performed in a TaKaRa PCR Thermal Cycler MP (TP-3000) using a PCR profile consisting of one cycle of 1 min at 94°C, 45 cycles of 10 sec at 94°C, 30 sec at 36°C , 60 sec at 72°C and one cycle of 7 min at 72°C. After amplification the products were separated on 2.0 per cent agarose gel by electrophoresis and stained with ethidium bromide. The gels were then photographed on a UV-transilluminator.

RESULTS AND DISCUSSION

PCRs were repeated twice for each sample to find out their RAPD fingerprints. Only bands reproducible on all runs were considered for analysis. One hundred and twenty primers used in this analysis yielded 386 scoreable bands with an average of 3.22 bands per primer. All but five markers were monomorphic across all the tested plants.

Somaclonal as well as standard lines showed morphological similarities to their respective source lines when cloned in vitro. Their genetic stability was also verified by RAPD patterns of these lines as no aberration in RAPD profiles of these lines was observed. Morphological observations on variant plants indicate that this variation could not likely be explained by alterations due to pathological or physiological factors as no signs of any disease or infection were observed; and control plants maintained exactly in the same conditions did not show any abnormal phenotypes. On the other hand narrow-leaf phenotypes showed different RAPD patterns vis-a-vis normal plants with primers OPG 12, OPQ 04, OPF 20 and OPT 02 (Table 1, Fig 3). Phenotypes A and B were distinguished from each other only by primer OPB 04. In this case phenotype A had an additional band at 350 bp. This band was missing in phenotype B as well as in normal plants. The presence or absence of genetic variation probably depends upon the source of explant and method of regeneration or on the source of regenerants (callus, protoplast and cell) (Larkin et al 1989). As callus culture a high risk method for genetic alteration (Shenoy and Vasil 1992) was used to propagate

Table 1. Results showing RAPD variation between normal and narrow-leaf phenotypes, and between narrow-leaf phenotypes A and B

Primer	Band size (kb)	Result
OPG 12	540	Band present in mutants only
OPQ 04	340	Band present in normal plants only
OPB04	350	Band present in mutant A only
OPF 20	550	Band present in normal plants only
OPT 02	360	Band present in mutants only

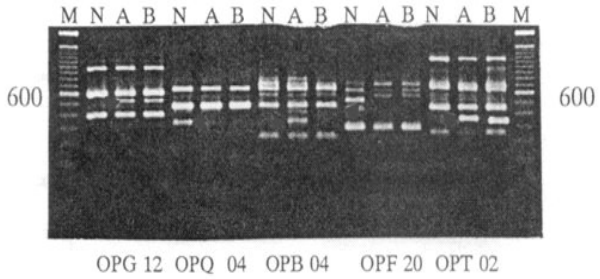

Fig 3. Agarose gel electrophoresis of amplified sequences from a RAPD reaction by using primers OPG 12, OPQ 04, OPB 04, OPF 20, and OPT 02. M, N, A, and B represent the 100-bp marker, normal plant, phenotype A, and phenotype B respectively

hybrid poplar; mutation(s) might have occurred in DNA sequence leading to narrow-leaf phenotypes.

Genetic molecular markers are considered to be reliable in monitoring variability in the DNA sequence of plants. Several authors have applied RAPD technique to investigate the genetic variability and found it very efficient and reliable (Brown et al 1993, Munthali et al 1996, Sanchez et al 2003, Sheidai et al 2008). Munthali et al (1996) compared the results obtained from RAPD analysis with those obtained with restriction fragment length polymorphism (RFLP) and isozymes (Sabir et al 1992) and found no difference in their results. Among poplars RAPD technique was used to study the somaclonal variation in P deltoides (Rani et al 1995) and many other cultivars of Populus (Torjek et al 2001). Molecular approaches such as DNA fingerprinting which were used to show that X-ray treated induced mutation in *Prunus avium* (Yang and Schmidt 1994) and somatic embryo derived variegata phenotypes in *Picea glauca* (Isabel et al 1996) could be correlated with the absence or presence of RAPD fragments. Different hypothesis might account for presence or

absence of a RAPD fragment. Generally changes in RAPD pattern are interpreted in favor of mutation at either one of the two priming regions or deletion/insertion/ inversion in the DNA intervening between priming sites.

The plants of hybrid poplar have been cultured in vitro since 1972 and remained stable since then. Although the genetic status of observed variations in hybrid poplar has not completely been established only 4 RAPD markers out of 386 screened in this study were found to be associated exclusively with the narrow-leaf mutants which suggests that these narrow-leaf mutants could share some link in the genetic basis of their phenotypes. Further studies of RAPD bands correlating with narrow-leaf mutants involving isolation sequencing and their subsequent matching with known gene sequences would make better understanding about these variations.

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