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Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*)

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Abstract Microsatellite DNA markers of ten simple sequence repeat (SSR) loci were used to examine somaclonal variation in randomly selected micropropagated plantlets derived from three different *Populus tremuloides* donor trees (genotypes). The plantlets were obtained from tissue cultures of dormant vegetative buds, and those derived from the same donor tree, grown in the greenhouse, did not exhibit any sign of visible morphological variation. No microsatellite DNA variation was observed among 13 somaclones of one tree and 4 somaclones of another tree at eight of the ten SSR loci. However, despite the small number of micropropagated progeny per tree sampled, microsatellite DNA variation was detected among the plantlets derived from the same donor trees at two SSR loci. The primer pair for the SSR locus *PTR5* revealed somaclonal variation in 1 out of the 13 plantlets obtained from one genotype, while the primer pair for the *PTR2* SSR locus revealed somaclonal variation in one out of the four plantlets obtained from another genotype. The variation at the *PTR2* locus resulted in the appearance of a new allele of increased size, possibly due to an addition of the repeat units, while the variation at the *PTR5* locus resulted in the appearance of third allele, presumably due to the presence of a single extra chromosome or duplication of a chromosomal segment. These results demonstrate that the genetic fidelity of micropropagated plants of *P. tremuloides* cannot always be assured and somaclonal variation can occur even when tissues of well organized vegetative buds are used for tissue cultures; that somaclonal variation cannot always be detected at the gross morphological level; and that microsatellite DNA markers provide useful and sensitive markers for determining the clonal fidelity and somaclonal variation in *P. tremuloides.*

Keywords Poplars · Microsatellite DNA markers · Tissue culture micropropagation · Somaclonal variation · Clonal fidelity

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Introduction

In vitro clonal propagation of forest trees, due to its high multiplication rate, is an attractive alternative for obtaining the high multiplication of elite genotypes of those species that could easily be clonally propagated through conventional methods. For example, a single bud of a mature eucalyptus tree may yield as many as 100,000 plants in vitro in 1 year (Gupta et al. 1981). Moreover, this method may be the only way to clonally propagate those species that cannot easily be cloned through conventional methods (Ahuja 1983, 1984, 1987). Somaclonal variation is of special relevance in perennial plants (Skirvin et al. 1994) and long-generation forest trees since occasional mutations can sometimes only be noticed at very late developmental stages, or even in their offsprings. The tissue culture environment may cause a general disruption of cellular controls, leading to numerous genomic changes in the tissue culture-derived progeny (Phillips et al. 1994). The occurrence of somaclonal variation is a potential drawback when the propagation of an elite tree is intended, where clonal fidelity is required to maintain the advantages of desired elite genotypes (e.g., superior growth, wood properties, disease resistance, and other quality traits). On the other hand, stable somaclonal variation of a specific type may be advantageous for the improvement of certain traits (Antonetti and Pinon 1993; Karp 1995; Jain et al. 1998).

Trembling aspen *(Populus tremuloides* Michx.) is a fast-growing, widespread tree species in North America

and an important source of wood for pulp and paper and oriented-stranded board industries. The increasing commercial demand for trembling aspen wood underlines the importance of improving the quality and productivity of regenerating stocks (Rajora 1991), making *P. tremuloides* an ideal candidate for genetic improvement through breeding and biotechnology. Although most poplars can be propagated easily by the rooting of shoot cuttings, *P. tremuloides*, like its sister aspen species, cannot be clonally propagated in this way. Therefore, it is an ideal candidate for tissue culture micropropagation, and methods for the in vitro multiplication of this species have already been developed (Winton 1970; Ahuja 1983, 1984, 1987; Noh and Minocha 1986; Rajora 1992). However, in order that the clonal fidelity of micropropagated aspen plants be assured, it is essential to determine the existence of any somaclonal variation in the regenerants.

Somaclonal variation can occur for various morphological, physiological, disease resistance, and other traits as well as for biochemical and molecular genetic markers (Larkin and Scowcroft 1981; Karp 1995; Rani and Raina 2000). As gross morphological variations are expected to occur at a much lower frequency than cryptic (e.g., DNA level) variations (Evans et al. 1984), the absence of visible variation does not preclude the absence of all variation among the micropropagated progeny. Allozyme markers can be used for examining cryptic somaclonal variation, but these markers are limited by both the number and amount of polymorphism and their developmentally regulated expression. DNA markers are a more attractive means for examining somaclonal variation since they are more informative and are not developmentally regulated, but techniques such as random amplified polymorphic DNA (RAPD) suffer from a lack of reproducibility (Riedy et al. 1992; Ellsworth et al. 1993). Moreover, RAPDs are dominant diallelic markers; thus, individual parental alleles cannot usually be differentiated by these markers in diploid organisms. Therefore, dominant markers, including amplified fragment length polymorphisms (AFLPs), are not quite informative enough for examining somaclonal variation. Co-dominant, simply inherited, highly polymorphic and reproducible DNA markers are the most suitable markers for detecting somaclonal variation. The sensitivity, reproducibility, co-dominance and strong discriminatory power of microsatellite DNA/SSR (simple sequence repeat) markers (Rajora et al. 2001) make them particularly suitable for detecting somaclonal variation, but their application in the study of somaclonal variation has been rather quite limited (Wang et al. 1996; Barrett et al. 1997; Chowdari et al. 1998).

In poplars (*Populus*), the occurrence of somaclonal variation among tissue culture-regenerated plants has been reported for various morphological, physiological, biochemical, disease resistance and herbicide tolerance traits (Fry et al. 1997). However, most of the studies employed callus cultures (Lester and Berbee 1977), and prolonged culture in the presence of synthetic growth regulators is known to induce somaclonal variation (Larkin and Scowcroft 1981). In trembling aspen, somaclonal variation has been reported for pigment and isozymes (Noh and Minocha 1990) and disease resistance (Valentine et al. 1988), but DNA markers have not yet been used for determining somaclonal variation in trembling aspen or, with one exception (Wang et al. 1996), in any other species of the genus *Populus*.

The frequency of somaclonal variation in *Populus* may be influenced by the regeneration methods employed and the tissue source (Ostry et al. 1994). Micropropagated plants from the cultures of pre-formed structures, such as shoot tips and axillary buds, and from the tissues of hardwood shoot cuttings have been reported to maintain clonal fidelity (Ahuja 1987; Wang and Charles 1991; Ostry et al. 1994), but there is still a possibility of generating somaclonal variants employing this method (Rani and Raina 2000).

Rajora (1992) developed a method for operational micropropagation of trembling aspen using dormant vegetative buds as the explant source. However, this method needs to be evaluated for the maintenance of clonal fidelity of the micropropagation-regenerated plants. The primary objective of the study reported here was to evaluate the usefulness of microsatellite DNA markers in detecting somaclonal variation in micropropagated plants of trembling aspen. We have employed recently developed microsatellite DNA markers (Dayanandan et al. 1998; Rahman et al. 2000) to determine the clonal fidelity of and to examine somaclonal variation in a small sample of micropropagated plantlets of trembling aspen derived from tissue cultures of dormant vegetative buds. This is the first report of the existence of microsatellite DNA somaclonal variation in trembling aspen.

Materials and methods

Several genotypes of trembling aspen (*P. tremuloides* Michx.) were micropropagated through aseptic tissue cultures of dormant vegetative buds as described in Rajora (1992). Briefly, the protocol was as follows. Dormant, healthy vegetative buds were collected from eight approximately 20- to 40-year-old trembling aspen donor trees growing in natural populations, surface-sterilized for 20 min in 25% commercial bleach containing a few drops of Tween 85, and rinsed thrice (10 min each) with sterile distilled water. The bud meristems with a few juvenile leaves were aseptically removed, surface-sterilized in 10% bleach for 5–10 min, washed at least four times with sterile distilled water, and then cultured on either ACM (aspen culture medium; Ahuja 1983, 1984), WPM (woody plant medium; Lloyd and McCown 1981), or MS medium (Murashige and Skoog 1962) containing hormonal supplements of ACM-1, ACM-2 or ACM-3 for bud break, shoot differentiation/multiplication, and root induction, respectively (Ahuja 1983, 1984). Although all of these three media were found to be adequate, the WPM and ACM were found to be better suited for trembling aspen micropropagation. The in vitro-established micropropagated plantlets were further multiplied through the repeated excision and subculture (4–10 times) of developing shoots and shoot primordia. Rooted individual plantlets were potted ex vitro, acclimated to ambient conditions and subsequently grown in a greenhouse, where they were assessed at the morphological level for the occurrence of off-types. Six of the eight trembling aspen trees could be successfully micropropagated.

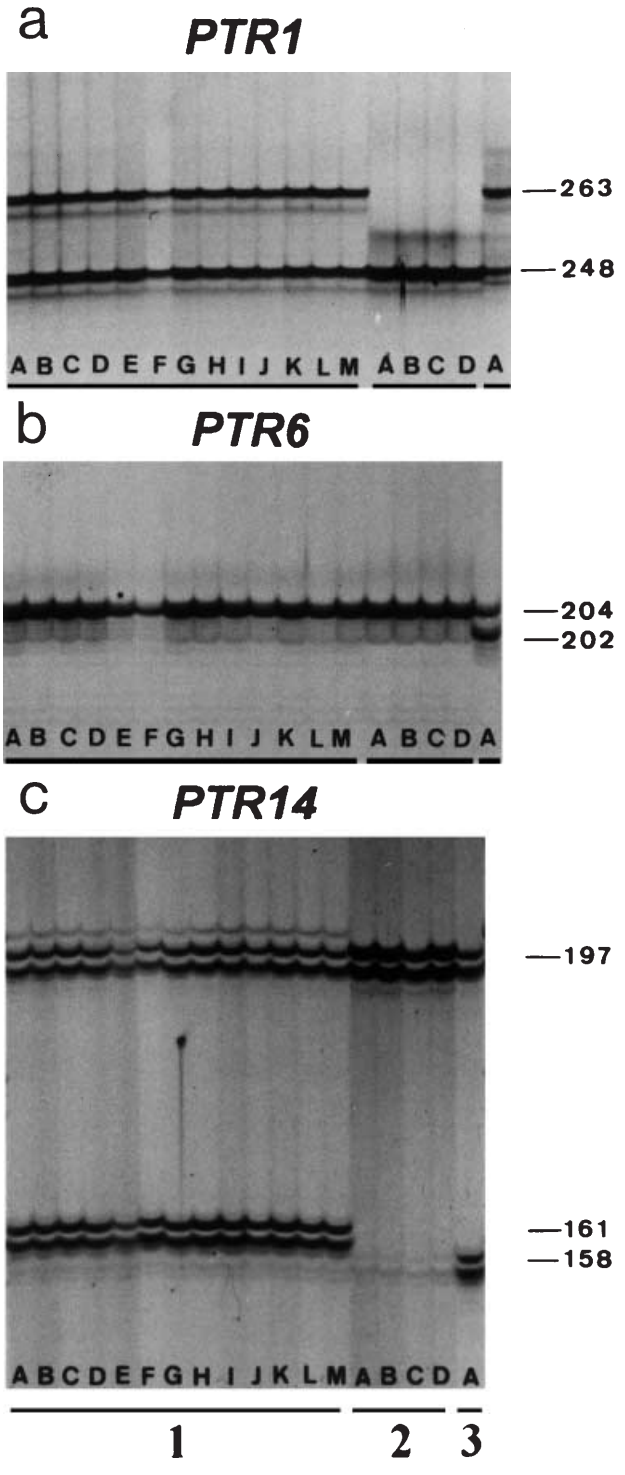

Fig. 1a–c Microsatellite DNA fingerprints of 13 somaclones of tree 1 (*lanes A–M*), 4 somaclones of tree 2 (*lanes A–D*), and 1 somaclone of tree 3 (*lane A*), showing the absence of somaclonal variation at microsatellite DNA loci *PTR1* (**a**), *PTR6* (**b**), and *PTR14* (**c**). The allele sizes are shown as lengths of bases/nucleotides

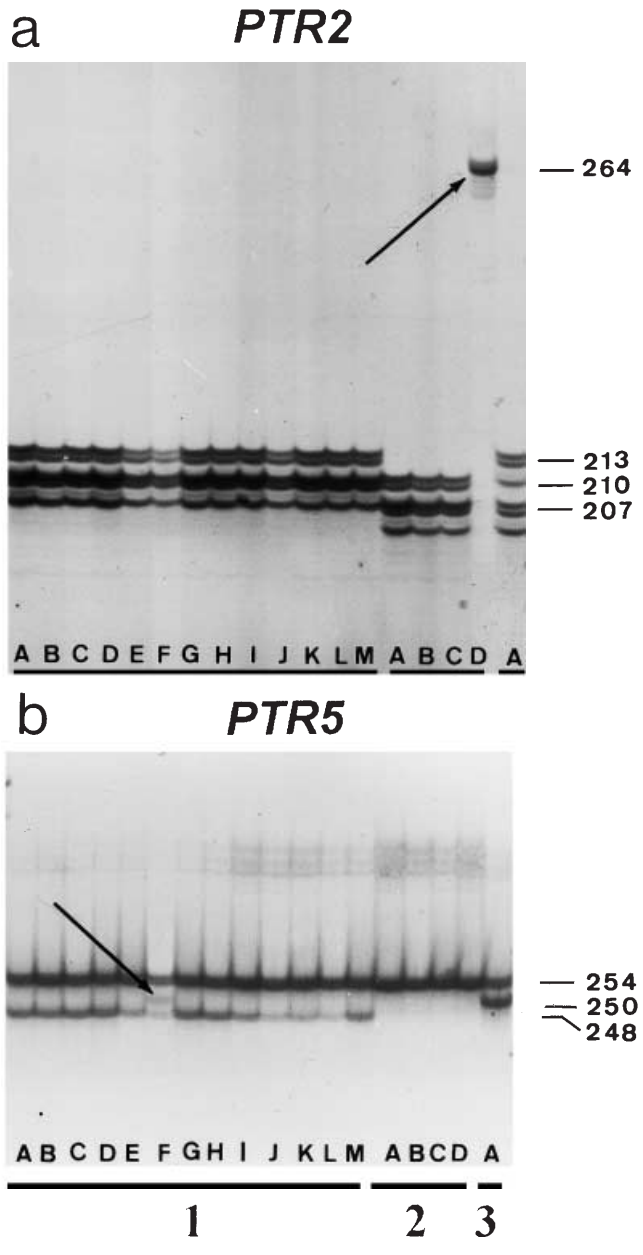

Fig. 2a, b Microsatellite DNA fingerprints of 13 somaclones of tree 1 (*lanes A–M*), 4 somaclones of tree 2 (*lanes A–D*), and 1 somaclone of tree 3 (*lane A*), showing microsatellite DNA somaclonal variation at: **a** the *PTR2* locus in one of the four plantlets (*lane 2D*) derived from tree 2, **b** the *PTR5* locus in 1 of the 13 plantlets (*lane 1F*) derived from tree 1. The alleles are labeled as their size in base pairs. The *arrows* indicate the microsatellite DNA somaclonal variants observed

Thirteen micropropagated plants from donor tree 1 (A–M), and four from donor tree 2 (A–D) were selected at random for determining the occurrence of microsatellite DNA somaclonal variation among the tissue culture-derived plants. In addition, one somaclone of donor tree 3 (A) was included in the study. DNA was extracted from the leaves of approximately 6-month-old individual plantlets following a modified CTAB protocol described in Rajora and Dancik (1995). Only 18 plants were selected for the study because this number best suited the experimental protocol.

Ten microsatellite DNA/SSR loci (*PTR1*, *PTR2*, *PTR3*, *PTR4*, *PTR5*, *PTR6*, *PTR7*, *PTR8*, *PTR12*, and *PTR14*) (Dayanandan et al. 1998; Rahman et al. 2000) were used to examine somaclonal variation. Microsatellite DNA markers were amplified using individual SSR primer pairs according to the 60°↓54°C touch down protocol (Rahman et al. 2000) using a GeneAmp 9600 (Perkin-Elmer, Applied Biosystems, Foster City, Calif.) thermal cycler.

Each polymerase chain reaction (PCR) experiment also contained a negative control consisting of a complete reaction mix, minus the template DNA from trembling aspen, to test for the presence of non-specific amplification. The negative control always failed to show the presence of any amplified product and is consequently not shown here in the results. The amplified products, along with 20 base-pair marker standards (GenSura) (not shown in Figs. 1 and 2), were separated on denaturing polyacrylamide gels and visualized by silver staining as described in Rajora et al. (2000). The gels were air-dried, and contact prints were made on APC film (Promega) according to manufacturer's instructions to obtain permanent images. Microsatellite DNA alleles at a locus were designated on the basis of their molecular sizes in nucleotides/base pairs (bp). The microsatellite DNA analysis for the SSR loci that revealed somaclonal variation was repeated at least three times, and consistent results were obtained.

Results and discussion

Visual assessments of approximately 6-month-old tissue culture-derived plantlets growing in a greenhouse failed to reveal any morphological differences among regenerated plantlets of the same explant trees, indicating the absence of major somaclonal variation among them. This is not surprising given the fact that organized structures such as apical and axillary meristems tend to produce micropropagated progeny with reduced or no variation among them due to their origin from pre-existing meristems and the absence of an intervening callus stage (Vasil and Vasil 1980; Wang and Charles 1991). However, the presence of RAPD polymorphisms in morphologically similar clones arising from the same donor plant of *Populus deltoides* (Rani et al. 1995) indicates that visible evaluation may not be sensitive enough to detect somaclonal variation.

No microsatellite DNA variation was observed among the somaclones at eight SSR loci (*PTR1*, *PTR3*, *PTR4*, *PTR6*, *PTR7*, *PTR8*, *PTR12*, and *PTR14*) (Table 1). Figure 1 depicts a representative sample of the microsatellite DNA profiles of the sampled micropropagated plantlets of three trembling aspen trees at three SSR loci (*PTR1*, *PTR6*, and *PTR14*), showing no somaclonal variation. The microsatellite DNA variants for the locus *PTR14* were able to differentiate among the three trembling aspen donor trees (Fig. 1, Table 1) by virtue of their allelic difference at this locus.

Microsatellite DNA somaclonal variation was detected at two SSR loci: *PTR2* and *PTR5* (Fig. 2, Table 2). The SSR locus *PTR2* showed allele size variation in one of the four micropropagated plants derived from tree 2 (Fig. 2a, Table 2), and this microsatellite DNA variation, with respect to the increased size of the allele, could have arisen as a result of the addition of the repeat units at this locus. This result is consistent with similar observations of microsatellite DNA allele size somaclonal variation reported for *Populus nigra* (Wang et al. 1996). Tandemly repeated sequences are known to show increased levels of instability in tissue culture, with the regenerants containing variable copy number (Landsmann and Uhrig 1985; Brettell et al. 1986). This is most likely affected by mitotic recombination due to inter-chromaid unequal crossing over or intra-chromatid exchange of repeats (Phillips et al. 1994). Similar phenomena may account for the variation observed at the *PTR2* locus in the present study.

At the *PTR5* locus,1 of the 13 somaclones derived from tree 1 showed microsatellite DNA somaclonal variation, whereas all four somaclones from donor tree 2 were identical with respect to their allelic constitution at this locus (Fig. 2b, Table 2). Although all 13 somaclones derived from donor tree 1 had the same allelic constitution of 254/248, one somaclone (lane 1F, Fig. 2b) also had an additional (third) 250-bp allele. Repeated analysis produced the same results. Since no more than one or two alleles were detected in this individual at the nine other SSR loci, as was expected for a diploid individual,

Table 1 Allelic constitution of trembling aspen (*Populus tremuloides*) micropropagated plants at eight SSR loci, showing no somaclonal variation

Donor tree	Micropropagated plants tested	*PTR1*	*PTR3*	*PTR4*	*PTR6*	*PTR7*[a]	*PTR8*	*PTR12*	*PTR14*
1	13	263/248	230/220	194/194	204/204	226/220/214	140/138	256/252	197/161
2	4	248/248	252/224	194/194	204/204	232/226	140/136	256/252	197/197
3	1	263/248	230/220	194/194	204/202	236/230/224	138/138	256/252	197/158

[a] Primers for *PTR7* sequences resolve two microsatellite DNA loci in *Populus tremuloides*, and alleles cannot be assigned to a specific locus. The table shows alleles observed at both microsatellite DNA loci resolved by the primers of *PTR7*

Table 2 Allelic constitution of trembling aspen micropropagated plants at two SSR loci, showing somaclonal variation

SSR locus	Donor tree	Micropropagated plant	Allelic constitution
PTR2	1	13 (A–M)	213/210
	2	3 (A–C)	210/207
		1 (D)	264/264
	3	1 (A)	213/207
PTR5	1	12 (A–E, G–M)	254/248
		1 (F)	254/248/250
	2	4 (A–D)	250/250
	3	1 (A)	254/250

the appearance of the third allele at *PTR5* does not support a triploid status of this individual somaclone. One likely explanation for the consistent presence of the third allele in this individual is probably the addition of a single extra chromosome or the duplication of a chromosome segment in this somaclone during the tissue culture propagation process. This individual somaclone of tree 1 may be an aneuploid based on the presence of the third allele in the progeny; however, in the absence of cytological analysis this cannot be ascertained. Since the sampled micropropagated plants were destroyed after DNA extraction, cytological analysis could not be carried out on those plants showing somaclonal microsatellite DNA variation. It should be noted that the correlation between cytological and molecular analysis with respect to observed variation is not always straightforward. For example, in Sitka spruce (*Picea sitchensis*) somatic embryos, no RAPD variation was observed after an extensive study even though cytogenetic analysis indicated a high level of aneuploidy (Fourre et al. 1997). On the other hand, normal cell cycle controls that prevent cell division before the completion of DNA replication are presumed to be disrupted by tissue culture, resulting in chromosome breakage that could subsequently reunite, thereby leading to duplicated regions (Phillips et al. 1994). It has also been postulated that the late replicating nature of heterochromatin can perturb the cell cycle and result in enhanced chromosome breakage when cells are induced to divide under the conditions of in vitro cultures (Lee and Philips 1988). Such events may also lead to the production of the additional allele at the *PTR5* locus observed in the present study.

Somaclonal variation may arise as a result of minor point mutations and rearrangements in nuclear or organellar DNA, the activation of transposable elements, polyploidy, aneuploidy, and epigenetic changes (Fry et al. 1997). Poplar somaclonal variation has generally been correlated with changes at the chromosome level; however, there is also evidence that Leuce somaclones may also arise without undergoing major chromosomal changes (Fry et al. 1997). Also, the poplars of the Leuce section have been reported to have a higher somaclonal variation rate than poplars belonging to the Aigeiros or Tacamahaca sections (Antonetti and Pinon 1993). In the present study, we observed somaclonal variation that presumably arose from the addition of repeat DNA sequences (*PTR2*) and from the addition of a single extra chromosome (aneuploidy) or duplication of chromosome segments arising from breakage and reunion (*PTR5*).

The detection of somaclonal variation using microsatellite DNA markers among morphologically indistinguishable micropropagated plants underlines the need for testing tissue culture-propagated plants at the molecular level. In general, the use of synthetic growth regulators (such as 2,4-dichlorophenoxyacetic acid), callus production, and long-term culture tend to produce genetic as well as epigenetic variations in many species (Larkin and Scowcroft 1981). Also, the departure from organized growth is a key element in inducing somaclonal variation, suggesting that in disorganized growth the constraints that act to eliminate genetic variations in normal meristems are either suppressed or that mechanisms of genetic instability are induced (Karp 1995). This may indicate a higher frequency of within-clone genetic variation in micropropagated trembling aspen plants than is assumed or generally observed in natural populations, particularly with respect to morphological and phenological traits. However, clonal uniformity based on morphological and phenological traits is not precise. In a recent study, all members of the morphologically and phenologically identified clones of trembling aspen surveyed from natural populations in Alberta were genetically distinct based on their RAPD or chloroplast DNA restriction fragment length polymorphism profiles (Rajora et al., in preparation). Microsatellite DNA markers provide an even higher discriminating power for detecting genetic variation in trembling aspen (Dayanandan et al. 1998; Rahman et al. 2000).

The occurrence of microsatellite DNA variation may not result in detrimental functional effects on somaclones, since microsatellites are generally considered to be selectively neutral. On the other hand, it has been suggested recently that microsatellite sequences primarily reside in the single-copy regions of the pine genome (Elsik et al. 2000), which may lead to microsatellites being linked to genes of selective value in the genome. However, de novo microsatellite DNA variants as observed in our study are unlikely to be linked with traits of selective value. We do not know the value of the somaclonal variants observed in trembling aspen with respect to tree improvement of this species since the relationship between microsatellite DNA somaclonal variation and various traits of interest is not known. Nevertheless, somaclonal variation related to disease resistance has been previously reported in trembling aspen (Valentine et al. 1988).

The results of our study demonstrate that somaclonal variation has occurred in the micropropagated plants of *P. tremuloides*, even though organized meristem tissues of vegetative buds were used as the explant source, and that this variation could be detected using microsatellite DNA markers in a very small sample size. This is not surprising given the high informativeness, variability, and resolution power of the microsatellite DNA markers in trembling aspen (Dayanandan et al. 1998; Rahman et al. 2000). The existence of somaclonal variation among micropropagated plants derived through the culture of organized meristems has been shown for various morphological, cytological, biochemical, and molecular traits (Rani and Raina 2000). The three donor plants had an unique allelic constitution at each of *PTR2*, *PTR5*, *PTR8*, and *PTR14* loci (Tables 1, 2; Figs. 1, 2), which enabled their genetic differentiation using only one primer pair and reinforcing the utility of microsatellite DNA markers for clonal fingerprinting in trembling aspen.

In conclusion, our study demonstrates that the clonal fidelity of micropropagated plants of *P. tremuloides* cannot always be assured even when organized tissues of dormant vegetative buds are used as the explant source, that somaclonal variation cannot always be detected at

the gross morphological level, and that microsatellites are useful and sensitive markers for determining the clonal fidelity and somaclonal variation in *P. tremuloides.*

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