Euphytica **85**: 341–345, 1995.

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Monitoring genetic fidelity *vs* somaclonal variation in Norway spruce (*Picea abies*) somatic embryogenesis by RAPD analysis

Berthold Heinze[1] & Josef Schmidt
Biotechnology Department, Austrian Research Centre, A-2444 Seibersdorf, Austria; [1] *present address: Institute of Forest Genetics, Federal Forest Research Centre, Hauptstrasse 7, A-1140 Vienna, Austria*

Key words: Picea abies, genetic stability, somatic embryogenesis, RAPD

Summary

Somaclonal variation, which is a welcome source of genetic variation for crop breeding, is unwanted when direct regenerants have to be used in tissue culture mass propagation (eg. in many forest trees), or in the regeneration of genetically transformed plants. Random amplified polymorphic DNA (RAPD) was used to analyse somatic embryos and plants regenerated from embryogenic cell lines in Norway spruce, *Picea abies* (L.) Karst. RAPD facilitated the identification of clones, as material from the same cell lines shared identical patterns of amplified fragments, whereas regenerants from different cell lines were easily distinguishable by their respective patterns. For comparisons with explant donor genotypes, cell lines were initiated from cotyledons. Some of the seedlings that had parts of their cotyledons removed were grown on as control plants. Somatic embryos regenerated from cotyledon cell lines showed no aberrations in RAPD banding patterns with respect to donor plants. We conclude that gross somaclonal variation is absent in our plant regeneration system.

Abbreviations: ESM – embryogenic suspensor mass, RAPD – random amplified polymorphic DNA, RFLP – restriction fragment length polymorphism, (2,4-dichlorophenoxy)acetic acid – 2,4-D, 1-naphthaleneacetic acid – NAA

Introduction

Somaclonal variation (Larkin & Scowcroft, 1981) is unwanted in clonal propagation, e.g. of genetically transformed plants, or for large-scale mass propagation programmes in general. In long-lived forest trees, especially where adverse effects are expressed only after years in the field, this may be economically disastrous. True-to-type propagation of the selected genotypes, therefore, is desired. We have been working on somatic embryogenesis in Norway spruce as a conifer model species for Austrian forest trees (Buttgereit, 1991), concentrating on high altitude material from the Austrian Alps, where seed availability is periodically insufficient. The use of reforestation material genetically well-adapted to the extreme climatic conditions there is vital, as many of these forests protect against landslides and avalanches. Genetic characterization of tissue culture material for reforestation may serve to shorten the extensive field-testing necessary. We have used RAPD for this purpose (Williams et al., 1990; Welsh & McClelland, 1990). RAPD analysis offers several advantages over restriction fragment length polymorphism (RFLP) analysis: no prior cloning of DNA sequences is necessary, very little starting material (plant tissue) is sufficient, and quick DNA extraction protocols are suitable (Rafalski et al., 1993).

In spruce species, somatic embryos have been tested for ploidy level (Lelu, 1987), nuclear DNA content (Mo et al., 1989), isozyme patterns (Eastman et al., 1991) and inheritance of RAPD fragments in cell lines from controlled crosses (Isabel et al., 1993). In contrast to the latter authors, we report here on the assessment of somatic embryos and plants on a larger scale. Furthermore, for comparison of propagules with the original genotype of the explant, seedling cotyledon cell

lines were initiated, and somatic embryos recovered were compared by RAPD analysis to those plants the cotyledons of which had been used for culture initiation. Growth and field performance studies involving somatic embryo-derived spruce plants are also underway (Attree & Fowke, 1993; Becwar, 1993; Roberts et al., 1993; Gupta et al., 1993).

Materials and methods

Seeds were obtained from the Federal Forest Research Centre (FBVA), Vienna, Austria: several Austrian high altitude seedlots (open-pollinated) from certified stands. Somatic embryogenesis (Buttgereit, 1991) proceeded through the stages of initiation of embryogenic suspensor masses (ESMs) from mature zygotic embryos or cotyledons (Hakman et al., 1985; Krogstrup, 1986; Lelu et al., 1987), proliferation of ESMs, somatic embryo maturation (Hakman et al., 1985; von Arnold & Hakman, 1988; Becwar et al., 1987; Roberts et al., 1990), controlled dehydration and germination (Roberts et al., 1990).

DNA was extracted from zygotic and somatic embryos as well as from plant needles using the standard protocol specified by Heinze et al. (submitted). In brief, plant tissue was homogenized in microcentrifuge tubes, incubated in extraction buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.25 M NaCl, 1% w/v SDS, 1% w/v PEG MW 8000, 0.2% v/v β-mercaptoethanol) and treated with Proteinase K, phenol and chloroform. DNA was precipitated in 10% PEG – 0.7 M NaCl, washed in 70% ethanol, and dissolved for RAPD analysis. RAPD reactions were performed in a total volume of 15 μl consisting of 1 × supplier's buffer containing 1.5 mM $MgCl_2$, approx. 5 ng DNA, 400 nM primers (10-mers from Operon (OP), Alameda, CA), RNase A (0.3 μg, Boehringer Mannheim, Pikaart & Villeponteau, 1993; Heinze, 1994), tetramethylammonium chloride (2×10^{-5} M, Vierling & Nguygen, 1992), and DNA polymerase (DynaZyme from Finnzymes Oy, 0.05 U/μl, or Taq from Promega, 0.067 U/μl) in the following programme in an MJ Research thermocycler: 3 cycles of 94° C for 1 min, 35° C for 3 min, slow heating (1° C per 4 sec) to 65° C, 72° C for 2 min; then 51 cycles of 94° C for 40 sec, 38° C for 1 min, 72° C for 2 min; and finally, further 10 min at 72° C, and 4° C until recovery and electrophoresis. Amplified DNA fragments were resolved in 1.25% agarose (UltraPure: NuSieve GTG, 4 : 1 w/w) in 0.5 × TBE, visualized by ethidium bromide staining, and photographed (Polaroid 655 films).

Results

Clones of somatic embryos or plants from somatic embryos from a given cell line were identical in their RAPD patterns, whatever primers were used. An example is shown in Fig. 1. No single aberrant band was detected in 50 somatic embryos from cell line 'J7', nor from 100 plants of line 'J1'. The same applied to somatic embryos and plants from other lines, where fewer propagules were analysed. This is in contrast to zygotic embryos from seeds, which could easily be distinguished by their RAPD patterns. Likewise, different clones showed abundant polymorphisms (Fig. 2). In most cases, the first primer applied allowed for genotype distinction; if not, calculating a simple similarity index from combined data of several primers resolved all genotypes and individuals (Heinze 1993).

Somaclonal variation is known to produce genetic differences within propagules of single cell lines (Larkin & Scowcroft, 1981). Mutations may also occur in conifer somatic embryogenesis in the cell line induction phase, where callus formation precedes ESM proliferation. Disorganized callus growth has been associated with somaclonal variation (Brown, 1991). Mutant propagules from developing ESMs could be genetically identical, differing from the parent. We tested this hypothesis by analysing somatic embryos from two lines obtained from seedlings, and comparing embryo RAPD patterns to those of the original plants. Again (Fig. 3), no significant pattern changes were evident.

Discussion

RAPD has been successful in genotype identification in cultivars of e.g. diploid wheat (Vierling & Nguyen, 1992), tomato (Williams & St.Clair, 1993; Klein-Lankhorst et al., 1991), papaya (Stiles et al., 1993), cocoa clones (Wilde et al., 1992) and rose (Torres et al., 1993). Markers were inherited in Mendelian fashion (Carlson et al., 1991; Heinze et al., submitted), and are therefore useful for constructing genetic maps (Tulsieram et al., 1992). Although it has not been possible to distinguish sports (arising from mutated branches) in certain apple cultivars (Harada et al., 1993; Mulcahy et al., 1993), Caetano-Anollés et al. (1993) were able

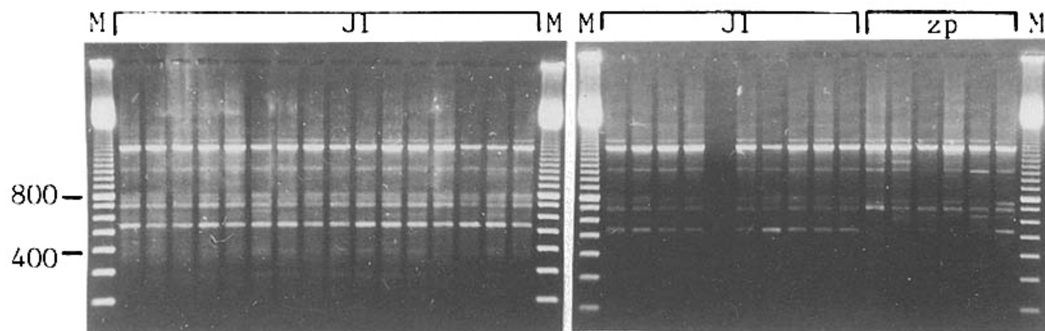

Fig. 1. Identical RAPD patterns from somatic embryo-derived plants of a single clone ('J1'), as opposed to variable patterns in individual 'zygotic' (seed-derived) plants (zp). Primer OPA-02. M, molecular weight markers (100 basepair ladder, Pharmacia).

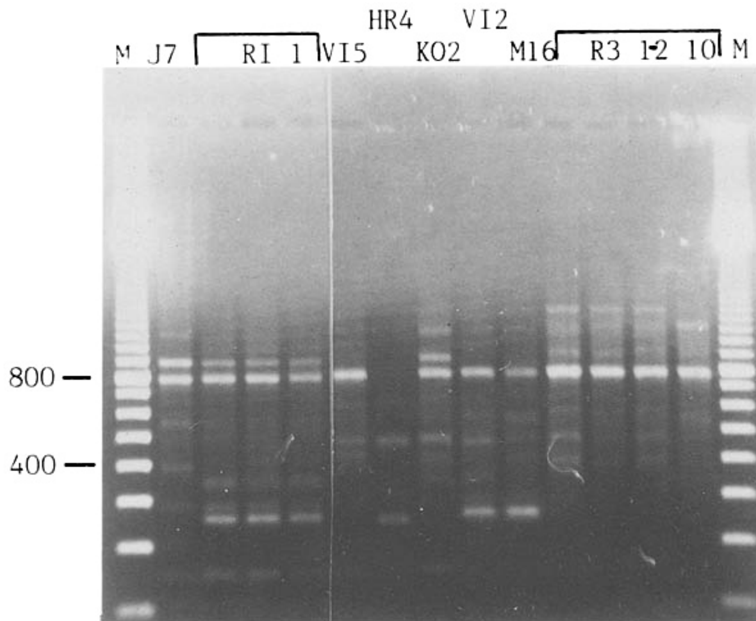

Fig. 2. Clone identification with RAPD patterns. Plant from different somatic embryogenesis-clones ('J1', 'RI 1', . . .) are compared with respect to the pattern obtained with primer OPA-08. M, molecular weight markers (100 basepair ladder, Pharmacia).

to fine-tune the technique for mutant detection in soybean (*Glycine max*). We employed RAPD analysis to screen for crude aberrants in somatic embryogenesis of Norway spruce, but failed to identify any changes in fragment banding patterns arising from tissue culture. The numbers of RAPD bands we scored was fairly high, so they should include markers for most parts of the genome (Tulsieram et al., 1992). This leads us to conclude that there were no losses of major chromosome segments in our system. Cytological analysis of regenerated rye plants (*Secale cereale* L.) by Bebeli et al. (1990) detected chromosome losses and rearrangements. Son et al. (1993) reported the same observations in poplar. In agreement with the conclusions of Shenoy & Vasil (1992), we think that somatic embryogenesis may be an effective sieve to eliminate genetically aberrant propagules, in contrast to reported cases of somaclonal variation identified after shoot regeneration in various crops (e.g. Son et al., 1992; Breiman et al., 1987; Brettell et al., 1986). Being a complicated developmental process, somatic embryogenesis requires the concerted action of many genes

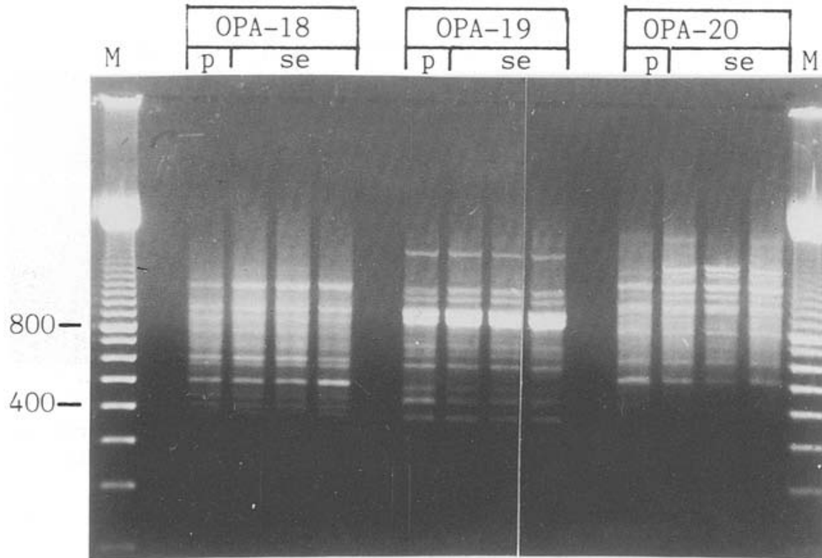

Fig. 3. Comparison of RAPD patterns obtained from explant donor plant (p) and somatic embryos (se) of cell line 'cPF 111'. Primers (Operon) indicated above lanes. M, molecular weight markers (100 basepair ladder, Pharmacia).

and may be sensitive to variation-induced malfunction in any of them. Furthermore, only a few polyploid conifer species are known: *Pinus* and *Picea* contain no such examples. These species may be regarded as not tolerating raised levels of ploidy.

Our cultures were derived from explants with a view to avoiding long phases of unorganised (callus) growth. Brown et al. (1991) detected considerable variation in RFLP banding patterns of *Zea mays* callus, but regenerated shoots showed much less variation. Conifer ESMs show structural organisation (Becwar, 1993), therefore they are unlikely to tolerate the same degree of variation as callus apparently does. In most tissue culture studies cited here, (2,4-dichlorophenoxy)acetic acid (2,4-D) was used as the auxin, and is considered to be a mutagen. However, we avoided the use of 2,4-D and replaced it by 1-naphthaleneacetic acid (NAA; Buttgereit, 1991), which is regarded as less mutagenic.

There are several forms of DNA variation that RAPD analysis is unlikely to detect, including rare point mutations and duplications of genes or chromosomes. Some of these variations, however, are hardly detectable by any current method of DNA analysis in similar settings.

We conclude that no somaclonal variation could be detected in our propagation system, using the very efficient (Brown, 1991; Heinze et al., submitted) RAPD analysis. Low frequencies of variants not detected by this screening method may be acceptable in forestry, where many plants do not survive the first cycles of thinning in any case.

Acknowledgements

We are grateful to Agnes Burg for tissue culture and plant caretaking assistance, to Roger Westcott at Unilever Research, Bedford, UK, for project collaboration, and to Josef Glössl at Universität für Bodenkultur, Vienna, for useful hints. Funds by Unilever Austria and the Austrian Federal Ministry for Agriculture and Forestry.

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