An improved procedure for production of white spruce (Picea glauca) transgenic plants using Agrobacterium tumefaciens

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Abstract

An efficient and reproducible procedure for the transformation of white spruce (Picea glauca [Moench] Voss) embryogenic tissues was developed using A. tumefaciens-mediated gene transfer. Rapidly dividing white spruce embryogenic tissues were co-cultivated with disarmed A. tumefaciens strains containing additional copies of the virulence regions from plasmid PToK47. The plasmid pBi121, containing the neomycin phosphotransferase II (nptII) gene providing kanamycin resistance as a selectable marker and the β-glucuronidase (uidA) reporter gene, was used as binary vector. The highest frequency of transformation (15 transformed tissues g\(^{-1}\) FW of treated embryogenic tissue) was obtained with 5-d-old tissues grown in liquid medium and co-cultivated with Agrobacterium for 2 d in the same medium but containing 50 μM acetosyringone. Recovery of kanamycin-resistant tissues was improved when tissues were first grown for 10 d on a timentin-containing medium (400 mg l\(^{-1}\)), to prevent bacterial overgrowth, before application of the selection pressure. After 6 weeks on kanamycin-selection medium, resistant tissues were obtained and showed stable uidA expression. The presence of the transgenes was demonstrated by PCR analysis and their integration into the genome was confirmed by Southern hybridization. Transgenic plants were regenerated from transformed tissues within 4 months after co-culture.

Key words: Agrobacterium tumefaciens, embryogenic tissue, transformation procedure, transgenic plants, uidA expression, white spruce.

Introduction

The development of tissue culture and transformation systems for tree species including conifers has advanced significantly in the past decade. Stable transformation was reported in Picea abies by the bombardment of somatic embryos (Robertson et al., 1992). Using the same technique, transgenic plants of Picea glauca have been regenerated (Ellis et al., 1993; Bommineni et al., 1993) and similar results have been reported for Picea mariana and Larix laricina (Charest et al., 1996; Klimaszewska et al., 1997). However, this direct procedure of transformation generally resulted in multiple copies of genes being integrated into the host genome, in fragmentation and gene rearrangements, in high frequency of sterile plants (Finnegan and McElroy, 1994; Flavell, 1994) and sometimes in a non-Mendelian inheritance of transgene(s) (Scheid et al., 1991; Peng et al., 1995). Furthermore, the transformation frequency was very low for coniferous species.

Genetic transformation using Agrobacterium tumefaciens is the most efficient procedure for introducing DNA into the genome of many monocotyledonous plants (reviewed by Smith and Hood, 1995). This system has been effective because only one or a few copies of the transfer DNA (T-DNA) are integrated into the host genome. The transgenic plants are generally fertile and the foreign genes are often transmitted to progeny in a Mendelian manner (Rhodora and Thomas, 1996). Recently, several publications have shown that transformation of previously recalcitrant species of monocotyledons (rice, corn and maize) can be accomplished through the use of disarmed A. tumefaciens strains to which additional copies of certain virulence genes were added (Liu et al., 1992; Gelvin and Liu, 1994; Hiei et al., 1994; Hansen et al., 1994; Li et al., 1996; Ishida et al.,...
Materials and methods

Plasmid and embryogenic tissue preparation

The binary vector pBI121 used in the co-cultivation experiments has been described in detail elsewhere (Jefferson et al., 1987). The neomycin phosphotransferase II (nptII) gene, providing resistance to kanamycin, and the β-glucuronidase (uidA) gene were used, respectively, as selectable marker and reporter genes. The binary vector pBI121 was introduced into the agropine strain EHA105 (Hood et al., 1993), the octopine strain LBA4404 (Hoekema et al., 1983) and the nopaline strain GV3101 (Koncz and Schell, 1986), all disarmed Agrobacterium strains containing an additional 15.8 kb fragment carrying extra copies of the virB, virC and virG regions from the supervirulent plasmid PToK47 (Jin et al., 1987).

Embryogenic tissues were initiated from mature zygotic embryos of white spruce (Picea glauca) as described previously (Tremblay, 1990). The embryogenic suspension cultures were grown in 125 ml Erlenmeyer flasks containing 50 ml of liquid maintenance HLM-1 medium (Tremblay, 1990). They were subcultured every week at a ratio 1:5 (v/v) under low light intensity (5 μmol m−2 s−1) with a 16 h photoperiod at 23 ± 2 °C on a rotary shaker at 100 rpm.

Prior to transformation experiments, different antibiotics were compared for their ability to control Agrobacterium growth, but also for their neutral reaction with white spruce embryogenic tissues during maintenance and maturation. For the maintenance experiment, 200 mg FW of embryogenic tissues grown as suspension culture were spread over a nylon membrane placed in Petri dishes on solid HLM-1 medium with timentin. After 5 weeks, mature somatic embryos were separated from the tissues and transferred onto a germination medium (Khlifi and Tremblay, 1995) containing either 0, 10, 25 or 50 mg l−1 kanamycin. Germination was performed under light (75 μmol m−2 s−1) with a 16 h photoperiod, at 23 ± 2 °C. After 5 weeks germination, the number of embryos showing no sign of growth (dead) or developing a root or showing a GUS positive coloration was recorded.

β-glucuronidase histochemical assay

Histochemical GUS assays were conducted essentially as described earlier (Jefferson, 1987). Briefly, kanamycin-resistant tissues or putative transgenic seedlings were incubated overnight at 37 °C in a X-Gluc solution composed of 0.1% (w/v) 5-bromo-4-chloro-3-indolyl-β-glucuronic acid, 100 μM sodium phosphate (pH 7), 0.5 μM potassium ferrocyanide, 0.5 μM potassium ferricyanide, and 10 μM ethylene diamine tetraacetic acid (EDTA). Plant cells and tissues were scored as GUS-positive if any deep indigo blue colour was present.

Genomic DNA extraction, Polymerase Chain Reaction (PCR) amplification, and Southern blot analysis

Genomic DNA was extracted from transformed and untransformed (control) tissues by using a DNeasy Plant Mini Kit (Qiagen) when used for PCR amplification (small-scale preparation) and a DNeasy plant Maxi-Kit (Qiagen) when used for Southern blot analysis (large-scale preparation) according to manufacturer's instructions. PCR amplification of the uidA and nptII genes was performed with the following specific primers: 5′-GTTAGCTTCTTAGAAAACCCCAACCCGTG-3′ and 5′-AAAGTCCCGCTAGGCTCTTTGGGCATG-3′ for the uidA gene, 5′-ACTGAAG-CCGGAAGGGAACCTGAGCTCTATT-3′ and 5′-GATACCG-TAAGACGAGGAAACCGTGCT-3′ for the nptII gene. Standard PCR was carried out in 15 μl containing 100–200 ng DNA, 15 pmol of each primer, 200 μM dNTP, 1× PCR buffer,
and 1 unit taq DNA polymerase (Boehringer, Mannheim). PCR was performed in a programmable thermocycler (Perkin Elmer Cetus) with the following conditions: 5 min at 95°C, followed by 34 cycles (denaturation for 1 min at 94°C, annealing for 1 min at 55°C and extension for 2 min at 72°C) and ended with 10 min at 72°C. Samples were then stored at 4°C. Amplified DNA was detected by ultraviolet light after electrophoresis on 0.8% agarose/ethidium bromide gels using 0.5× TRIS-borate (TBE) as running buffer.

For Southern blot analysis, isolated DNA (100 µg) was cleaved overnight at 37°C with 300 units of HindIII and EcoRI before separation by electrophoresis on 1% agarose gel (Sambrook et al., 1989). Digested DNA was denatured, neutralized and then transferred overnight onto a positively charged nylon membrane (Boehringer, Mannheim) in 4×SSC (Sambrook et al., 1989). DNA was bound to the membrane using a UV Stralinker (Fotodyne Inc.) and then probed with the 3 kb fragment containing the uidA coding sequence, which was obtained by digestion of pBil21 with HindIII and EcoRI. The probe was labelled with 32P-dCTP using T7 Quick Prime kit (Pharmacia). Hybridization was performed overnight at 65°C followed by washes in 2×SSC and 1×SSC+1% SDS for 30 min each. The membrane was then exposed to X-ray film at −80°C with two intensifying screens for 3–4 d.

Results

Choice of selective agent and antibiotic against bacterial overgrowth

The tolerance of embryogenic tissue to kanamycin in the maintenance medium is presented in Fig. 1. A 10-fold increase in fresh weight was recorded after 1 week for embryogenic tissue on non-selective medium (0 mg l−1). Since no tolerance was recorded for the tissues growing on 25 mg l−1 kanamycin, this concentration was chosen for the selection medium.

To select for the antibiotic most effective at preventing bacterial overgrowth while having a minimal effect on white spruce, embryogenic tissues were cultured on HLM-1 medium containing different concentrations of timentin, cefotaxime and carbenicillin. Addition of 100–500 mg l−1 cefotaxime or carbenicillin or of 100–400 mg l−1 timentin did not significantly affect tissue growth compared to the control without antibiotic. However, timentin at 400 mg l−1 provided a better protection against bacterial overgrowth than cefotaxime and carbenicillin even at higher concentration. When 250 mg l−1 of each antibiotic was added to the maturation medium, mature embryonic growth as well as the germination rate of the embryos did not significantly differ from the controls without antibiotic.

Improvement of transformation procedure

The sensitivity of different genotypes (G-165, G-192, G-205, G-305, G-362) of white spruce to different A. tumefaciens strains (LB4404, EHA105, GV3101) was investigated. Among the different strains of Agrobacterium tested, only strain EHA105 successfully produced transformed tissues. Additionally, positive results were only observed with line G-205, which was then used in subsequent experiments.

Different parameters affecting the efficiency of the transformation procedure (solid and liquid co-cultivation, duration of co-cultivation, physiological age of tissue, and timing of application of selective agent) were tested. The optimal results were obtained with 5-d-old tissues grown in liquid culture and subsequently co-cultivated with Agrobacterium for 2 d in a maintenance medium containing 50 µM acetosyringone. The highest yield of kanamycin-resistant tissues was observed when selection was applied just 10 d after co-cultivation.

Transformed cells and transformed immature embryos were observed within 10 d of culture on the maintenance medium containing 400 mg l−1 timentin (Fig. 2A, B). Kanamycin at 25 mg l−1 was sufficient as the selective agent for putatively transformed tissues which were out-growing on the selection medium within 6 weeks, while untransformed tissues died (Fig. 2C). The kanamycin-resistant tissues were screened for uidA expression. They showed numerous portions of tissue expressing GUS (Fig. 2D). GUS-like staining was never observed in untransformed embryogenic tissue.

Kanamycin-resistant embryogenic tissues produced high numbers of cotyledonary embryos after 5 weeks on a maturation medium without kanamycin with at least 50% of them expressing GUS (Fig. 2E). GUS activity was detected histochemically in both globular and cotyledonary transformed embryos of white spruce (Fig. 2F). Mature somatic embryos were transferred

![Fig. 1. Kanamycin toxicity curves for white spruce embryogenic tissue. Growth rate was measured on five replicated samples of 10 pieces of embryogenic tissue after 8 d on HLM-1 medium containing 0, 5, 10, 20, and 25 mg l−1 kanamycin. Data are means ± SD, n = 5.]
onto germination medium supplemented with kanamycin to select resistant (putatively transformed) plantlets. GUS-histochemical assays demonstrated the presence of deep indigo blue colour in all parts of the plantlets (root, hypocotyl, cotyledon and needles) (Fig. 2G, H). After greenhouse acclimatization, transformed plantlets showed normal growth and phenotype (Fig. 2I).

**Screening of stable transformants through germination of somatic embryos on kanamycin**

The effect of kanamycin addition into the germination medium on the recovery of somatic plantlets is shown in Table 1. Most untransformed and transformed cotyledonary embryos developed into seedlings on a germination medium without kanamycin. However, germination on a medium containing 50 mg l\(^{-1}\) kanamycin resulted in 57% mortality for the untransformed somatic embryos with only 6% of them being able to develop a radicle. The opposite result was obtained for the putatively transformed embryos, with 63% of them able to develop a root, all of them expressing \(\textit{uidA}\) (Table 1). Figure 2H shows the difference between an untransformed seedling and a transformed seedling on the germination medium supplemented with 50 mg l\(^{-1}\) kanamycin.

**Screening of stable transformants through molecular analysis**

PCR analysis was used to detect the presence of T-DNA in the kanamycin-resistant and GUS-positive tissues. Genomic DNA from independently transformed and untransformed (control) tissues was subjected to PCR. Figure 3 shows that the samples from transformed tissues gave the predicted DNA fragment bands of 0.5 kb for \(\text{nptII}\) gene (lanes 3 and 4) and 0.8 kb for \(\text{uidA}\) gene (lanes 5, 6), whereas no amplification was detected in the sample from untransformed tissue (lane 2). Southern analysis with the \(^{32}\text{P}\)-labelled \(\text{uidA}\) probe showed a hybridization signal in the three transformed tissues.
This paper presents a high transformation frequency of white spruce embryogenic tissue using a disarmed *A. tumefaciens* strain. Indeed, the frequency of 15 transformants g\(^{-1}\) FW of embryogenic tissues co-cultivated with *Agrobacterium* is high in comparison with the frequency of 1 transformant 10 g\(^{-1}\) FW of bombarded embryogenic tissues obtained by direct gene transfer (Ellis *et al.*, 1993; Bommineni *et al.*, 1993; Charest *et al.*, 1996). The frequency obtained in the present study is also higher than those obtained in similar experiments on hybrid larch (1–2 transformants g\(^{-1}\) FW) (Levée *et al.*, 1997) and white pine (4 transformants g\(^{-1}\) FW) (Levée *et al.*, 1999).

Among the parameters evaluated in this study, *Agrobacterium* strains, plant genotype and physiological state of embryogenic tissue were the most important factors for the success of transformation. With the three *Agrobacterium* strains tested, no transformed tissue was obtained if additional copies of the *virB*, *virC* and *virG* genes from plasmid pToK47 were not added. When additional copies of these virulence genes were added to the strains, transformed tissues were only obtained with the agropine strain (EHA 105) while no transformation was obtained with octopine (LBA4404) or nopaline (GV3101) strains. As shown for other species such as lettuce (Curtis *et al.*, 1994), rice (Hiei *et al.*, 1994; Rhodora and Thomas, 1996), and maize (Ishida *et al.*, 1996), the use of a supervirulent strain of *A. tumefaciens* is essential to facilitate gene insertion into white spruce embryogenic tissue. The requirement for supervirulent plasmid pToK47 containing the *virB*, *virC* and *virG* regions suggests that virulence induction may be a limiting factor for spruce transformation. Using this system, it was possible to show transient expression of the *uidA* gene in Sitka spruce (*Picea sitchensis*), but no stable transformants were produced (Drake *et al.*, 1997). Transient expression of the *uidA* gene has also been observed in both loblolly pine (*Pinus taeda*) and Norway spruce (*Picea abies*) and transformed embryogenic tissues were obtained from Norway spruce (Wenck *et al.*, 1999).

As reported for other species (Sangwan *et al.*, 1992; Bergmann and Stomp, 1992), the physiological status of the tissue was also considered to be an important factor for successful transformation of white spruce. The results of these studies showed that rapidly dividing embryogenic suspension culture, obtained 5 d after a 7 d subculture, provides suitable material to get a high frequency of transformation. Additionally, co-cultivation in liquid medium and addition of 50 \(\mu\)M acetosyringone were essential for successful transformation. Acetosyringone is one of the low molecular weight phenolic compounds naturally released by wounded plant cells and

### Table 1. Recovery of transformed and untransformed seedlings of white spruce after 5 weeks on a germination medium containing different concentrations of kanamycin

| Kanamycin concentration (mg l\(^{-1}\)) | Initial number of embryos | Percentage (%) of germinating embryos
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<tr>
<td></td>
<td>With root</td>
<td>Without root</td>
</tr>
<tr>
<td>0</td>
<td>113 C(^a)</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>115 T</td>
<td>82</td>
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<tr>
<td>10</td>
<td>112 C</td>
<td>45</td>
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<td></td>
<td>117 T</td>
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<td>25</td>
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<td>50</td>
<td>108 C</td>
<td>6</td>
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<td></td>
<td>119 T</td>
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\(^{a}\)C, Control (untransformed); T, Transformed.

\(^{b}\)Calculated from the initial number of embryos in each treatment.
acts as an inducer of the virulence genes. For white spruce, the use of acetosyringone probably increases T-DNA transfer, as reported in a wide range of plant species (Stachel et al., 1985; Godwin et al., 1992). It was also shown that timentin, when used at a high concentration (400 mg l\(^{-1}\)), provided better protection against bacterial overgrowth than cefotaxime and carbenicillin. By using timentin instead of cefotaxime or carbenicillin, the costs associated with antibiotic utilization are reduced. Finally, a two-step procedure for screening transformants, that is a first selection of transformed tissues and a second selection during the germination of putatively transformed embryos on a kanamycin-containing medium (25 and 50 mg l\(^{-1}\), respectively), improved the rapidity and efficiency of the transformation procedure allowing recovery of transgenic plants growing in soil 3–4 months after co-culture. Additionally, the kanamycin concentration used for the selection of transformed embryogenic tissues of white spruce is low in comparison with those used for other woody plants (Le et al., 1996; Franche et al., 1997; Levée et al., 1997).

In conclusion, a simple and efficient transformation procedure has been developed for embryogenic tissue of white spruce using disarmed *Agrobacterium* strain EHA105 containing copies of *virB*, *virC* and *virG* genes from the supervirulent plasmid pToK47. The procedure described here was successfully applied to produce 1200 transgenic white spruce plants containing insecticidal genes from *Bacillus thuringiensis* from 15 independent transformation events. This procedure will therefore facilitate white spruce improvement via genetic engineering and as well as facilitate physiological studies through the use of genetic manipulation.

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**References**


