

Stable *Agrobacterium*-mediated transformation of Norway spruce embryogenic tissues using somatic embryo explants

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ABSTRACT: In conifers and other plants with long reproductive cycles, transformed embryogenic tissues can serve as a convenient source of plant material for the testing of insecticidal or fungicidal transgene efficiency. In this report, transgenic embryogenic tissue was obtained after the transformation of somatic embryos of Norway spruce (*Picea abies* (L.) Karst.) by *Agrobacterium tumefaciens* with the *gus*-intron chimeric gene. The stable integration of transgenes was confirmed by PCR and Southern hybridization. The transformation was successful only in a suitable embryogenic cell line sensitive to *Agrobacterium*. Out of the nine embryogenic lines tested only one gave transgenic callus.

Keywords: *Agrobacterium tumefaciens*; genetic engineering; GUS activity; *Picea abies* (L.) Karst.

Conventional plant breeding methods have resulted in significant genetic gains in some conifers (SHELBOURNE et al. 1989). The long reproductive cycles of conifers, however, render conventional breeding techniques highly time consuming, and some desirable traits of commercial value, such as insect and fungal resistance, are not available in the breeding populations. The genetic engineering methods and tissue culture technologies offer faster and more efficient introduction of desired attributes.

Genetic transformation of plants by *Agrobacterium tumefaciens* is the preferred method of transgene integration into plant genome. A stable transformation procedure has been developed also for various forest tree species (e.g. BAJAJ 2000); the first transgenic tree was described in 1987 (FILLATTI et al. 1987). Transgenic conifers were reported about 15 years ago (HUANG et al. 1991) and to date there have been only a few reports of stably transformed conifers using *Agrobacterium* (e.g. KLIMASZEWSKA et al. 2003; CHARITY et al. 2005; HENDERSON, WALTER 2006).

The Norway spruce (*Picea abies* [L.] Karst.) is an important source of timber in Central Europe.

Nevertheless, the damage caused by bark beetles (*Scolytidae*) entails significant economic losses. The production of transgenic trees with increased insect resistance is one of the possibilities which can solve this problem. However, the effective method of genetic transformation of spruce is necessary. KLIMASZEWSKA et al. (2001) obtained transgenic spruce plants after the co-cultivation of embryogenic tissues with *Agrobacterium tumefaciens*. The possibility of *Agrobacterium*-mediated transformation of spruce embryogenic tissues was described also by WENCK et al. (1999) and LE et al. (2001); non-embryonic tissues do not usually have a sufficient regeneration capacity for transgenic plant regeneration. Particle bombardment is another method how to obtain transgenic spruce. One may use either embryogenic masses (ELLIS et al. 1993; CHAREST et al. 1996; TIAN et al. 2000) or somatic embryos (ROBERTSON et al. 1992; BOMMINENI et al. 1993) as biolistic target.

In this paper we report a novel method of genetic transformation of spruce, namely the *Agrobacterium tumefaciens*-mediated transformation of cotyledonary somatic embryos.

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MATERIAL AND METHODS

Plant material and transformation procedure

The embryogenic cell lines of Norway spruce (*Picea abies* [L.] Karst.) were obtained from Forestry and Game Management Research Institute, Strnady, Czech Republic (MALÁ 1991; MALÁ et al. 1995). Embryogenic tissues were maintained in the dark and at 23°C on half-strength Litvay medium including vitamins (Duchefa) (LITVAY et al. 1985) containing 400 mg·l⁻¹ L-glutamine and 400 mg·l⁻¹ casein hydrolysate (L1 medium), supplemented with 2.2µM BAP, 4.5µM 2,4-D, 2.3µM kinetin, 2 mg·l⁻¹ glycine, 20 g·l⁻¹ sucrose and 2 g·l⁻¹ gelrite (L2 medium).

Not fully developed cotyledonary-stage somatic embryos were collected 4–6 weeks after the transfer of embryogenic tissues to L1 medium supplemented with 50µM ABA, 6% sucrose and 6 g·l⁻¹ Phytigel™ (Sigma) according to TIAN et al. (2000).

The transformation of somatic embryos of Norway spruce was carried out by *Agrobacterium tumefaciens* strain LBA4404 containing the helper plasmid pAL4404 and binary vector with the *gus*-intron chimeric gene and *nptII* selectable gene (VANCANNEYT et al. 1990). An overnight liquid culture of *A. tumefaciens* was pelleted by centrifugation, resuspended in 10mM MgSO₄ to an optical density of OD_{600 nm} 0.9 and a sterile solution of acetosyringone was added to a final concentration of 50µM. The somatic embryos were cultivated in this solution for 45 min at 23°C on a shaker (100 rpm) and then they were transferred onto L2 medium. After 48 hours, the somatic embryos were placed onto L2 medium supplemented with 400 mg·l⁻¹ Timentin. Reinduced embryogenic tissues were carried onto L2 medium supplemented with 200 mg·l⁻¹ cefotaxime and 25 mg·l⁻¹ kanamycin.

Detection of *gusA* and *nptII* genes in transgenic embryogenic tissues

Kanamycin-resistant embryogenic tissues were screened for the presence of *gusA* gene by polymerase chain reaction (PCR). The DNA samples for PCR were prepared with Extract-N-Amp™ Plant PCR Kit (Sigma). The primers GUS1 5'-TCGAT-GCGGTCACCTATTAC-3' and GUS2 5'-CCACG-GTGATATCGTCCAC-3' which amplify a 495 bp fragment were used. This fragment consists of a part of the *gusA* gene including an intron in nucleotide position 263–757. The samples were

heated to 94°C for 4 min, followed by 35 cycles of 94°C for 45 s, 55°C for 30 s, 72°C for 2 min, with a final extension step of 72°C for 10 min. The absence of residual bacterial contaminants was demonstrated in all tested embryogenic tissues by PCR using primers for *virA* gene, located outside of the T-DNA. The primer sequences used 5'-AATTC-ACCGACGCGGCAGGATTTTAAGACAG-3' and 5'-AGCTTTGGTACGAGAGACTATTTTCGCGTAG-3' amplified DNA fragment of 1093 bp.

Southern blot analysis

Genomic DNA for Southern blot analysis was extracted from kanamycin resistant embryogenic tissues as described by TAI and TANKSLEY (1991). About 15 µg of DNA were digested with *Hind*III restriction enzyme, resolved overnight in 1% agarose gel with TBE buffer (SAMBROOK et al. 1989) and transferred onto nylon Hybond-N membrane. Southern hybridizations were performed according to CHURCH and GILBERT (1984). The membrane was probed with the 699 bp fragment of the *nptII* gene. The probe was labelled with [α -³²P] dCTP (3,000 Ci·mmol⁻¹) using a random priming kit, Rediprime™ II, and membranes were autoradiographed for 5 h using a phosphorimager Typhoon system (Amersham Pharmacia Biotech).

GUS assay

GUS activity was determined using a histochemical assay with X-gluc as substrate (JEFFERSON 1987).

RESULTS AND DISCUSSION

We report a procedure for the testing of *Picea abies* embryonic tissue susceptibility to *Agrobacterium tumefaciens* and for the production of transgenic embryogenic tissues from transformed somatic embryos. Using the *gus* gene transient expression assays followed by selection of kanamycin resistant tissues we could confirm the finding of KLIMASZEVSKA et al. (2001) that the success of spruce embryogenic tissue transformation is dependent on the choice of embryogenic cell line sensitive to *Agrobacterium*. Starting with embryos developed from nine embryogenic cell lines we found that seven lines never responded to *Agrobacterium*, showing neither transient expression in embryos nor growth of kanamycin resistant tissue.

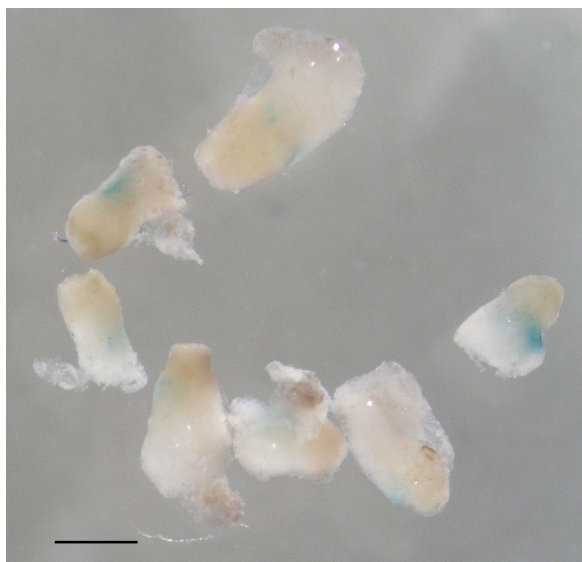


Fig. 1. The transient expression of *gusA* gene in transformed somatic embryos of S10 line. Blue sectors correspond to the GUS activity

Two lines only (S10 and S13) showed the transient expression of *gusA* gene (Fig. 1). Two independent experiments were performed and some variability in transient expression was also recorded. Still, the transient expression of a marker gene closely linked to a selectable gene facilitates the identification of *Agrobacterium*-responsive embryonic lines.

We verified in previous experiments that a sufficient concentration of kanamycin for the selection of spruce transformed embryogenic tissues is $25 \text{ mg} \cdot \text{l}^{-1}$ (MALÁ et al. 2009) and that the Timentin concentration of $400 \text{ mg} \cdot \text{l}^{-1}$ followed by cefotaxime $200 \text{ mg} \cdot \text{l}^{-1}$ reliably kills *Agrobacterium* in the course of a few months. The absence of bacteria was confirmed by PCR.

To apply more stringent selection and to avoid toxic effects of dying non-transformed cells on transgenic embryo viability (MIHAJLEVIC et al. 2003), the transformed embryos were transferred to a dedifferentiating medium and embryogenic

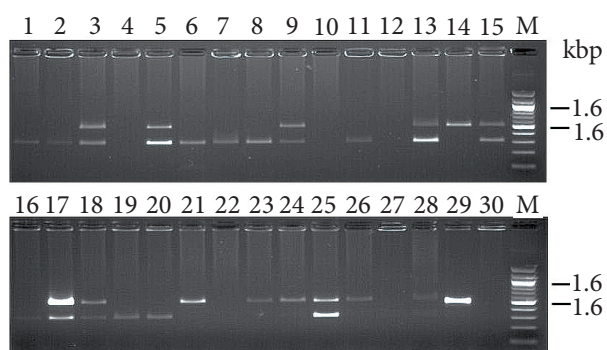


Fig. 2. An example of PCR analyses for the detection of 495 bp fragment of *gusA* gene in transformed embryogenic tissue

tissues were obtained that were further selected on kanamycin and then reinduced. The screening of reinduced embryogenic tissues growing on a medium with kanamycin $25 \text{ mg} \cdot \text{l}^{-1}$ affirmed the presence of *gusA* gene in many of them (Fig. 2).

The growth of reinduced embryogenic tissues was initially very slow, as probably only a small part of cells was transformed. The heterogeneity of obtained tissues during the first six months of growth was also confirmed by PCR; the samples taken from various places of one embryogenic tissue showed different results.

Based on PCR assays 27 positive tissues were chosen and cultivated gradually on 50 , 75 and $100 \text{ mg} \cdot \text{l}^{-1}$ kanamycin. A stronger selective pressure was used to eliminate nontransgenic cells in embryogenic tissues. The best growing tissue on a medium with $100 \text{ mg} \cdot \text{l}^{-1}$ kanamycin that was obtained from the S10 line embryo transformation was selected and the stable integration of the transgene was proved there by Southern blot analysis (Fig. 3).

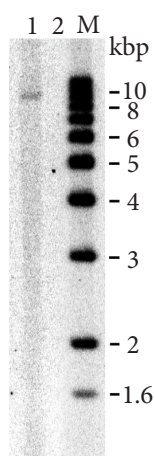


Fig. 3. Southern hybridization analysis of *Hind*III-digested DNA from transformed embryogenic tissue of spruce. DNAs were hybridized with 699 bp *nptII* probe

lane 1 – transformed embryogenic tissue,
lane 2 – non-transformed control

Southern hybridization using the *nptII* gene derived probe and *Hind*III digested genomic DNA allowed us to estimate the number of inserted copies of T-DNA. The fragment size of 1.4 kb at least was expected for transgenic tissue. Fig. 1 documents that the transgenic callus harboured a single copy of T-DNA.

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