

## Norway spruce (*Picea abies*) genetic transformation with modified *Cry3A* gene of *Bacillus thuringiensis*

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**Modified versions of the *Cry3A* gene of *Bacillus thuringiensis* (Bt) were transferred into Norway spruce (*Picea abies*). Both the biolistic approach and *Agrobacterium tumefaciens* mediated procedure were employed for transformation of embryogenic tissue (ET) cultures. The latter method proved to be more efficient yielding 70 transgenic embryogenic tissue lines compared with 18 lines obtained by biolistics. The modified *Cry3A* genes were driven by a 35S promoter and the *nptII* screenable selection marker gene was used in all vectors. The transgenic ETs were molecularly characterized and converted into mature somatic embryos. Germinating embryos formed plantlets which were finally planted into perlite and their *Cry3A* gene transcription activities were demonstrated by RT-PCR.**

**Key words:** *Cry3A* gene modification, *Picea abies*, *Agrobacterium tumefaciens* and biolistic transformation, embryogenic tissue, somatic embryogenesis

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### INTRODUCTION

Traditional tree breeding programs proceed at a slow rate due to long maturation times and the slow growth rate of trees. Nevertheless, biotechnological approaches bring in many new ideas how to achieve significantly quicker improvements of economically important tree traits. The first transgenic trees (*Populus* spp.) have been generated by Fillatti *et al.* (1987) and the first reports about transgenic conifers emerged during the subsequent decade (e.g. Huang *et al.*, 1991; Ellis *et al.*, 1993). Stable transformation of embryogenic tissue cultures of Norway spruce using biolistic and *Agrobacterium* mediated methods and production of transgenic plants from transformed cultures have been first reported by Walter *et al.* (1999) and Klimaszewska *et al.* (2001), respectively. Clapham *et al.* (2000) have published a paper on the development of a particle bombardment method enabling the production of transgenic plantlets of *P. abies* (L.) Karst. at suitable efficiency for practical use.

Most of the early papers dealing with conifer transformation used reporter and selection genes only. The more recent publications report the use of some important genes such as those conferring insect and herbicide resistance or controlling lignin biosynthesis. This demonstrates that conifer biotechnology is entering a phase of prospective commercial applications in plantation forestry. However, more research and development is necessary for both the validation of candidate gene func-

tions and economic production of transgenic conifers for commercial deployment (Henderson & Walter 2007).

One of the most obvious goals of Norway spruce transformation is to achieve spruce bark beetle resistance. Norway spruce is the most important tree species in Central and Northern Europe, which is, however, massively attacked by spruce bark beetles (*Ips* sp.), causing damage of sometimes disastrous dimensions. Classical bioinsecticide *B. thuringiensis* delta-endotoxin *Cry3A* was found inefficient in combating the pest because of low toxicity against *Ips* species and problematic application. Accordingly, we developed modified *Cry3A* genes and produced modified *Cry3A* toxins that showed increased toxicity against spruce bark beetle (Vlasák *et al.*, 2012).

In this paper, the modified versions of the *Cry3A* gene were introduced into the spruce embryogenic tissues derived from elite Norway spruce trees growing at different parts of the Czech Republic and the resulting transgenic lines were characterized by Southern and Northern blots.

### MATERIALS AND METHODS

**Plasmid construction.** Construction and structure of all plasmid vectors used were described elsewhere (Vlasák *et al.*, 2012). Briefly, the *Cry3A* gene was redesigned for high expression in Norwegian spruce and the sequence of the terminal regions as well as of loop 1 of domain II responsible for interaction with insect gut cells were modified. Modified *Cry3A* proteins were expressed in *E. coli* and tested on spruce bark beetle larvae. Genes producing the most toxic proteins were inserted in the *Agrobacterium tumefaciens* transformation vector (Fig. 1).

**Plant material and genetic transformation.** Twenty three embryogenic tissue lines (ETs) were derived at The Forestry and Game Management Research Institute, Strnady, Czech Republic, from open-pollinated immature cones of Norway spruce (*Picea abies*) trees (Malá 1991; Malá *et al.*, 1995) growing at different parts of the Czech Republic. Established ETs were maintained at 23°C in the dark on proliferation L medium containing half-strength of Litvay (Litvay *et al.*, 1985) medium (Duchefa, The Netherlands) with supplementation of 400 mg/l L-glutamine, 400 mg/l casein hydrolysate, 20 g/l sucrose, 2 mg/l glycine, 2 g/l Gelrite™ (Duchefa), 2.2 µM BAP, 4.5 µM 2,4-D, and 2.3 µM kinetin.

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**Abbreviations:** 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; ET(s), embryogenic tissue culture(s); f.w., fresh weight; *GUSint*, β-glucuronidase gene with intron; *nptII*, neomycin phosphotransferase II gene; SE(s), somatic embryo(s)

To evaluate the competence of particular ET genotypes to be genetically transformed transient expression of the *GUSint* gene (Vancanneyt *et al.*, 1990) was employed. ETs were re-activated as described by Klimaszewska *et al.* (2001). Briefly, proliferating ET was suspended in liquid medium L (5 ml of medium for each 350 mg ET) in a 50 ml centrifuge test tube and vigorously shaken to obtain fine suspension without substantial tissue pieces. Five ml of the suspension was poured onto Whatman Grade 2 filter paper (diameter 7 cm) in a Büchner funnel using a wide-mouth 5 ml pipette and a short (about 5 s) low-pressure pulse was used to remove the liquid. The filter paper with attached cells spread out over the whole filter surface was transferred to the fresh semi-solid L medium and cells were cultured in the dark at about 23°C for 2 weeks. This procedure was repeated 3 times and 6 days after the last re-activation ET was ready to use. About 125 mg of such ET was applied *via* cell suspension procedure mentioned above onto filter paper (Whatman Grade 2, 70 mm diameter) in a circle with a diameter of about 2 cm and the filter was placed on L medium in a 9 cm Petri dish. Particle delivery system (PDS 1000/He, BioRad) with 1100 psi rupture discs, 28 in. Hg air pressure reduction, 9 cm of object distance and 0.5 mg 1 µm Au particles coated with 0.8 µg plasmid DNA, was used. Transient expression of the *GUSint* gene was assessed histochemically according to Jefferson (1987) 24 h after shooting.

The stable transformation of ETs was carried out according to Klimaszewska *et al.* (2005) using *Agrobacterium tumefaciens* strain LBA4404 containing the helper plasmid pAL4404 and binary vector harbouring a modified version of the *Cry3A* gene equipped with a 35S promoter and a transcription termination sequence. After 2 days of ETs and *A. tumefaciens* co-cultivation, bacterial cells were washed out by liquid L medium, ETs were placed onto the L medium supplemented with kanamycin (25 mg/l) and cefotaxime (200 mg/l) and exchanged every 2 weeks. Another transformation approach was biolistic with the same parameters as for transient expression assays. After bombardment, the ETs on filter paper were cultured at 22°C in the dark on L medium and 2–3 days later they were transferred onto the same medium supplemented with kanamycin (25 mg/l) and cultured at the same conditions with a transfer on fresh medium every 2 weeks.

#### Maturation of transgenic ETs, desiccation of somatic embryos and their conversion to plants.

Transformed ETs were sub-cultured on proliferation L medium supplemented with 25 mg/l kanamycin at 14-day intervals (22°C, dark) for about 4 months. To initiate the maturation of SEs, ETs were re-activated 3 times on kanamycin containing L medium firstly. Subsequently, 350 mg suspended ETs were placed onto a filter paper (Whatman Grade 2, 7 cm in diameter) in a 9 cm Petri dish with 25 ml of maturation medium and were maintained at 22°C and in the dark for 6 weeks without medium change. The maturation medium with pH 5.8 consisted of half strength L medium, 68 g/l sucrose, 6 g/l Gelrite™, 0.4 g/l L-glutamine, 0.4 g/l casein hydrolysate, 4 g/l glycine, and 50 µM ABA [(+)-abscisic acid] (Sigma). Cotyledonary SEs were picked up and transferred into a growth chamber for partial desiccation performed essentially as described by Schwarzerová *et al.* (2010). Briefly, the embryos were cautiously put onto dry filter paper in 6 cm Petri dishes and the open dishes were put onto 2 filter paper layers wetted with sterile water in 20 cm Petri dishes. After the large dishes were covered with a lid and sealed with parafilm the humidity

inside the dish space was above 98%. The dishes were kept at 18°C, light/dark regime 16/8 h and light intensity 30 µmol/m<sup>2</sup> per s for 16–18 days.

Germination of desiccated SEs was carried out in 9 cm Petri dishes filled with 25 ml of germinating medium consisting of half strength McCown woody plant (MWP) medium (Lloyd & McCown, 1980) containing vitamins, 30 g/l sucrose, 0.4 g/l casein hydrolysate, 0.4 g/l glutamine, and 7 g/l agarose with pH 5.8. Petri dishes, each with about 30 SEs, were tilted vertically at an angle of about 40°C and placed in a plant culture chamber at 22°C and with continual irradiation of 65 µmol/m<sup>2</sup> per s for 2–3 weeks. When roots reached 1–2 cm, plantlets were transferred into plant culture dishes (100×40 mm, PAA Laboratories, Germany) filled with 100 ml fine-grained sterile perlite soaked with half strength vitamin containing MWP medium supplemented with 30 g/l sucrose, and 0.5 µM indolylbutyric acid (IBA), pH 5.8. The plantlets were cultured at 22°C and continual lighting of 100 µmol/m<sup>2</sup> per s until the terminal sprouts reached approximately 5–10 mm.

**Molecular analyses of putative transgenic ETs.** All kanamycin-resistant ETs were screened for the presence of both the *np1II* marker gene and the gene of interest (i.e. *Cry3A* gene) by PCR. Template DNAs were isolated with Extract-N-Amp™ Plant PCR Kit (Sigma) and primers NPT1 (5'-ACG CAG GTT CTC CGG CCG CTT G-3') and NPT2 (5'-GAA GCG GTC AGC CCA TTC GCC G-3') amplified a 699 bp fragment of the *np1II* gene, whilst Tox802A (5'-TAG GAA CAG CAT GCC TAG CTT C-3') and ToxSB (5'-AGA TTT GTG TTA GCC ACA GC-3') primers amplified a 693 bp fragment in all *Cry3A* gene versions used in transformation vectors. The absence of residual bacterial contaminants in kanamycin resistant ETs arising after *Agrobacterium*-mediated transformation was PCR assayed using primers for the *virA* gene (5'-AATTCACCGACGCGGCAG-GATTTTAAGACAG-3' and 5'-AGCTTTGGTACGAGAGACTATTTTCGCGTAG-3') located outside of the T-DNA and amplifying a DNA fragment 1093 bp long. The PCR cycling conditions were as follows: 94°C for 4 min followed by 35 cycles of 94°C for 45 s, 55°C for 30 s and 72°C for 2 min, and completed with a final extension step of 72°C for 10 min. For PCR analyses of the emblings simple alkaline lysis (Klimyuk *et al.*, 1993) of one or two small (2 mm) pieces of cotyledon was used as a DNA extraction method. To release DNA polymerase from the inhibition by compounds present in the crude DNA extracts 0.1% (w/v) bovine serum albumin and 1% (w/v) polyvinylpyrrolidone (40 kDa) were added to the PCR mixture (Xin *et al.*, 2003).

Genomic DNA for Southern blot analyses was isolated from about 500 mg f.w. of ETs essentially as described by Tai & Tanksley (1991) and two 15 µg samples of each DNA were digested with restriction endonucleases — either with *Bam*HI or *Xba*I. DNAs were electrophoresed overnight in 1% TBE agarose gels (Sambrook *et al.*, 1989) and transferred onto nylon Hybond-N<sup>+</sup> (GE Healthcare, UK) membranes. The membranes harbouring DNAs after *Bam*HI restriction were probed with the 699 bp fragment of the *np1II* gene whilst the 693 bp probe derived from the *Cry3A* gene was used for membranes carrying *Xba*I digested DNAs. The probes were labelled with [ $\alpha$ -<sup>32</sup>P]dCTP (1.11 10<sup>8</sup> MBq/mmol) using a random priming kit, Rediprime™ II (GE Healthcare, UK), and a hybridization protocol of Church & Gilbert (1984) was applied. Washed membranes were scanned after 5 h exposure using a phosphorimager Typhoon system (Amersham Biosciences, Little Chalfont, UK).

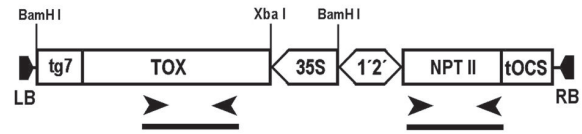


The total RNA was extracted from 100 mg f.w. of selected transgenic ETs using a RNeasy Plant Mini Kit (Qiagen) and used for Northern blotting analyses. 25 µg of each RNA were fractionated in a 1% (w/v) agarose gel in MOPS running buffer with 1.2 M formaldehyde, transferred onto nylon Hybond-N<sup>+</sup> membrane and probed with 693 bp *Cry3A* gene derived probe labelled with [ $\alpha$ -<sup>32</sup>P]dCTP. The hybridisation buffer and temperature as well as conditions during membrane washing and detection were the same as for the Southern hybridisation (Church & Gilbert, 1984).

For two-step reverse transcription PCR (RT-PCR), total RNA extracted by the RNeasy Plant Mini Kit (Qiagen) from 20–30 mg of cotyledons of germinating somatic embryos was used. Enhanced Avian HS RT-PCR Kit (Sigma, Germany) was employed in accordance with manufacturer's recommendations. Briefly, 20 µl of the first step mixture containing, among others, 20 U of enhanced AMV reverse transcriptase and 2.5 µg total RNA was incubated at 42°C for 50 min for the first strand cDNA synthesis. Subsequently, 2 µl of this mixture was used as a template in a second-step reaction with specific primers and JumpStart AccuTaq LA DNA polymerase allowing amplification of the relevant transgene fragment if specific mRNA was present in the analysed RNA sample.

## RESULTS AND DISCUSSION

The competence of the lines for genetic transformation was estimated by transient expression assay of the *GUSint* gene. We found that the number of blue spots in ETs that underwent 3 cycles of reactivation was about an order of magnitude higher than in non-reactivated cultures (data not shown). The average numbers of blue spots per shot and per 125 mg of reactivated ET showed considerable differences among the genotypes used (Supplementary Table 1 at [www.actabp.pl](http://www.actabp.pl)). We also estimated the ability of ETs to reach the maturation stage of somatic embryogenesis which is, according to Walter *et al.* (1999) or Wang *et al.* (2009) determined, among others, by the genotype. It is evident (Supplementary Table 1 [www.actabp.pl](http://www.actabp.pl)) that the lines split in two groups: one which formed tens or hundreds of mature SEs per one Petri dish (with 350 mg ET), and the second group



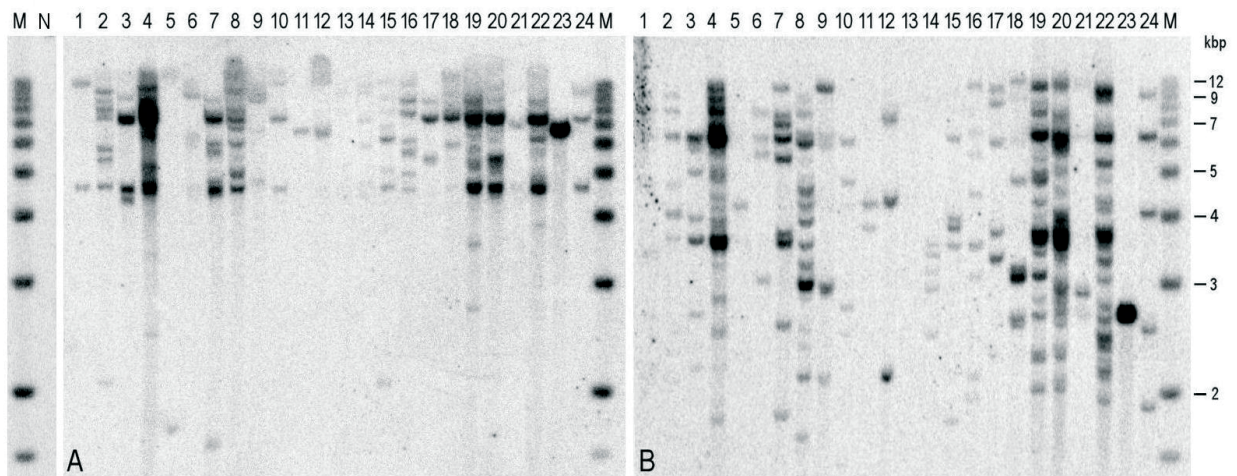
**Figure 1. General structure of T-DNA used in all transformation vectors**

TOX, modified *Cry3A* gene; NPTII, neomycin phosphotransferase II marker gene; 35S, 35S promoter from CaMV; 1'2', dual promoter from the manopine synthase of *A. tumefaciens* T-DNA; tg7, termination sequence from gene 7 of *A. tumefaciens* T-DNA; tOCS, termination sequence from the octopine synthase of *A. tumefaciens* T-DNA; LB, RB left and right border, respectively, of T-DNA. The arrowheads represent primers used for the PCR and RT-PCR analyses, the black lines show the positions of Southern probes.

which was able to produce mature SEs in very low numbers or not at all. Only the first group ET lines are a suitable target for genetic transformation.

Both previously reported successful Norway spruce transformation procedures, i.e. indirect gene delivery (Klimaszewska *et al.*, 2001) and direct transformation method (Walter *et al.*, 1999), were used. The use of ETs as target materials for gene delivery has several advantages (Trontin *et al.*, 2007). ETs consist of actively dividing cells that are the most competent material for genetic transformation. The whole process from ET to transgenic plantlet(s) takes significantly less time than organogenesis from other explants. In addition, transformed ETs can be easily cryopreserved without compromising re-growth and transgene expression (Tereso *et al.*, 2006). This is an important advantage because some *P. abies* ET cultures progressively lose their maturation potency already after 6 months (Högberg *et al.*, 1998). According to Walter *et al.* (1999), the non-transformed tissue of *Picea abies* is highly resistant to kanamycin but our results (Malá *et al.*, 2009) showed that kanamycin concentrations of 50 mg/l and 100 mg/l appeared toxic in all lines used. Therefore, 25 mg kanamycin/l was applied as a starting selection concentration.

Transformation of ETs mediated by *A. tumefaciens* as well as by biolistics was demonstrated at first by PCR analyses (data not shown). In total, 185 putatively transgenic ETs were assayed and 88 showed the presence of both transgenes. No residual bacterial contaminants



**Figure 2. Example of Southern blot analysis of transgenic ETs**

DNA from ETs transformed via *A. tumefaciens* was digested with *Bam*HI (panel A) or *Xba*I (panel B) and hybridised with *nptII* gene derived probe (A) or *Cry3A* probe (B). Lanes 1–24 — embryogenic tissues after *A. tumefaciens*-mediated transformation; lane M — 1 kb DNA Ladder (Gibco BRL). No hybridisation signal was found in non-transformed ET (lane N).

**Table 1. Copy number of the left and right border regions of T-DNA**

The copy number was determined from Southern blots. Three samples after *A. tumefaciens*-mediated transformation and 1 sample after biolistics did not show any hybridisation signal (e.g., lane 13 on Fig. 2) even though they were PCR positive.

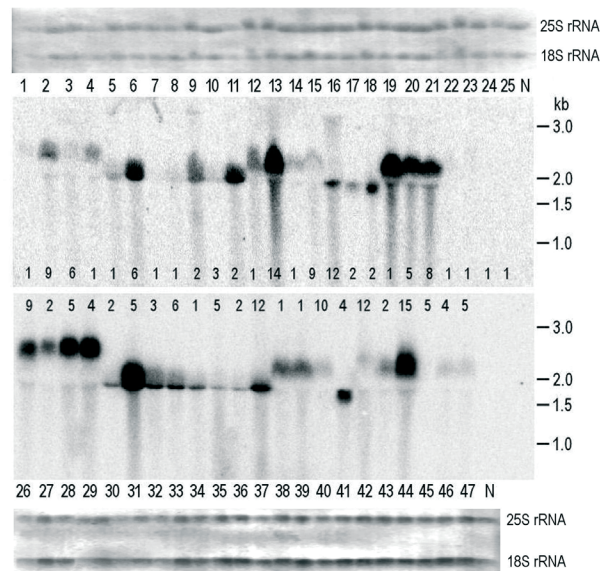
Number of copies	Transformation method			
	<i>A. tumefaciens</i>		Particle bombardment	
	LB adjacent sequence	RB adjacent sequence	LB adjacent sequence	RB adjacent sequence
1–3	32 (48%)	43 (64%)	12 (71%)	10 (59%)
4–8	26 (39%)	24 (36%)	3 (17%)	5 (29%)
> 8	9 (13%)	0	2 (12%)	2 (12%)

were detected by PCR in kanamycin resistant ETs transformed with *A. tumefaciens* about 2 months after transformation (data not shown).

Finally, all 88 kanamycin resistant ETs were transferred to L medium with higher concentration of the antibiotic (50 mg/l) for stronger selection, maintained for a 2–3 week subculture period, and their genomic DNAs were analysed by Southern hybridisation. The DNA samples were digested with two different enzymes and hybridised with two probes (Fig. 1) with the aim to assess the number of copies of the left or right border sequences of T-DNA integrated into spruce DNA. The copy number was usually high reaching up to 15 copies (Fig. 2). In addition, the copy numbers of the left and right border sequences were relatively often not identical in individual ETs indicating a complicated process of T-DNA integration. It is evident that the indirect method of transformation resulted, in our hands, in a higher copy number of inserted sequences than biolistics (Table 1). These findings are surprising because the indirect transformation method usually results in a low copy number of transgene(s) in *Picea* species (Wenck *et al.*, 1999; Klimaszewska *et al.*, 2001). On the contrary, the biolistic transformation procedure is known to introduce a high copy number of the genes transferred into plant cells. For example, Clapham *et al.* (2000) reported 36% transgenic ETs of *P. abies* with a low copy number (1–3) of transgenes but 64% of ETs carrying up to 15–20 copies after gene transfer by particle bombardment. However, there are also reports about a low copy number of a transgene introduced into *P. abies* via biolistics (Wadenbäck *et al.*, 2008; Elfstrand *et al.*, 2001).

For Northern blotting, 47 ETs were selected which harboured a low (1–2) or high (> 5) copy number of the *Cry3A* transgene, and carried all modified versions of *Cry3A*. Transcription of the *Cry3A* gene was not detected in a few transgenic ETs (Fig. 3); the rest showed transgene expression at very different levels with the size of mRNAs corresponding to the type of the *Cry3A* gene modification. We identified ETs exhibiting extremely high expression (lane 13, 19, 31, 44) and bearing both a high copy number ( $\geq 5$ ) and a single copy of the transgene. At the same time, we found low or no expression in ETs with a high copy number (e.g. lane 3, 15, 40, 42) or a single copy (e.g. 1, 7, 22–25) of the transgene. We tried to correlate the expression level with the copy number of the transgene but we could not find a clear correlation. That is not surprising because many reports dealing with the correlation between transgene copy number and expression levels have been published without obtaining unambiguous results. For *P. abies*, Klimaszewska *et al.* (2003) described that the coefficient of the regression of the logarithm of GUS activity versus the number of inserted *gus* copies was not statistically different from zero. However,

one copy of the transgene was found in 22 of 24 translines. On the other hand, they showed a strong positive correlation between the *gus* copy number and the level of GUS in a subset of translines of black spruce (*P. mariana*) in the same report. Nevertheless, this correlation became only modest when all the black spruce translines were analysed. Transgene silencing due to its high copy number has been repeatedly described (Graham *et al.*, 2011) but there are also reports that lines harbouring single copy insertion have been silenced (De Wilde *et al.*, 2001; Mourrain *et al.*, 2007). As stated by Graham *et al.* (2011), in overall the silencing data are in accordance with the idea of a threshold for total or aberrant transcripts, above which the gene silencing mechanism is triggered, rather than with some direct mechanism sensing the genomic copy number. The potential to generate aberrant RNAs, i.e. double-stranded RNA, hairpin RNAs or tandem repeat RNAs resulting from integration events such as partial deletions and/

**Figure 3. Northern blot analyses of transgenic ETs**

About 25 µg of total RNA were probed with 693 bp *Cry3A* gene derived probe. Lanes 1–4 and 26–29 — translines harbouring vector with expected mRNA size of 1914 bases+poly(A); lanes 5–11 and 30–37 — translines with expected mRNA size of 1646 b+poly(A); lanes 12–15 and 38–40 — translines with expected mRNA size of 1737 b+poly(A); lanes 16–18 and 41 — translines with expected mRNA size of 1362 b+poly(A); lanes 19–22 and 42–47 — translines with expected mRNA size of 1716 b+poly(A); 23–25 — translines with expected mRNA size of 2354 b+poly(A); lanes N — non-transformed embryogenic tissue; RNA size marker — RNA Marker 0.2–10 kb (Sigma). Numbers inside the picture represent the number of *Cry3A* gene copies in given ET samples estimated from Southern blot analyses. 25S and 18S rRNA bands indicate the commensurate loading of total RNA.



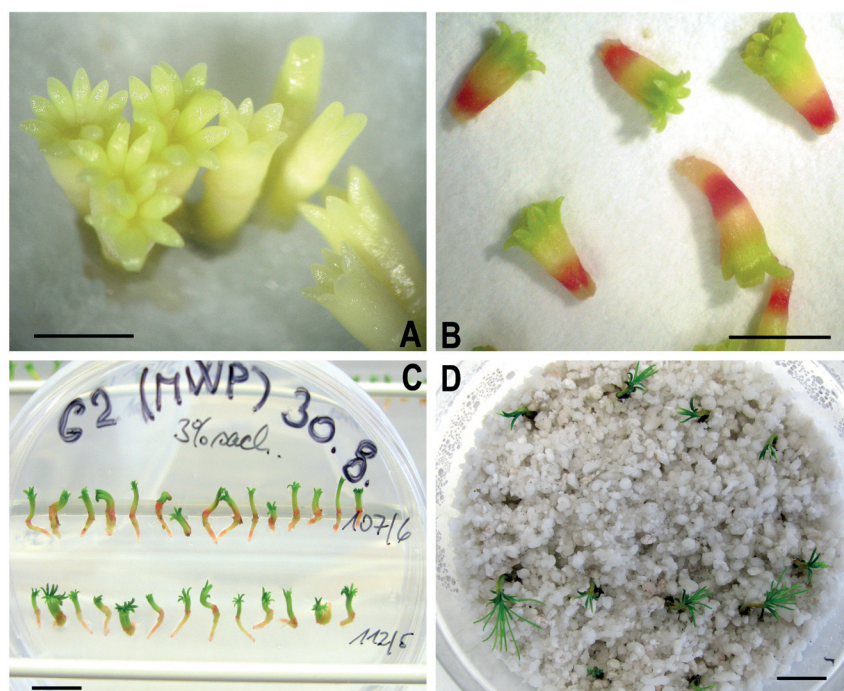
**Table 2. Maturation and germination of Norway spruce somatic embryos (SE) from the control (S35) and 19 transgenic lines**  
Two P. dishes (each with 350 mg ET) were scored for each line.

Line	No.s of SE	SE germination	
		Total No.	%
S35	23	19	83
103/15	24	0	0
104/1	4	2	50
106/1	128	73	57
107/5	11	11	100
108/5	15	10	67
109/2	37	25	68
110/5	5	2	40
111/1	15	8	53
112/3	59	36	61
103/16	89	60	67
104/4	39	31	79
104/6	1	0	0
105/8	18	14	78
107/6	32	30	94
108/1	20	18	90
108/2	1	1	100
108/8	11	11	100
112/3	3	1	33
112/5	27	25	93

or duplications of transgene sequences was reported (Waterhouse *et al.*, 1998; Ma & Mitra 2002). Of course, the probability of at least one such aberrant integration event increases with the transgene copy number.

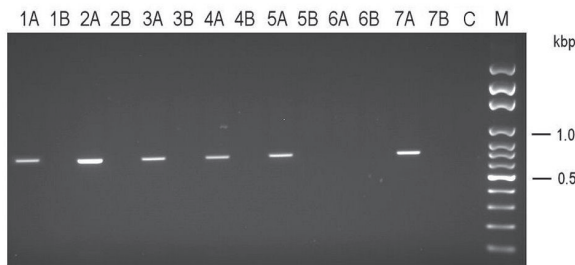
Twenty two ETs with relatively high *Cry3A* gene transcription according to Northern analyses were transferred to the maturation medium (without kanamycin) and 18 of them could be converted into somatic embryos (Table 2, Fig. 4A). The number of developed SEs was considerably different in individual transgenic lines even though they originated from a single S35 line. At the moment we can only speculate that either different transgene product levels in particular lines or the effects of T-DNA insertion mutagenesis could interfere with somatic embryogenesis. Desiccated somatic embryos (Fig. 4B) usually germinated very well (Fig. 4C) and continued to grow when transferred into perlite (Fig. 4D). One or two cotyledon leaves were cut from individual emblings of the transgenic lines for the *Cry3A* gene PCR assay. Out of 122 emblings tested 103 (84%) harboured the *Cry3A* gene (Supplementary Fig. 2 at [www.actabp.pl](http://www.actabp.pl)). It is evident that kanamycin resistant ETs consisted of both transformed and non-transformed cells with higher frequency of the former ones. Such chimerical ETs arising after transformation experiments were also reported by Tereso *et al.* (2006) in *Pinus pinaster* or Salaj *et al.* (2009) in hybrid firs. We also analysed the emblings of some transgenic lines for *Cry3A* gene transcription activities by RT-PCR. Each of the 18 samples analysed consisted of total RNA isolated from 2-6 individual emblings of one transgenic line. Twelve samples gave clear evidence of transcription activity of the *Cry3A* gene in the emblings (Fig. 5).

In this paper, the modified versions of the *Cry3A* gene exhibiting enhanced toxicity to spruce bark beetle were introduced into spruce embryogenic tissues derived from elite Norway spruce trees growing at different parts of the Czech Republic. The transgenic ETs developed into mature somatic embryos which were converted into emblings exhibiting expression of modified *Cry3A* genes.



**Figure 4. Somatic embryogenesis and conversion into emblings in transgenic spruce ETs**

(A) Cotyledonary SEs of transgenic line 103/16 on the maturation medium. (B) The SEs of the 112/5 line after 16 days of desiccation. (C) Somatic embryo germination of the 107/6 and 112/5 lines after 14 days on germinating medium. (D) Culture of emblings of the 103/16 line after 28 days in perlite soaked with MWP medium. Bars: A 2 mm; B 3 mm; C and D 1 cm.



**Figure 5. RT-PCR analysis of transgenic emblings**

Total RNAs were DNase I treated and the samples in lanes A were used for cDNA production by reverse transcriptase. Following amplification by DNA polymerase carried out both in samples A and in samples where no reverse transcriptase was used (lanes B). Lanes 1–6 — different transgenic emblings; lane 7 — positive control (transgenic plant material); lane C — negative control (non-transgenic embling); lane M — 100 bp DNA ladder (New England Biolabs).

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