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BRIEF COMMUNICATION

Production of transgenic *Pinus armandii* plants harbouring *btCry*III(*A*) gene

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Abstract

A synthetic chimeric gene *SbtCryIII(A)* encoding the insecticidal protein btCryIII(A), was transformed into *Pinus armandii* embryos and embryogenic calli using *Agrobacterium tumefaciens*. Polymerase chain reaction and genomic DNA Southern blot analysis showed that the *SbtCryIII(A)* gene was integrated into the genome of transgenic *Pinus armandii* plants, and Northern blot analysis indicated that the *SbtCryIII(A)* gene was transcribed.

Additional key words: embryo, gene for insect resistance, Northern blot, PCR, Southern blot.

Pinus armandii Franch, an economically and ecologically important forest tree, is widely planted in Centre and Southwest of China. Conventional breeding failed in producing pest resistant genotypes of *Pinus armandii* due to the lack of resistance genes among the cultivated *Pinus armandii*.

Genetic transformation has the potential to allow the selective improvement of individual traits in elite clones while still maintaining the existing combination of genes responsible for the superior phenotype (Shin *et al*. 1994, Klimaszewska *et al*. 1997, Wenck *et al*. 1999). Many of the woody species have appeared to be recalcitrant to genetic manipulation. Despite this, progress has been made in a number of species, for example, some of the *Populus* hybrids (Martín-Trillo and Martínez-Zapater 2002), sour orange (Ghorbel *et a*l. 2000), sweet orange (Cervera *et al*. 2005), tea (Lopez *et al*. 2004), water melon (Cho *et al*. 2008), tomato and potato (Bříza *et al*. 2008). In addition, attempts have been made to produce insect-resistant transgenic plants through the introduction and expression of foreign genes encoding insecticidal or insect-inhibiting proteins, such as Bt endotoxins, protease inhibitors and lectins (Zhou *et al*. 1998, Kota *et al*. 1999, Corbin *et al*. 2001, Guo *et al*. 2004, Jube and Borthakur 2007). *Agrobacterium*-mediated transformation is one of the favoured methods for introduction of foreign genes into higher plants. Inoculation with *Agrobacterium* resulted in tumor development in numerous coniferous species (Stomp *et al*. 1990, Bergmann and Stomp 1992). Several reports are available on the *Agrobacterium*-mediated transformation of *Pinus halepensis*, *Pinus taeda*, and *Pinus pinea* (Tzfira *et al*. 1996, Humara *et al*. 1999, Wenck *et al*. 1999). To date, there is no report of the successful genetic transformation of *Pinus armandii* and therefore the aim of our research was to develop a new protocol for the integration of foreign *SbtCryIII(A)* gene into *Pinus armandii* embryos and embryogenic calli using *Agrobacterium tumefaciens*.

Mature zygotic embryos were aseptically removed from the megagametophytes and cultured on pretreatment medium consisted of Murashige and Skoog (MS) medium supplemented with 10 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D), 4 mg dm^{-3} 6-benzyladenine (BA), and 4 mg dm^{-3} kinetin for 1 to 3 weeks. Embryos and embryogenic calli were used in transformation experiments.

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; BA - 6-benzyladenine; BSA - bovine serum albumin; CTAB - cetyltriethylammonium bromide; IBA - indole-3-butyric acid; MS - Murashige and Skoog; NAA - 1-naphtaleneacetic acid; PCR - polymerase chain reaction; SDS - sodium dodecyl sulphate; SSC - salt sodium citrate; YEP - peptone yeast extract.
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Escherichia coli DH5α, *Agrobacterium tumefaciens* LBA4404 containing helper plasmid pAL4404, pUCSbtCryIII(A) (Zhang *et al*. 2004), and pBin513 (Zhang *et al*. 2007) were preserved or constructed in our laboratory. The chimeric *SbtCryIII(A)* gene was isolated from pUCSbtCryIII(A) as a *Bam*HI and *Sal*I fragment and inserted into the *Bam*HI and *Sal*I sites of the binary vector pBin513 (Zhang *et al*. 2007) to form the plant expression vector pBSbtCryIII(A) (Fig. 1).

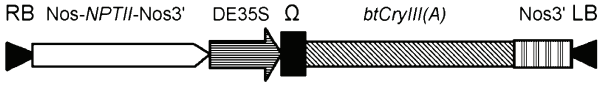

Fig. 1. Gene structure of plant expression vector pBSbtCryIII(A).

The plasmid is a dual-expression vector containing the selectable *nptII* gene encoding the kanamycin resistance and a synthetic chimeric gene *SbtCryIII*, encoding the insecticidal protein CryIII(A). This expression vector was transformed into *Agrobacterium tumefaciens* as previously described (Zhang *et al*. 2007). Infection was performed by dipping embryos or embryogenic calli into bacterial suspension for 30 to 60 min followed by transfer, without rinsing, to the MS medium. After co-cultivation for 2 d in the dark at 28 °C, the infected embryo or calli were rinsed in sterile water, blotted dry with sterile filter paper to remove an excess of bacteria, and then transferred onto the selective medium. The selective medium was Murashige and Skoog (MS) medium containing 500 mg dm^{-3} carbenicillin and kanamycin at 10, 50, 100 or 150 mg dm^{-3}. After selection for 9 - 12 weeks, the resistant calli were transferred to the MS medium containing 2 mg dm^{-3} BA and 0.5 mg dm^{-3} IBA. Plantlets arising from resistant calli were transferred to MS medium containing 1 or 2 mg dm^{-3} pacclobutrozol (*PP333*) and 0.5 mg dm^{-3} NAA for rooting, and then potted in soil.

DNA was extracted from shoots by the CTAB method as described before (Zhang *et al*. 2005). Gene specific primers for *CryIII(A)* amplification were $P_1^{(+)}$ (5' CTG ACG TAA GGG ATG ACG CAC) and $P_2^{(-)}$ (5' CAG TGA TCA GTG TAC TCT TGC G). A fragment of 740 bp should be amplified by using these two primers. Amplifications were carried out in 0.025 cm^3 of reaction mixture containing 0.0025 cm^3 10× PCR buffer, 0.2 mM dNTPs, 2 mM of $MgCl_2$, 1 U Taq DNA polymerase (*Sangon*, Shanghai, China), 20 ng of each primer (*Sangon*) and 20 ng of template DNA. The reaction mixture was incubated at 94 °C for 120 s, followed by 35 cycles of 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 60 s. The PCR reactions were terminated following incubation at 72 °C for 6 min, electrophoresed with 0.002 cm^3 loading buffer through 1.0 % (m/v) agarose gel containing 0.5 μg cm^{-3} ethidium bromide, and visualized and photographed on an UV transilluminator.

*Bam*HI~*Xho*I fragment of *SbtCryIII(A)* gene were ^{32}P-labelled using random-primed labelling (Feinberg and Vogelstein 1983) and used as probes in Southern blot analysis. The digested samples were electrophoresed on 1 % agarose gels and transferred onto a nylon membrane, as described by Sambrook *et al*. (1989). This nylon was baked at 80 °C for 2 h to immobilize transferred DNA. The prehybridization blots were hybridized with *btCryIII(A)* gene probe overnight at 65 °C. They were then washed 3 times with sodium phosphate buffer (pH 7.2) and 1 % SDS at 65 °C for 5, 30, and 15 min. The blot was subsequently analyzed by means of autoradiography with X-ray films (*Kodak*).

RNA was isolated from leaves using a method outlined by Schultz *et al*. (1994) with slight modifications. Hybridization was conducted by pre-hybridizing the membrane in hybridization buffer (0.5 M sodium phosphate, pH 7.2, 7 % SDS, 1 mM EDTA, 1 % BSA) supplemented with 0.5 dm^3 of 10 mg cm^{-3} denatured herring sperm DNA (*Invitrogen*, Paisley, UK), for 4 h at 60 °C. The denatured probes were then added to the buffer and hybridized at 60 °C overnight in hybridization oven. Thereafter, the membrane was washed twice in 40 mM sodium phosphate buffer (pH 7.2) with 1 % (m/v) SDS for 10 min at room temperature and washed in 40 mM sodium phosphate (pH 7.2) with 5 % (m/v) SDS at the hybridization temperature for 15 min. A series of stringency washes in 2× SSC, 0.1 % SDS followed by 1× SSC, 0.1 % SDS were depending on the binding strength of the probes to the templates. After the membrane was exposed overnight to a *Fuji* phosphor imager plate, the image was scanned using *Fuji* film *3000 PhospoImager*.

Nine to twelve weeks after the kanamycin resistant calli were transferred to differentiation medium, putative transgenic adventitious shoots were formed on the surface of kanamycin resistant calli. The frequency of adventitious bud formation was 8.5 - 21.9 % on the differentiation medium supplemented with BA and IBA in the 12th week of culture and 5.4 to 16.5 % of shoots were rooting. With this system, a total of about 300 independently transformed plants were obtained.

PCR analysis was carried out as a rapid identification for the presence of the *SbtCryIII(A)* gene in kanamycin resistant putative transgenic plants of *Pinus armandii*. The expected 740 bp band was amplified in some of the kanamycin resistant transgenic plants. No 740 bp band was amplified in the non-transformed regenerated plants (Fig. 2*A*). Thus, it indicated that some of putatively transformed regenerated plantlets from inoculation might be transformants.

It was worth noting that not all of the kanamycin resistant transformants showed the expected 740 bp-fragment when we performed PCR analysis. It is partly because some mutations happened during the treatment with the small calli. The PCR-undetected callus could not grow well anymore after keeping on selections in subculture. The positive ones were screened and used for further analysis.

Both growth and phenotype of most regenerated plantlets appeared to be similar to the untreated control. However, about 2 % of transformants appeared different in morphology, such as curled leaves, longer leaves,

shorter knots or curled stems comparing with the control ones. All the PCR possitive plants could grow well on kanamycin containing medium, however, the control explants died during the first three weeks of culture on kanamycin containing medium. Some PCR positive plants were selected for Southern blot hybridization and Northern blot hybridization. Fig. 2*B* showed the results of the transgenic plant DNA hybridized with ^{32}P-dCTP labelled *SbtCryIII(A)* gene probes. The patterns of hybridization with *Bt* gene probe suggested that the insect-resistant gene have been inserted into the genome of some plants (Fig. 2*B*). Northern blot hybridization analysis showed that the expression of *SbtCryIII(A)* was detected in some of the transgenic plants but absent in the controls (Fig. 2*C*).

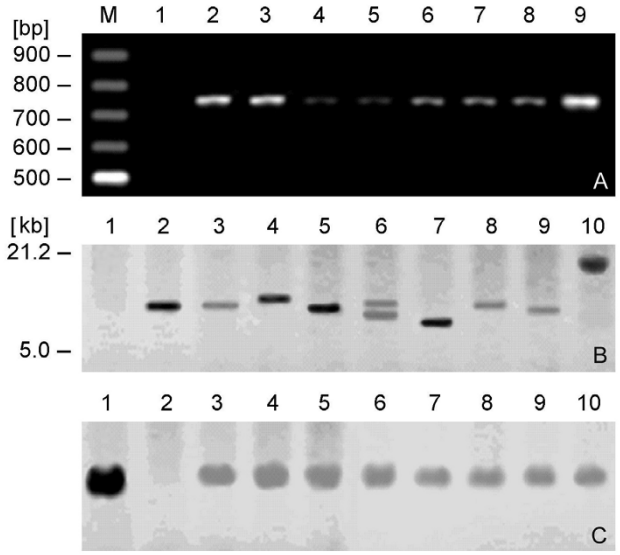

Fig. 2. PCR analysis, Southern-blot analysis and Northern blot analysis of *P. armandii* transgenic lines. *A* - *Line* 1: negative control, non-transformed regenerated plant; *lines* 2 - 8: candidate transgenic lines 3#, 7#, 14#, 15#, 18#, 22#, 25#, 31#; *line* 9: positive control, pBSbtCryⅢ(A). *B* - Genomic DNA was digested with the restriction enzyme *Hin*dIII and hybridized with *SbtCryIII(A)* probes labelled with ^{32}P. *Line* 1: negative control, non-transformed regenerated plant; *lines* 2 - 9: candidate transgenic lines 3#, 7#, 14#, 15#, 18#, 22#, 25#, 31#; *line* 10: positive control, pBSbtCryⅢ(A) (5 pg). *C* - Each lane contains 20 μg of total RNA. *Line* 1: positive control, total RNA from *Escherichia coli* DH5α containing pBSbtCryⅢ(A); *line* 2: negative control, non-transformed regenerated plant; *line*s 3 - 10: candidate transgenic lines 3#, 7#, 14#, 15#, 18#, 22#, 25#, 31#.

It was evident that the original starting material for *Agrobacterium*-mediated transformation is the most crucial factor for increased efficiency of transformation. Successful transformation of *Pinus armandii* was dependent on many factors. The choice of starting concentration of kanamycin for the transformation of *Pinus armandii* calli is very important. The frequency of resistant calli was 6.2 - 21.4 % when 10 mg dm^{-3} kanamycin was used for the first and/or second selection, but only 1.5 - 2 % on a starting selection medium containing 50 and 150 mg dm^{-3} kanamycin. The concentration of *Agrobacterium* is also very important. In the present study, it was observed that the use of a lower concentration of *Agrobacterium* suspended in YEP medium and co-cultivation on media overlaid with *Whatman No. 1* filter paper, reduced the browning of calli after co-cultivation. Possibly, this is because of reduced damage to explants during *Agrobacterium* infection, which resulted in less phenolic production and better recovery of callus during selection. The length of co-cultivation period affected the efficiency of transformation. A co-culture time of 2 - 3 d was needed so that there were enough bacteria to cover the calli, and transformed plants could be obtained. Similar results were also reported by Chen *et al*. (1997). At last, the states of callus also affected the efficiency of transformation. In our observation, any callus that turned brown within 10 d and new callus growth appearing after 20 d during the third round of selection were found to be untransformed. The calli that grow actively during the third round of selection were found to be transformed.

It is hard to find a properly insect-resistant parent among the existed *Pinus armandii* germplasms. Introducing a foreign gene against the insect into the certain *Pinus armandii* lines is a good way. In this study, a new expression vector was constructed and transgenic *Pinus armandii* plants, which contained *SbtCryIII(A)* gene, were obtained. It was the first time to transfer foreign genes into the genome of *Pinus armandii.*

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