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Full Length Research Paper

Transgenic *Pinus armandii* plants containing BT obtained via electroporation of seed-derived embryos

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A genetic transformation system for *Pinus armandii* was presented using electroporation for gene delivery and mature embryo as the gene target. Plasmid DNA (pBSbtCryⅢ(A)), which contained a selectable *npt//* gene for resistance to the kanamycin and a synthetic chimeric gene SbtCryⅢ(A) encoding the insecticidal protein btCryⅢ(A), was delivered into mature P. *armandii* embryos via electroporation. Transformed plants were identified by their ability to grow on a selective medium containing 50 mg/L of kanamycin. Plant resistance to the application of kanamycin, PCR and southern hybridization indicated that *SbtCryⅢ*(A) genes had integrated into the P. *armandii* genome. Results of Southern blot hybridization indicated that some PCR positive plants might be inlaid.

Key words: Electrotransformation, insect-resistant gene, mature embryo, DNA hybridisation, *Pinus armandii.*

INTRODUCTION

Pinus armandii Franch, an economically and ecologically important forest tree, is widely planted in Centre and Southwest of China (Liu et al., 2010). However, insect pest among *P. armandii* causes a lot of economic loss every year. Genetic improvements of forest plants were studied in the past two decades (Bergmann and Stomp, 1992; Humara et al., 1999; Wenck et al., 1999; Liu et al., 2010), and genetic transformation had become one of the most important tools. There were a lot of methods for production of transgenic plants such as using Agrobacterium (Zhang et al., 2007), polyethelene glycol (Choi et al., 1998), microprojectile bombardment (Vasil et al., 1992; Becker et al., 2000), and electroporation (Fromm et al., 1986). Different explant types were used in the transformation of plants, including mature seed-derived calli, immature embryo-derived calli, immature embryos, and shoot apex (Hiei et al., 1994; Aldemita and Hodges, 1996; Park et al., 1996). However, the improvement of *P. armandii* via biotechnological approaches is limited by the lack of a transformation system.

We present results of genetic transformation of mature *P. armandii* embryos containing a synthetic chimeric gene SbtCryⅢ(A) encoding the insecticidal protein btCryⅢ(A) using electroporation. The results indicated that foreign DNA could be integrated into the host genome.

MATERIALS AND METHODS

Materials

Mature embryos, isolated from field-grown *P. armandii* were used in transformation experiments. Mother plants are all elite clones, collected from deferent places of Yunnan Province (China), and preserved in Zixi mountain (Yunnan, China) in 1987 (Zhu, 2006).

Agarose (spain subpackage) and the HexaLabel™ DNA Labeling Kit was purchased from Huamei Bioengineering Co. (China) and Fermentas (Hanover, MD) respectively. Carbenicillin, kanamycin and HEPES were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). PCR primers were synthesised by Shanghai Sangon (China). Endonucleases (BamHI and XhoI), dNTPs and Taq DNA polymerase, KCl, CaCl2 and all other chemicals were

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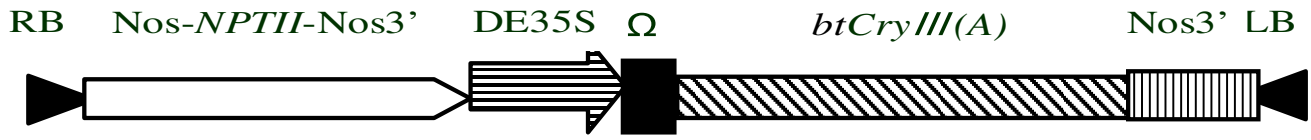

Figure 1. Gene structure of plant expression vectors.

bought from Shanghai Sangon (China).

Plasmid DNA

Escherichia coli DH5α, 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) (Figure 1). It is a dual-expression vector containing the selectable *nptII* gene encoding the kanamycin gene and a synthetic chimeric gene *SbtCryIII(A)*, encoding the insecticidal protein CryIII(A).

Electroporation

Seed capsules of dried mature *P. armandii* seeds were moved. Before use, the embryos were immersed in 70% ethanol for 8 to 10 s and then in 0.1% $HgCl_2$ for 5 to 8 min (depending on degree of tenderness). Finally, the explants were washed with 4 or 5 rinses of sterile water to eliminate the sterilization agent. The embryos were then carefully transferred into a disposable microcuvette containing 200 µl of *P. armandii* electroporation buffer (80 mmol/L KCl, 5 mmol/L $CaCl_2$, 10 mmol/L Hepes, and 0.5 mol/L mannitol [pH 7.2]). To each cuvette, 50 µg of plasmid DNA was added and co-incubated with embryos for 1 h. The cuvettes were then transferred to ice bath for 10 min. Thereafter, the electroporation was carried out by discharging one pulse with field strength of 375 v/cm from a 900-µF capacitor. The pulse strength, capacitance, and electroporation apparatus were described by Dekeyser et al. (1990).

Regeneration and selection of transgenic lines

Plantlets were regenerated from electroporated embryos by culturing onto a hormone-free MS medium supplemented with 500 mg/L carbenicillin and 50 mg/L kanamycin. Then, the *NPTII*r plantlets were transferred to pots with pasteurized soil. The pots were kept in a greenhouse at 18 to 22℃ with natural light–dark cycles. The relative humidity varied between 50 and 95% (between 60 and 80% during 80% of the time), and soil moisture was maintained with steriled water.

PCR

DNA was extracted from shoots by the CTAB method (Zhang et al., 2008). DNA quantity was estimated spectrophotometrically by measuring absorbance at 260 nm. DNA samples were diluted in sterile deionized water and maintained at -20℃. Gene specific primers for *SbtCryIII(A)* amplification are $P_1^{(+)}$ (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 25 µl of reaction mixture containing 2.5 µl 10 × PCR buffer, 0.2 mmol/L dNTPs, 2mmol/L of $MgCl_2$, 1U of Taq DNA polymerase (Shanghai Sangon, China), 20 ng of each primer pairs (Shanghai Sangon, China) and 20 ng of template DNA. The reaction mixture was incubated at 94℃ for 120 s, followed by 35 cycles of 94℃ for 45 s, 57℃ for 45 s, and 72℃ for 60 s. The PCR was terminated following incubation at 72℃ for 6 min. The amplifications added with 2 µl loading buffer were electrophoresed through 1.0% in an agarose gel containing 0.5 µg/ml ethidium bromide, and were visualized and photographed on an UV transilluminator.

Southern blot

*Bam*HI ~*Xho*I fragment of *SbtCryIII(A)* gene was ^{32}P-labled 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 experiment was conducted with a view to estimate the transgene copy number in the putative transformants. The blot was subsequently analyzed by means of autoradiography with x-ray films (Kodak, Japan).

RESULTS

Kanamycin resistant shoots were rooted on medium containing 50 mg/L kanamycin. About 70 to 80% of the shoots produced roots and regenerated into a complete plantlet. With this system, a total of about 150 independently transformed plants were obtained. However, the transfer of *NPTII*r plantlets to the greenhouse and soil leads to loss of some transgenic plants. The results of PCR analysis revealed that more than 80% of the regenerated plants could produce the expected gene-specific PCR product, implying that they are possibly transgenic. Portions of gels showing typical amplification products are shown in Figure 2.

Some PCR positive plants were selected for Southern blot hybridization to confirm their transgenic nature. Figure 3 showed the results of the transgenic plant DNA hybridized with ^{32}P-dCTP labeled *SbtCryIII(A)* gene probes. The patterns of hybridization with *SbtCryIII(A)* gene probe suggests that the insect-resistant genes have been inserted into the genome of some plants. Results showed that there were no copy in one of them ($^{\#}$21), a single copy in three of them ($^{\#}$2, $^{\#}$13, $^{\#}$27), two copies in three of them ($^{\#}$7, $^{\#}$15, $^{\#}$32) , and three copies in one of

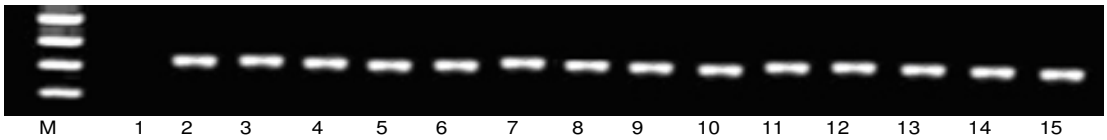

Figure 2. PCR analysis of *P. armandii* transgenic lines. Line 1: Negative control, non-transformed regenerated plant; Line 2-15: Candidate transgenic lines.

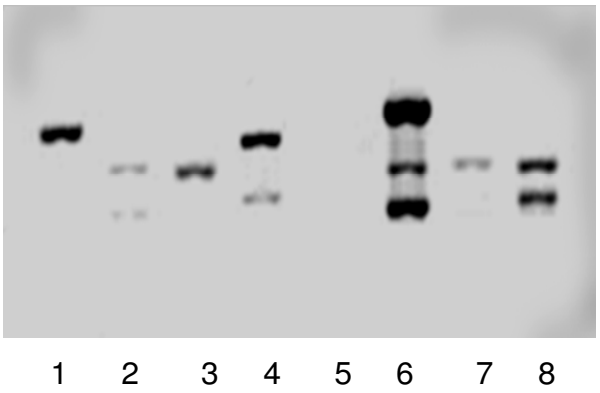

Figure 3. Southern blot hybridization patterns of pBSbtCryIII(A) transformed *P. armandii* plants. Lane1～8, pBSbtCryIII(A) transgenic plants#2, #7, #13, #15, #21, #26, #27 and #32, which were all PCR positive plants grown in greenhouse.

them (#26). The different size of the hybridization bands for different plants (Figure 3) reflected the different integration sites of *SbtCryIII(A)* gene on plant genome. It was possible that a single copy of *SbtCryIII(A)* gene had been integrated into different cells of the same plant, or two or three copies of gene had been inserted into the genome of the same cell, which caused that there were different integration sites in one plant. The reason that there was no copy of gene in the PCR positive plant #21 might be that the plant was inlaid, and the genome of some leaves had been inserted into the gene, some were not. The results of Southern blot hybridization indicated that some PCR positive plants might be inlaid.

DISCUSSION

Cry genes from the cosmopolitan soil bacterium *Bacillus thuringiensis* (*Bt*) encoded a diverse group of crystal-forming proteins that exhibit insecticidal activity, particularly against the larvae of coleopteran, lepidopteran, and dipteran insects, are becoming a cornerstone of insect pest management (Baum et al., 1996). These toxins kill insects by binding to and creating pores in midgut membranes (Gill et al., 1992). Unlike many conventional insecticides, *Bt* toxins do not harm people, arthropod natural enemies, or most other nontarget organisms (Tabashnik et al., 1997). Recent biotechnological developments offer the promise of even greater use of *Bt* toxins in genetically transformed pest-resistant plants (Baum et al., 1996). The *SbtCryIII(A)* gene encodes a protein, which forms flat rectangular crystals, particularly active against coleopteran species. Coleopteran pests are a constant threat to *P. armandii*. This study provided an alternative to chemical insecticides for controlling coleopteran pest s in *P. armandii*.

Although, there were a lot of methods for production of transgenic forest plants, transformation systems of forest plants were regarded as tedious, irreproducible, time consuming, labour intensive, and genotype dependent (Fourre et al., 1999; Tang 2000; Liu et al., 2010). With these gene transfer systems, the transgenic plants were often founded to be phenotypic abnormalities or fertility reduced (Sawahel, 1997a; Fourré et al., 1999). These problems may be due to long tissue culture periods and use of immature embryos, which are less stable than mature embryos and somaclonal variations (Sawahel, 1997b; Mo et al., 2009). In the present study, the mature embryos were used for transformation of foreign gene. Results revealed the use of electroporation for gene transfer into mature embryos facilitated integration of foreign DNA. This transformation approach offered an attractive system for the transformation of *P. armandii*. The gene transfer system presented in this manuscript can be performed with a simple and commonly electroporation apparatus, which had a high penetration power into adjacent cell layers. The advantages of electroporation for gene transfer into mature embryos over other gene transfer methods included no requirement for expensive apparatus like gene gun, no DNA-binding tungsten particles required, no time-consuming tissue culture required, easy to operate. However, this method had been discouraged for getting the inlaid plants instead of pure ones, and it is very hard to divide up the inlaid plants.

Conclusions

1. A genetic transformation system for *P. armandii* was set up using electroporation for gene delivery.

2. Plasmid DNA (pBSbtCryIII(A)) had been delivered into

mature *P. armandii* embryos.
3. Results of PCR and Southern analysis indicated that *SbtCryIII(A)* genes had integrated into the *P. armandii* genome and some transformed plants might be inlaid.

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