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Original Research Article

Agrobacterium-mediated Transformation via Somatic Embryogenesis System in Korean fir (*Abies koreana* Wil.), A Korean Native Conifer

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Abstract - This study was conducted to establish an efficient transformation system by using somatic embryogenesis in an important Korean native conifer, Korean fir (*Abies koreana*). Embryogenic masses were induced from mature zygotic embryos of the Korean fir on Schenk and Hildebrandt medium, which was supplemented with thidiazuron. For genetic transformation, the embryogenic masses were co-cultivated with a disarmed *Agrobacterium tumefaciens* strain C58/pMP90 containing the plasmid vector pBIV10 or LBA4404 containing the plasmid vector MP90. Both vectors contain the kanamycin resistance and beta-glucuronidase (*GUS*) reporter genes. A total of 48 lines of embryogenic masses were selected on mLV medium containing 50 μg/mL of kanamycin after 4 weeks of culture, following 3 days of co-cultivation with *A. tumefaciens* strain C58/pMP90 carrying pBIV10 (none of the lines was cultivated with strain LBA4404 carrying MP90). Quantitative real-time PCR was performed, and high levels of GUS transcripts were observed in the 48 putative transgenic lines; however, the control (non-transgenic line) showed negative results. Results of histochemical staining showed that the expression of the *GUS* reporter gene was observed in somatic embryos that developed from the embryogenic masses of all 48 lines. Stably transformed cultures were successfully produced by co-cultivation with *A. tumefaciens* strain C58/pMP90 carrying pBIV10 in Korean fir. Here, we have reported an *Agrobacterium*-mediated gene transfer protocol via somatic embryogenesis that may be helpful in developing breeding and conservation strategies for the Korean fir.

Key words - Transformation, Somatic embryogenesis, Conifer, Embryogenic masses, Korean fir

Introduction

Korean fir (*Abies koreana*) belonging Pinaceae, one of the important Korean native conifers, distributes in the southern regions of Korea, especially on the tops of the Mts. Halla, Jiri and Songni. In recent years, *Abies* genus, especially *A. koreana*, has been degenerating possibly due to climate change (Lee *et al*., 2008), and now the tree is one of rare plant which is needed to be conserved (Korea Diversity Information System, Korea Forest Service). Recently somatic embryogenesis system was established in Korean Forest Research Institute for conservation of this valuable species (Park *et al*., 2010).

Somatic embryogenesis has been found to be a useful system for plant regeneration (Gupta and Conger, 1999). In conifers, somatic embryogenesis represents the best system for plant regeneration and gene transfer technology (Find *et al*., 2005).

Genetic transformation is a very powerful tool to transfer genes from other sources to the conifers (Klimaszewska *et al*., 1997; 2001). A range of targets are of interest for genetic engineering in conifers such as pest and herbicide resistance, improving growth rate, wood properties and quality, and tolerance to the stresses (Lelu and Pilate, 2000; Newton *et al*., 2001; Tang and Newton, 2003; van Raemdonck *et al*., 2001). The most successful method in conifer was the biolistic technique until late 1990' (Wenck *et al*., 1999). Currently *Agrobacterium*-mediated gene transfer is the useful method for most of plant biotechnology laboratories because of the high percentage of single-copy and single-locus insertion events compared to other plant transformation techniques (Aronen *et al*., 2007; Klimazewska *et al*., 1997; 2001; Newton, 2001; Parasharami *et al*., 2006; Pavingerová *et al*., 2011).

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Recently *Agrobacterium*-mediated genetic transformation has been successfully performed using embryogenic masses in several conifers such as *Picea glauca*, *P. mariana*, *P. abies* (Klimaszewska *et al*., 2001), *Pinus radiata* (Grace *et al*., 2005), *Pinus patula* (Nigro *et al.,* 2004), *Pinus roxburghii* (Parasharami *et al*., 2006).

However, conifer transformation has been limited by difficult propagation of explant material, selection efficiencies and low transformation frequency (Grant *et al*., 2004; Find *et al*., 2005). Moreover, most of gymnosperm transformation protocols was established in non-Korean native conifers (Grace *et al*., 2005; Klimaszewska *et al*., 2001; Nigro *et al.,* 2004) which are difficult to apply in Korean species. Thus, there is need for a high efficiency transformation protocol of Korean native conifer for molecular breeding strategy and further biotechnological application.

The aim of the study reported here was to establish an efficient *Agrobacterium tumefaciens*-mediated transformation protocol for Korean fir that is degenerated by climate change. To the best of our knowledge, this is the first report on transformation of this conifer in the world, and the first report on gymnosperm transformation in Korea.

Materials and Methods

Embryogenic mass induction from mature zygotic embryo cultures

Embryogenic masses (EMs) of Korean fir were initiated from mature zygotic embryos collected from Mt. Halla in 2007 on SH (Schenk and Hildebrant, 1972) medium supplemented with 5.0 mg/L thidiazuron (TDZ), 100 mg/L myo-inositol, 0.5 g/L L-glutamine, 1 g/L casein hydrolysate, 3% (w/v) sucrose, and 0.3% (w/v) gelrite (Aldrich, USA). EMs (150−200 mg fresh weight) were plated on petri-dish containing EMs induction medium for proliferation following Klimazewska's method (2001), and subcultured on fresh medium every 2-week. The pH of the medium was adjusted to 5.8 before autoclaving. The cultures were maintained in the dark at 24 ± 1℃ for proliferation and maintenance of EMs.

Agrobacterium-mediated transformation

The disarmed *A. tumefaciens* strain C58/pMP90 containing the binary vector pBIV, which is a pBIN plus derivative was kindly provided by Dr. Klimaszewska (Natural Resources of Canada, Canadian Forest Service), and used for transformation in this study. The plasmid pBIV carried the 35S-35SAMVgus::nptII fusion derived from pBI426. As a control, disarmed *A. tumefaciens* strain LBA4404 containing binary vector carrying selective nptII genes, which is generally used in poplar transformation in Korea Forest Research Institute (KFRI), was used.

The bacterial culture was grown in LB liquid medium containing 50 μg/mL rifampicin, 50 μg/mL kanamycin sulfate and 20 μg/mL gentamicin sulfate (Sigma-Aldrich) on a shaker at 250 rpm at approximately 28℃ for 16 h. The bacterial cultures were pelleted by centrifugation and resuspended in the LB medium to an optical density of 0.6 (OD 600 nm).

Prior to the transformation experiments, EMs were prepared and cultured on filter papers as described by Klimaszewska *et al*. (2001). For the transformation experiments, the EMs was harvested from the filter papers and suspended in half strength of LV (mLV, Litvay *et al*., 1985) liquid medium containing 100 μM acetosyringone. An equal volume of *A. tumefaciens* culture in mLV was added to the cell suspension, resulting in 50 mg EMs suspended in 1 ml of bacterial culture at OD 600 nm 0.3 in mLV medium in a 50 ml falcon tube. The control EMs were treated the same way except that no *A. tumefaciens* was added to the cultures. To select stable transformants, 3-4 ml of suspension cultures was directly plated on proliferation medium containing kanamycin (50 μg/mL). After 3 weeks, putative transformants were transferred to fresh kanamycin-containing plates. After another 3 weeks, surviving EMs were transferred to fresh plates. After a third round of selection, EMs were again transferred to fresh plates, and at this time, a portion of each clump of EMs were sampled for GUS staining and for genomic DNA extraction.

Maturation of somatic embryos and conversion

For the maturation of somatic embryos (SEs), EMs confirmed as transformants by GUS staining and PCR were plated on mLV medium containing 60 μM abscisic acid (ABA) and 20 g/L sucrose, and cultures were maintained in the dark at 24 ± 1℃ for 8 weeks. After 8 weeks of culture, fully developed SEs (cotyledonary stage) were transferred on mLV medium

containing 1.0 mg/L GA_3 for conversion into plantlets. Cultures were maintained under dim light (< PPFD 10 μmol$m^{-2}s^{-1}$) for 1 week and then transferred normal culture condition (PPFD 35~40 μmol$m^{-2}s^{-1}$) for 8 weeks.

GUS histochemical assay

EMs and mature somatic embryos were soaked in GUS reaction buffer modified from Jefferson *et al*. (1987) (20 mM Na_2HPO_4 + NaH_2PO_4, pH 7.0, 10 mM K_3 [$Fe(CN)_6$], 10 mM K_4 [$Fe(CN)_6$×$3H_2O$], 10 M Na_2EDTA, 0.1% Triton X-100, 1.5 mM X-Glucuronide). After incubation at 37℃ for 24 h, the number of stained embryos, or embryogenic masses was determined using a dissecting microscope at 40x magnification.

Genomic DNA extraction and PCR for npt activity

For the polymerase chain reaction (PCR) analysis, genomic DNA was isolated from embryogenic masses (EMs) of individual lines (putative transgenic EMs) using Qiagen genomic tips (Valencia, CA, USA) following the manufacturer's protocol adapted for plants. Two combinations of primers were used for PCR amplification of fragments from gene neomycin phosphotransferase II (*npt*II) which is encoding for kanamycin resistance. For PCR reactions, each reaction mixture contained 100 ng of genomic DNA, 1 PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer and 0.03 U ll) 1 Taq DNA polymerase (Invitrogen, Carlsbad, California, USA). The PCR conditions were 94℃ for 5 min followed by 30 cycles of 94℃ for 1 min, 56℃ for 1 min and 72℃ for 1 min and a final extension step at 72℃ for 5 min. The amplified PCR products were separated in 2% agarose gel stained with ethidium bromide, and observed under UV illumination.

RNA extraction and qRT-PCR analysis

Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH). These RNA samples were converted into cDNA, and quantitative real-time RT-PCR was performed followed by Grant *et al*. (2004). The synthesized cDNA samples served as templates, and gene-specific primers were employed to amplify approximately 110 to 130-bp fragments unique to the gene. For the qRT-PCR experiments, SYBR Premix ExTaq was used according to the manufacturer's instructions (Takara, Japan) using the Mx3000P® QPCR System (Stratagene, USA). PCR conditions consisted of 95℃ for 2 min, followed by 25 cycles at 95℃ 30 s, 60℃ 30 s, 72℃ 30 s, and final extension at 72℃ 30 s. Normalization was done to UBC28, which showed stable expression as a housekeeping gene for calculating the relative expression levels of the GUS gene. (Primer used for detection UBC28 gene: 5' to 3' *UBC28q_for* TCCAGAAGGATCCTCCAACTTCCTGCAGT, *UBC28q_rev* ATGGTTACGAGAAAGACACCGCCTGAATA).

Results and Discussion

Agrobacterium-mediated transformation

Putative transformants appeared within 3 weeks of plating onto kanamycin containing plates (Fig. 1). Between two strains of *A. tumefaciens,* C58/pMP90/pBIV10 and LBA440/MP90/PAT, higher transformation efficiency was achieved with the former strain on the selection medium containing 50 μg/mL kanamycin. On the contrary, the other strain, LBA440/MP90/PAT, which was often used for poplar transformation, failed to get a transgenic line.

Agrobacterium mediated gene transfer is a frequently used method for transformation because of the high efficiency as compared to other plant transformation techniques such as biolistics (Wenck *et al*., 1999). In most of conifer species, the embryogenic system for genetic transformation is obtained from EM derived from immature zygotic embryos and they served as the best starting material for the successful transformation (Klimaszewska *et al*., 1997, 2001; Nigro *et al*., 2004; Grace *et al*., 2005; Tereso *et al*., 2006; Tang *et al*., 2007).

On the selection medium, 48 lines of EMs survived on mLV medium containing 50 μg/mL kanamycin after 12

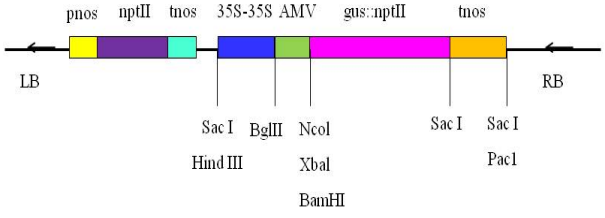

Fig. 1. Vector construction of C58/pMP90/pBIV10 for *Agobacterium*-mediated transformation of Korean fir using somatic embryo system.

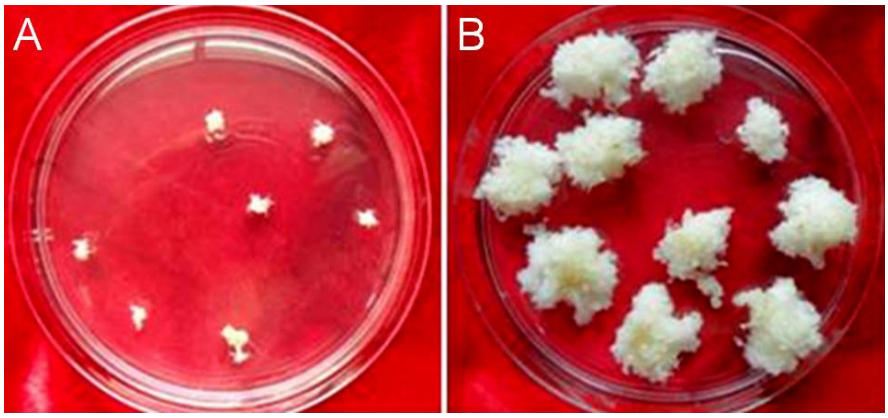

Fig. 2. Selection of embryogenic masses (EMs) by *Agobacterium*-mediated transformation on mLV medium containing 50 μg/mL kanamycin (selection medium). A. Non-transformed EMs on selection medium, B. Transformed EMs on selection medium.

weeks of co-culture with *A. tumefaciens* strain C58/pMP90 carrying pBIV10, and none of them from strain LBA4404 carrying MP90 (Fig. 2).

Almost EMs including control were survived in 1st week after transferring onto kanamycin medium, however survival rate of EMs co-cultivated with strain LBA 4404 and control were drop down to 4.3% and 9.7% at 3rd week in kanamycin containing medium, respectively while EMs co-cultivated with strain C58 survived 43.2% at the same time point (Fig. 3).

PCR was used to examine some of the selected lines for the presence of the T-DNA. Of the lines selected, all analyzed showed the presence of T-DNA using the NPT (Fig. 4). The bacterial *npt*II gene is the most frequently used selectable marker gene for generating transgenic plants in conifers (Klimaszewska *et al*., 1997, 2001; Grant *et al*., 2004; Grace *et al*., 2005). The *npt*II gene confers kanamycin resistance by inhibiting protein synthesis and is a conditional positive selection system that is toxic to untransformed cells (Miki and McHugh, 2004).

After using PCR to verify the insertion, GUS transcription in the transgenic EM lines were assessed by quantitative real time-PCR to quantify GUS transcription levels in the transgenic EM lines (Fig. 5). High levels of GUS transcripts were observed in the putative transgenic lines, and the level of expression was varied depending on the lines (Fig. 5).

Portions of each EMs were screened for GUS expression and 100% was found to express GUS throughout all of the masses (Fig. 6A), while GUS staining was never observed in untransformed cultures. It shows that kanamycin is a suitable

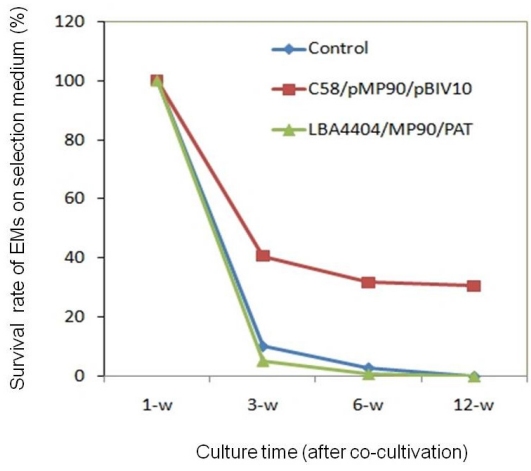

Fig. 3. Survival rate of embryogenic masses (EMs) on selection medium containing 50 μg/mL kanamycin for 12 weeks after co-culture with 2 strains of *Agrobacterium tumefaciens*.

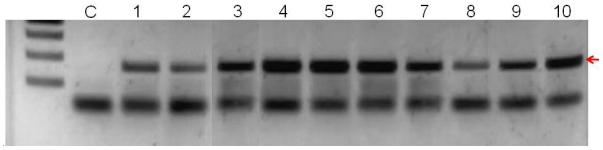

Fig. 4. PCR detection of T-DNA sequences. C: control (non-transformed EMs), 1-10: putative transgenic EM lines survived on selection medium containing 50 μg/mL kanamycin (arrow : npt).

antibiotic for selection of transformants in Korean fir. Wenck *et al*. (1999) reported that efficiencies were the same for kanamycin and hygromycin selection in *Agrobacterium*-mediated transformation of Norway spruce and loblolly pine.

The bacterial enzyme b-glucuronidase coded by *E. coli* uid A gene is the most widely used reporter gene in many plant

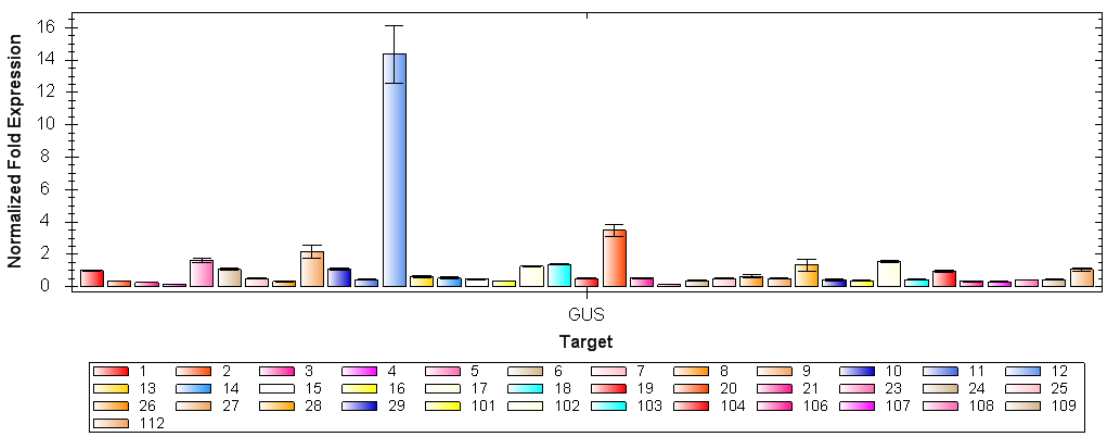

Fig. 5. Relative expression levels of *β*-glucuronidase (GUS) gene determined in the putative transgenic EM lines by real-time qRT-PCR.

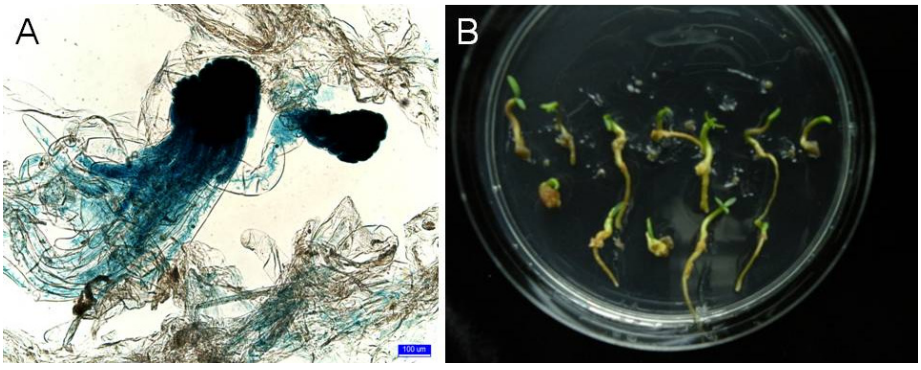

Fig. 6. GUS stained somatic embryos (line 12) (A) and developed somatic seedlings of korean fir after 8 weeks of culture on conversion medium (B).

species including conifers (Tang *et al.,* 2007; Tang and Newton, 2005; Tereso *et al*., 2006). The GUS enzyme is very stable within plants and non-toxic when expressed at high levels. A useful feature of GUS as a reporter gene is that it can be fused with other proteins (Jefferson *et al*., 1987).

The mechanism for DNA transfer to a plant cell as well as targeting of the DNA to a complex tissue or organ competent for regeneration is a major issue to be considered for effective and successful transformation. The source material used in this study is rapidly dividing embryogenic cultures. Embryogenic cultures were also utilized for *Agrobacterium*-mediated transformation of *Pinus patula* (Nigro *et al*., 2004) and *P. radiate* (Grant *et al*., 2004). Although currently several methods exist for transferring foreign genes into plants, but somatic embryogenesis has proved to be the best method to produce transgenic plants in a number of conifer species that ultimately leads to their application in commercial forestry. Our result using EMs of Korean fir supports that rapid growth of the source material is essential for transformation of conifer.

EMs confirmed as transformants by GUS staining and RT-PCR were developed into somatic embryos (SEs) on mLV medium containing 60 μM abscisic acid (ABA). Fully developed SEs were transferred on mLV medium containing 1.0 mg/L GA_3. After 8 weeks on conversion medium, approximately 65% SEs were converted into somatic seedling which having radicle and cotyledons (Fig. 6B). However, they were failed for further development.

Several factors are important for *Agrobacterium* mediated transformation of plants. These include plant material, Agrobacterium strain and co-cultivation time and so on. Rapid growth of the source material is important for transformation

of Norway spruce (Pavingerová *et al*., 2011), *Pinus pinaster* (Tereso *et al*., 2006), and chir pine (Parasharami *et al*., 2006), and may contribute to the success observed in this study. Along with proper source material, strain selection is clearly important as has been shown for other species (Wenck *et al*., 1999). Herein Agrobacterium C58/pMP90 was suitable strain for Korean fir. However, we failed to obtain acclimatized transgenic plant, and it remains for further study.

In conclusion, *Agrobacterium*-mediated gene transfer using somatic embryogenesis system is a useful technique for large-scale generation of transgenic Korean fir and may prove useful for other native conifer species. To the best of our knowledge, this is the first report on the transformation of Korean native conifer using *Agrobacterium*.

This work thus provides a high efficiency *A. tumefaciens*-mediated transformation protocol for *A. koreana* using somatic embryogenesis system. It may be helpful for breeding and conservation strategies, and to introduce foreign genes for the improvement of this plant.

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