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Thermotolerant Transgenic Ginseng (*Panax ginseng* C.A. Meyer) by Introducing Isoprene Synthase Gene through *Agrobacterium tumefaciens*-mediated Transformation

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ABSTRACT : The cost of conventional cultivation of ginseng (*Panax ginseng* C.A. Meyer) is very expensive, because shadow condition should be maintained during cultivation periods owing to inherently weak plant for high-temperature. Therefore, application of plant biotechnology may be possible to overcome these difficulties caused by conventional breeding of ginseng. Transgenic plants were produced via *Agrobacterium tumefaciens* GV3101, both carrying the binary plasmid pBI121mLP*ISO* with *nptII* and *Iso* (isoprene synthase) gene. Integration of the transgenes into the *P. ginseng* nuclear genome was confirmed by PCR analysis using *nptII* primers and *Iso* primers. RT-PCR result also demonstrated the foreign isoprene synthase gene in three transgenic plant lines (T1, T3, and T5) which was expressed at the transcriptional level. When whole plants of transgenic ginseng were exposed to high temperature at 46 ℃ for 1 h, a non-transformed plant was wilted from heat shock, whereas a transgenic plant appeared to remain healthy. We suggest that the introduction of exogenous isoprene synthase is considered as alternative methods for generating thermotolerance ginseng.

Key words **:** *Agrobacterium tumefaciens*, *Panax ginseng*, isoprene synthase, thermotolerance

INTRODUCTION

Panax ginseng C.A. Meyer, commonly known as ginseng, one of the most medicinally important genera in the Orient. This plant have been applied as tonic, anti-cancer, vaccine for common cold and/or influenza, prophylacetic, anti-stress, and anti-aging agents (See *et al.*, 1997; Scaglione *et al.*, 1996; Takahashi, 1992). The cultivation of ginseng is troublesome and a period of 4-6 years is required to harvest the roots. The cost of conventional cultivation of ginseng is very expensive, because shadow condition should be maintained during cultivation periods owing to inherently weak plant for high-temperature. Therefore, application of plant biotechnology may be possible to overcome these difficulties caused by conventional breeding of ginseng.

Until now, the best characterized aspect of acquired thermotolerance has been production of heat shock protein (HSP) (Vierling, 1991; Howarth & Skot, 1994; Burke, 2001). Majority of HSPs are functionally linked to cellular chaperon activities and maintain homeostasis of the cell. Recently, it has been reported that isoprene emission against environmental stress contributes to thermotolerance in plants. Isoprene, made from dimethylallyl diphosphate by isoprene synthase, is an important biological process because it plays a large role in atmospheric chemistry (Thompson, 1992). Several studies have independently confirmed the hypothesis (Singsaas & Shakey, 2000; Shakey *et al.*, 2001; Velikova & Loreto, 2005), plants make isoprene for thermotolerance, especially protection from damage caused by rapid temperature fluctuation (Shakey & Singsaas, 1995). The study of characterization of *Populous alba* isoprene synthase (*PaIspS*) showed the gene expression was strongly induced by heat stress (40 ℃) (Sasaki *et al.*, 2005). We suggest that the introduction of exogenous isoprene synthase is considered as alternative methods for generating thermotolerance ginseng.

A few research studies have been conducted to improve its tolerance to environmental stress such as high temperature using molecular breeding technology. But the generation of transgenic ginseng plants shown tolerance for high-temperature stress has not been reported. One of several reasons still has been remain to understand the mechanism of environmental stress, which is intricate. The present study was aimed at

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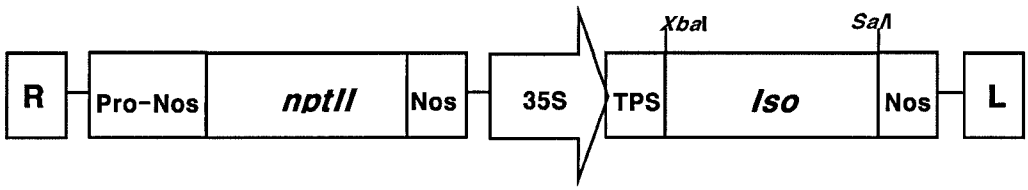

Fig. 1. A vector construction with pBI121mLP*ISO* containing neomycin phosphotransferase (*nptII*) and *Populous alba* × *Populous tremula* isoprene synthase genes (*ISO*, Accession number: AJ294819). For *P. ginseng* transformation, Locations are indicated for the nopaline synthase (NOS term), transit peptide sequence (TPS), CaMV 35S promoters (35S), left (L) and right (R) borders.

exploiting transgenic plants for thermotolerance. This work firstly describes the possibility that the introduction of isoprene synthase in ginseng plants gene plays important role on thermotolerance.

MATERIALS AND METHODS

Plant material

Stratified *P. ginseng* seeds were immersed in 70% ethanol for 1 min, then in 3% sodium hypochlorite solution for 10 min, followed by three washes in sterile distilled water. Carefully dissection of the zygotic embryos from seeds was performed as described by Choi *et al.*, (1998). The cotyledons as an explant were prepared for *Agrobacterium* transformation.

Conditions of Co-cultivation

A single clone of the disarmed *A. tumefaciens* strain GV3101 containing the binary vector pBI121mLP*ISO* (Fig. 1) consisting of neomycin phosphotransferase (*nptII*) gene as a selectable marker and *Populous alba* × *Populous tremula* isoprene synthase genes (*ISO*, Accession number: AJ294819) controlled under CaMV 35S promoter was selected. *A. tumefaciens* cultures were grown at 28℃ on a gyratory shaker at 220 rpm in liquid YEP medium containing 50 ㎎/ℓ kanamycin. The bacterial cells were collected by centrifugation for 5 min at 3000 rpm and resuspended in liquid inoculation medium (MS medium, Murashige & Skoog, 1962).

Production of transgenic plants

Excised cotyledons were dipped into the *A. tumefaciens* culture in liquid inoculation medium 20 min, blotted dry on steril filter paper, and incubated in the dark at 20℃ on callus induction medium (MS medium containing 3% sucrose, 1 ㎎/ℓ 2,4-D, 0.8% agar). After 3-days of co-cultivation with *A. tumefaciens*, the cotyledons were transferred to fresh same medium except added 400 ㎎/ℓ cefotaxim to eliminate bacteria. After 4 month of inoculation, embryogenic calli were subcultured on the MS medium supplemented with 100 ㎎/ℓ kanamycin without plant growth regulators for selecting putative transgenic somatic embryos. Induced somatic embryos for germination were cultured on the MS medium containing 5 ㎎/ℓ GA_3, 3% sucrose, 100 ㎎/ℓ kanamycin, and 0.8% agar. Regenerated putative transgenic plantlets were grown in the growth chamber at 25℃ under 16 h photoperiod. Plantlets were then transferred to plastic pots containing soil, sand, and peat 4 : 4 : 3 (v/v) in a greenhouse. In vitro culture of transformed *P. ginseng* plantlet (over 10 ㎝ in height) was used for investigating high temperature stress. The plantlet was cultured in a bottle (7 × 12 ㎝) containing 70 ㎖ MS medium at 46℃ for 1 h under continuous light conditions. The treated plants were transferred to normal conditions (25℃) for recovery from the stress. After 3 weeks of transfer to normal conditions, changes of morphological characterization were observed.

DNA analysis in transgenic plants

Genomic DNAs of the non-transformed leaves and transformed plants were isolated with DNeasy Plant Mini Kit (Qiagen). PCR was performed to detect the *nptII* and *Iso* genes in both the T-DNA of the *Agrobacterium* plasmid and genomic DNAs of the non-transformed leaves and hairy roots. The plasmid DNA of *A. tumefaciens* GV3101 strain containing pBI121mLP*ISO* was used as a positive control. Primers for PCR were designed according to the DNA sequence of the *nptII* gene. The primer pair used for *nptII* gene amplification of an 700-bp fragment was: 5'-GAGGCTATTCGGCTATAGCTG-3' and 5'-ATCGGGAGCGGCGATACCGTA-3'; for the *Iso* gene amplification of an 810-bp fragment was: 5'-TCAATACAGTGATTGCCGTAATTCA-3' and 5'-TTATCTC TCAAAGGGTAGAATAGG-3'. The PCR mixture consisted of DNA, 0.4 mM of each primer, 0.2 mM of each dNTP, 2.5U Taq DNA polymerase (5U/㎕, Takara, Shiga, Japan), and 10 ㎕ 10× buffer in a final volume of 100 ㎕. The PCR was run under the following conditions: an initial denaturation at 94℃ for 5 min; denaturation at 94℃ for 1 min; primer annealing at 58℃ for 1 min; elongation at 72℃ for 1 min, 30 cycles; a final extension at 72℃ for 10 min. The amplification PCR products were examined by electrophoresis on a 1.5% (w/v) agarose gel.

RT-PCR analysis

First strand cDNA was synthesized using AMV reverse transcriptase (Promega) from 2 microgram total RNA plants that were isolated from the leaves of transgenic ginseng. For each reaction, 1 $\mu\ell$ of the RT reaction was used as template for PCR. For normalization, an actin fragment amplified by the primer designed from *P. ginseng* was employed as an internal standard. The forward and reverse sequences used for PCR amplification of actin were 5'-GATGACATGGAAAAGAT TTGGCATC-3' and 5'-AAGGATGGCATGAGGGAGGGCG-TAA-3', respectively. PCR conditions were as follows: 95℃ for 5 min followed by 95℃, 1 min; 58℃, 1 min; 72℃, 1 min; 30 cycles. The amplification PCR products were examined by electrophoresis on a 1.5% (w/v) agarose gel.

RESULTS AND DISCUSSION

Excised cotyledon explants were used to facilitate infection with *Agrobacterium tumefaciens* and the induction of transformed primary callus. The formation of primary callus was induced on MS medium containing 1 ㎎/ℓ 2,4-D, 3% sucrose, 0.8% agar, and 400 ㎎/ℓ cefotaxim. To select transgenic lines, callus induced from explants was cultured on the medium containing 100 ㎎/ℓ kanamycin. At high concentration of kanamycin, the growth of callus was not inhibited (Fig. 2A). After 2 month growth in the selection medium, callus was developed to somatic embryogenic cells (Fig. 2B). Putative transgenic somatic embryos were transferred to the regeneration medium containing 5 ㎎/ℓ GA_3 to germinate them. After this treatment, a root and shoot were formed and most of them grew to plantlet (Fig. 2C, D). To avoid infection by fungus or other microorganism, we used autoclaved soil including sand, and peat as described by Choi *et al.* (1998). Choi *et al.* (2003) reported that not all of the plantlets survived in soil that had not been autoclaved because of fungal infection. As shown in Fig. 2E, a transgenic ginseng plant was successfully trans-

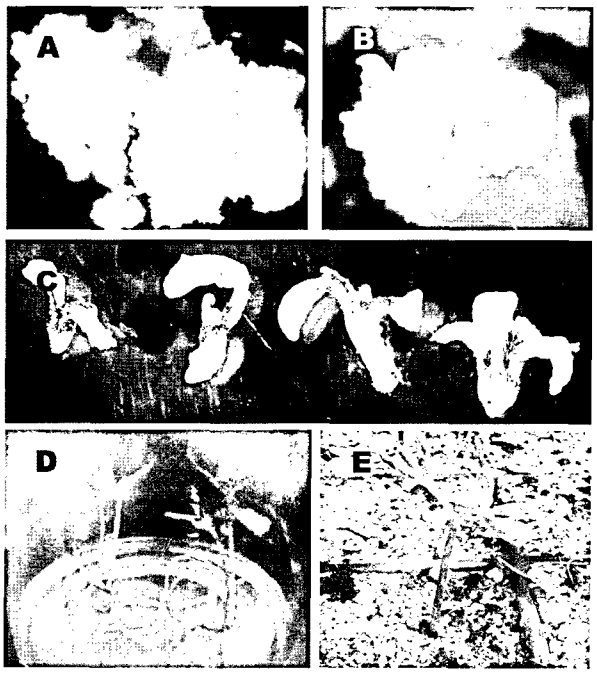

Fig. 2. Transgenic plants of *P. ginseng*. A, Embryogenic callus on MS medium supplemented with 3% sucrose, 1% agar, 400 ㎎/ℓ cefotaxim, and 100 ㎎/ℓ kanamycin; B, Surviving embryos on the medium containing high concentration of kanamycin (120 ㎎/ℓ); C, Transgenic plantlets on the medium supplemented with 150 ㎎/ℓ kanmycin.; D, Successful transfer to the soil.

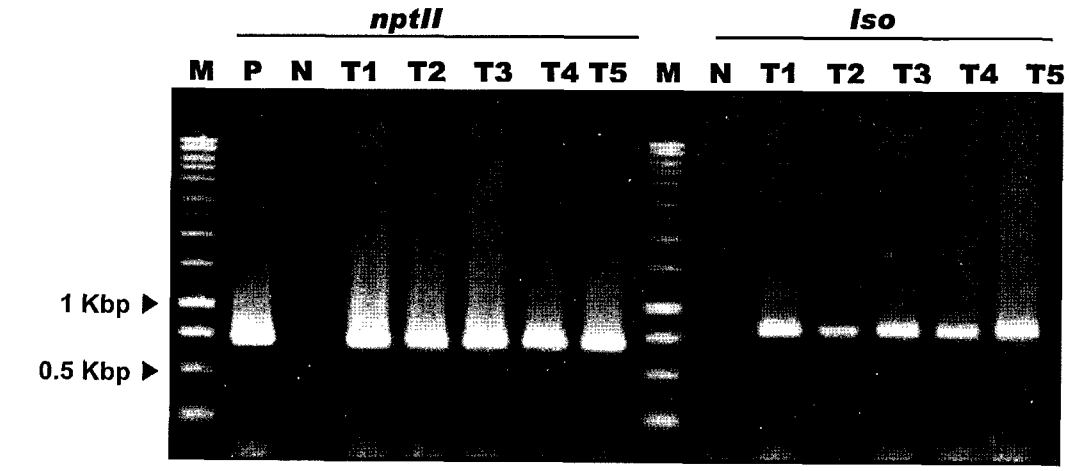

Fig. 3. PCR analysis of the *nptII* and *Iso* genes in *P. ginseng* transgenic plants. Lanes: P, plasmid DNA isolated from *A. tumefaciens* GV3101 strain as a positive control; T1, T2, T3, T4, and T5, DNA isolated from leaves of transgenic plants; N, DNA isolated from non-transformed leaves as a negative control.

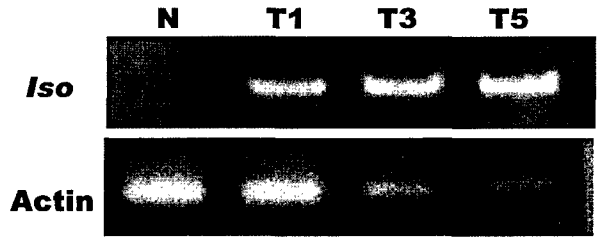

Fig. 4. RT-PCR analsysis of *Iso* gene in leaves of wild-type and transgenic *P. ginseng*. Total RNA extracted from non-transformed leaves (N) and transgenic plants (T1, T3, and T5) were subjected to RT-PCR analysis. An actin fragment was amplified as internal loading control.

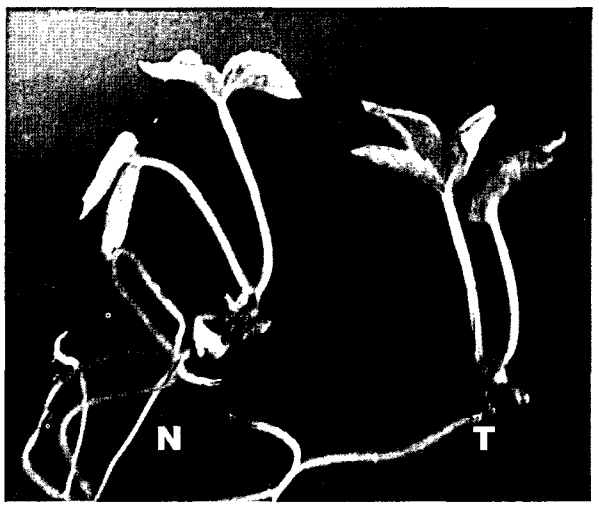

Fig. 5. Effects of high temperature (46℃) on transgenic ginseng plant. After high temperature treatment for 1 h, plants were transferred to normal conditions (25℃) for recovery from the stress. Visible differential damages in the leaves of a non-transformed plant (N) and a transformed plant (T).

ferred to the soil.

The PCR analysis of ginseng transgenic plants showed that they contained the *nptII* and *Iso* genes (Fig. 3). Amplification with the *nptII* primers showed a 740-bp band for the transgenic plants (lane T1-T5) and for the pBI121mLP*ISO* plasmid (lane P), while no band was observed for the untransformed plants (lane N). When the *Iso* primers were used for amplification, a 800-bp band was visualized only for transgenic plant clones (lane T1-T5). This result demonstrated that transformation of ginseng was confirmed by polymerase chain reaction analysis.

To investigate whether *Iso gene* transcription is tissue specific, total RNA was isolated from leaves of transformed ginseng plantlets. For reverse transcriptase-polymease chain raction (RT-PCR) analysis, we designed two primers to amplify a 800-bp fragment corresponding to *Populous alba* × *Populous tremula* isoprene synthase cDNA. As shown in Fig. 4, only *Iso* gene transcripts were observed in leaves of transgenic plants, but were not detected in a non-transgenic plant. RT-PCR result demonstrated the foreign isoprene synthase gene in three transgenic plant lines (T1, T3, and T5) which were expressed at the transcriptional level.

When whole plants of transgenic ginseng were exposed to high temperature at 46 ℃ for 1 h, a non-transformed plant was wilted from heat shock, whereas a transgenic plant appeared to remain healthy (Fig. 5). This result revealed that transgenic plants exhibited a tolerance to high temperature. Several genes for environmental stress of high temperature have been employed to generate transgenic plants. Tang *et al.* (2006) demonstrated enhanced tolerance to high temperature by introducing the genes, which consist of Cu/Zn superoxide dismutase and ascorbate peroxidase expressed in chloroplasts under the control of an oxidative stress-inducible *SWPA2* promotor, in potato. Although our first attempt could show the possibility that isoprene synthase gene plays important role on thermotolerance in ginseng plants, further experiments should be performed to exactly demonstrate thermotolerance of transgenic ginseng.

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LITERATURE CITED

Burke JJ (1994) Identification of genetic diversity and mutations in higher plant acquired thermotolerance. Physiol. Plant. 112:167-170.

Choi YE, Yang DC, Park JC, Soh WY, Choi KT (1998) Regenerative ability of somatic single and multiple embryos from cotyledons of Korea ginseng on hormone-free medium. Plant Cell Rep. 17:544-551.

Choi YE, Jeong JH, In JK, Yang DC (2003) Production of herbicide-resistant transgenic *Panax ginseng* through the introduction of the phosphinothricin acetyl transferase gene and successful soil transfer. Plant Cell Rep. 21:563-568.

Howarth CJ, Skot KP (1994) Detailed characterization of heat shock protein synthesis and induced thermotolerance in seedlings of *Sorghum bicolor* L. J. Exp. Bot. 48:1353-1363.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Sasaki K, Ohara K, Yazaki K (2005) Gene expression and characterization of isoprene synthase from *Populus alba*. FEBS Letters 579:2514-2518.

Scaglione F, Cattaneo G, Alessandria M, Cogo R (1996) Effi-

cacy and safety of the standardized ginseng extract G 115 for potentiating vaccination against common cold and/or influenza syndrome. *Drugs Exp. Clin. Res. 22:65-72.*

See DM, Brounmand N, Sahl L, Tilles JG (1997) In vitro effects of Echinacca and ginseng on natural killer and antibody-dependent cell cytotoxicity in healthy subjects and chronic fatique syndrome or acquired immunodeficiency syndrome patients. *Antimicrob. Agent Chemother. 41:961-964.*

Shakey TD, Chen XY, Yeh S (2001) Isoprene increase thermotolerance of fosmidomycin-fed leaves. Plant Physiol. 125: 2001-2006.

Shakey TD, Singsaas EL (1995) Why plants emit isoprene. Nature 374:769.

Singsaas EL, Shakey TD (2000) The effects of high temperature on isoprene synthesis in oak leaves. Plant Cell Environ. 23:751-757.

Takahashi M (1992) Anti-stress effect of ginseng on the inhibition of the development of morphine tolerance in stressed mice. Japan J. Pharmacol. 59:399-404.

Tang L, Kwon SY, Kim SH, Kim JS, Choi JS, Cho KY, Sung CK, Kwak SS, Lee HS (2006) Enhanced tolerance of transgenic potato plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against oxidative stress and high temperature. Plant Cell Rep. 25:1380-1386.

Thompson AM (1992) The oxidizing capacity of the Earth's atmosphere: probable past and future changes. Sicence 256: 1157-1165.

Velikova V, Loreto F (2005) On the relationship between isoprene emission and thermotolerance in *Phragmites australis* leaves exposed to high temperatures and during the recovery from heat stress. Plant Cell Environ. 29:1820-1828.

Vierling E (1991) The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol. Biol. 42:579-620.