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Growth Pattern and Content of Tropane Alkaloids of Metabolic Engineered *Scopolia parviflora* Hairy Root Lines

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ABSTRACT : Hyoscyamine and scopolamine are two most common tropane alkaloids found in the Solanaceae. The pEB expression vector carrying Ns*pmt* gene was transformed to *Agrobacterium rhizogenes*. The growth of transgenic hairy roots were approximately to 80% of the wild type root. Transgenic hairy roots are less developed on only their branch roots than those of the wild type root. The extracts from Ns*pmt* transgenic hairy root lines, 3 and 5 contained between 3.52 and 4.23 ㎎/g dry weight as hyoscyamine and did between 5.23 to 6.40 ㎎/g dry weight as scopolamine. These results showed that the overexpression of the *pmt* gene enhanced tropane alkaloids production of *S. parviflora* transformed roots and this improvement affected both hyoscyamine and scopolamine production.

Key words : root growth index, morphology, metabolic engineering, tropane alkaloids

INTRODUCTION

Hyoscyamine and scopolamine are two most common tropane alkaloids found in the Solanaceae. These alkaloids possess therapeutic properties and have been used for various medicinal applications (Hashimoto *et al.*, 1993). Hyoscyamine functions as an anticholingergic and antispasmodic agent, while scopolamine is a powerful hypnosis inducer (Waller & Nowacki, 1978).

Plant cell and tissue cultures for the production of secondary metabolites, in general, have met with limited success due to their low yields (Cusido *et al.*, 1999). In order to overcome the low production yield, studies in plant cell cultures focused on the optimization of the culture conditions and of the high yielding cell lines. Also, genetic engineering of secondary metabolic pathways aimed to either increase or decrease the quantity of a specified compound or group of compounds (Dixon, 2001). The use of root cultures for physiological and biochemical studies, in particular hairy roots obtained by transformation with *Agrobacterium rhizogenes*, has steadily increased in long time ago (Tepfer, 1984). This has been particularly true for studies at the molecular and genetic levels.

The complete biosynthesis pathway of tropane alkaloids is not yet fully understood, and only a few enzymes have been isolated and their corresponding genes cloned (Oksman & Arro, 2000). Recent efforts have aimed at increasing the flux through the biosynthetic pathway (Sato *et al.*, 2001). Tropane alkaloids are derived from putrescine by way of N-methylputrescine (Fig. 1).

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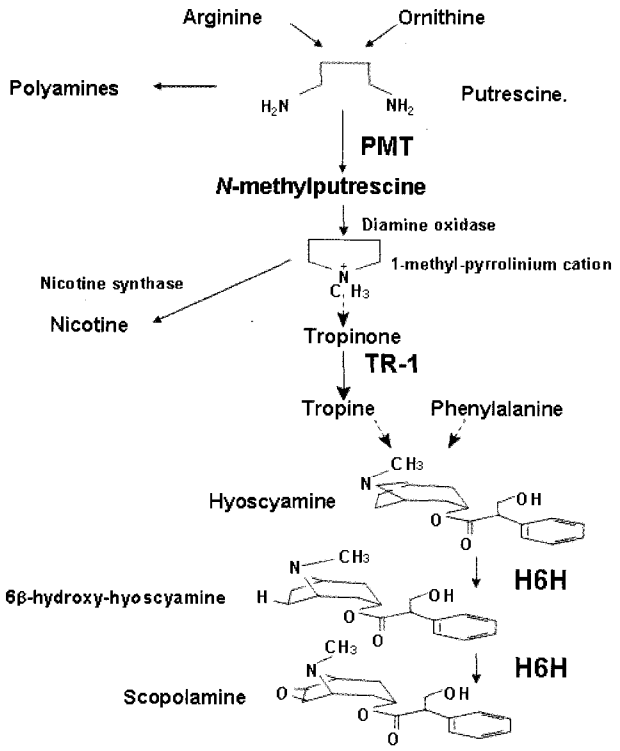

Fig. 1. Biosynthesis pathways of tropane alkaloids. The tropane ring system arises from ornithine and /or arginine via the formation of putrescine. This is then methylated by putrescine N-methyl transferase (PMT) to tropinone. Tropine formed by tropinone reductase (TR-1) is esterified with phenylalanine to give the derivative hyoscyamine. Hyoscyamine 6β-hydroxylase (H6H), a bifunctional enzyme, is finally converted to scopolamine from hyoscyamine.

Because putrescine is metabolized to polyamines such as spermidine and spermine, the N-methylation of putrescine catalyzed by PMT is the first committed step in the biosynthesis of these alkaloids. However, the overexpression of the tobacco *pmt* gene in *Scopolia parviflora* hairy root cultures has not been studied. Thus, the high demand for these tropane alkaloids from *S. parviflora* has made necessity to search for an alternative, biotechnological approach for their production.

MATERIALS AND METHODS

Plant material and culture conditions

S. parviflora was provided from the Gwangreung Arboretum, Korea. The rhizome of plant was cut into 1~3 ㎝ pieces with sterile knife and surface sterilized with 1% (v/v) NaOCl and 0.5% (v/v) Triton X-100 mixture. After they were rinsed in sterile distilled water, the rhizome was subjected to root induction on B5 basal agar medium (Gamborg *et al.*, 1968) with 3 % (w/v) sucrose and 0.75% (w/v) agar. Shoot was induced with incorporation of 1.0 ㎎/ℓ GA to this medium. Subculturing was conducted every 4 weeks. All cultures were maintained at 25±1℃ with 16/8 h (light/dark) cycle. Shoot culture was performed according to the method of Jung *et al.* (2002).

Construction of expression vector

Nicotiana sylvestris cDNA encoding putrescine N-methyltransferase (Ns*pmt*) was cloned in pcDNAII that was gifted by Dr. Hashimoto from Nara Institute of Science and Technology in Japan. The Ns*pmt* cDNA from pcDNAII was cut with XbaI and BamHI to release Ns*pmt* gene. The BamHI-generated gaps were filled in using Klenow DNA polymerase and the resulting fragment was subcloned into SmaI site between sequence and Tnos (polyadenylation signal of the gene for nopaline synthase in the Ti plasmid) terminator of pEB vector, to obtain pEB*pmt* expression vector (Fig. 2). All DNA manipulations were performed according to Sambrook *et al.* (1989) and vector construction was carried out according to the methods of Mitsuhara *et al.* (1996).

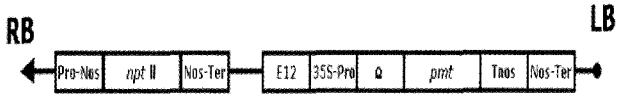

Fig. 2. Construction of pEB pmt expression vector. The pmt fragment was inserted between Ω sequences of TMV and nopaline synthase terminators. CaMV 35S promoter drives pmt gene. 35S-Pro: 5'-upstream sequence of CaMV 35S promoter, Tnos: polyadenylation signal of the gene for nopaline synthase in the Ti plasmid, E12: 5'-upstream sequence of CaMV 35S promoter, Ω: 5'-upstream sequence of TMV. cDNA size of Nspmt is 1062 bp. Plasmid total size is about 13 Kb.

Genetic transformation

The pEB*pmt* expression vector was transformed to *A. rhizogenes* strain KCTC (Korean Collection for

Type Cultures) 2703 by direct transfer method. Leaf discs were inoculated with *A. rhizogenes* carrying Ns*pmt* gene. Hairy roots that appeared on the edges of leaf discs were independently transferred and propagated in B5 agar medium containing 3% sucrose and 1.0 ㎎/ℓ IBA. Culture media contained 100 ㎎/ℓ of kanamycin and 250 ㎎/ℓ cefotaxime for selection of transgenic hairy root lines. The hairy root lines which grew with no bacterial contamination were used to establish the cultures of transgenic hairy root lines. Hairy root lines were selected and transferred to B5 liquid medium with antibiotics. Each hairy root clone was also cultured in B5 medium without growth regulators, maintained on a shaker at 100 rpm at 25℃ in the dark, and subcultured every 4 weeks. Hairy roots induced after 4 weeks of incubation were cultured separately on B5 basal solid medium supplemented with 500 ㎎/ℓ carbenicillin to eliminate the bacteria. Rapidly growing clones were used to establish the cultures of hairy roots. After several subcultures, transformed hairy roots were transferred to half-strength B5 solid medium with 0.75% agar and then also cultured in B5 liquid medium without growth regulators. All the cultures were maintained as described above conditions.

Polymerase chain reaction

The presence of the transferred Ns*pmt* gene in the transgenic roots was confirmed by PCR. The genomic DNA of kanamycin resistant root was extracted from the putative engineered hairy roots (Sambrook *et al.*, 1989). The oligonucleotide primers were used for amplication. The genomic DNA of kanamycin resistant roots was extracted and subjected to PCR amplification with primers; P1(F)Ns*pmt*: 5'-CCCACCCACGAGGA GCATC-3, P2(R)Ns*pmt*: 5'-GAGCTAGTATGAAGA CCG-3' for 30 cycles of 1 min. at 94℃, 1 min. at 55℃ and 1 min. at 72℃ to confirm the Ns*pmt* gene incorporation. The complete PCR mixture contained 200 ng of total DNA, 12.5 pmol/㎕ of each oligonucleotide primer, 200 ㎛M dNTPs, 1.5 U Taq polymerase (Pharmacia Biotech) and buffer supplied by the enzyme manufacture in a total volume of 25 ㎕. Confirmed hairy roots were transferred on the B5 medium contained 100 ㎎/ℓ kanamycin and 250 ㎎/ℓ cefotaxime.

Growth index and morphology in transgenic hairy root lines

Each hairy root lines was also cultured in B5 solid medium, at 25℃ in the dark, and subcultured every 4 weeks. The roots were separated from the medium and then weighed. The growth index (GI) was calculated using the following equation; (harvest weight − inoculum weight)/ inoculum weight.

The roots were dipped and rinsed a few times with distilled water to visualize root hair formation and measured with the light microscope (Olympus Co.). All experiments were done at the same time under the same conditions. After 4 weeks of growth, the roots were removed from the Petri dishes and photocopied with a black background. The surface of roots were determined by use of light microscope equiped with digital photo camera (Olympus Co.).

Extraction of tropane alkaloids in hairy root lines

Contents of tropane alkaloids were determined according to the method of Jung *et al.* (2002). Transgenic and wild type roots were oven-dried at 40℃, then powdered and soaked overnight in EtOH and 28% NH_4OH (19:1, v/v) mixture, after which these macerated materials were centrifuged for 10 min. at 6,000 rpm. Treatment with the MeOH was repeated twice, and then the combined alcohol extracts were dried by evaporation to dryness at 45℃. The dried residue was dissolved in 1 ㎖ of 0.1 N HCl and the flask washed with 0.5 ㎖ of HCl. The acidic aqueous solution was filtered through No. 2 filter paper into a 10 ㎖, glass stoppered centrifuge tube and made alkaline with diluted KOH (final pH 8.0 to 9.0). After being added with 6 ㎖ of $CHCl_3$ and vigorously shaked the tube on a shaker (Thermonic Co. Tokyo) for 30 s, it was centrifuged for 2 min. at 1,500 rpm. The lower layer containing the alkaloids was pipetted into a 30 ㎖ round-bottomed flask. Pipetting was repeated twice with 6 ㎖ $CHCl_3$, and then the combined $CHCl_3$ extracts were dried by evaporation at 50℃. Samples were taken up in 1 ㎖ MeOH (HPLC grade), filtered through a 0.45 ㎛m nylon membrane and stored at −20℃ until analysis.

The hyoscyamine and scopolamine contents were measured by HPLC on a TSK gel column (10 ㎕, 3.2 ×250 ㎜, Tosho). A mobile phase consisting of acetonitrile/dipotassium phosphate (22:78, v/v), at a flow rate of 0.8 ㎖/min. was applied. Alkaloids were detected by monitoring absorbance at 215 ㎚ using a UV detector. Hyoscyamine and scopolamine were supplied from Sigma. All chemicals used in this study were reagent grade. After separation by HPLC, the proposed hyoscyamine and scopolamine peaks were collected, and analyzed by using EI mass spectrometer (JMS-AX505WA).

Statistical analysis

The experiments were repeated for a minimum of three times. Each numeral value represents the mean and standard deviation (SD) by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Plant culture and genetic transformation

The plasmid containing cDNA encoding Ns*pmt* driven by a CaMV 35S promoter with a selectable marker (*npt*II) gene was introduced into *S. parviflora* shoot tissue using *A. rhizogenes* harboring pEB*pmt*. The hairy root lines were subcultured in B5 medium containing 1.0 ㎎/ℓ IBA for at least 1 month. Hairy root clones were first screened for the kanamycin-resistant phenotype. The hairy root lines were obtained and cultured in a hormone-free half-strength B5 solid medium without antibiotics. The presence of Ns*pmt* gene in several transgenic plant was confirmed by PCR amplication (Fig. 3). The genomic DNA of vector control root carrying *A. rhizogenes* rol gene but not the 35S-*pmt* transgene did not show any amplified band (data not shown). In addition, the genomic DNA of wild type root did not show any amplified band but all the putative engineered hairy roots gave a band of 1.3 Kb size, corresponding to the Ns*pmt* gene fragment, which consequently confirmed the presence of the transgenes in the hairy root lines.

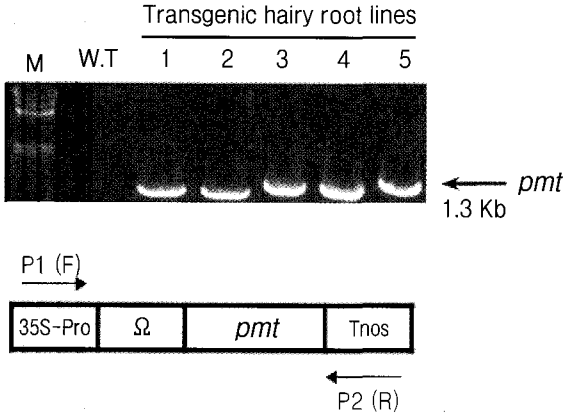

Fig. 3. PCR analysis of *S. parviflora* transgenic hairyroot lines. Genomic DNA from transgenic hairy root lines and wild type root of *S. parviflora* was extracted and subjected to PCR amplification with primers P1(F)Ns*pmt*: 5′-CCCACCCACGAGGAGCATC-3′, P2(R)Ns*pmt*:5′-GAGCTAGTATGAAGACCG-3′. The amplified products were separated by agarose gel electrophoresis. Lane 1: molecular weight marker and lanes 2~7 contained wild type root and transgenic hairy root lines (1~5), respectively.

Growth and morphology in transgenic hairy root lines

The growth index of both 3 and 5 transgenic hairy roots were approximately to 80% of the wild type root (Fig. 4). Retardation of growth in transgenic hairy roots may be caused by introduction of Ns*pmt*

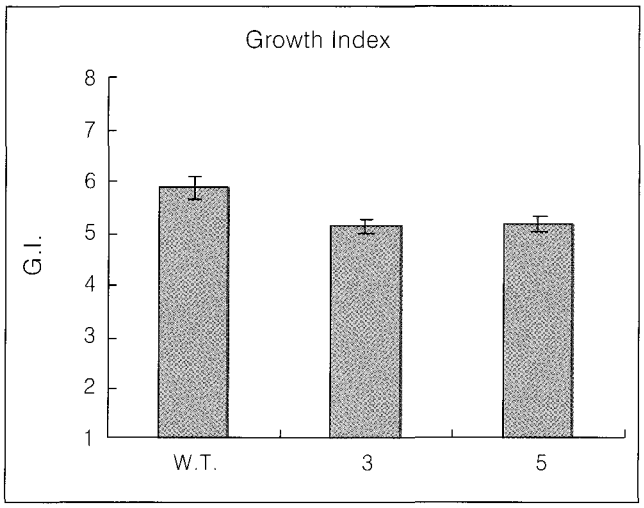

Fig. 4. Growth of wild type root and transgenic hairy root lines. The root growth was measured by the growth index (harvest weight - inoculum weight/ inoculum weight). W.T.: wild type root, 3 and 5: Transgenic hairy root lines.

gene. Canel *et al.* (1998) also reported that tdc overexpressed *Catharanthus roseus* cells showed signals of stress and poor growth. In our experiment, introduction of foreign gene had a negative effect on growth of transgenic hairy roots.

The morphology of the *S. parviflora* hairy root lines carrying the Ns*pmt* gene differed from wild type root. These transgenic hairy roots slowly grew and lately appeared branching roots (Fig. 5). Insertion of T-DNA identified as loci *rolA*, *rolB* and *rolC* are affected on hairy root induction (Schmulling *et al.*, 1989). It was also shown that *rol* genes play the most important role in hairy root induction. In particular, *rolB* seems to be the most crucial in the differentiation process of transformed cells, while *rolA* and *rolC* provide accessory functions (Schmulling *et al.*, 1989).

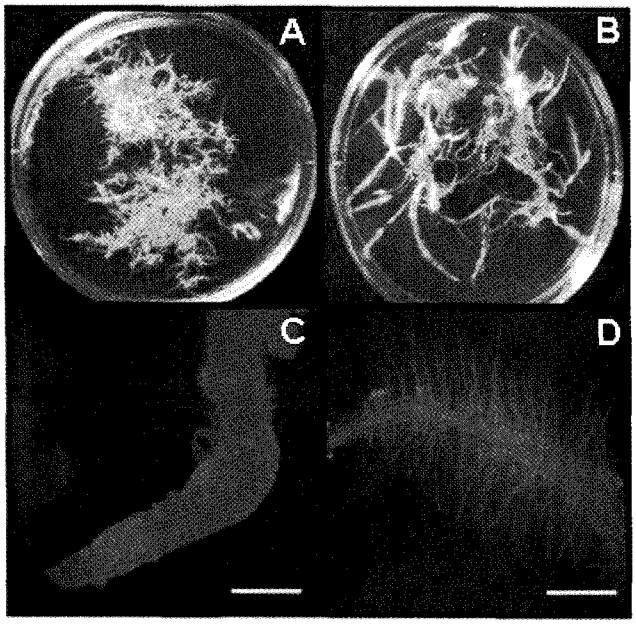

Fig. 5. Differences of morphology in wild type root and transgenic hairy roots. The morphology of the *S. parviflora* hairy root lines carrying the Ns*pmt* gene differed from wild type roots. A and C: wild type root, B and D: Nspmt hairy root, Bars in the dark field images (C and D are 0.4 ㎝).

Tropane alkaloids content in metabolic engineered hairy root lines

Contents of tropane alkaloids in four-week-old transgenic hairy roots were determined by HPLC analysis. Considerable variation between the transgenic hairy roots was observed in hyoscyamine and scopolamine production. The levels of hyoscyamine and scopolamine in extracts of the wild type root were 1.51 and 1.02 ㎎ per g dry weight, respectively (Fig. 6). The extracts from transgenic hairy root lines, #3 and #5 contained between 3.52 and 4.23 ㎎/g dry weight in hyoscyamine and between 5.23 to 6.40 ㎎/g dry weight, as the levels of scopolamine, respectively. The hairy roots of other transgenic root lines (#1, #2 and #4) contained a comparable level of hyoscyamine and scopolamine compared to the wild type. The best transgenic hairy root line (#5) produced above five times more scopolamine production than the wild type root after 4 weeks of culture. The Ns*pmt*-transformed hairy root lines of *S. parviflora* displayed an alkaloid profile similar to that of the wild type root.

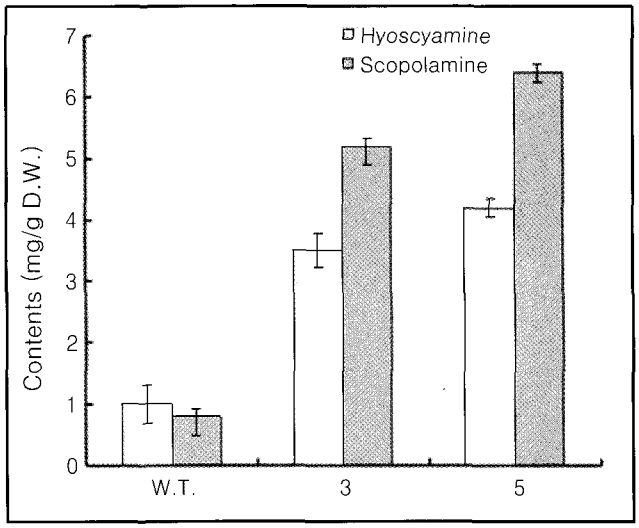

Fig. 6. Contents of tropane alkaloids in wild type root and transgenic hairy root lines. Tropane alkaloid content was determined using HPLC. W.T.: wild type root, 3 and 5: Transgenic hairy root lines.

Therefore, we suggest that the overexpression of the upstream regulator of the tropane alkaloid pathway activated the biosynthesis of the final product. Many studies reported that Solanaceae plants changed the tropane alkaloids profiles for *pmt* overexpressing roots, and the endogenous polyamine pool in the whole plant (Hashimoto *et al.*, 1993;

Matsuda *et al.*, 1998; Zabetakis *et al.*, 1999). Our results showed that the overexpression of the Nspmt gene enhanced alkaloid production of *S. parviflora* transformed roots and this improvement affected both hyoscyamine and scopolamine production. In this study, these values are two to three times higher than those reported in which adventitious root cultures from Korean native growing *S. parviflora* were cultured in B5 medium (Jung *et al.*, 2002). Although metabolic engineering in *S. parviflora* could improve the tropane alkaloids yields, it is comparable or lower than those of other species. There is needed for extensive experimental trials to produce the secondary metabolites on commercial scales. The establishment of optimal culture condition and more efficient gene expression system could enhance the tropane alkaloid production. Metabolically engineered medicinal plants will complement conventional breeding efforts for the production of tropane alkaloids with quantitatively and qualitatively improved pharmacological properties.

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