Overexpression of Hyoscyamine 6β-Hydroxylase (h6h) Gene and Enhanced Production of Tropane Alkaloids in Scopolia parviflora Hairy Root Lines

KANG, YOUNG MIN[1], OK SUN LEE[2], HEE YOUNG JUNG[1], SEUNG MI KANG[1], BYUNG HYUN LEE[2], CHANDRAKANT KARIGAR[3], THEERTHA PRASAD[4], JUNG DONG BAHK[2], AND MYUNG SUK CHOI[1,5]*

[1]Division of Forest Science, Gyeongsang National University, Jinju 660-701, Korea
[2]Division of Applied Life Science, Gyeongsang National University, Jinju 660-701, Korea
[3]Division of Biochemistry, Department of Chemistry, Central College Campus, Bangalore University, Bangalore, 560001, India
[4]Department of Biotechnology, University of Agricultural Science, GKVK, Bangalore, 560065, India
[5]Environmental Biotechnology Research Center, Gyeongsang National University, Jinju 660-701, Korea

Abstract The hyoscyamine 6β-hydroxylase (h6h) gene was introduced into the genome of Scopolia parviflora through the Agrobacterium rhizogenes binary vector system. The enzyme was expressed ally and tissue specific selectively in roots, resulting in five transgenic hairy root lines. The presence of the h6h gene in kanamycin-resistant hairy roots and its overexpression were confirmed by polymerase chain reaction (PCR), Northern blotting, and Western blotting, respectively. In the transgenic hairy root lines which constitutively expressed the H6H enzyme, hyoscyamine and scopolamine accumulated in high concentration. Among the transgenic hairy root lines that expressed the H6H enzyme, only two were more productive. The levels of tropane alkaloids in transgenic hairy root varied greatly: The best transgenic line (#5) contained 8.12 mg of scopolamine per g dry weight, which produced the compound three times more than wild-type root. These results suggest a possibility of improving the yield of tropane alkaloids in hairy root lines by genetic and metabolic engineering.

Key words: Hyoscyamine 6β-hydroxylase, Scopolia parviflora, tropane alkaloids, hairy root, metabolic engineering

Tropane alkaloids, including hyoscyamine and scopolamine, are medicinally and commercially important secondary metabolites that are produced in several Solanaceae. Physiologically, these alkaloids affect the parasympathetic nervous system and are amongst the oldest drugs used in traditional oriental therapy. Due to their medicinal value the commercial demand for tropane alkaloids has recently increased by ten-fold [9]. Usually, tropane alkaloids have been obtained from cultivated leaves of Solanaceae [1]. Since conventional plantations have slow growth rate, they are considered to be undesirable as mass production systems. However, this problem can be overcome by using in vitro culture with high growth and production rates. In order to overcome the low production yield, studies in plant cell cultures have mainly been focused on the optimization of the culture conditions and the high yielding cell lines. Also, genetic engineering of secondary metabolic pathways has been attempted to either increase or decrease the quantity of any specified compounds or group of compounds [7].
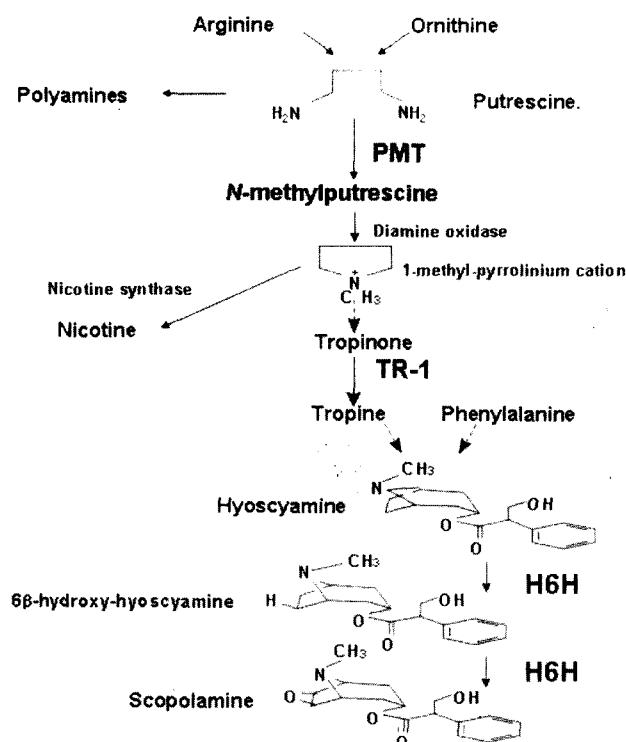
The complete biosynthetic pathway of tropane alkaloids is not yet fully understood, and only a few enzymes have been isolated and their corresponding genes were cloned [4]. The biosynthetic pathway of tropane alkaloids initiates either ornithine or arginine, and hyoscyamine 6β-hydroxylase (H6H), a bifunctional enzyme, whose function farther down the pathway is known to catalyze the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine, and the epoxidation to scopolamine [16]. Hence, H6H appears to be a promising enzyme for metabolic and genetic engineering to enhance the yields of scopolamine in plants (Fig. 1).

Scopolia parviflora, which was used in the present study, is a Solanaceae perennial plant. It is endemic to Korea and has recently been classified as being a rare endangered species. However, there is little research on its metabolic engineering and the in vitro culture for the production of its tropane alkaloids. In the present study, introduced gene constructs containing cDNA clones of h6h, by way of a powerful CaMV promoter with a Ω

*Corresponding author
Phone: 82-55-751-5493; Fax: 82-55-753-6015;
E-mail: mschoi@nongae.gnsu.ac.kr

**Fig. 1.** Biosynthetic 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 bifuntional enzyme, finally converts hyoscyamine to scopolamine.

sequence, resulted in overexpression of the *h6h* gene in hairy root lines culture.

MATERIALS AND METHODS

Construction of H6H Expression Vector

Hyoscyamus niger cDNA encoding *h6h*, which was cloned in pBluescriptII SK+, was kindly provided by Dr. Hashimoto
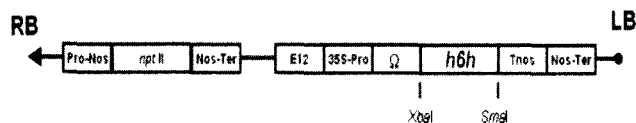
Fig. 2. Construction of pBEh6h expression vector.

cDNA encoding *h6h* cloned in pcDNAII was digested with *Xba*I and *Bam*HI restriction enzymes, and the fragments were subcloned into the *Sma*I site of pBE2113 vector to obtain pBE*h6h*. The *h6h* fragment was inserted between sigma sequence TMV (Tabacco Mosaic Virus) and nopaline syntheses terminator. E12: 5'-upstream sequence of CaMV 35S promoter × 2, 35S-Pro: 5'-upstream sequence of CaMV 35S promoter; Ω: 5'-upstream sequence of TMV; Tnos: polyadenylation signal of the gene for nopaline synthase in the Ti plasmid. H6H cDNA size is 1,035 bp. Plasmid total size is about 13 kb.

from Nara Institute of Science and Technology, Japan. The *h6h* cDNA from pcDNAII was cut with *Xba*I and *Bam*HI restriction enzymes to release *h6h* gene. The *Bam*HI-generated gaps were filled by using Klenow DNA polymerase, and the resulting fragment was subcloned into a *Sma*I site between Ω sequence and Tnos (polyadenylation signal of the gene for nopaline synthase in the Ti plasmid) terminator of pBE2113, to finally obtain pBE*h6h* construct (Fig. 2). All DNA manipulations were performed according to the manual of Sambrook [19], and vector construction carried out by the modified method of Mitsuhara [12, 15, 17].

Hyoscyamine Feeding Experiments

The adventitious roots (0.5 g) were cultured in 30 ml of B5 liquid medium supplemented with 30 g/l sucrose and 0.1 mg/l IBA (indole butyric acid). Hyoscyamine (Sigma), H6H substrate, was added to B5 liquid medium at 0.1 to 1.0 mM concentrations. The cultures were grown in dark at 25±1°C at 100 rpm on a rotary shaking incubator. Roots were harvested after 4 weeks, and then analyzed for tropane alkaloids by HPLC.

Plant Material and Genetic Transformation

Mother plant of *S. parviflora* was obtained from the Gwangreung Arboretum, Korea. The rhizome was cut into 1-3 cm pieces with a sterile knife and the surface was sterilized with a mixture of 1% NaClO and 0.5% Triton X-100 by vigorous shaking for 25 min. After a rinse in sterile distilled water, the rhizomes were subjected to root induction on B5 basal agar medium [3]. The medium included 30 g/l of sucrose and 7.5 g/l of agar. Shoot induction was initiated with incorporation of 1.0 mg/l of GA to this medium. Subculturing was carried out after every 4 weeks. All cultures were maintained at 25±1°C with a 16 h/8 h (light/dark) cycle.

The vector pBE*h6h* was transformed to *Agrobacterium rhizogenes* strain KCTC (Korean Collection for Type Cultures) 2703 by the direct transfer method. Leaf discs were inoculated with *A. rhizogenes* carrying the *h6h* gene. Hairy roots that appeared on the edges of leaf discs were independently transferred and propagated in B5 agar medium containing 30 g/l sucrose and 1.0 mg/l IBA. Culture medium contained 100 mg of kanamycin and 250 mg/l cefotaxime for selection of transgenic hairy root lines. Hairy root lines 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 without antibiotics. Each hairy root clone was also cultured in B5 medium without growth regulators, maintained on a shaker at 100 rpm and 25°C in dark, and subcultured every 4 weeks. The genomic DNA of kanamycin-resistant roots was extracted and subjected to PCR amplification with primers, P1 (F): 5'-CCCACCCACGAGGAGCATC-3', P2

(R):5'-AATCTGTAACTA AAATATACC-3', for 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C to confirm the *h6h* gene incorporation.

Northern Blotting

Transformants confirmed by PCR (more than 10 lines) were transferred to the B5 medium with 1.0 mg/l of IBA. After 4 weeks, the regenerated transgenic hairy roots (5 lines) were ground to fine powder in liquid nitrogen, and the RNA was isolated by using RNase Kit (Qiagen) [19]. RNA fractions were separated on formaldehyde agarose gel (1.2%), followed by blotting them onto a nylon membrane (Millipore) by capillary action. Membranes were hybridized with [^{32}P]-dCTP (Amersham, Pharmacia Biotech) labeled cDNA probes at 65°C in the solution containing 7% SDS, 0.25 M sodium phosphate (pH 7.2), 1% BSA, and 1 mM EDTA. After hybridization, the membranes were washed for 20 min at 65°C in 0.1 SSC (1× SSC in 0.15 M NaCl and 15 mM sodium citrate) and 0.1% SDS. The membranes were washed for 20 min at 65°C in 0.5× SSC and 0.1% SDS, followed by washing for 20 min at 65°C in 0.1× SSC and 0.1% SDS. The blots were then exposed to photographic film (X-Omat AR-5, Eastman Kodak) with an intensifying screen at -70°C for 30 min to 48 h. The amount of [^{32}P]-cDNA probe, hybridizing to specific mRNAs, was semi-quantitatively determined by exposing the membrane to a phosphor image screen and by laser densitometry (Molecular Dynamics, CA, U.S.A.).

SDS-PAGE and Western Blotting

Leaves, stem, main roots, and branch roots of native growing plant, and transgenic roots were collected, frozen with liquid nitrogen, and then homogenized. The cell homogenate was suspended in a protein extraction buffer (300 mM Tris-HCl and 1 mM EDTA) and centrifuged at 12,000 rpm for 15 min. Protein concentration of the supernatant was measured by the BioRad protein assay kit. The crude cell extracts (20 µg/lane) were evolved on 12.5% separating gel by SDS-PAGE [19]. After electrophoresis, the proteins were transferred to a nitrocellulose membrane by electroblotting: Electrotransfer was performed at 13 mA for 1 h in an SDS electrotransfer buffer (2.9 g of glycine, 5.8 g of Tris base, 3.7 ml of 10% SDS, and 200 ml of MeOH). Immunoreactions were carried out by using H6H antibodies as the primary mono-antibodies, which were kindly provided by Dr. Hashimoto of Nara Institute of Science and Technology, Japan. Unbound antibodies were removed by three washes of TTBS (500 mM Tris-HCl, 1.5 M NaCl, and 1% Tween 20) and the antibodies bound to the membranes were detected by using peroxide-conjugated antimouse IgG as the secondary antibody (Sigma). The antigen protein was detected by chemiluminescence by using an ECL-detecting reagent (Amersham, U.K.), according to the manufacturer's protocol [12, 15].

Determination of Root Growth

Each hairy root was also cultured in B5 medium, maintained on a shaker at 100 rpm at 25°C in dark, and subcultured every 4 weeks. The roots were separated from the medium and then blotted and weighed fresh. The growth index (GI) was calculated by the following equation; (harvest weight-inoculum weight)/inoculum weight.

Extraction and Analysis of Tropane Alkaloids

Extraction and determination of tropane alkaloid were performed according to the method of Kang *et al.* [14]. Harvested hairy roots were oven-dried at 50°C, and then powdered and soaked overnight in a mixture of 95% EtOH-28% NH$_4$OH (19:1, v/v), after which this macerated material was centrifuged for 3 min at 1,500 rpm. Treatment with the basic methyl alcohol was repeated twice, and the combined methyl alcohol extracts were then dried by evaporation to dryness at 45°C. The dried residue was dissolved in 1 ml of 0.1 N HCl, and the flask was rinsed once with 0.5 ml of HCl. The acidic aqueous solution was filtered through No. 2 filter paper into a 10-ml glass-stopper centrifuge tube, and was made alkaline with diluting KOH (final pH 8-9). Six milliliters of CHCl$_3$ were added, and after shaking the tube vigorously on a shaker (Thermonics Co. Tokyo) for 30 s, it was centrifuged for 10 min at 6,000 rpm. The lower layer, containing the alkaloids, was pipetted into a 30-ml round-bottomed flask. Extraction with 6 ml of CHCl$_3$ was repeated twice, and then the combined CHCl$_3$ extracts were dried by evaporation to dryness at 50°C. Samples were taken up in 1 ml of MeOH (HPLC grade), filtered through a 0.45 µm nylon membrane, and stored at -20°C until required for analysis.

The contents of hyoscyamine and scopolamine were measured by HPLC on a TSK gel (3.2×250 mm, Tosho) column. A mobile phase, consisting of acetonitrile/dipotassium phosphate (22:78), at a flow rate of 0.8 ml/min was applied. Tropane alkaloids were detected by monitoring absorbance at 215 nm. The correlation coefficient (R) of standards was 99.7% for hyoscyamine and 99.9% for scopolamine. The concentrations of tropane alkaloids were calculated by using a calibration curve of standards and samples. Hyoscyamine and scopolamine were supplied from Sigma. All chemicals used in this study were of reagent grade.

After separation by HPLC, the hyoscyamine and scopolamine peaks were collected, and analyzed by using EI mass spectrometery (JMS-AX505WA). The experiments were repeated a minimum three times.
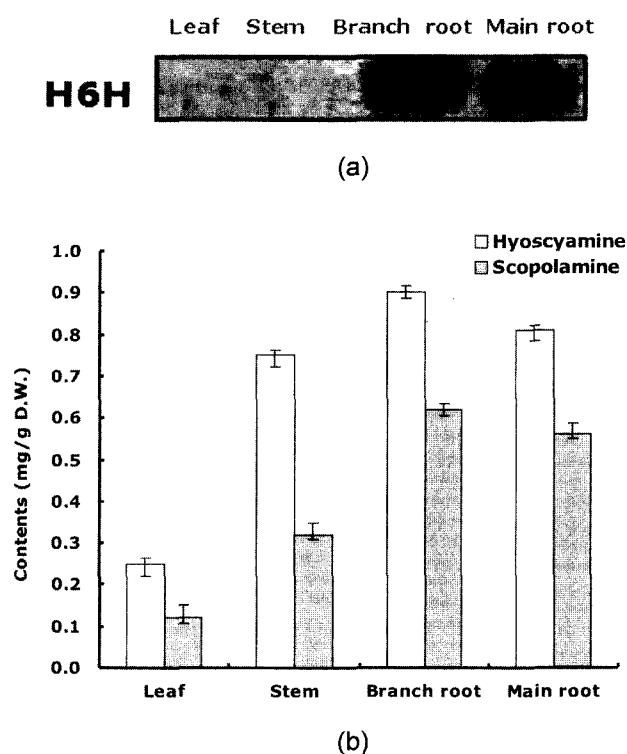
Statistical Analysis

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

RESULTS AND DISCUSSION

Tissue Specific Expression of H6H and Contents of Tropane Alkaloids

To determine the H6H protein level in *S. parviflora*, we examined the expression of H6H enzyme by Western blotting by using protein extracted from various tissues - leaf, stem, branch root, and main root (Fig. 3a). The H6H protein expression was found only in the roots; there was no expression in the leaf and stem. As for the hairy roots, *h6h* transcript levels were higher in branch roots than in main roots.

We used HPLC analysis to confirm the contents of tropane alkaloid in various tissues of *S. parviflora*. Hyoscyamine and scopolamine were found to be distributed variously in all plant parts, the levels being the highest in root followed by stem and leaf. It was found that the contents of both hyoscyamine and scopolamine in the roots were almost two to three times higher than in the leaf and stem (Fig. 3b). Western blot and HPLC analysis clearly showed that root is the primary tissue for tropane alkaloid biosynthesis with participation of H6H enzyme and they are likely translocated to other parts of the plant. This observation is in agreement with the reported localization of H6H in pericycle [5].

Recently, an immunohistochemical study using a highly specific monoclonal antibody against H6H revealed this enzyme to be localized in the pericycle tissue of several scopolamine-producing plants [6]. The leaf, stem, branch root, and main root of native plant contained 0.25, 0.75, 0.91, and 0.81 mg of hyoscyamine per g dry weight and 0.12, 0.32, 0.62, and 0.56 mg of scopolamine per g dry weight, respectively. The pattern of H6H protein expression paralleled the accumulation of tropane alkaloids in the roots. These data corresponded well to the study of Hashimoto *et al.* [9] that tropane alkaloids are mainly synthesized in the cultured roots. Furthermore, as shown by our results, these recently suggest that tropane alkaloid is actively more biosynthesized in branch root than main root.

Feeding of Hyoscyamine to Root Cultures and Increased Scopolamine Production

Because scopolamine is more widely used than hyoscyamine, scopolamine is more expensive than hyoscyamine. However, hyoscyamine is produced about 2-3 fold more than that of scopolamine in *S. parviflora*. Therefore, in order to enhance scopolamine production, we carried out an experiment to feed hyoscyamine to roots in the hope of bioconversion: Since H6H catalyzes the conversion of hyoscyamine to scopolamine in the tropane alkaloid biosynthetic pathway, the exogenous substrate feeding should likely increase the flux of final products. Indeed, feeding of hyoscyamine to *S. parviflora* roots consistently resulted in a dramatic increase of scopolamine content. As shown in Fig. 4, when

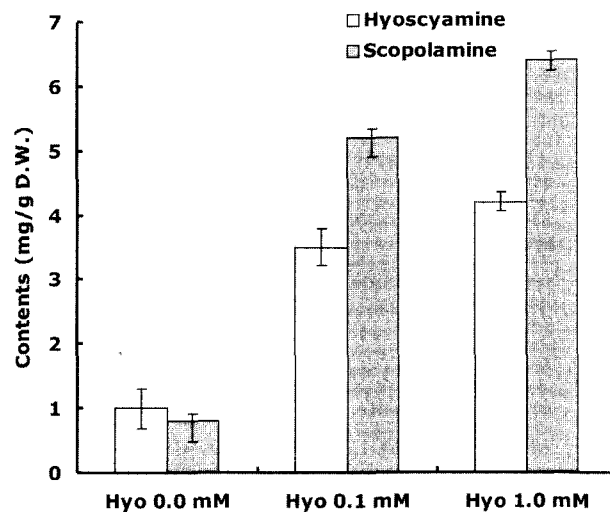Leaf   Stem   Branch root   Main root

(a)

(b)

Fig. 3. Tissue specific expression of H6H enzyme (a) and contents of tropane alkaloid in *S. parviflora* tissues (b).

Protein was extracted from wild-type *S. parviflora* and subjected to SDS-PAGE. The proteins were electroblotted onto nitrocellulose membrane. H6H was detected immunologically with mouse monoclonal antibodies. The alkaloids were extracted from the plants with chloroform, and their concentrations were determined by HPLC.


Fig. 4. Effect of hyoscyamine feeding to wild-type hairy root of *S. parviflora*.

The hairy roots were cultured in 30 ml of B5 liquid medium supplemented with 30 g/l sucrose and 0.1 mg/l IBA. 0.1 to 1.0 mM concentration of hyoscyamine, H6H substrate, was fed to B5 liquid medium. The cultures were grown in dark at 25±1°C on a rotary shaking incubator at 100 rpm. Roots were harvested after 4 weeks, and then analyzed for tropane alkaloids by HPLC.

cultured in B5 liquid medium supplemented with 0.0 to 1.0 mM hyoscyamine, that in wild-type roots had increased, ranging from 0.8 to 6.4 mg/g dry weight. These results indicate that the H6H enzyme is able to convert exogenously supplied hyoscyamine to scopolamine. Furthermore, as seen in Fig. 2, scopolamine content was high when hyoscyamine content was abundant and scopolamine contents remained high throughout the culture period of 4 weeks. Since scopolamine is the final product of the tropane alkaloid pathway, we speculate that hyoscyamine is metabolized quickly to stable terminal metabolite. These results suggest that increased flux of hyoscyamine may result in more production of scopolamine.

Genetic Transformation

We developed transgenic plants overexpressing *h6h* genes. A plasmid containing cDNA, which encoded *h6h* driven by a CaMV 35S promoter with a Ω sequence, and a plasmid containing a selectable marker (*npt*II) gene were simultaneously introduced into *S. parviflora* shoot tissue by using *A. rhizogenes* 2703 harboring pBE2113. Hairy roots were induced after infection of *S. parviflora* plants with *Agrobacterium*, and adventitious hairy roots were regenerated from shoot tissues. Subsequently, the hairy root lines were subcultured for at least 1 month in a B5 medium containing 1.0 mg/l IBA, and hairy root clones were first screened for the kanamycin-resistant phenotype. Kanamycin-resistant hairy root lines obtained were then cultured in a hormone-free half-strength B5 solid medium without antibiotics. The *h6h* gene, when subcloned into pBE*h6h* and transformed to *S. parviflora* via *A. rhizogenes*, resulted in several transgenic hairy root lines that were confirmed by PCR amplification (Fig. 5). The genomic DNA of vector control root carrying *Agrobacterium rol* gene and the genomic DNA of wild-type root did not show any
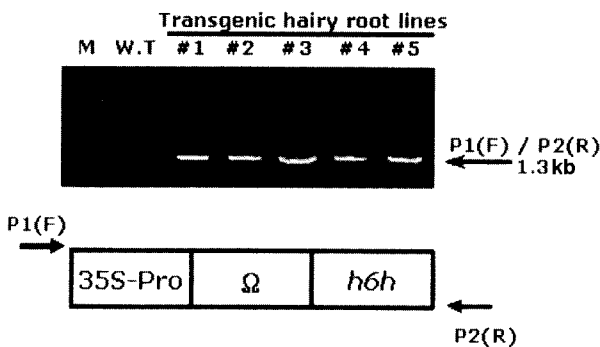
Fig. 5. PCR amplification was performed by using P1 (F), P2 (R) to confirm transgenic hairy root lines.

Electrophoresis on 1.0% agarose gel was performed to detect *h6h* gene amplified by PCR. The PCR product was obtained with P1 (F): 5'-GCTCTAGAATGGCTACTTTTGTGT-3'; P2 (R): 5'-CGGGATCCTTAG-ACAGTGATTTTA-3'. M: λDNA digested with *Hind*III; lanes 2-7: wild-type and transgenic hairy root lines.
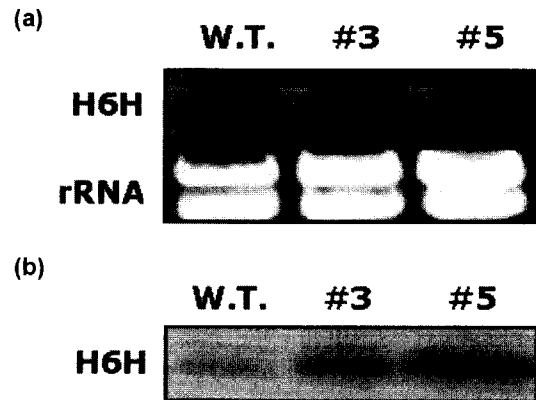
Fig. 6. Northern blotting (a) and Western blotting (b) of transgenic hairy root lines.

The upper panel shows total mRNA extracted and separated on formaldehyde agarose. After blotting RNA onto a nylon membrane, the *h6h* specific mRNA species were identified by ^{32}P-labeled cDNA probe and semiquantitatively quantified by phosphor imaging and laser densitometry. The lower panel indicates the H6H specific proteins, SDS-PAGE separated and Western blotted and in the wild-type root and transgenic hairy root lines. W.T.: wild-type root; #3 and #5: transgenic hairy root lines.

amplified band. However, the 35S *h6h* transgene and all the putative engineered hairy roots gave a band of 1.3 kb size, corresponding to the *h6h* gene fragment and confirming the presence of the transgene in the hairy root lines.

Overexpression of *h6h* Gene in Transgenic Hairy Roots

Northern blotting showed the presence of the corresponding mRNA in all the root lines tested which carried the 35S-*h6h* gene (Fig. 6a), confirming the capacity of the transgene to be expressed in the transformed hairy roots. The levels of *h6h* transcripts in the transgenic hairy root lines were higher than in the wild-type root.

Promoters regulate gene expression both quantitatively and qualitatively: the cauliflower mosaic virus 35S promoter, which has frequently been used as a constitutively strong promoter in plants, and the tobacco mosaic virus (TMV) have a unique G-free sequence (Ω sequence) in the 5'-untranslated region (UTR) of its genomic RNA [8]. Thus, this promoter with a Ω sequence should also be useful for the production of tropane alkaloids in hairy root cultures.

The protein extracts prepared from the transgenic hairy root lines were subjected to SDS-PAGE for Western analysis, and a 36 kDa protein band, that crossreacted with H6H specific antibodies, was found in the extracts: Western blotting with a H6H specific monoclonal antibody showed that the H6H polypeptide of 36 kDa was abundant in several transgenic hairy root lines and that the levels of *h6h* expression were generally 2-3 fold higher then that in wild-type root. The levels of H6H expression in transgenic hairy root lines varied considerably among the 10 lines, and Jouhikainen *et al.* [10] have shown that the expression

levels of H6H also varied among *H. muticus* hairy root cultures. Functional expression of the *H. niger h6h* gene has been shown by the enhanced contents of tropane alkaloids in *Nicotiana tabacum* [18]. These different levels of scopolamine together with the presently decided studies are highly suggestive of the relationship between enzyme expression levels and alkaloid production among the species [21]. In addition, the expression levels of H6H protein in the transgenic hairy root lines were two or more times greater than in the wild-type (Fig. 6b).

Hashimoto *et al.* [9] for the first time were able to produce about 3.2 mg of tropane alkaloids per g dry weight of *Atropa belladonna* by employing metabolic engineering technique; that yield of tropane alkaloids in *A. belladonna* was lower than that of other Solanaceae. However, our results showed that the overexpression of the *h6h* gene increased the contents of tropane alkaloids in *S. parviflora*, and

this could be due to a different, specific post-translational regulation of the endogenous enzyme, in contract to the exogenous enzyme.

Different Root Growth in Hairy Root Lines

Transgenic hairy roots have less developed root hair only on their branch roots, compared with the wild-type root (Fig. 7a), and the morphology of the *S. parviflora* hairy root lines carrying the *h6h* gene was different from that of the wild-type root. These transgenic hairy roots grew slowly and lateral branching appeared. When the transgenic roots were cultured on B5 basal liquid medium for 8 weeks, the root growth index decreased, compared to the wild-type root (Fig. 7b). Therefore, our experiment indicated that the foreign gene had a negative effect on growth of transgenic hairy roots in almost all hairy root lines, and in the root growth index, and decreased and did not stimulate root browning. Canel *et al.* [2] also reported that *tdc* overexpressed *Catharanthus roseus* cells showed signs of stress and poor growth.

Tropane Alkaloid Contents in Overexpressed Hairy Roots

Concentrations of tropane alkaloids from regenerated transgenic hairy root lines were determined by HPLC analysis. Considerable variation of both morphology and tropane alkaloids production was observed in the transgenic hairy roots. The levels of hyoscyamine and scopolamine in the extracts of the wild-type plant were 1.51 and 1.02 mg/g dry weight, respectively (Fig. 8). In the transgenic hairy root lines which constitutively express the H6H enzyme, high concentrations of both hyoscyamine and scopolamine were observed: The extracts from the hairy root lines

(a)

(b)
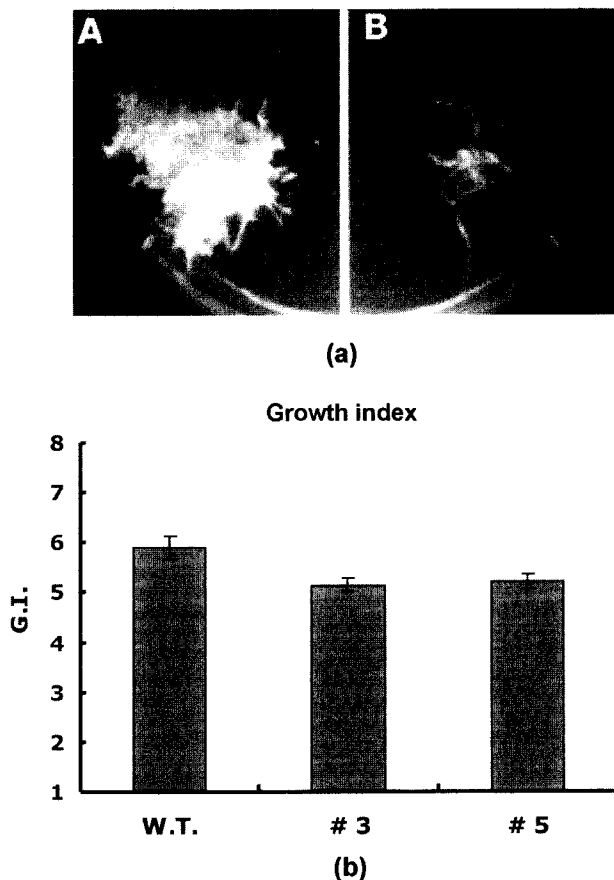
Fig. 7. Different morphology (a) and growth index (b) of wild-type root and transgenic hairy root lines. The morphology of the *S. parviflora* hairy root lines carrying the *h6h* gene was different from that of wild-type roots. A: wild-type root; B: transgenic hairy root line #5.

The roots growth was measured by the growth index (harvest weight - inoculum weight/ inoculum weight). These transgenic hairy roots grew slowly and lateral branching appeared. W.T.: wild-type root; #3 and #5: transgenic hairy root lines.
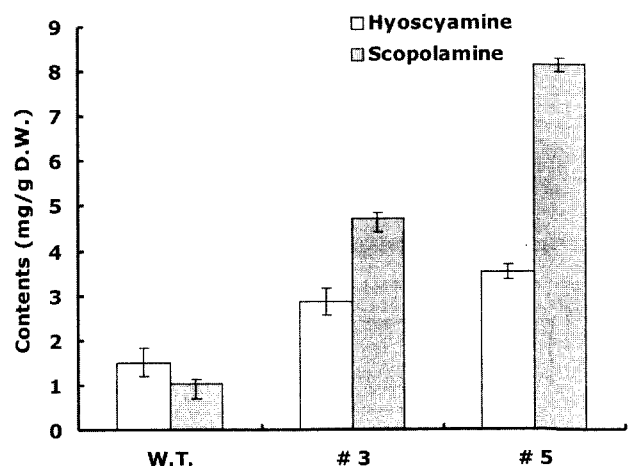
Fig. 8. Tropane alkaloids contents of wild-type and transgenic hairy root lines.

The rhizomes were cultured for 4 weeks, and the alkaloid concentrations were determined by using HPLC. W.T.: wild-type root; #3 and #5: transgenic hairy root lines.

numbers 3 and 5 contained between 2.85 and 3.52 mg of hyoscyamine per g dry weight, respectively. The levels of scopolamine production in the transgenic hairy roots varied greatly, ranging from 4.72 mg/g dry weight to 8.12 mg/g dry weight, and its levels were lower than those of hyoscyamine, except in the transgenic hairy root lines numbers 3 and 5. The best transgenic hairy root line (#5) produced over three times more scopolamine than the wild-type plants after 4 weeks of culture. Although transgenic lines showed improved yields of alkaloid, they were less than the metabolite feeding experiments. Therefore, there is a need for extensive experimental trials to produce the secondary metabolites on a commercial scale. As of today, the traditional methods are discredited, because of low productivity and lack of appropriate downstream processing to obtain desired products. In addition, there is also no clear understanding about the complete secondary metabolic pathways, and only a few enzymes and intermediates have been well characterized [11, 20].

Many studies reported that *h6h* overexpressing roots of Solanaceae plants have various alkaloids profiles, in addition to the endogenous polyamine pool in the whole plant [10, 13, 16, 18, 20]. However, our results showed that the overexpression of the *h6h* gene enhanced alkaloid production of *S. parviflora* transformed roots, improving the production of both hyoscyamine and scopolamine.

CONCLUSION

Studies on the production of tropane alkaloids in Solanaceae have received considerable intensity during the past. Since H6H has key roles in the secondary metabolism of hyoscyamine and scopolamine, manipulation or engineering of this pathway would be worthwhile to enhance their production. The H6H enzyme in *S. parviflora* hairy root cultures was proved to be a rate limiting enzyme. The feeding of exogenous hyoscyamine to tissue cultures as additional substrates enhanced scopolamine production. Because H6H catalyzes the hydroxylation and epoxidation to hyoscyamine from scopolamine, enhanced H6H enzyme levels favored the accumulation of scopolamine [3].

In conclusion, high concentrations of tropane alkaloids were accumulated in the transgenic plants that constitutively expressed the H6H enzyme. The *h6h* gene of overexpressing transgenic hairy root cultures aged faster and accumulated higher amounts of tropane alkaloids than wild-type roots. Thus this study showed that the yields of tropane alkaloids could be enhanced by genetic and metabolic engineering of the secondary metabolic pathways. In this respect, the genetic approach appears to be a promising one. Further studies on plant tissue cultures in combination with pathway engineering strategy can enhance the production of useful secondary metabolites on a commercial scale.

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