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MOLECULAR FARMING/METABOLIC ENGINEERING/SECONDARY METABOLISM

Enhanced production of tropane alkaloids in transgenic *Scopolia parviflora* hairy root cultures over-expressing putrescine *N*-methyl transferase (PMT) and hyoscyamine-6β-hydroxylase (H6H)

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Abstract *Scopolia parviflora* adventitious roots were metabolically engineered by co-expression of the two gene putrescine *N*-methyl transferase (PMT) and hyoscyamine-6β-hydroxylase (H6H) cDNAs with the aid of *Agrobacterium rhizogenes*. The transformed roots developed into morphologically distinct *S. parviflora* PMT1 (Sp*PMT1*), *S. parviflora* PMT1 (Sp*PMT2*), and *S. parviflora* H6H (Sp*H6H*) transgenic hairy root lines. Consequent to the introduction of these key enzyme genes, the production of the alkaloids hyoscyamine and scopolamine was enhanced. Among the transgenic hairy root lines, Sp*PMT2* line possessed the highest growth index. The treatment of transgenic hairy roots with growth regulators further enhanced the production of scopolamine. Thus, the results suggest that PMT1, PMT*2*, and H6H genes may not only be involved in the metabolic regulation of alkaloid production but also that these genes may play a role in the root development.

Keywords Hyosamine-6β-hydroxylase (H6H) · Putrescine *N*-methyl transferase (PMT) · Hairy roots · Phytohormones · *Scopolia parviflora* · Tropane alkaloids

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Introduction

Hyoscyamine and scopolamine are two of the most important tropane alkaloids (TA) found as secondary metabolites in the *Solanaceae* family members (Hashimoto and Yamada 1987). Since these alkaloids possess various therapeutic properties, they are used in different medical applications. The general route for the production of such secondary metabolites on a commercial scale is to employ plant cells and their tissue cultures. Such approaches, however, have found limited success owing to low yields (Cusido *et al.* 1999). The metabolic engineering strategies thus can be developed to overcome the limitations of the traditional *in vitro* methods (Dixon 2001). Recently, efforts along these lines have been conducted, and a TA biosynthetic pathway has been manipulated for enhanced metabolite productions (Sato *et al.* 2001; Kang *et al.* 2005).

The amino acids arginine and ornithine are the starting substrates for the biosynthesis of TA. The critical step

during the TA biosynthesis (Lee *et al.* 2005) is the *N*-methylation of putrescine by PMT (EC 2.1.1.53), which diverts putrescine toward the production of the alkaloids (Fig. 1). Further along the metabolic pathway is a bifunctional enzyme H6H (EC 1.14.11.11) that hydroxylates hyoscyamine to 6β-hydroxyhyoscyamine, which subsequently undergoes epoxidation to scopolamine (Fig. 1). Thus, PMT and H6H enzymes are important targets for metabolic engineering in order to improve the yields of scopolamine. Also, as roots are the main sites of alkaloid biosynthesis in *Scopolia parviflora*, the generation of hairy roots followed by their mass culture would provide increased TA productions (Min *et al.* 2007a; Palazon *et al.* 2008).

The hairy root cultures have proven efficient systems for the production of secondary metabolites that are normally biosynthesized in roots (Hu and Du 2006). It has also been shown that transformed root cultures possess certain advantages such as rapid growth, genetic stability, and autotrophy with respect to plant hormones. Many physical and chemical factors such as media components, light, temperature, and exogenous phytohormones can influence the growth and metabolism of hairy roots. In general, hairy roots are cultured in phytohormone-free media, but several recent works have shown that phytohormone feeding has a positive influence on the production of secondary metabolites. However, the reports concerning the responses of metabolically engineered plants to phytohormone treatments are scanty.

In this study, we report the effects of introducing two exogenous genes of the TA pathway into *S. parviflora* adventitious roots. We have also investigated the effects of phytohormone treatments on metabolically engineered hairy roots.

Materials and Methods

Plant material, adventitious root, and bacterial strain. *S. parviflora* was obtained from the National Arboretum in Gyeonggi Province, Korea. The rhizome segments of 2–3 cm were collected, surface-sterilized with 70% (*v/v*) ethanol for 2 min, and then followed by 5% (*v/v*) NaClO treatment for 15 min. After a fourth wash with sterile distilled water, rhizomes were transferred to the sterile B5 (Gamborg *et al.* 1968) basal liquid medium pH 5.6–5.8 containing 3% (*w/v*) sucrose and cultured by incubating under dark conditions at 25±2°C on a rotary-shaking incubator at 100 rpm (JeioTech, Seoul Korea).

The genetic transformations and root culturing were performed according to the method of Kang *et al.* (2004a). *Escherichia coli* XLl-Blue MRF (Stratagene, La Jolla, CA) was used in DNA cloning experiments. *Agrobacterium rhizogenes* KCTC 2703 (Korean Type Cultures Collections; Taejon, South Korea) was employed for root induction.

Gene constructs and transformation of S. parviflora with PMT and H6H cDNAs. The PMT1, PMT2, or H6H cDNAs cloned into pcDNAII were released on treatment with the *Xba*I and *Bam*HI restriction enzymes. *Bam*HI-generated gaps were filled in using Klenow DNA polymerase. The resulting fragments were subcloned into the *Sma*II site of pBE2113 between its Ω sequence and its nopaline synthase terminator to obtain the corresponding pBE gene constructs (Fig. 2). In general, DNA manipulations were performed according to Sambrook *et al.* (1998), and vector construction was carried out according to Mitsuhara *et al.* (1996).

The pBE expression vector bearing corresponding cDNA was transformed into *A. rhizogenes* KCTC 2703 by the direct transfer method. The bacterium delivered the constructs into *S. parviflora* rhizomes. The genes for transformation were obtained from a cultured root cDNA library of *S. parviflora* by probing with PMT cDNA probe (*Nicotiana sylvestris* NCBI accession: AB004322) and H6H cDNA probe (*Hyoscyamus niger* NCBI accession: D26583; Kim 2003).

Hairy roots that formed after 4 wk of incubation were transferred to solid B5 medium supplemented with 500 mg/L carbenicillin to inhibit bacterial growth. Sterile and rapidly growing root clones were selected and utilized to establish hairy root cultures. After a second subculture, transformed hairy roots were shifted to 1/2-strength B5 agar medium (0.75% *w/v*) or B5 liquid medium without growth regulators. All the cultures were maintained as described earlier (Kang *et al.* 2005).

Identification of transgenics S. parviflora roots. The integration of the transferred genes into *S. parviflora* genome was confirmed by polymerase chain reaction (PCR). The genomic DNA from kanamycin-resistant roots was extracted from the putative transgenic hairy roots (Sambrook *et al.* 1998). The oligonucleotide primers for amplification of the introduced genes were: P1 (F) 5'-cccacccacgaggagcatc-3', P2 (R) Sp*PMT1*: 5'-gagctctcaaaactcaaccaaatc-3', P2 (R) Sp*PMT*2: 5'-aagcttacgccttcagacgatcga-3', and P2 (R) Sp*H6H*: 5'-gtactgcagcataattgctctgac-3'. The complete PCR mix contained 200 ng of template DNA, 12.5 pmol μl^{-1} of each oligonucleotide primer, 200 μM dNTPs, 1.5 U Taq polymerase (Pharmacia Biotech, Seoul, Korea), and buffer supplied by manufacturer, in a total volume of 25 μl. PCR was carried out for 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. The amplified PCR products (~1.3 kb each) were separated and analyzed with 1.2% agarose gel. Authentic transgenic hairy roots were further transferred and cultured in B5 medium containing kanamycin (100 mg l^{-1}) and cefotaxime (250 mg l^{-1}).

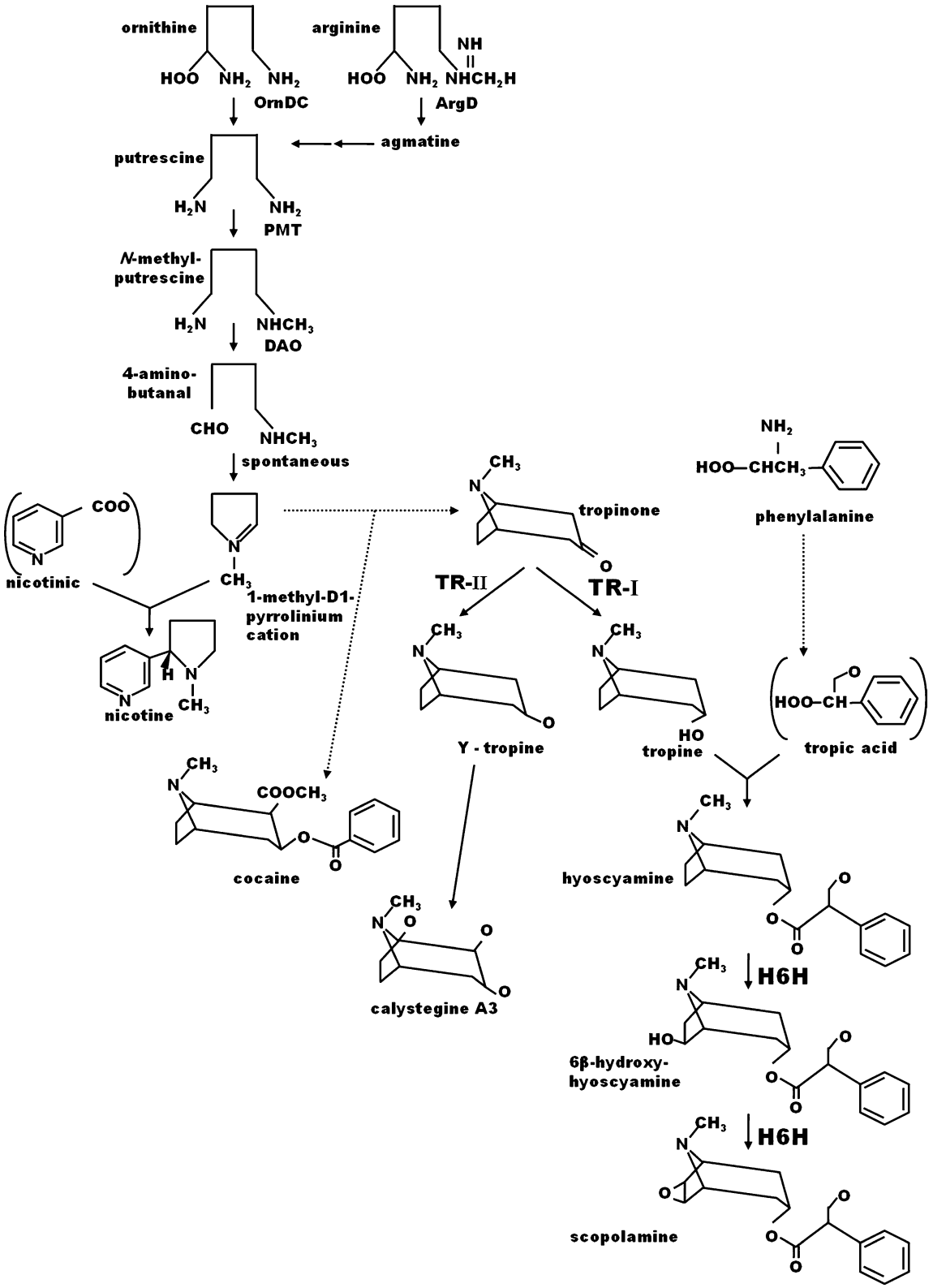

Figure 1. Biosynthetic pathway of tropane alkaloids. The tropane ring system arises from ornithine and/or arginine via the formation of putrescine. This is then methylated by PMT to tropinone. Tropine formed by tropinone reductase (TR-1) is esterified with phenylalanine to give the derivative hyoscyamine, H6H, a bifunctional enzyme that converts hyoscyamine to scopolamine (Lee *et al.* 2005).

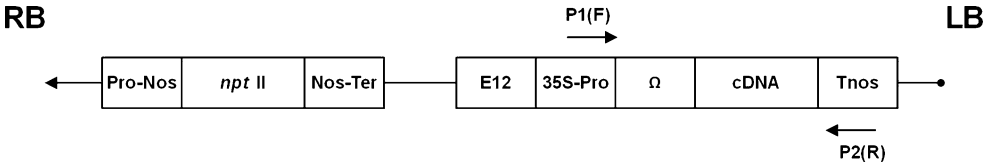

Figure 2. Construction of pBEPMT*1,* pBEPMT*2*, and pBEH6H expression vectors. *S. parviflora* root mRNA population was screened to obtain PMTs(PMT1 and PMT2) and H6H-specific mRNAs. The cDNAs corresponding to PMTs (PMT1, PMT2) and H6H genes were inserted between sigma sequence of TMV and nopaline syntheses terminator. *E12*: 5'-upstream sequence of cauliflower mosaic virus (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).

Phytohormone treatment of transgenic hairy roots. The effects of various phytohormones(N_6-(2-isopentyl)adenine (2iP), kinetin (kin), gibberellic acid (GA_3), benzyladenine, 3-indolebutyric acid (IBA), 3-indoleacetic acid (IAA), and 1-naphthylacetic acid (NAA) on transgenic hairy root growth and TA production were investigated. Each phytohormone was added into the media at various concentrations. Among the phytohormones, 2ip and GA_3 were filter-sterilized through a 0.22-μm filter (Millipore S.A. Co., Molsheim, France) and included into the media after autoclaving, while others were incorporated into the media before autoclaving. The adventitious roots and transgenic hairy roots were inoculated into 20 ml media contained in 100-ml Erlenmeyer flasks. The culture flasks were incubated at 25±1°C on a rotary shaker (100 rpm) in the dark for 4 wk.

Determination of root growth. The cultured roots and transgenic hairy roots were harvested at regular intervals. The roots were washed twice with sterile distilled water, placed between the folds of filter paper, and blotted dry in order to record fresh weight per culture flask. The root growth index (GI) was calculated using the equation: (harvest weight−inoculum weight)/inoculum weight).

Morphological observations of roots. Growth of the wild-type roots and transgenic hairy roots was measured with a ruler. The microscopic observation and sample preparations were according to the modified protocols (Ursula *et al.* 2001). The tissue specimens for microscopy were either fresh or fixed in glutaraldehyde and in 1,4-piperazinediethanesulfonic acid buffer (0.1 M; pH 7.2) containing 2.5% (*w/v*) glutaraldehyde under vacuum (three times for 30 s each), and finally placed in a trough at atmospheric pressure for 1 h. Fixed samples were rinsed three times with buffer for 1 h each, embedded in 3% (*w/v*) agarose, and sliced in a DTK-1000 micro-slicer (Ted Pella, Redding, CA). The light micrographs of specimens were captured with a digital camera fitted to a light microscope (Nikon CoolPix 5700, Toyko, Japan).

Transmission electron microscopy (TEM) of samples was carried out after initial fixing of plant material in an aqueous solution of 1% (*w/v*) formaldehyde and 2% (*w/v*) glutaraledehyde for 1 h. Following fixation, samples were dehydrated in a graded ethanol series; dried in carbon dioxide; coated with gold particles; and examined at ×5,000 magnification with energy filtering-transmission electron microscopy (EF-TEMEM 912; Omega, Carl Zeiss, Stuttgart, Germany).

Extraction and estimation of tropane alkaloids. Determination of alkaloids, hyoscyamine, and scopolamine was performed according to the method of Jung *et al.* (2002). The explants (1.0 g) were oven-dried at 50°C, pulverized, and soaked overnight in 10 ml EtOH-28% NH_4OH (19:1, *v/v*) mixture. After centrifugation for 10 min at 12,000×*g*, the supernatant solution was subjected to vacuum evaporation. The resulting extract was solubilized in methanol (HPLC-grade), filtered through a prefilter (Ψ0.2 μl Supelco, Bellefont, PA), and analyzed by High Pressure Liquid Chromatography (HPLC) (Gilson, Seoul, Korea) equipped with a TSK gel ODS-80Ts (4.6 mm×25 cm, 5 μm, Tosoh, Tokyo, Japan) column and a UV 3000 detector (Gilson).

The separation of the metabolites was effected with an isocratic mobile phase consisting of a mixture of acetonitrile/50 mM potassium phosphate (22:78 *v/v*) pH-adjusted to 3.0. A 20-μl sample was injected into the port with a flow rate of 0.8 ml min^{-1}. The system was calibrated with commercial hyoscyamine and scopolamine as standards (Sigma-Aldrich Co., St. Louis, MO). The quantification of TA was achieved by comparing the retention times obtained by co-chromatography with standards and samples.

The metabolite estimations were repeated for a minimum of three times. The correlation coefficients (*R*) with the standards were 99.84% for hyoscyamine and 99.87% for scopolamine with deviations of three replicates as ±0.001.

Statistical analysis. Three replicates for each treatment were used in analysis of growth parameters such as root and root hair formation. The results shown are mean± standard error. The significance of differences between mean values was determined by analyses of variance.

Results

Genetic transformation of S. parviflora with PMT and H6H genes. The integration of *PMT1, PMT2*, and *H6H* cDNAs was confirmed by PCR analysis; however, the electrophoretograms did not reveal bands corresponding to endogenous PMT and H6H genes probably because of the unspliced introns (Fig. 3). The full-length clone of Sp*PMT1* contained 1,389 bps, with an open reading frame encoding 338 amino acids. The Sp*PMT*2 cDNA was 1,361 bps to encode Sp*PMT2* of 359 amino acids, and full-length Sp*H6H* cDNA possessed 1,297 bps to encode 344 amino acids.

The hairy root lines were subcultured in B5 medium with 1.0 mgL^{-1} IBA for at least 1 mo. The transgenic hairy roots were further cultured in a hormone-free 1/2-strength B5 solid medium without antibiotics.

Growth and tropane alkaloid levels in S. parviflora transgenic hairy roots. An increase in the growth of transgenic hairy roots compared with the wild-type roots was observed in B5 basal liquid medium (Table 1). The GI of Sp*PMT1*, Sp*PMT2*, and Sp*H6H* hairy roots varied. The GI for transgenic hairy roots in the study ranged between 200% and 300% as compared with wild-type. The maximum GI observed was 28.65 for Sp*PMT2*–6 hairy root line. In general, all hairy root lines showed increased GI without phytohormone sensitivity.

The production of scopolamine and hyoscyamine also varied among the adventitious roots and transgenic hairy roots (Table 1). The production of scopolamine in SpPMT lines remained unaffected. However, the production of hyoscyamine in SpPMT and Sp*H6H* transgenic hairy root lines was slightly different. In transgenic hairy roots, the scopolamine level was more than that of hyoscyamine. The amounts of hyoscyamine and scopolamine in wild-type adventitious root

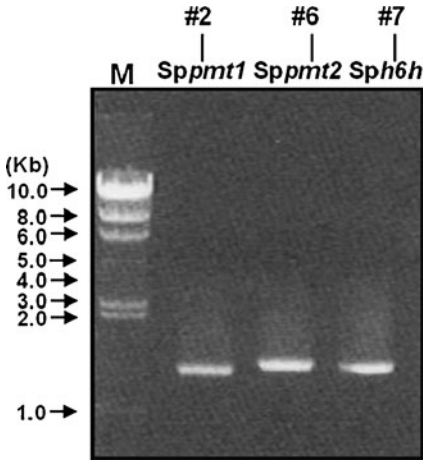

Figure 3. PCR amplification of PMT*1,* PMT*2*, and H6H. The hairy root line number *2* represents Sp*PMT1*, number *6* corresponds to Sp*PMT2*, and number *7* to Sp*H6H.*

Table 1. Differences in growth and tropane alkaloid production by wild-type and transgenic roots

Root lines	Growth index	Scopolamine[z]	Hyoscyamine[z]
Wild-type	5.82±0.23	1.06±0.15	1.91±0.29
Sp*PMT1*–2	18.03±2.40	3.00±0.01	2.92±0.01
Sp*PMT1*–8	17.37±1.00	3.03±0.84	2.93±0.62
Sp*PMT1*–12	16.85±0.97	3.05±0.85	2.79±0.18
Sp*PMT1*–13	17.06±0.85	3.15±1.03	2.63±0.15
Sp*PMT1*–15	24.03±6.71	3.10±0.33	2.82±0.27
Sp*PMT1*–16	17.30±1.51	3.43±0.66	2.91±0.08
Sp*PMT2*–6	28.65±8.61	3.31±0.18	2.74±0.66
Sp*PMT2*–7	23.54±6.73	3.09±0.15	3.00±0.34
Sp*PMT2*–12	22.20±6.13	3.18±1.00	2.75±0.27
Sp*H6H*–7	20.60±2.49	2.95±0.54	2.69±0.18
Sp*H6H*–12	17.50±2.60	2.79±0.18	2.56±0.07

[z] In milligrams per gram dry weight

were 1.91 and 1.06 mg g^{-1} DW, respectively. The transgenic hairy root lines accumulated more of hyoscyamine (2.69–3.00 mg g^{-1} DW) and scopolamine (2.95 to 3.43 mg g^{-1} DW). It was further noted that these hairy roots could retain the alkaloid production ability consistently even after several subcultures.

Growth pattern and morphology of transgenic hairy roots. After 4 wk of infection of the adventitious roots by *A. rhizogenes,* numerous hairy roots began to emerge. The morphology of the *S. parviflora* hairy root lines carrying the Sp*PMT1*, Sp*PMT2*, and Sp*H6H* genes differed from that of adventitious roots (Fig. 4). Wild-type adventitious root formed thick primary roots without branching and secondary roots. However, transgenic hairy root lines presented the characteristic traits of the primary roots but with an extensive lateral branching and profuse root hairs. This is in agreement with the fact that, when *A. rhizogenes* infects the plant, the T-DNA between TR and TL regions of the Ri plasmid in the bacterium is transferred and integrated into the nuclear genome of the plant. At the same time, this transformation event directs the production of hairy roots (Chilton *et al.* 1982). After subcultures, the wild-type adventitious roots increased in length without branching points and turned brown except for newly developing yellowish parts. However, transgenic hairy roots remained stable on successive subcultures for 1 yr.

The TEM study of the morphological characteristics of transgenic hairy roots (Fig. 5) displayed characteristic features. In hairy roots, the adjacent cells were smaller; cell organelles were more condensed as compared with the wild-type roots. Another interesting feature observed in transgenic hairy roots was the presence of a large spherical-shaped idioblast cells (Fig. 5*a, b*).

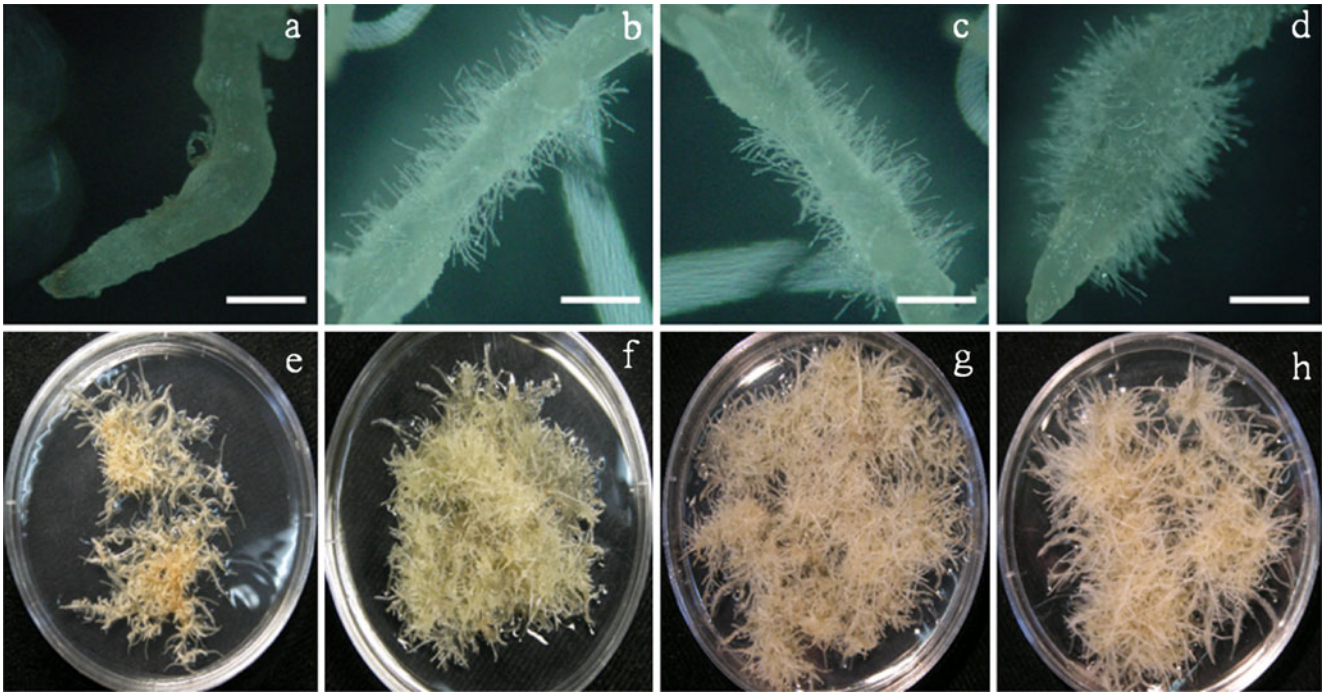

Figure 4. The morphology of wild-type adventitious roots and hairy root lines of *S. parviflora*. *a*, *e* are of wild-type adventitious root; *b*, *f* correspond to Sp*PMT1* hairy roots; *c*, *g* for Sp*PMT2* hairy roots; and *d*, *h* represent Sp*H6H* hairy roots. *Bars* in the dark field images are 0.4 cm.

Influence of phytohormones on transgenic root growth and alkaloid production. The phytohormones at low concentrations (5 mg L^{-1}) had a minor influence on the growth of both adventitious and transgenic hairy roots (data not shown). Also, the pattern of root growth with either cytokinins or auxins was not much different. The cytokinins 2ip and kin promoted adventitious root growth. Among the phytohormone-treated hairy roots, GI of Sp*H6H* line increased by six times against wild-type adventitious roots exposed to 2.0 mg L^{-1} IAA. Phytohormone treatments in general enhanced the production of scopolamine and hyoscyamine (data not shown) in both wild-type and transgenic hairy roots. Phytohormone treatment at all levels enhanced scopolamine and hyoscyamine accumulation in transgenic hairy roots only. Sp*PMT1* transgenic line produced highest quantities of alkaloids at 2.0 mg L^{-1} of kin among all other treatments (scopolamine, 7.97; hyoscyamine, 2.92 mg g^{-1} DW). Sp*PMT2* hairy roots produced 6.98 and 2.58 mg g^{-1} hyoscyamine after exposure to 0.5 mg L^{-1} 2ip. However, the TA levels in Sp*H6H* hairy roots were comparable to the wild-type adventitious roots.

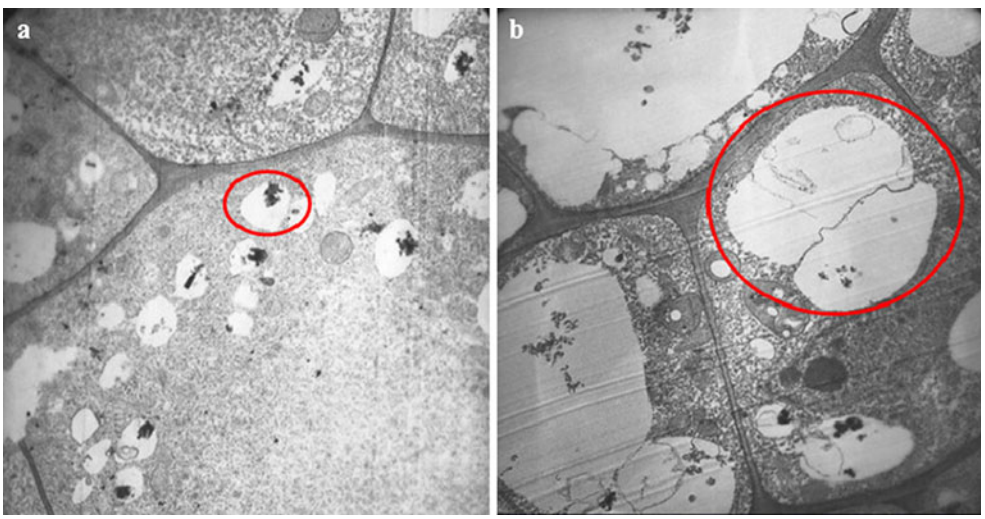

Figure 5. Electron micrographs of wild-type and transgenic hairy root cells (at ×5,000 magnification). *a*, Wild-type root; *b*, transgenic hairy root (Sp*PMT1*). *Circle* represents an idioblast.

Discussion

S. parviflora adventitious roots transformed into hairy roots under the influence of *A. rhizogenes*. The growth rate of the transgenic hairy roots was higher than that of the wild-type adventitious roots. The observed acceleration in growth of transgenic hairy roots may be due to the introduction of highly expressibleSp*PMT1*, Sp*PMT2*, and Sp*H6H* genes via *A. rhizogenes*. In a similar study, Canel *et al.* (1998) have reported that tryptophan decarboxylase (*tdc*) gene over-expressed *Catharanthus roseus* cells show signals of stress and poor growth. However, in our experiments, owing to the introduction of endogenous genes, the transgenic hairy roots remained healthy.

The root phenotype of the hairy root lines carrying Sp*PMT1*, Sp*PMT2*, and Sp*H6H* genes differed from wild-type adventitious root (Fig. 4). Transgenic hairy root lines showed the characteristic traits like primary roots with extensive lateral branching and profuse root hairs. Hairy root formation is a consequence of the integration of the T-DNA of Ri plasmid of *A. rhizogenes* into the plant genome (Chilton *et al.* 1982; Spena *et al.* 1987). Moyano *et al.* (1999) have found that *aux*plays a significant role in the morphology and alkaloid production of transformed roots in *Datura metel* and *Duboisia* hybrid. In transformed roots of cucumber, the phenotype of roots is influenced by the expression of auxin synthesis genes of TR-DNA, resulting in roots with two distinct morphologies (Rhodes *et al.* 1994).

The transgenic hairy roots showed large spherical idioblast cells in TEM micrographs (Fig. 5). Such idioblast cells have been demonstrated to participate in the biosynthesis and accumulation of TA (Constabel 1983). They have also been observed in *C. roseus* cultures (Mersey and Culter 1986) and *Datura stramonium* callus (Iranbakhsh *et al.* 2006). Such an observation in *S. parviflora* hairy roots supports our studies concerning elevated levels of the TA. This increase is also attributed to the overexpression of key TA pathway enzymes by translation of *PMT1, PMT2*, and *H6H* genes in transgenic hairy roots. Similar observations have been made in transgenic hairy root of *S. parviflora* cultures by Kang *et al.* (2005) and *Atropa belladonna* cultures by Rothe *et al.* (2003).

The phytohormone treatments did not influence the growth of transgenic hairy roots a great deal. However, they influenced the production of scopolamine and hyoscyamine. Rhodes *et al.* (1994) have reported that the hormonal regimes influence the pattern of growth of the culture and may directly or indirectly affect the operation of secondary metabolites pathways.

Washida *et al.* (2004) have reported that the IBA and NAA combination promotes ginsenoside and hairy root formation. Thus, the enhancements in alkaloids in cultured hairy roots are indicative of the phytohormones acting as stress factors to elicit and stimulate secondary metabolism. Surprisingly, phytohormone treatment on the hairy root lines examined had marginal influence on improvements in hyoscyamine content. Thus, exogenous phytohormones may change the endogenous hormone balance, leading to elevated scopolamine production in transgenic hairy root lines.

The feeding of phytohormones to metabolic-engineered hairy roots enhanced TA levels in comparison to other culture methods. Table 2 shows the TA production methods and their contents from previous studies. It decried various approaches

Table 2. Methods for producing tropane alkaloids with *S. parviflora* (various approaches by Myung-Suk Choi's group)

Method	Materials	TA content	Reference
Bacterial elicitors	Gram-negative strain (*Pseudomonas aeruginosa*) Gram-positive strains (*Bacillus cereus* and *Staphylococcus aureus*)	*S*=2.8 mg g^{-1} DW *H*=3.7 mg g^{-1} DW	Jung *et al.* 2003
Signaling elicitors	Methyl jasmonate and salicylic acid	*S*=0.7 mg g^{-1} DW *H*=2.0 mg g^{-1} DW	Kang *et al.* 2004a
Tissue cultures	Adventitious roots and shoots through rhizome cultures	*S*=1.3 mg g^{-1} DW *H*=1.7 mg g^{-1} DW	Kang *et al.* 2004b
Metabolic engineering (HnH6H)	Transgenic hairy roots using H6H of *H. niger*	*S*=8.0 mg g^{-1} DW *H*=4.0 mg g^{-1} DW	Kang *et al.* 2005
Metabolic engineering (NsPMT)	Transgenic hairy roots using PMT of *N. sylvestris*	*S*=6.4 mg g^{-1} DW *H*=4.2 mg g^{-1} DW	Lee *et al.* 2005
Bioreactor (scale-up)	Small-scale bubble column bioreactor cultures	*S*=1.6 mg g^{-1} DW *H*=3.4 mg g^{-1} DW	Min *et al.* 2007a
Selection and single cell cloning	Tropane alkaloids high-producing *H. niger* root	*S*=16.64 mg g^{-1} DW *H*=26.64 mg g^{-1} DW	Min *et al.* 2007b
Metabolic engineering (SpPMTs and Sp*H6H*)	Transgenic hairy roots using PMTs of *S. parvilflora* and H6H of *S. parvilflora*	*S*=3.4 mg g^{-1} DW *H*=3.0 mg g^{-1} DW	Kang *et al.* (this study)
	After responding to phytohormones in these root lines	*S*=8.0 mg g^{-1} DW *H*=3.0 mg g^{-1} DW	

to produce TA from *S. parviflora* by only Myung-Suk Choi's group. Although non-transgenic rhizome cultures produced only 3.0 mg g^{-1} DW tropane alkaloids (S, 1.3 mg g^{-1} DW^{-1}; H, 1.7 mg g^{-1} DW; Kang *et al.* 2004b), transformed hairy root clones produced more tropane alkaloids (Kang *et al.* 2005; Lee *et al.* 2005). However, the tissue culture methods provide only minor enhancements (Jung *et al.* 2003; Kang *et al.* 2004a; Min *et al.* 2007b).

In conclusion, these metabolic-engineered transgenic hairy roots produced higher scopolamine and hyoscyamine. The supplementation of phytohormones into the hairy root culture media enhanced the scopolamine production. Thus, such studies can form the basis for the development of industrial process for TA production from metabolic-engineered hairy roots.

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