Planta (2007) 225:887–896
DOI 10.1007/s00425-006-0402-1

ORIGINAL ARTICLE

# Tropane alkaloids production in transgenic *Hyoscyamus niger* hairy root cultures over-expressing Putrescine *N*-methyltransferase is methyl jasmonate-dependent

**Lei Zhang · Bin Yang · Beibei Lu · Guoyin Kai · Zinan Wang · Yang Xia · Ruxian Ding · Hanming Zhang · Xiaofen Sun · Wansheng Chen · Kexuan Tang**

Received: 8 August 2006 / Accepted: 30 August 2006 / Published online: 27 September 2006


**Abstract** The cDNA from *Nicotiana tabacum* encoding Putrescine *N*-methyltransferase (PMT), which catalyzes the first committed step in the biosynthesis of tropane alkaloids, has been introduced into the genome of a scopolamine-producing *Hyoscyamus niger* mediated by the disarmed *Agrobacterium tumefaciens* strain C58C1, which also carries *Agrobacterium rhizogenes* Ri plasmid pRiA4, and expressed under the control of the CaMV 35S promoter. Hairy root lines transformed with *pmt* presented fivefold higher PMT activity than the control, and the methylputrescine (MPUT) levels of the resulting engineered hairy roots increased four to fivefold compared to the control and wild-type roots, but there was no significant increase in tropane alkaloids. However, after methyl jasmonate (MeJA) treatment, a considerable increase of PMTase and endogenous H6Hase as well as an increase in scopolamine content was found either in the transgenic hairy roots or the control. The results indicate that hairy root lines over-expressing *pmt* have a high capacity to synthesize MPUT, whereas their ability to convert hyoscyamine into scopolamine is very limited. Exposure to MeJA strongly stimulated both polyamine and tropane biosynthesis pathways and elicitation led to more or less enhanced production simultaneously.

**Keywords** *Agrobacterium rhizogenes* · Hairy roots · *Hyoscyamus niger* · *pmt* gene · Methyl jasmonate · Scopolamine · Tropane alkaloids

**Abbreviations**

| | |
|---|---|
| H6H | Hyoscyamine-6β-hydroxylase (enzyme) |
| *h6h* | Hyoscyamine-6β-hydroxylase (cDNA) |
| JA | Jasmonic acid |
| MeJA | Methyl jasmonate |
| MPUT | *N*-methylputrescine |
| PCR | Polymerase chain reaction |
| PMT | Putrescine *N*-methyltransferase (enzyme) |
| *pmt* | Putrescine *N*-methyltransferase (cDNA) |
| PUT | Putrescine |
| RT-PCR | Reverse transcription PCR |
| SD | Spermidine |
| SM | Spermine |

L. Zhang · Z. Wang · Y. Xia · X. Sun · K. Tang (✉)
State Key Laboratory of Genetic Engineering,
School of Life Sciences, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, Fudan University,
Shanghai 200433, People's Republic of China
e-mail: kxtang@fudan.edu.cn

L. Zhang · B. Yang · B. Lu · R. Ding · H. Zhang · W. Chen
Department of Pharmacognosy, School of Pharmacy,
Second Military Medical University,
Shanghai 200433, People's Republic of China

G. Kai
Laboratory of Plant Bioengineering,
College of Life and Environment,
Shanghai Normal University,
Shanghai 200234, People's Republic of China

W. Chen (✉)
Department of Pharmacy, Changzheng Hospital,
Second Military Medical University,
Shanghai 200003, People's Republic of China
e-mail: chenws@vnet.citiz.net

K. Tang
Plant Biotechnology Research Center, School of Agriculture and Biology, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, Institute of Systems Biology,
Shanghai Jiao Tong University,
Shanghai 200030, People's Republic of China

## Introduction

A few genera of plant family *Solanaceae*, including *Hyoscyamus, Duboisia*, *Atropa* and *Scopolia*, are able to produce biologically active tropane alkaloids such as hyoscyamine and scopolamine simultaneously (Evans 1979; Endo et al. 1991; Christen et al. 1993; Hashimoto and Yamada 1994). Tropane alkaloids are widely used as parasympatholytics that competitively antagonize acetylcholine with great commercial demands; thus there has been a long-standing interest in increasing the content of this kind of alkaloids, especially the much more valuable scopolamine in cultivated medicinal plants.

New ways for medicinal plant improvement and for the production of plant drugs have opened up as the result of advances in plant biotechnology and increasing interest in plant-derived pharmaceuticals (Parr 1989). Recent re-evaluation of root culture has led many laboratories to exploit this organ culture system for the production of plant secondary metabolites. Rapidly growing root cultures can be established either by manipulation of auxin levels in the culture medium or by the genetic transformation of plants with *Agrobacterium rhizogenes*. The *Agrobacterium*-mediated transformation has an advantage that any foreign genes of interest placed in a binary vector can be simultaneously transferred to the transformed hairy root clones. Since tropane alkaloids are mostly synthesized in the young root cells and translocated to the aerial parts of solanaceous plants (Hashimoto and Yamada 1987; Hashimoto et al. 1991), the application of hairy root technology has been proved beneficial. Small-scale jar fermenters for several *Solanaceous* species hairy roots have been developed as prospective in vitro systems for commercial production of tropane alkaloids (Yamada and Hashimoto 1982; Oksman-Caldentey et al. 1991; Sevón et al. 1995, 1997), but until very recently the lack of understanding of the regulation of secondary metabolite pathways has limited the general use of metabolic engineering in medicinal plants. Hyoscyamine is usually the main alkaloid in transgenic root cultures of many *Solanaceae* plants including *Hyoscyamus* (Moyano et al. 2003).

Tropane alkaloids are derived from a common intermediate, the *N*-methylpyrrolinium cation (Fig. 1). Putrescine (PUT) is a common precursor of both polyamines such as spermidine (SD) and spermine (SM) and tropane/pyridine alkaloids (Guggisberg and Hesse 1983; Hashimoto et al. 1989; Hibi et al. 1994). Putrescine *N*-methyltransferase (PMT; EC.2.1.1.53) is the enzyme involved in the removal of PUT from the polyamine pool, since it catalyzes the *N*-methylation of this diamine to form *N*-methylputrescine (MPUT). Because the tropane ring moiety of the tropane alkaloids is derived from PUT by way of MPUT synthesis, the *N*-methylation of PUT catalyzed by PMT is the first committed step in the biosynthesis of these alkaloids (Hibi et al. 1992). A variety of chemical, environmental and developmental cues have been implicated in its regulation (Leete 1979; Ghosh 2000).

Some researches have already been done on the genetic engineering of pharmaceutically important tropane alkaloids (Zhang et al. 2005; Oksman-Caldentey and Arroo 2000). Because the conversion of hyoscyamine to the much more valuable scopolamine is the major goal of these studies, more workers paid their attention to the *h6h* gene coding for the last enzyme involved in the tropane alkaloid biosynthesis (Fig. 1) (Hashimoto et al. 1993; Oksman-Caldentey et al. 1997; Jouhikainen et al. 1999; Palazón et al. 2003; Zhang et al. 2004).

Recent efforts have been aimed at increasing the flux through the biosynthetic pathways. It was reported that over-expression of PMT in transgenic plants of *Nicotiana sylvestris* (Sato et al. 2001) increased the nicotine content, whereas suppression of endogenous PMT activity severely decreased the nicotine content and induced abnormal morphologies. However, over-expression of PMT in *A. belladonna* L. has not affected tropane alkaloid levels either in transgenic plants or in hairy roots (Sato et al. 2001). Moyano et al. (2002) inserted the *N. tabacum pmt* gene into the hairy roots of a hybrid of *Duboisia*, and the MPUT levels of the resulting engineered hairy roots increased (two–four-fold) compared to wild-type roots, but there was no significant increase in either tropane or pyridine-type alkaloids. They also introduced the T-DNA of the Ri plasmid together with the tobacco *pmt* gene into the genome of *D. metel* and *H. muticus* in order to influence the production of tropane alkaloid (Moyano et al. 2003). This was one of the few reports showing that the over-expression of the tobacco *pmt* gene improved tropane alkaloid production in hairy root cultures in a plant species-dependent manner. Hairy root cultures over-expressing the *pmt* gene aged faster and accumulated higher amounts of tropane alkaloids than control hairy roots. Both hyoscyamine and scopolamine production were improved in hairy root cultures of *D. metel*, whereas in *H. muticus* only hyoscyamine contents were increased by *pmt* gene over-expression (Moyano et al. 2003). The results indicate that the same biosynthetic pathway in two related plant species can be differently regulated, and over-expression of a given gene does not necessarily lead to a similar accumulation pattern of secondary metabolites. Furthermore, it may also

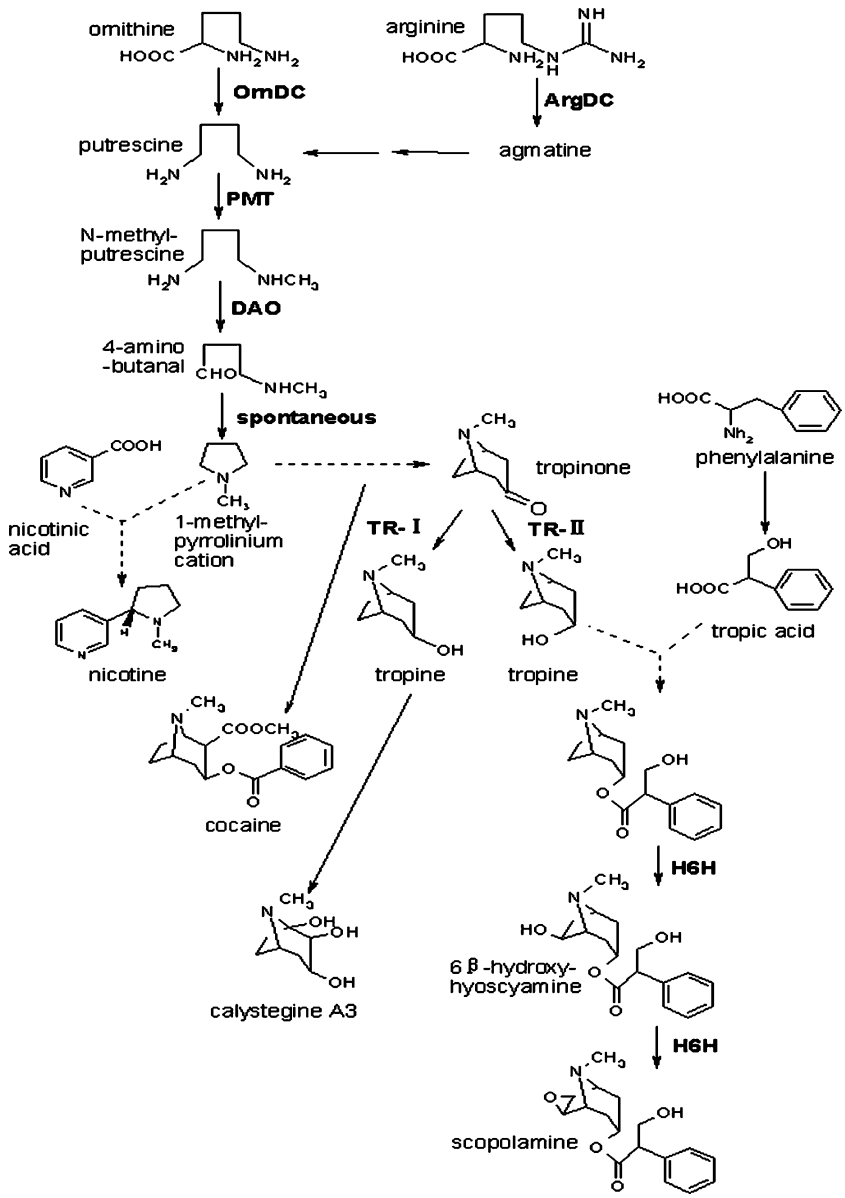


**Fig. 1** Biosynthetic pathways of pyridin and tropane alkloids. *ArgDC* arginine decarboxylase, *OrnDC* ornithine decarboxylase, *PMT* putrescine *N*-methyltransferase, *DAO* diamine oxidase, *TR* tropinone reductase, *H6H* hyoscyamine 6β-hyroxylase

indicate that the transgene allows bypassing the endogenous control of metabolic flux to the alkaloids that would take place at the level of the first committed enzymatic step in their biosynthesis. Previous experiments with *Datura* hairy roots showed that the transcription of the gene encoding PMT is sensitive to the culture media conditions in which the roots grow. It seems that PMT synthesis ceases or is diminished in response to any stress that may induce an alteration in polyamine metabolism (Robins et al. 1991a). Hence, PMT appears to be flux-limiting and consequently a very good candidate for genetic manipulation.

The *pmt* gene is regulated by plant hormones, light, different kinds of stress and elicitors like jasmonates and their strong expression is primarily in the cultured roots (Ghosh 2000). Jasmonates are lipid-derived plant hormones involved in the regulation of a number of processes such as development, senescence, secondary metabolism and response to wounding and pathogen attack (Creelman and Mullet 1997). In response to wounding, jasmonates are synthesized and activate a subset of defence genes, through a signaling pathway (Pieterse et al. 1998; Schenk et al. 2000; Turner et al. 2002). The action of jasmonic acid (JA) and methyl jasmonate (MeJA) as elicitors of secondary metabolites in plants is well known. Furthermore, the accumulation of several classes of alkaloids is strongly stimulated upon treatment with MeJA (Fits and Memelink 2000; Biondi et al. 2002). It was reported that *N. sylvestris* PMT gene conserved 5'-flanking region contained JA-responsive elements and PMT could be up-regulated by MeJA treatment in the root

of *N. sylvestris* (Shoji et al. 2000). Moreover, Biondi et al. (2002) have documented a considerable increase of PMT activity as well as an increase in polyamine contents and tropane alkaloids after MeJA treatment in transformed roots of *H. muticus*. On the contrary, after treatment of *A. belladonna* roots (Suzuki et al. 1999) and *H. muticus* callus culture (Biondi et al. 2002) with MeJA, secondary metabolism was not affected. These results indicate that multiple intersecting signal transduction pathways and different transcriptional regulatory factors are involved in mediating JA-responsiveness of *pmt* expression in plants.

In order to shed some light on the secondary metabolism response to *pmt* over-expression and MeJA in *H. niger*, the *N. tabacum* PMT gene has been inserted into the hairy roots of *H. niger* and treated with MeJA. The activities of enzymes involved in PUT and tropane alkaloid biosynthesis pathway, polyamine and alkaloid production capacities of these engineered hairy root lines have been compared with the respective ones of blank transformed hairy roots and the wild-type root.

## Materials and methods

### Construction of *pmt* expression vector

The plasmid pTVPMT carrying the tobacco PMT cDNA (Hibi et al. 1994) with an introduced *Nco*I site at the first ATG was kindly provided by Professor T. Hashimoto of the Nara Institute of Science and Technology (Kyoto, Japan). The 1,400 bp *Nco*I-*Bam*HI fragment of the cDNA insert was isolated from pTVPMT and subcloned in pET-3d (Novogen, North Ryde NSW, Australia). The resulting plasmid pET-3d-PMT was introduced into competent cells of *E. coli* DH5α strain. The 1,439 bp *Xba*I-*Bam*HI fragment of the cDNA insert was isolated from pET-3d-PMT and subcloned into the plasmid pRoc2275-C between the CaMV 35S promoter and the nopaline synthase terminator, resulting in pBMI (Moyano et al. 2002). Disarmed *A. tumefaciens* strain C58C1 (Parr 1989) harboring both pBMI and *A. rhizogenes* Ri plasmid pRiA4, containing the *pmt* gene, was used for plant transformation.

### Plant transformation and root cultivation

Sterile leaf sections of *H. niger* were inoculated with *A. rhizogenes* strain A4 or *A. tumefaciens* strain C58C1 (pRiA4, pBMI) carrying the *pmt* gene. Wild-type plants were grown in the same growth chamber. Roots generated at cutting edges three to four weeks after co-cultivation were excised and cultivated individually on solid, hormone-free half-strength B5 medium (Gamborg et al. 1968) supplemented with 500 mg/l carbenicillin to eliminate excess bacteria and, in the case of transformed roots carrying the *pmt* gene, with 100 mg/l kanamycin. Rapidly growing lines that showed kanamycin resistance with no bacterial contamination were used to establish hairy root lines. About 100 mg of fresh roots of about 3 cm in length were inoculated into 150 ml conical flasks containing 40 ml of liquid half-strength B5 medium and maintained on an orbital shaker at 100 rpm and 25°C in the dark. The hairy root clones were routinely sub-cultured every 2 weeks as described earlier (Oksman-Caldentey et al. 1991).

### Elicitation culture of MeJA treatment

Randomly selected three root clones from each culture lines (transgenic hairy root and blank transformed hairy root, respectively) and incubated in liquid half-strength B5 medium, with 50 μM MeJA. The culture conditions were same as described above.

### RNA and DNA extraction

Total RNA was extracted from the putative engineered hairy root clones, control hairy roots and wild-type root after 3 weeks of cultivation with Plant RNA Mini Kit (Watson, China) according to the manufacturers' handbook. Genomic DNA was isolated from the putative engineered hairy root clones, control hairy roots and wild-type root using the CTAB method (Ausubel et al. 1995). The quality and concentration of RNA and DNA samples were examined by agarose gel electrophoresis and spectrophotometer analysis.

### Polymerase chain reaction (PCR) analysis

The presence of the transferred *Agrobacterium rol* and *pmt* genes was detected by PCR as described previously (Zhang et al. 2004). PCR primers for *pmt* checking were FPMT (5′-GCCATTCCCATGAACGGCC-3′) and RPMT (5′-CCTCCGCCGATGATCAAAACC-3′). In a 200 μl thin-wall PCR tube, the 50 μl reaction mixture contained 1 μl of each PCR primer (10 μmol/l), 1 μl of 10 mmol/l dNTPs, 5 μl 10 × PCR buffer ($Mg^{2+}$ plus) and 2.5 units of *Taq* DNA polymerase (TaKaRa) with 200 ng genomic DNA as template. PCR was carried out on a Thermo Hybaid. Amplification procedure for *pmt* was: 5 min of predenaturation at 94°C, 35 cycles of amplification (1 min at 94°C, 1 min at 60°C, 45 s at 72°C), followed by 5 min at 72°C. The amplified samples were electrophoresed on 1% agarose gel.

### Southern blot analysis

After *Hin*dIII (Biolabs, USA) digestion, the digested DNAs were blotted on Hybond N membranes (Amersham Pharmacia, USA) and hybridized with the cDNA fragment encoding PMT from tobacco amplified by PCR (Sambrook et al. 1989). Hybridization, washing and signal detection were done as reported (Zhang et al. 2004).

### Reverse transcription PCR (RT-PCR) analysis

RNA expression of the tobacco PMT and endogenous H6H were carried out with randomly selected independent transgenic and at least two untransformed hairy root clones of *H. niger*. All RNA templates were digested with DNase I (RNase-free). Primers FPMT and RPMT and *h6h* gene-specific primers FH6H (5′-TGCTTAGACATTGATTTTATATGGC-3′) and RH6H (5′-GAGACATTTGATGGCTACTTTT-3′) synthesized according to the published sequence data of the *H. niger h6h* gene (Hashimoto and Yamada 1987) were used to amplify *pmt* and the endogenous *h6h* genes, respectively, with One Step RNA PCR Kit (TaKaRa, Japan). Two primers, 18SF (5′-ATGATAACTCGACGGATCGC-3′) and 18SR (5′-CTTGGATGTGGTAGCCGTTT-3′) were used to amplify 18S rRNA gene as controls. Ten microlitres out of 25 μl was loaded on an ethidium bromide-stained 1.2% (w/v) agarose gel. RT-PCR was performed at 50°C for 30 min followed by 94°C for 2 min and 23 cycles of amplification (94°C for 30 s, 56°C for 30 s and 72°C for 30 s) in a 25-μl reaction mixture containing 0.25 μg RNA sample. The relative level of H6Hase mRNA in each sample was calculated by comparing their band strength by scanning the gels with the ultraviolet and visible light automobile analyzing instruments of FR-200A (FuRi, China) and using the image smart view software.

### Enzyme activity assay

PMT activity was evaluated using the method of Feth et al. (1985) with some modifications. The reaction conditions were described before (Zhang et al. 2004).

### Polyamine and alkaloids analysis

Polyamines were first divided into their trichloroacetic acid-soluble and -insoluble forms. These then were quantified separately by HPLC after conversion to their dansyl derivatives (Tassoni et al. 2000; Bagni and Tassoni 2001). Cellular polyamine contents were expressed as the sum of both forms. Standard polyamines were subjected to the same procedure. Extraction of tropane alkaloids (hyoscyamine, scopolamine) was based essentially on the method described by Yamada and Hashimoto (1982) and analyzed by HPLC as described by Collinge and Yeoman (1986). Alkaloid levels were determined both in the roots and in the culture medium. Standards for hyoscyamine and scopolamine were prepared in methanol at a final concentration of 1 mg/ml.

### Statistical analysis

The statistical significance of the differences in distribution of PMT activity, combined (free plus conjugated forms) polyamine and alkaloid content among various root cultures at the end of culture period, and time courses of relative endogenous H6H mRNA expression with and without MeJA treatment were calculated using Statgraphics® Plus 5.0 (Statistical Graphics Corp.). Error bars on graphs indicate standard deviations (SD) calculated from at least three replicate samples.

## Results and discussion

A binary vector was constructed to introduce the tobacco *pmt* gene with a selectable marker (*nptII*) gene and the T-DNA of pRiA4 of *Agrobacterium rhizogenes* into *H. niger* leaf explants using disarmed *A. tumefaciens* C58C1 strain. Control hairy root lines generated from blank transformations were denoted as KO lines. Forty-one hairy root lines, which appeared 2–3 weeks after the *Agrobacterium* inoculation (83% of all the leaf explants), survived the transfer to the antibiotic-containing selective medium (Fig. 2). Twenty-eight kanamycin resistant hairy root lines were obtained and cultivated in hormone-free half-strength B5 solid medium without antibiotics, all of which were confirmed to be positive for the *pmt* transgene by PCR analysis. Some transgenic hairy root lines turned brown and aged considerably faster when cultured on solid medium. According to Moyano et al. (2003), an undesirable effect of metabolic engineering is the promotion of metabolic flux alterations that can induce cell death. This fact produces metabolic changes not directly related to the transgene presence. For this reason, brownish root clones of *H.niger* were discarded and only root cultures showing a normal growth capacity were considered for further experiments and named OP lines. Both KO and OP lines were sub-cultured for 4 weeks in hormone-free half-strength B5 liquid medium with or without MeJA treatment.

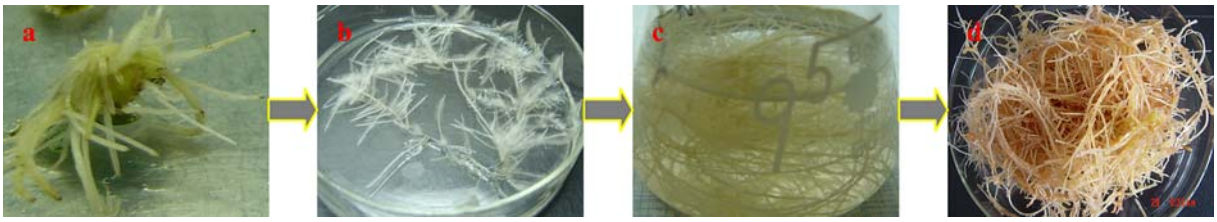


**Fig. 2** Development of transformed hairy root lines. **a** Induced roots emerging from an inoculated 1 $m^2$ leaf explant. **b** Fast growing transformed roots with profuse branching. **c** Hairy roots growing in liquid half strength B5 medium in a 250 ml Erlenmeyer flask. **d** Harvested hairy root after 4 weeks of liquid culture

The phenotype and morphology of root lines carrying the *pmt* gene differed from the control clones and the wild-type root. Control lines grew fast and vigorously, with thick and multiple branches, while OP lines grew relatively slowly and aged rapidly . At the beginning of the culture period on the solid medium, the color of the OP lines was white and later turned brown. Plasmid pRiA4 in this plant species might cause the peculiar color formation in the hairy roots. MeJA added to liquid cultures decreases the growth rate of all root lines and the presence of phenolic compounds was more evident. Rapid ageing of transformed roots has previously been connected with high alkaloid production (Jouhikainen et al. 1999), while the altered phenotype obtained in this study might be most probably due to the accumulation of phenolic compounds (Moyano et al. 2003). On the other hand, due to the role of polyamines in plant development (Martin-Tanguy 1997), prematured senescence of root cultures and slowered growth capacity in *Hyoscyamus* can be caused by *pmt* over-expression in polyamine metabolism, because the substrate of PMT enzyme (PUT) is shared with polyamine metabolism (Sato et al. 2001).

The presence of *rol* C and *pmt* transgene in the genome of hairy roots was checked by PCR (Fig. 3a). DNA template from wild-type *H. niger* did not show any amplified band, while 100% of the engineered hairy roots (OP lines) as well as the plasmid pBMI-RiA4 yielded the target bands: 461 bp corresponding to the *pmt* cDNA fragment and 626 bp corresponding to the *rol*C gene, preliminarily proving the integration of *pmt* into the target hairy root lines.

Rearrangements of the input plasmid(s) and/or mutations and methylation within the transforming expression cassettes may be responsible for the apparent silencing of specific transgenes (Meyer 1999; Rocha et al. 2002). We investigated *pmt* integration pattern in transgenic root lines by Southern hybridization using gene-specific probe. The result obtained from 17 such root lines showed that the complexity of the hybridizing bands was different in different root lines (Fig. 3b). Each of these lines displayed 1–3 hybridization signals.

RT-PCR was carried out to analyze *pmt* transcript accumulation using 1.0 μg of total RNA from wild-type and independently transformed hairy root line OP12 at the third week of cultivation. All transgenic lines showed a *pmt* band (Fig. 3c). Low levels of MeJA elicited maximal effects already after MeJA treatment and the amount of detectable mRNA represented a 1.5-fold increase compared with samples not treated with MeJA.

PMT activity in transgenic hairy root cultures (OP12) (10.73 pkat/mg ± 4.31) was significantly ($P < 0.01$) higher than that in the wild-type root (6.25 pkat/mg ± 1.25) and in A4 blank transformation lines (KO) (1.99 pkat/mg ± 0.46) in the absence of elicitor. MeJA enhanced enzyme activity both in KO and OP lines (Fig. 4). In the presence of 50 μM MeJA, there was ~twofold increase in PMT activity in the KO lines and a 1.5-fold increase in the OP lines, respectively. MeJA has been previously shown to up-regulate gene expression and activities of biosynthetic enzymes in *H. muticus* (Biondi et al. 2000), tobacco (Imanishi et al. 1998) and rice (Lee et al. 1996). The concentration of *pmt* mRNA corresponded to enzyme activity and all transgenic lines achieved maximum PMT activity at the end of the culture period (data not shown).

Wild-type (WT) and A4 blank transformation lines (KO) displayed comparable levels of polyamines, including PUT, MPUT, SD and SM (Fig. 5). In hairy roots over-expressing *pmt* tobacco gene (OP), four–five-fold increase of MPUT was observed, the average content of KO being 4.24 nmol/mg dry wt and that of OP12 being 17.52 nmol/mg dry wt. After treatment with 50 μM MeJA, the production of MPUT was boosted either in KO or OP lines. This compound is directly catalyzed by the action of the enzyme encoded by the *pmt* gene and represents the first specific precursor of tropane alkaloids in several plant species including *H. niger* (Oksman-Caldentey and Arroo 2000). Obviously, *pmt* gene over-expression could induce a deviation of polyamine metabolic flux toward MPUT biosynthesis. On the other hand, there was a slight decrease in SD and SM content followed by *pmt* over-expression and/or MeJA elicitor

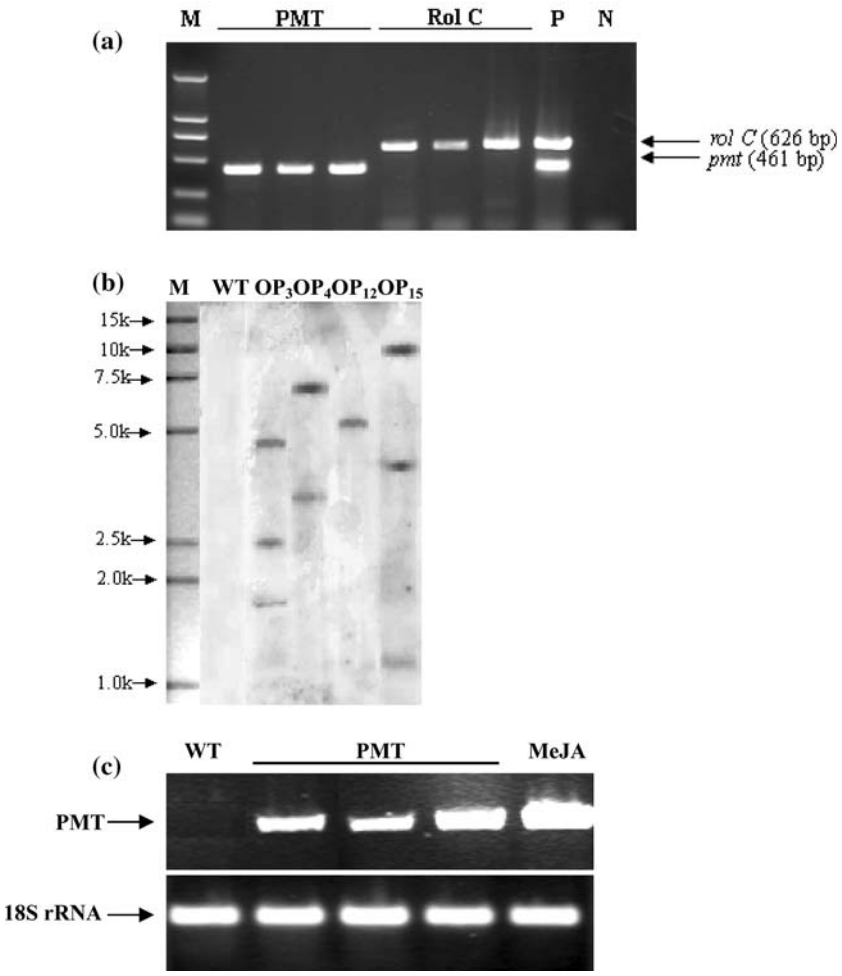


**Fig. 3** Molecular analysis of transgenic hairy root lines of *H. niger*. **a** PCR analysis for the presence of *rol*C and *pmt* genes in independently transformed hairy root lines. *M* DL-2000 Marker (100–2,000 bp); *PMT and Rol C* PCR amplified DNA bands of *pmt* and *rolC* genes from transformed roots induced by *A. tumefaciens* strain C58C1 (pRiA4, pBMI) respectively; *P* plasmid pBMIRiA4 as positive control; *N* wild-type root as negative control. **b** Representative Southern blot hybridization analysis. Genomic DNA was digested with *Hin*dIII overnight and probed with *pmt* gene-specific probe. *WT* wild-type root as negative control. *OP3, OP4, OP12* and *OP15* independent transgenic hairy root lines. Four of 17 transgenic hairy root lines analyzed are shown. **c** Representative RT-PCR analysis for the expression of *pmt* in wild-type root (*WT*), in independent transgenic hairy root lines (*PMT*) and in transgenic hairy root line under MeJA treatment (*MeJA*). *M* Molecular marker; the 18S rRNA is used as internal control

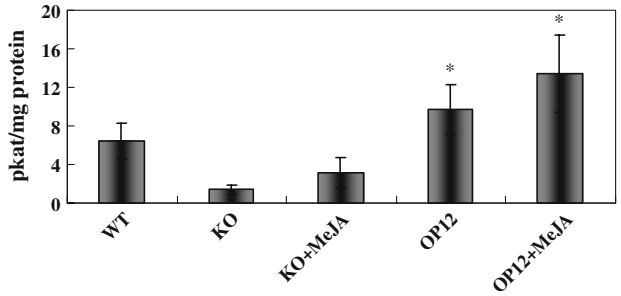


**Fig. 4** Distribution of PMT activity among various root cultures at the end of the culture period (28 days). *WT* wild-type root of *H. niger*; *KO* a control transformed root line induced by *A. rhizogenes* A4 strain; *KO + MeJA* a control root line treatment with 50 μM MeJA; *OP12 pmt* over-expression hairy root line; *OP12 + MeJA* transgenic hairy root line treatment with 50 μM MeJA. Error bars indicate SD calculated from at least three replicate samples. *: vs. KO and WT, $P < 0.01$

culture compared with their respective control. A rational explanation is that in *H. niger* roots, PUT biosynthesis is probably not a rate-limiting step in polyamine metabolism and an increase in MPUT levels could not lead to an alteration of free PUT, SD and SM endogenous pool. The same phenomenon was observed in *pmt* over-expression *N. sylvestris*, but contrary to what happened in *A. belladonna* roots, there was an increase in the endogenous polyamine pool (Sato et al. 2001). The fact that treatment with MeJA failed to induce SD and SM accumulation in *H. niger* hairy root is in accordance with its lower PUT biosynthetic activities. Robins et al. (1991b) obtained the same conclusion that SD and SM were maintained at constant levels, irrespective of the exposure of *Datura stramonium* L. roots to either DL-α-difluoromethylornithine or DL-α-difluoromethylarginine.

Figure 5 also shows the capacity of transgenic root lines and elicitor cultures to biosynthesize hyoscyamine and scopolamine. All lines produced tropane alkaloids in which scopolamine was the major compound. OP lines produced alkaloid quantitatively and qualitatively similar to the KO controls. The unchanged alkaloid profiles in the OP line indicated that the increase of MPUT in *H. niger* hairy roots carrying *pmt* transgene was not converted into the alkaloids hyoscyamine and

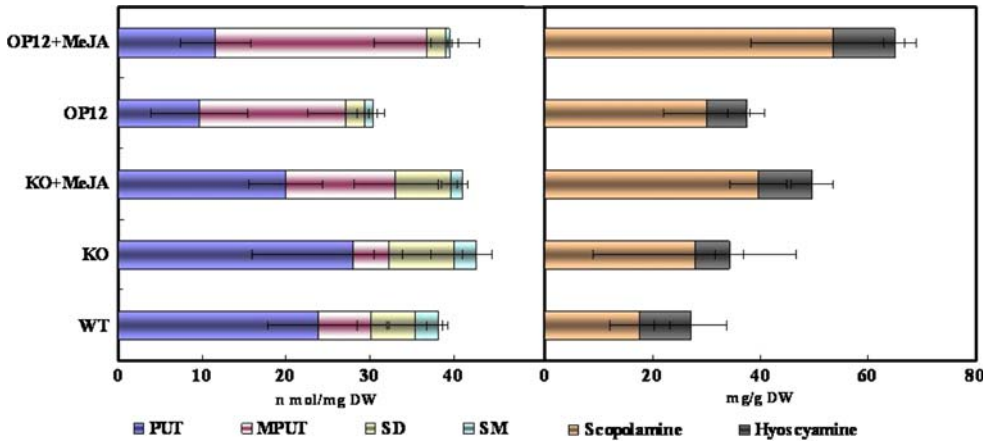


**Fig. 5** Combined (free plus conjugated forms) polyamine and alkaloid content analysis among various root cultures (denotes are the same as above) at the end of the culture period (28 days). *PUT* putrescine; *MPUT* methylputrescine; *SD* spermidine; *SM* spermine. *Error bars* indicate SD calculated from at least three replicate samples

scopolamine. Our results showed that a several-fold enhancement in *pmt* expression in the hairy roots was not sufficient to boost scopolamine biosynthesis, suggesting that in *H. niger* transformed roots, alkaloid synthesis is downstream-limited rather than limited at the conversion of PUT to MPUT. When *A. belladonna*, *N. sylvestris* (Sato et al. 2001) and *Duboisia* (Moyano et al. 2002) were adopted to over-express *pmt*, the authors reported contrasting effects of the transgene expression on alkaloid synthesis, since no changes in tropane alkaloids content were observed. The authors suggested how PMT expression in the roots was not sufficient to boost tropane alkaloid synthesis in these *Solanaceae* plant. But these facts contradict previous results obtained in *H. albus* (Hibi et al. 1992) and *Datura* hybrid (Robins et al. 1990), two plant species also known to produce tropane alkaloids. A good correlation between PMT activity and the capacity to produce nicotine and tropane alkaloids was observed in these two species and potato (Stenzel et al. 2006). Also *pmt*-transformed root cultures of *H. muticus* and *D. metel* were able to produce higher alkaloids (Moyano et al. 2003). It is apparent that the inter-relationships between alkaloid and polyamine metabolism in plants, where putrescine acts as a common intermediate, involves regulatory mechanisms acting to ensure that the biosynthesis of polyamines is maintained, when needed, at the expense of alkaloid formation (Robins et al. 1991b). The response of plants of *Solanaceae* to *pmt* over-expression seems to be species-related. This may be partly due to a different, specific post-translational regulation of the endogenous enzyme in respect to the foreign one. Further individual flux limitation by subsequent biosynthetic steps should also be considered.

An interesting feature reported here and previously observed in root and callus cultures of *H. muticus* in response to MeJA (Biondi et al. 2002) was the marked MeJA eliciting increased ability to convert hyoscyamine into scopolamine. Because of the low activity of H6H which catalyzes hyoscyamine to scopolamine (Jouhikainen et al. 1999), the capacity to synthesize scopolamine was very limited when single *pmt* gene was introduced into root cultures of various plants (Moyano et al. 2002, 2003). In order to investigate whether or not an increase in scopolamine content was due to MeJA enhancing the endogenous H6H expression, we analyzed time courses of relative endogenous H6H mRNA level upon MeJA with and without 50 μM MeJA in transgenic lines (OP + MeJA), blank transformed lines (KO + MeJA) and control (KO) (Fig. 6). The data showed that MeJA stimulated the endogenous H6H activity in both KO (8.00 ± 2.03) and OP (8.44 ± 0.96) lines, and then the biosynthesis pathway from hyoscya-

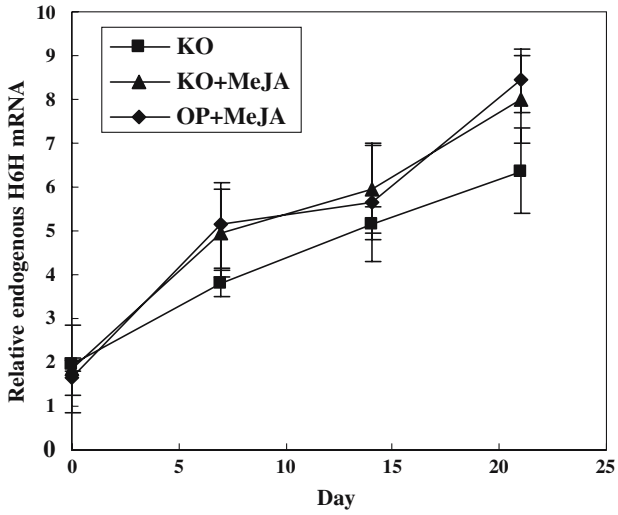


**Fig. 6** Time courses of relative endogenous H6H mRNA expression with and without 50 μM MeJA treatment in transgenic hairy root lines (*OP + MeJA*), blank transformed lines (*KO + MeJA*) and control (*KO*). *Error bars* indicate SD calculated from at least three replicate samples

mine to scopolamine was activated. Consequently, MeJA treatment promoted the yield of scopolamine.

This is an insight report on the *pmt* bioengineering into *H. niger* cultured hairy root lines. Successful engineering of scopolamine biosynthesis is the basis of utilizing the possible variation among the transgenic root clones as bioreactors to produce scopolamine on large scale. Detailed knowledge at the level of the enzymes will help us to develop models simulating the fluxes through the metabolic networks. When the steps of the biosynthetic pathway are relatively clarified and the respective genes have been cloned, exact regulation toward enhanced productivity of medicinal natural products is quite desirable. Results of the present work indicate that metabolic engineering, either alone or in combination with elicitation by MeJA, provides practical means to stimulate valued secondary metabolites production.

**Acknowledgments** The authors are very grateful to Professor T. Hashimoto of the Nara Institute of Science and Technology (Kyoto, Japan) for supplying the pTVPMT plasmid carrying the tobacco *pmt* cDNA and to Professor M.T. Piñol of Sección de Fisiología Vegetal, Facultad de Farmacia, Universidad de Barcelona (Barcelona, Spain) for his generous gift of the plant expression vector pBMI. This research was supported by National Natural Science Foundation of China (30371746, 20572130), Shanghai Natural Science Foundation (04ZR14051, 05ZR14093), Shanghai Science and Technology Committee and Shanghai Rising-Star Program (06QB14010).

## References

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1995) Short Protocols in Molecular Biology. Wiley, New York

Bagni N, Tassoni A (2001) Biosynthesis, oxidation and conjugation of aliphatic polyamines in higher plants. Amino Acids 20:301–317

Biondi S, Fornale S, Oksman-Caldentey K-M, Eeva M, Agostani S, Bagni N (2000) Jasmonates induce over-accumulation of methylputrescine and conjugated polyamines in *Hyoscyamus muticus L*. root cultures. Plant Cell Rep 19:691–697

Biondi S, Scaramagli S, Oksman-Caldentey K-M, Poli F (2002) Secondary metabolism in root and callus cultures of *Hyoscyamus muticus* L.: the relationship between morphological organisation and response to methyl jasmonate. Plant Sci 163:563–569

Christen P, Roberts MF, Phillipson JD, Evans WC (1993) Alkaloids of *Erythroxylum zambesiacum* stem-bark. Phytochemistry 34:1147–1151

Collinge MA, Yeoman MM (1986) The relationship between tropane alkaloid production and structural differentiation in plant cell cultures of *Atropa belladona* and *Hyoscyamus muticus*. In: Morris P, Scragg A, Fowler M (eds) Secondary metabolism in plant cell cultures. Cambridge University Press, Cambridge, pp 82–88

Creelman RA, Mullet JE (1997) Biosynthesis and action of jasmonate in plants. Ann Rev Plant Physiol Plant Mol Biol 48:355–381

Endo T, Hamaguchi N, Eriksson T, Yamada Y (1991) Alkaloid biosynthesis in somatic hybrids of *Duboisia leichhardtii* F. Muell. and *Nicotiana tabacum*. Planta 183:505–510

Evans WC (1979) The biology and taxonomy of the *Solanaceae*. In: Hawkes JG, Lester RN, Skelding AD (eds) Tropane alkaloids of the Solanaceae. Academic, London, pp 241–254

Feth F, Arfmann HA, Wray V, Wagner KG (1985) Determination of putrescine *N*-methyltransferase by high performance liquid chromatography. Phytochemistry 24:921–923

Fits LV, Memelink J (2000) ORCA3, a jasmonate-responsive ranscriptional regulator of plant primary and secondary metabolism. Science 289:295–297

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Ghosh B (2000) Polyamines and plant alkaloids. Indian J Exp Biol 38:1086–1091

Guggisberg G, Hesse M (1983) Putrescine, spermidine, spermine, and related polyamine alkaloids. In: Brossi A (ed) The alkaloids, vol 22. Academic, New York, pp 85–188

Hashimoto T, Hayashi A, Amano Y, Kohno J, Iwanari H, Usuda S, Yamada Y (1991) Hyoscyamine 6 beta-hydroxylase, an enzyme involved in tropane alkaloid biosynthesis, is localized at the pericycle of the root. J. Biol Chem 266:4648–4654

Hashimoto T, Yamada Y (1987) Purification and characterization of hyoscyamine 6β-hydroxylase from root cultures of *Hyoscyamus niger* L. Hydroxylase and exoxidase activities in the enzyme preparation. Eur J Biochem 164:277–285

Hashimoto T, Yamada Y (1994) Alkaloid biogenesis: molecular aspects. Ann Rev Plant Physiol Plant Mol Biol 45:257–285

Hashimoto T, Yukimune Y, Yamada Y (1989) Putrescine and Putrescine *N*-methyltransferase in the biosynthesis of tropane alkaloids in cultured roots of *Hyoscyamus albus* I. Biochemical studies. Planta 178:123–130

Hashimoto T, Yun D-J, Yamada Y (1993) Production of tropane alkaloids in genetically engineered root cultures. Phytochemistry 32:713–718

Hibi N, Fujita T, Hatano M, Hashimoto T, Yamada Y (1992) Putrescine *N*-methyltransferase in cultured roots of *Hyoscyamus albus*. Plant Physiol 100:826–835

Hibi N, Higashiguchi S, Hashimoto T, Yamada Y (1994) Gene expression in tobacco low-nicotine mutans. Plant Cell 6:723–735

Imanishi S, Hashizume K, Nakakita M, Kojima H, Matsubayashi Y, Hashimoto T, Sakagami Y, Yamada Y, Nakamura K (1998) Differential induction by methyl jasmonate of genes encoding ornithine decarboxylase and other enzymes involved in nicotine biosynthesis in tobacco cell cultures. Plant Mol Biol 38:1101–1111

Jouhikainen K, Lindgren L, Jokelainen T, Hiltunen R, Teeri TH, Oksman-Caldentey K-M (1999) Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures by genetic engineering. Planta 208:545–551

Lee TM, Lur HS, Lin YH, Chu C (1996) Physiological and biochemical changes related to methyl jasmonate-induced chilling tolerance of rice (*Oryza sativa* L.) seedlings. Plant Cell Environ 19:65–74

Leete E (1979) Biosynthesis and metabolism of the tropane alkaloids. Planta Med 36:97–112

Martin-Tanguy J (1997) Conjugated polyamines and reproductive development: biochemical, molecular and physiological approaches. Physiol Plant 100:675–688

Meyer P (1999) Stabilities and instabilities in transgenic expression. In: Lindsey K (eds) Transgenic plant research. Harwood, Zurich, pp 263–275

Moyano E, Fornalé S, Palazón J, Cusidó RM, Bagni N, Piñol MT (2002) Alkaloid production in *Duboisia* hybrid hairy root cultures overexpressing the *pmt* gene. Phytochemistry 59:697–702

Moyano E, Jouhikainen K, Tammela P, Palazόn J, Cusidό RM, Piñol MT, Teeri TH, Oksman-Caldentey K-M (2003) Effect of *pmt* gene overexpression on tropane alkaloid production in transformed root cultures of *Datura metel* and *Hyoscyamus muticus*. J Exp Bot 54:203–211

Oksman-Caldentey K-M, Arroo R (2000) Regulation of tropane alkaloid metabolism in plants and plant cell cultures. In: Verpoorte R, Alfermann AW (eds) Metabolic engineering of plant secondary metabolism. Kluwer, Dordrecht, pp 253–281

Oksman-Caldentey K-M, Kivelä O, Hiltunen R (1991) Spontaneous shoot organogenesis and plant regeneration from hairy root cultures of *Hyoscyamus muticus*. Plant Sci 78:129–136

Oksman-Caldentey K-M, Teeri TH, Hiltunen R (1997) Enhanced scopolamine production in *Hyoscyamus* plants and hairy root cultures (Patent application PCT/FI98/00577)

Palazón J, Moyano E, Cusidó RM, Bonfill M, Oksman-Caldentey K-M, Piñol MT (2003) Alkaloid production in *Duboisia* hybrid hairy root and plants overexpressing the *h6h* gene. Plant Sci 165:1289–1295

Parr AJ (1989) The production of secondary metabolites by plant cell cultures. J Biotechnol 10:1–26

Pieterse CMJ, Wees SCM, Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ, Loon LC (1998) A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell 10:1571–1580

Robins RJ, Parr AJ, Payne J, Walton NJ, Rhodes MJC (1990) Factors regulating tropane-alkaloid production in a transformed root culture of a *Datura candida* × *D. aurea* hybrid. Planta 181:414–422

Robins RJ, Parr AJ, Bent EG, Rhodes MJC (1991a) Studies on the biosynthesis of tropane alkaloids in *Datura stramonium* L. transformed root cultures. 1. The kinetics of alkaloid production and the influence of feeding intermediate metabolites. Planta 183:185–195

Robins RJ, Parr AJ, Walton NJ (1991b) Studies on the biosynthesis of tropane alkaloids in *Datura stramonium* L. transformed root cultures. 2. On the relative contributions of L-arginine and L- ornithine to the formation of the tropane ring. Planta 183:196–201

Rocha P, Stenzel O, Parr A, Walton N, Christou P, Dräger B, Leech MJ (2002) Functional expression of tropinonereductase І (*tr І*) and hyoscyamine-6ß-hydroxylase (*h6h*) from *Hyoscyamus niger* in *Nicotiana tabacum*. Plant Sci 162:905–913

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Sato F, Hashimoto T, Haciya A, Tamura, Choi KB, Morishige T, Fujimoto H, Yamada Y (2001) Metabolic engineering of plant alkaloid biosynthesis. Proc Natl Acad Sci USA 98:367–372

Schenk PM, Kazan K, Wilson I, Anderson JP, Richmond T, Somerville SC, Manners JM (2000) Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proc Natl Acad Sci USA 97:11655–11660

Sevón N, Därger B, Hiltunen R, Oksman-Caldentey K-M (1997) Characterization of transgenic plants derived from hairy roots of *Hyoscyamus muticus*. Plant Cell Rep 16:605–611

Sevón N, Oksman-Caldentey K-M, Hiltunen R (1995) Efficient plant regeneration from hairy root-derived protoplasts of *Hyoscyamus muticus*. Plant Cell Rep 14:738–742

Shoji T, Yamada Y, Hashimoto T (2000) Jasmonate Induction of Putrescine *N*-methyltransferase Genes in the Root of *Nicotiana sylvestris*. Plant Cell Physiol 41:831–839

Stenzel O, Teuber M, Dräger B (2006) Putrescine *N*-methyltransferase in *Solanum tuberosum* L., a calystegine-forming plant. Planta 223:200–212

Suzuki K, Yamada Y, Hashimoto T (1999) Expression of *Atropa belladonna* putrescine *N*-methyltransferase gene in root pericycle. Plant Cell Physiol 40:289–297

Tassoni A, van Buuren M, Franceschetti M, Fornalè S, Bagni N (2000) Polyamine content in *Arabidopsis thaliana* and effect of spermidine on plant development. Plant Physiol Biochem 38:383–393

Turner JG, Ellis C, Devoto A (2002) The jasmonate signal pathway. Plant Cell 14:153–164

Yamada Y, Hashimoto T (1982) Production of tropane alkaloids in cultured cells of *Hyoscyamus niger*. Plant Cell Rep 1:101–103

Zhang L, Kai GY, Zhang HM, Tang KX, Jiang JH, Chen WS (2005) Metabolic engineering of tropane alkaloid biosynthesis in plants. J Integrative Plant Biol 47:136–143

Zhang L, Ding RX, Chai YR, Bonfill M, Moyano E, Oksman-Caldentey K-M, Xu TF, Pi Y, Wang ZN, Zhang HM, Kai GY, Liao ZH, Sun XF, Tang K (2004) Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc Natl Acad Sci USA 101:6786–6791