Molecular Analysis of Tropane Alkaloid Biosynthesis

in *Atropa belladonna*

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Abbreviations

BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
dCTP	deoxycytosine triphosphate
DEPC	diethyl pyrocarbonate
DIG	digoxigenine
GUS	ß-glucuronidase
H6H	hyoscyamine 6ß-hydroxylase
ORF	open reading frame
PCR	polymerase chain reaction
PMT	putrescine N-methyltransferase
RACE	rapid amplification of cDNA ends
RT-PCR	reverse transcription-polymerase chain reaction
SAM	S-adenosylmethionine
SDS	sodium dodecylsulfate
SPDS	spermidine synthase
SSC	sodium chloride / sodium citrate
X-Gluc	5-bromo-4-chloro-3-indolyl-ß-D-glucuronide

INTRODUCTION

The alkaloids are one of the most diverse groups of secondary metabolites found in living organisms and have an array of structure types, biosynthetic pathways, and pharmacological activities. It may be assumed that plants developed a wide variety of secondary metabolites during hundreds of millions of years of evolution as a means of defending themselves against herbivores, microorganisms, viruses, and other plants. Among the more than 50,000 natural products that are known today, over 12,000 are alkaloids (Wink 1998). The tropane alkaloids are a group of structurally related secondary products that possess the azabicyclo[3.2.1]octane ring system. Well over 150 tropane alkaloids have now been isolated from plants or chemically synthesized (Lounasmaa 1988), and are usually found as esters between organic acids with either 3α- or 3ß-hydroxytropane derivatives.

Hyoscyamine and scopolamine are the two most common tropane alkaloids found in the Solanaceae, which include *Atropa belladonna* and *Hyoscyamus niger*, and plants containing these alkaloids have been used for their medicinal, hallucinogenic, and poisonous properties (Wink 1998). Both compounds are isomerized to the racemic forms during storage or alkaline isolation. The racemic mixtures of D- and L-hyoscyamine is called atropine, of which only the L-hyoscyamine is synthesized in plants and pharmacologically active. Hyoscyamine and scopolamine are competitive antagonists of the actions of acetylcholine and other muscarine agonists, both have similar peripheral, parasympatholytic properties, but they differ quantitatively in antimuscarinic actions and in action on the central nervous system. Hyoscyamine first stimulates and then depresses the central nervous system. Scopolamine tends to be more central nervous system-depressant (Neuwinger 1998). Because scopolamine is more powerful than hyoscyamine (Schmeller and Wink 1998), currently there is a 10-fold higher commercial demand for scopolamine, in the *N*-buthylbromide form, than there is for hyoscyamine and atropine combined.

Tropane alkaloids, nicotine, and polyamines are all synthesized from putrescine (Figure 1), which is formed from either ornithine or arginine (Hashimoto and Yamada 1994).
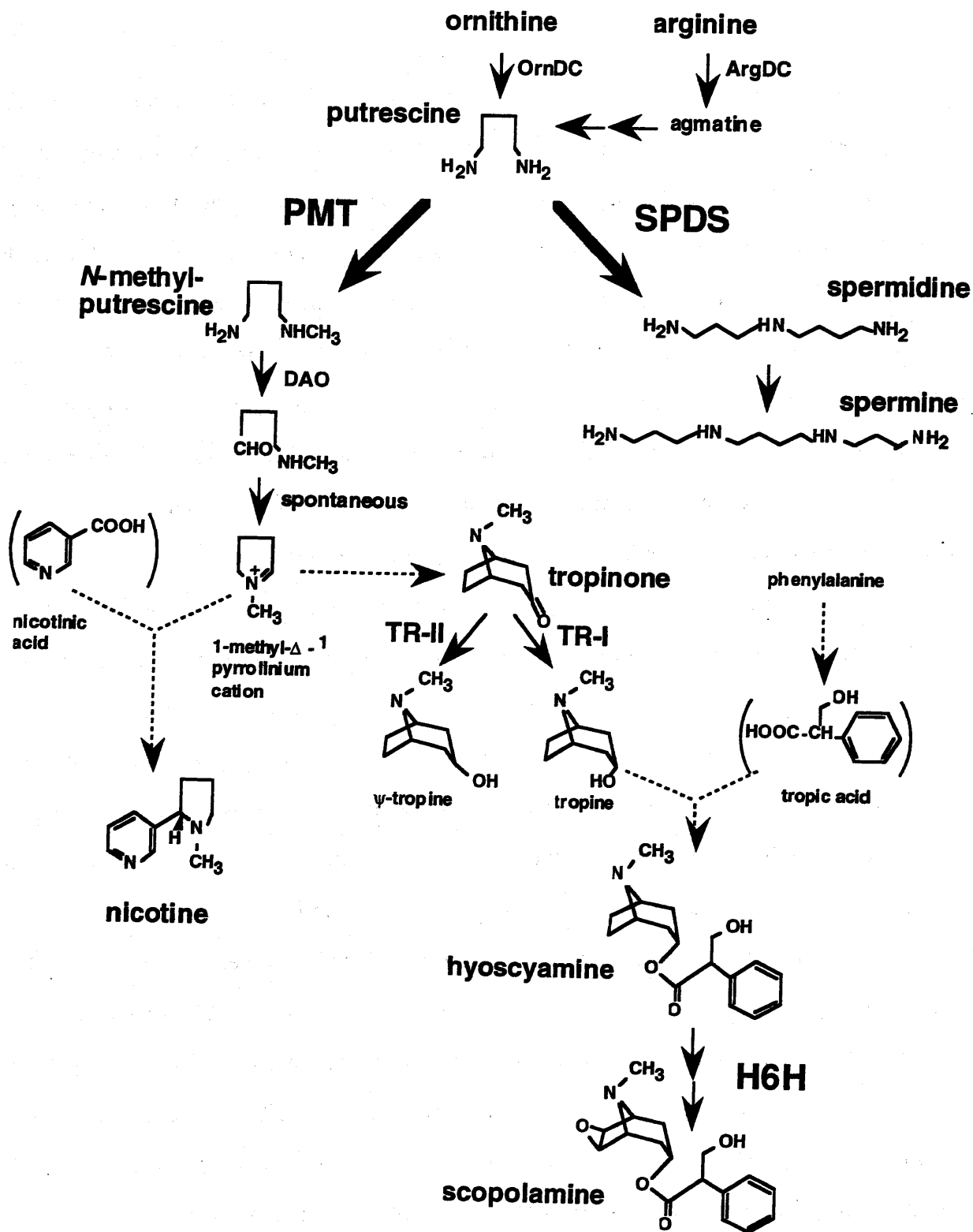
Figure 1 Biosynthetic pathways of tropane alkaloids and nicotine. OrnDC, ornithine decarboxylase; ArgDC, arginine decarboxylase; PMT, putrescine N-methyltransferase; SPDS, spermidine synthase; TR, tropinone reductase; H6H, hyoscyamine 6ß-hydroxylase.

Putrescine *N*-methyltransferase (PMT; EC 2.1.1.53) catalyzes the *S*-adenosylmethionine

(SAM)-dependent *N*-methylation of putrescine at the first committed step in the biosynthetic

pathways of tropane alkaloids and nicotine (Figure 2, Hibi *et al.* 1992), while spermidine

synthase (SPDS; EC 2.5.1.16) transfers the aminopropyl group of decarboxylated SAM to

putrescine, producing spermidine in the polyamine biosynthetic pathway (Hashimoto *et al.*

1998b). The biosynthetic pathway branches off to tropane alkaloids and nicotine pathways at

1-methyl-Δ^1-pyrrolinium cation. As a final product in the biosynthetic pathway of tropane

alkaloid, scopolamine, a 6,7-epoxide of hyoscyamine, is formed from hyoscyamine via 6ß-

hydroxyhyoscyamine (Figures 2 and 3). Hyoscyamine 6ß-hydroxylase (H6H; EC 1.14.11.11)

belongs to the 2-oxoglutarate-dependent dioxygenases (Hashimoto *et al.* 1986), and catalyzes

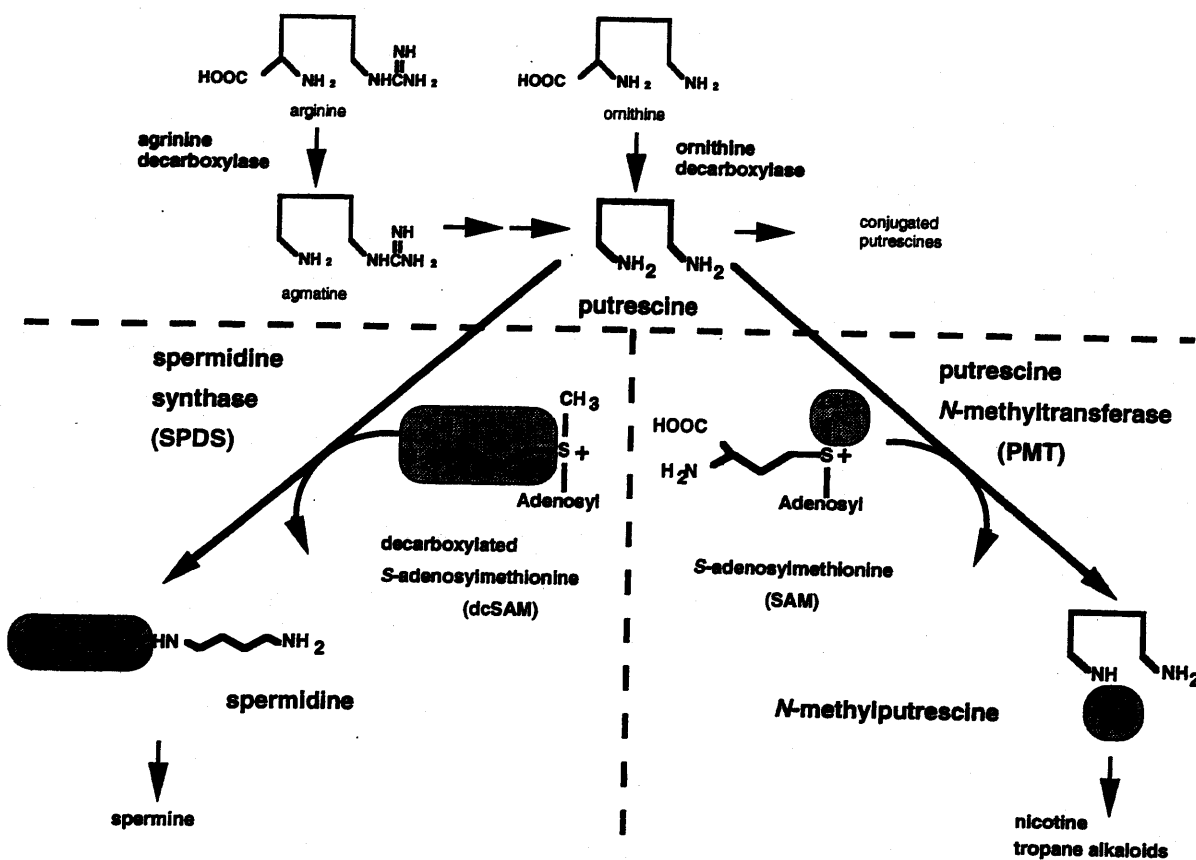**Figure 2** Biosynthesis of polyamines and plant alkaloids derived from putrescine. Spermidine is synthesized from putrescine by SPDS which requires dcSAM as a cofactor, whereas in several plants PMT transfers the methyl moiety of SAM to putrescine in the biosynthesis of nicotine and tropane alkaloids. Spermidine is further metabolized to spermine by spermine synthase. The groups transferred from the cofactors to putrescine are shaded.

two consecutive oxidation reactions: the hydroxylation of hyoscyamine to 6ß-hydroxyhyoscyamine, and the epoxidation of 6ß-hydroxyhyoscyamine to scopolamine (Figure 3, Hashimoto et al. 1993).
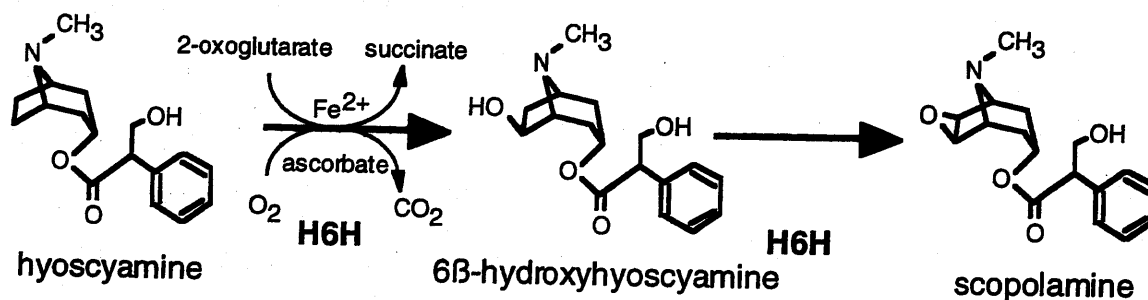
Figure 3 Biosynthetic pathway from hyoscyamine to scopolamine.

Nicotiana PMTs, which encode a protein with distinct sequence similarity to plant and animal SPDSs, probably originated from SPDSs during diversification of the Solanaceae (Hashimoto et al. 1998a, 1998b). A unique feature of *Nicotiana* PMTs is the N-terminal tandem repeat array consisting of different numbers of a conserved 11 amino acid element. This repeat array was found in all *Nicotiana* PMTs so far studied but is absent in all SPDSs in the database. Moreover, it can be removed from tobacco PMT without affecting the enzymatic property (Hashimoto et al. 1998a). Southern hybridization analysis indicated that the genomes of *A. belladonna* and *H. niger* do not contain sequences homologous to the tandem repeats found in *Nicotiana* PMTs (Hashimoto et al. 1998a). Nicotine has an insecticidal activity which may function as part of a defense response of tobacco against insect attack (McCloud and Baldwin 1997), whereas such protective activity against insects has not been found in either hyoscyamine or scopolamine. Tobacco *PMT* is up regulated by jasmonic acid (Imanishi et al. 1998) and down regulated by auxin (Hibi et al. 1994). Whether *PMT*s involved in tropane alkaloid biosynthesis are similarly regulated by plant hormones is an open question.

H6H cDNA was first isolated from *Hyoscyamus niger*, and was used to demonstrate

that *H6H* mRNA is abundant in cultured roots and present in plant roots, but absent in stems and leaves of *H. niger* (Matsuda *et al.* 1991). Immunohistochemical analysis using a monoclonal antibody against H6H protein further indicated that H6H is localized specifically to the pericycle of roots of various scopolamine-producing plants (Hashimoto *et al.* 1991). The 5'-upstream region of the *H. niger H6H* gene (*HnH6H*) was found to drive expression of a downstream GUS reporter gene in the pericycle cells of transgenic hairy roots from *H. niger* and *A. belladonna*, however the critical *cis*-acting elements required for this cell-specific expression have yet to be identified (Kanegae *et al.* 1994).

A.belladonna (Figure 4A) belongs to the subtribe Lyciinae of the tribe Solaneae of the Solanaceae family, according to the classification of Wettstein. Most members of the Lyciinae, which includes 14 genera, produce alkaloids of the hyoscyamine type (Evans 1979). In contrast, *H, niger* belongs to the subtribe Hyoscyaminaae of the same tribe Solaneae, produce alkaloids of the scopolamine type. All members of the tribe Cestreae, which includes 19 genera,
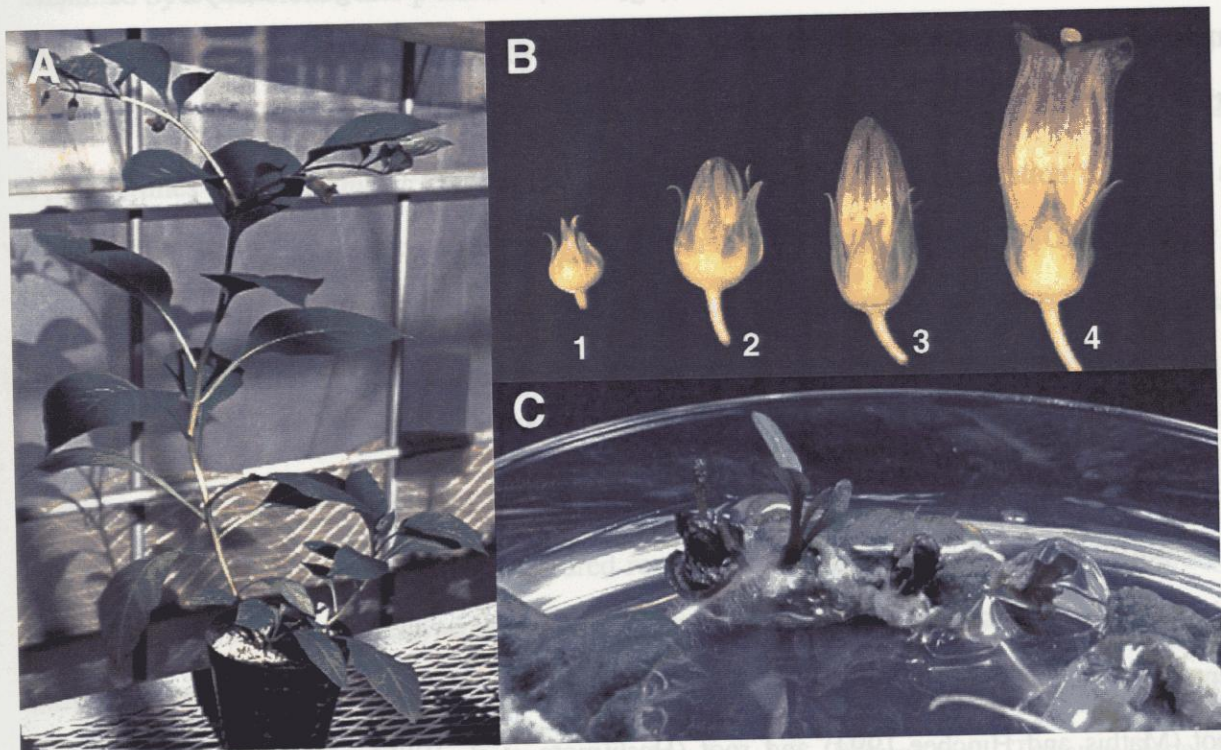
Figure 4 Illustrated are *Atropa belladonna*. mature plantlet (A) and different stages (1-4) in its flower development (B). (C) Regeneration of kanamycine resistant shoots from *A. belladonna* leaf sections after inoculation of *Agrobacterium tumefaciens*.

do not produce any tropane alkaloid, and *Nicotiana*, one of the genera of this tribe, produces nicotine as an alkaloid. Because the regeneration of fertile plants from tissue culture is much easier for *A. belladonna* (Figure 4C) than other tropane alkaloid-producing plants (e.g. *H. niger*), *A. belladonna* has been used for the production of somatic hybrids (Bajaj and Simola 1991) and transgenic plants with herbicide-resistance (Saito *et al.* 1992) or with improved alkaloid composition (Yun *et al.* 1992). To study the molecular regulation of genes involved in secondary metabolism, it is often important, and sometimes critical, to use a homologous system for experimental analysis, whereby the expression of cloned genes is studied in the same plant species from which the target genes were isolated. To take advantage of its facile genetic manipulation, I decided to study the regulation of alkaloid biosynthetic genes in *A. belladonna*.

In this report, I focused on *PMT* and *H6H* genes of *A. belladonna* at the first and last commited step in the biosynthetic pathway of scopolamine. I isolated two *AbPMT* cDNAs and *AbPMT* gene and *AbH6H* gene, and examined their tissue-specific expression patterns by in situ hybridization, immunohistochemistry and promoter::GUS transgene analyses. I then compared the gene expression of *AbPMT* and *AbH6H* with tobacco *PMT* and *H. niger H6H* with respect to cell-specificity and hormonal regulation.

MATERIALS AND METHODS

Plant materials

Atropa belladonna L., *Hyoscyamus niger* L. and *Nicotiana tabacum* L. cv. SR1 are maintained in our laboratory for studies on alkaloid biosynthesis, being grown in a greenhouse. Shoot (Mathis and Hinchee 1994) and root (Hashimoto *et al.* 1986) cultures were grown as reported.

Genomic DNA blots

Total genomic DNA was isolated from the leaves of *N. tabacum* cv. SR1, *A. belladonna* and *H. niger*, as described (Hibi *et al.* 1994), and 20 μg of genomic DNA was digested with restriction enzymes and electrophoresed through 0.8% agarose. The gel was denatured, neutralized, and blotted onto Hybond-N nylon membrane (Amersham) by standard procedures (Sambrook *et al.* 1989).

The blotted membrane was hybridized with a full-length *NtPMT1* cDNA probe radiolabeled with $[\alpha-^{32}P]dCTP$ (random primer DNA labeling kit; Takara) in 50% formamide, 2 x SSC, 10% dextran sulfate, 1% SDS, and 100 μg ml^{-1} sonicated salmon sperm DNA at 42°C for 16 h, and then washed three times with 1 x SSC, 1% SDS at 42°C for 1 h, 0.5 x SSC, 1% SDS at 55°C for 1 h, and 0.1 x SSC, 1% SDS at 65°C for 1 h. The membrane was stripped of the initial probe in 0.1 x SSC and 0.1% SDS at 100°C until no signal was detected, and subsequently rehybridized with the following probe specific for the tandem repeat which was labelled as above. The probe DNA fragment which included the *N*-terminal repeat array was amplified by PCR, cloned into pGEM-T (Promega), and excised as a *Bam*H I/*Eco*R I fragment. The PCR procedure used a thermocycler (Model 9600; Perkin-Elmer), two primers (5'-TGAAAATGGATCCCATATCTACC-3' and 5'-GCGAATTCTTAGTCGGAGCTTCCCAG-3'; underlines denote *Bam*H I and *Eco*R I sites, respectively), and amplification for 3 min at 94°C, followed by 15 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and finally a 10-min incubation at 72°C. Subsequent procedures were done as described above, except that the hybridized membrane was washed only once with 1 x SSC, 1% SDS at 42°C for 1 h.

Construction and screening of cDNA and genomic DNA libraries

Total RNA was isolated from cultured roots of *A. belladonna* and *H. niger* as reported (Kanegae *et al.* 1994), from which poly (A)$^+$ RNAs were purified by using an mRNA purification kit (Pharmacia). cDNA libraries were constructed by using a ZAP-cDNA Synthesis Kit (Stratagene) and an *in vitro* packaging kit Lambda Inn (Nippongene, Toyama, Japan). Screening of the cDNA libraries was performed according to Sambrook *et al.* (1989) with some modifications. Duplicate plaque filters (Hybond-N+; Amersham) were hybridized with a tobacco *PMT* cDNA (Hibi *et al.* 1994) probe labeled by using a Random Primer DNA Labeling

Kit (Takara) in 50% formamide, 2x SSC, 10% dextran sulfate, 1% SDS and 0.1 mg ml^{-1} salmon sperm DNA at 42°C for 14 h. Washing was performed in 0.1x SSC and 0.1% SDS at 65°C. After a second round of screening, cDNA inserts were excised *in vitro* from positive phage clones as pBluescript SK$^-$ plasmids.

Genomic DNA was prepared from leaf tissue of *A. belladonna* by CTAB extraction (Murry and Thompson 1980), and further purified by CsCl/EtBr ultra-centrifugation. Purified DNA was digested with *Sau*3A I, fractionated (9-23kb) on NaCl gradients, and then cloned into the *Xho* I site of λFixII (Stratagene). Screening of an *A. belladonna* genomic DNA library was performed as described above by using the full-length *AbPMT1* cDNA as a probe. The *H. niger H6H* cDNA (Matsuda *et al.* 1991) was used as the hybridization probe. The screening was done as described above, except for duplicate plaque filters (Gene Screen Plus; NEN) were hybridized in 50% formamide, 6x SSC, 5x Denhardt's solution, 0.5% SDS and 0.1mg/ml salmon sperm DNA. After a second round of screening, genomic DNA inserts from positive phage clones were subcloned into pBluescript II SK$^-$ (Stratagene). Nested-deletion clones were made (Sambrook *et al.* 1989), and DNA sequences were obtained by using ABI DNA sequencers (373A and 377A).

RNA blot hybridization

Total RNA (10 μg) isolated from several tissues of mature *A. belladonna* was separated by electrophoresis on 1.2% formaldehyde agarose gel and blotted onto a Hybond-N membrane (Amersham). The blot was hybridized with a ^{32}P-labeled 3' region of either *AbPMT1* cDNA (310-bp long) or *AbPMT2* cDNA (345-bp long) under the same conditions as described above. Washing was performed in 2x SSC and 0.1% SDS at 65°C for 3 h. These 3'-region fragments were obtained by PCR amplification of the *AbPMT* cDNAs from the respective plasmids, followed by removal of a vector sequence by *Xho* I digestion and agarose gel separation. The PCR primers used were the M13M4 primer (Takara) and the PMT2F primer (5'-GGACCTTTGAAGTTCTAC), which was designed based on the conserved nucleotide sequences between *AbPMT1* cDNA and *AbPMT2* cDNA. The blot was also hybridized with a ^{32}P-labeled coding region of *AbH6H* (270-bp long), which was obtained by RT-PCR

amplification of the total RNA isolated from *A. belladonna* root with H6H-F and H6H-R primers (see below), under the same conditions as described above.

PCR and RT-PCR

AbH6H (λ8) and *Abψ6H* (λ14), which had been cloned into pBluescript II SK⁻ (Stratagene), as well as *A. belladonna* genomic DNA, were used as the templates for PCR. PCR (thermal cycler model 9600; Perkin-Elmer) was done using two *H6H*-specific primers (H6H-F: 5'-CACTTTGGCTCATGGTTGTCA-3' and H6H-R: 5'-CCATCATAGTGTCCTC-CTGATCC-3'), and the following protocol: amplification for 3 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C, and finally a 10 min incubation at 72°C.

First-strand cDNA was synthesized from total RNA isolated from several tissues of *A. belladonna* by using a cDNA synthesis kit (Takara) and the oligo-d(T) primer provided. At 1 week after subculture, cultured roots were treated with 0 or 20 μM methyl jasmonate for 4 h and subsequently harvested. The first-strand cDNA (50 ng) was amplified using two *PMT*-specific primers (AbP-F: 5'-ATTGTTCATCTCCCACTTGG and AbP-R: 5'-TCTTTTGCTGGACC-AATAGG) or two *H6H*-specific primers (H6H-F and H6H-R). PCR was done as described above, except for 35 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. The amplification products with *H6H*-specific primers were digested with *Bam*H I, followed by electrophoresis on a 1.2% agarose gel. The gel was subsequently denatured, neutralized and blotted onto a Hybond-N membrane (Amersham) by standard procedures (Sambrook *et al.* 1989). The blotted membrane was hybridized with radio-labeled *H. niger H6H* cDNA. As quantification controls, *AbPMT1* and *AbPMT2* cDNAs were also amplified in different concentration combinations (see Figure 9B). The amplification products were digested with *Cla* I and/or *Pvu* II and subsequently electrophoresed on a 2% agarose gel. A partial *A. belladonna SPDS* cDNA was also obtained by RT-PCR according to Hashimoto *et al.* (1998b).

5'-RACE

The gene-specific primers, 5R-H1 (5'-GAAAGAGACCAAAATCTTGACAAGC-3'),

5R-H2 (5'-G<u>GAATTC</u>-<u>GTCGAC</u>GATTTGTTGAACAACAACAAGG-3'), and 5R-H3 (5'-AG<u>ACTAGT</u>CGAACATCATTTTCTGCCC-3'), were designed based on the DNA sequence of *AbH6H* (underlines denote restriction sites for *Eco*R I, *Sac* I, and *Spe* I, respectively). First-strand cDNA was synthesized as described above with the 5R-H1 primer, and tailed with polyadenines by terminal deoxynucleotidyl transferase (Takara). The first round of PCR was performed with a *Sac* I-*Xba* I-*Bam*H I-d(T)17 primer (5'-CTGAGCTCTAGAGGATCC-TTTTTTTTTTTTTTTTTT-3'), a *Sac* I-*Xba* I-*Bam*H I adapter (5'-CTGAGCTCTAGAGG-ATCC-3') and the 5R-H2 primer. Amplification products were purified with a Chroma Spin 400 column (Clontech) and used as the template for a second round of PCR, which was performed with the *Sac* I-*Xba* I-*Bam*H I adapter and the 5R-H3 primer. Amplification products were cloned into pBluescript II SK⁻ between the *Bam*H I and *Spe* I sites. The DNA sequences from ten clones were determined.

In situ hybridization

In situ hybridization was done according to (Meyerowitz 1987) with some modifications. Two primers, 5'-AA<u>GAATTC</u>AGTGGAAGTGAGGAAGTTGA-3' and 5'-TT<u>GGTACC</u>CAGGCAAGTTTTGTTGAAGC-3' (underlines denote restriction sites for *Eco*R I and *Kpn* I, respectively) were designed to amplify part of the third *AbH6H* exon. A 210 bp amplification product was cloned between the *Eco*R I and *Kpn* I sites of pBluescript II SK⁺ in which the *Sac* I site had been changed to an *Eco*R I site. The plasmid was digested with either *Eco*R I or *Asp*718 I to make digoxygenin-labeled antisense or sense RNA probe using the DIG-RNA labeling kit (Boehringer). Cultured roots in the rapid growing phase (3 to 4 days after transfer to a fresh liquid medium) and flowers were fixed for 2 to 3 h in an ice-cold solution of 3% (w/v) paraformaldehyde, 10% (v/v) glutaraldehyde and 33mM phosphate buffer (pH7.4), followed by dehydration in an ethanol series and a xylene series. Dehydrated samples were embedded in Histoprep 580 (Wako Pure Chemical Industries, Osaka, Japan), and stored until use at 4°C. Samples were sectioned at a thickness of 10μm, and mounted onto glass slides. After dewaxing and hydration, sections were treated with 0.05N HCl for 10 min, washed with 2x SSPE for 5 min and then with DEPC-treated water for 5 min, followed by Proteinase K

(2µg/ml) treatment at 37°C for 30 min. The sections were then dehydrated and vacuum-dried. RNA probes were hybridized with the sections at a 1/200 dilution in hybridization solution (Duck 1994) at 45°C for 16 to 20 h. The slides were then rinsed with 2x SSPE and buffer A (0.5M NaCl, 10mM Tris-HCl, pH7.5, 1mM EDTA), and then treated with RNase A (1-2µg/ml in buffer A) at 37°C for 30 min. Excess probe was removed by three additional washes in 2x SSPE and three washes in 0.2x SSPE at 50°C, for 20 min each. Sections were then blocked at room temperature with 1% Blocking reagent (DIG Nucleic Acid Detection Kit, Boehringer) in buffer 1 (100mM Tris-HCl, pH7.5, 150mM NaCl), and with 3% BSA in buffer 2 (buffer 1 plus 0.3% Triton X-100), followed by a 2 h incubation in diluted alkaline phosphatase-conjugated antibody solution (1:2500 in buffer 2 containing 1% BSA). Excess antibody conjugate was removed by three washes in buffer 2, and three washes in buffer 1, for 15 min each. For flower sections, 1% BSA was included in buffer 2 during washing. Slides were then rinsed once with 1% BSA in buffer 2 and twice in buffer 3 (100mM Tris-HCl, pH9.5, 100mM NaCl, 50mM MgCl2). Colors were developed for 1 to 2 days in buffer 3 containing 0.34mg/ml nitroblue tetrazolium salts and 0.175 mg/ml 5-bromo-4-chloro-3-indoyl phosphate toluidium salt. The reaction was stopped with a buffer containing 10mM Tris-HCl (pH8.0) and 1mM EDTA. Sections were passed through ethanol and xylene series, and mounted in Eukitt (O. Kindler, Germany).

Immunohistochemistry

Flower tissues were fixed and sectioned as described for in situ hybridization. The 10µm flower sections, after dewaxing with xylene and hydration, were blocked with 1% BSA in buffer 2, and incubated with anti-H6H monoclonal antibody mAb5 (Hashimoto et al. 1991) at 2.5µg/ml in buffer 2 containing 1% BSA at room temperature for 1 h. Sections were washed four times with buffer 2 for 15 min each, and treated with 3% BSA in buffer 2 for 1 h. Alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Kirkegaard & Perry Laboratories Inc.) was applied at a 1:3000 dilution to the sections. The rest of the procedures were done as described for in situ hybridization, except that color development was stopped after 4 h. As a negative control, the primary antibody mAb5 was omitted from the corresponding solution.

Construction of the promoter::GUS fusion vectors and plant transformation

The *Hin*d III site (from -2 to +4) in the 5'-flanking region of *AbPMT1* (λ1 clone), which had been subcloned in pBC SK⁻ (Stratagene), was changed to a *Bam*H I site by *Hin*d III digestion, filling-in by a Klenow fragment, *Bam*H I-linker ligation, and self ligation. The resultant plasmid was cut with *Xho* I, filled in by a Klenow fragment, and then digested with *Bam*H I to generate an *AbPMT1* fragment containing a 1373-bp upstream region from the 5'-end of the longest *AbPMT1* cDNA (designed as *AbP-1373*). The *AbP-1373* fragment was introduced between the *Eco*R V and *Bam*H I sites of pBluescript II SK⁻. The *AbP-748* fragment and the *AbP-295* fragment were similarly obtained by using the *Bst*X I and *Bam*H I sites and the *Eco*R I and *Bam*H I sites, respectively, except for the addition of a step of blunt ending after *Bst*X I digestion in the *AbP-748* fragment construction by exonuclease reaction of T4 DNA polymerase. These shorter 5'-upstream fragments were also introduced between the *Eco*R V and *Bam*H I sites of pBluescript II SK⁻. Subsequently, *AbP-1373*, *AbP-748*, and *AbP-295* were subcloned between the *Hin*d III and *Bam*H I sites in pBI 101 (Clontech, Figure 13F).

The 5'-flanking region of *AbH6H*, which was subcloned into pBluescript II SK⁻, was isolated as a *Sal* I-*Acc* I fragment spanning from -671 to +103 bp relative to the transcription initiation site, and introduced between the *Acc* I and *Bam*H I sites of pBluescript II SK⁻ after linker ligation. Subsequently, the *Sal* I-*Bam*H I fragment was cloned into pBI 101, digested with *Bam*H I, and then filled-in to make an in-frame fusion with the GUS open reading frame (ORF).

The resulting binary vectors were transferred into *Agrobacterium rhizogenes* strain 15834 and *Agrobacterium tumefaciens* strain LBA 4404 by electroporation (Nagel *et al.* 1990). Leaf disc transformation was perfomed according to Kanegae *et al.* (1994). Transgenic hairy roots were selected for kanamycin-resistance (250 mg l⁻¹) and subcultured on solid culture medium every 4 weeks. Transgenic *A. belladonna* and *N. tabacum* plants were regenerated from leaf discs and grown in a greenhouse according to Mathis and Hinchee (1994).

Histochemical analysis of ß-glucuronidase (GUS) expression

Histochemical staining for GUS activity was performed according to Jefferson *et al.* (1987) with some modifications. Plant tissues were soaked in a solution composed of 1 mM 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide (X-Gluc), 50 mM K-phosphate buffer (pH 7.0), 0.1% (v/v) Tween 20, 20% (v/v) methanol, and 5 mM dithiothreitol at 37°C for periods ranging from 4 h to overnight. After staining, samples were treated with chlorallactophenol (Beeckman and Engler 1994) to enhance tissue transparency.

In another set of experiments, root cross sections of 80 μm-thickness were prepared with a DTK-1500 microslicer (Dohan EM, Kyoto, Japan). Sections were stained in a solution containing 0.1 mM X-Gluc, 200 mM Na-phosphate buffer (pH 7.0), 0.1% (v/v) Triton X 100, 0.1 mM K-ferricyanide and 0.1 mM K-ferrocyanide.

RESULTS

1. Molecular analysis of *PMT* genes

Tandem repeats are absent in *Hyoscyamus* and *Atropa* PMTs

The *PMT* gene whose cDNA was previously cloned from *Nicotiana tabacum* root by subtractive hybridization (Hibi *et al*. 1994) is referred here as *NtPMT*. Although four repeats of the heptapeptide were previously noted at the *N*-terminus of NtPMT (Hibi *et al*. 1994), closer inspection revealed that the repeat unit actually consists of a DNA sequence 33 bp long that encodes 11 amino acids with a consensus of NGHQNGTSEHQ (Figure 8). To verify whether a similar repeat array is also present in the *PMT* genes of other plants, I hybridized a genomic blot of *Hyoscyamus niger*, *Atropa belladonna* (two Solanaceae species known to contain PMT for the biosynthesis of tropane alkaloids; Hashimoto *et al*. 1994) and tobacco, successively with
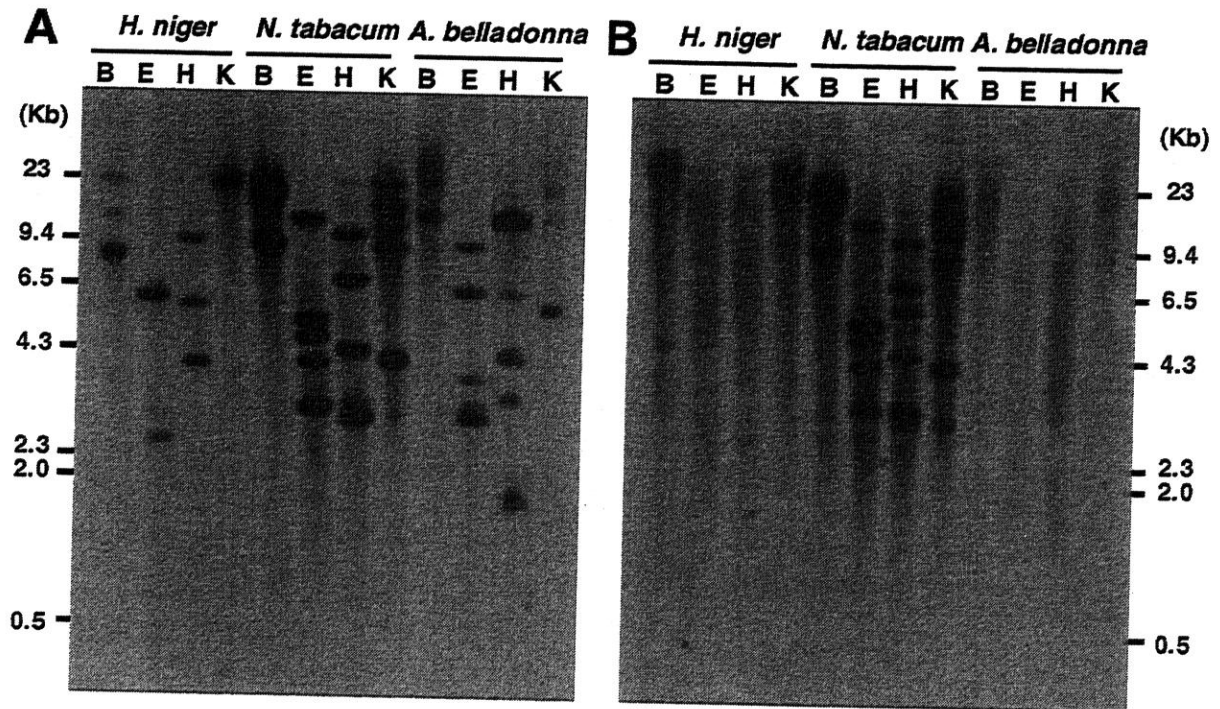
Figure 5 Genomic DNA blot analysis of *PMT* genes in three Solanaceae spiecies. (A) Genomic DNAs of *H. niger*, *N. tabacum*, and *A. belladonna* were digested with the indicated restriction enzymes, and probed with a full-length *NtPMT* cDNA. Restriction enzymes are indicated by B (*BamH* I), E (*EcoR* I), H (*Hind* III), and K (*Kpn* I). The numbers on the left show marker fragment sizes in kb. (B) The same membrane used in (A) was stripped of probe and rehybridized with an *NtPMT* PCR fragment that contained only the tandem repeat array.

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the full-length *NtPMT* cDNA probe and the *N*-terminal probe specific for the repeat array. In tobacco, five *EcoR* I, five *Hind* III and five *Kpn* I genomic fragments hybridized to the full-length probe (Figure 5A), whereas the same restriction fragments also hybridized to the repeat-specific probe (Figure 5B). This indicates that the tobacco genome contains five *PMT*-related DNA sequences, each with the repeat array.

Up to three and five fragments, respectively, hybridized to the full-length probe when *H. niger* and *A. belladonna* genomes were digested with *BamH* I, *EcoR* I, *Hind* III, and *Kpn* I (Figure 5A). None of these restriction fragments, however, hybridized to the repeat-specific probe, which only gave smears on the blot. Thus, *H. niger* and *A belladonna* genomes have *PMT*-related sequences, but these *PMT* homologues do not contain the tandem repeats found in *NtPMT*.

Isolation and characterization of *PMT* and *SPDS* cDNAs

Screening of approximately 3×10^4 independent clones from a cultured root cDNA library of *A. belladonna* with a tobacco *PMT* cDNA probe resulted in eight *PMT* cDNA clones (Figure 6). The eight clones were classified into two groups according to restriction enzyme digestion and nucleotide sequencing. Six clones contained the *AbPMT1* cDNA, and two remaining clones contained the *AbPMT2* cDNA. The longest *AbPMT1* cDNA (Ab8 clone) was 1305-bp long and encoded AbPMT1 of 336 amino acids (Figure 7A), whereas the longest *AbPMT2* cDNA (Ab3 clone) was 1294-bp long and encoded AbPMT2 of 340 amino acids (Figure 7B).

From a cultured root cDNA library of *H. niger*, one *PMT* cDNA (*HnPMT*) and two *SPDS* cDNAs (Hashimoto *et al.* 1998b) were isolated (Figure 6). The *HnPMT* cDNA was 1350-bp long and encoded HnPMT of 338 amino acids (Figure 7C). The *HnSPDS1* cDNA was 1231-bp long and encoded HnSPDS1 of 315 amino acids (Figure 7D), and the *HnSPDS2* cDNA was 1281-bp long and encoded HnSPDS2 of 308 amino acids (Figure 7E).

To determine whether *A. belladonna* have any *SPDS* gene, I cloned one RT-PCR fragment amplified from *A. belladonna* leaf total RNA, in which *AbPMT* RNA was absent (see below, Figure 9C), with *SPDS*-specific primers designated by Hashimoto *et al.* (1998b). This

AbPMT1
(1305bp)
ATG
TGA
1   69
1079   1305

* Ab8   (1-1305)
Ab4   (1-1303)
Ab6   (1-1296)
Ab1   (1-1248)
Ab5   (1-1239)
Ab12   (2-1259)

AbPMT2
(1294bp)
ATG
TAA
1 20
1042   1294

* Ab3   (1-1294)
Ab2   (114-1274)

HnPMT
(1350bp)
ATG
TGA
1   124
1140   1350

* Hn10   (1-1324)
Hn4   (62-1324)
Hn3   (67-1340)
Hn12   (539-1350)

HnSPDS1
(1231bp)
ATG
TGA
1 53
1000   1231

* Hn1   (1-1231)

HnSPDS2
(1281bp)
ATG
TGA
1   89
1010   1281

* Hn5   (1-1281)
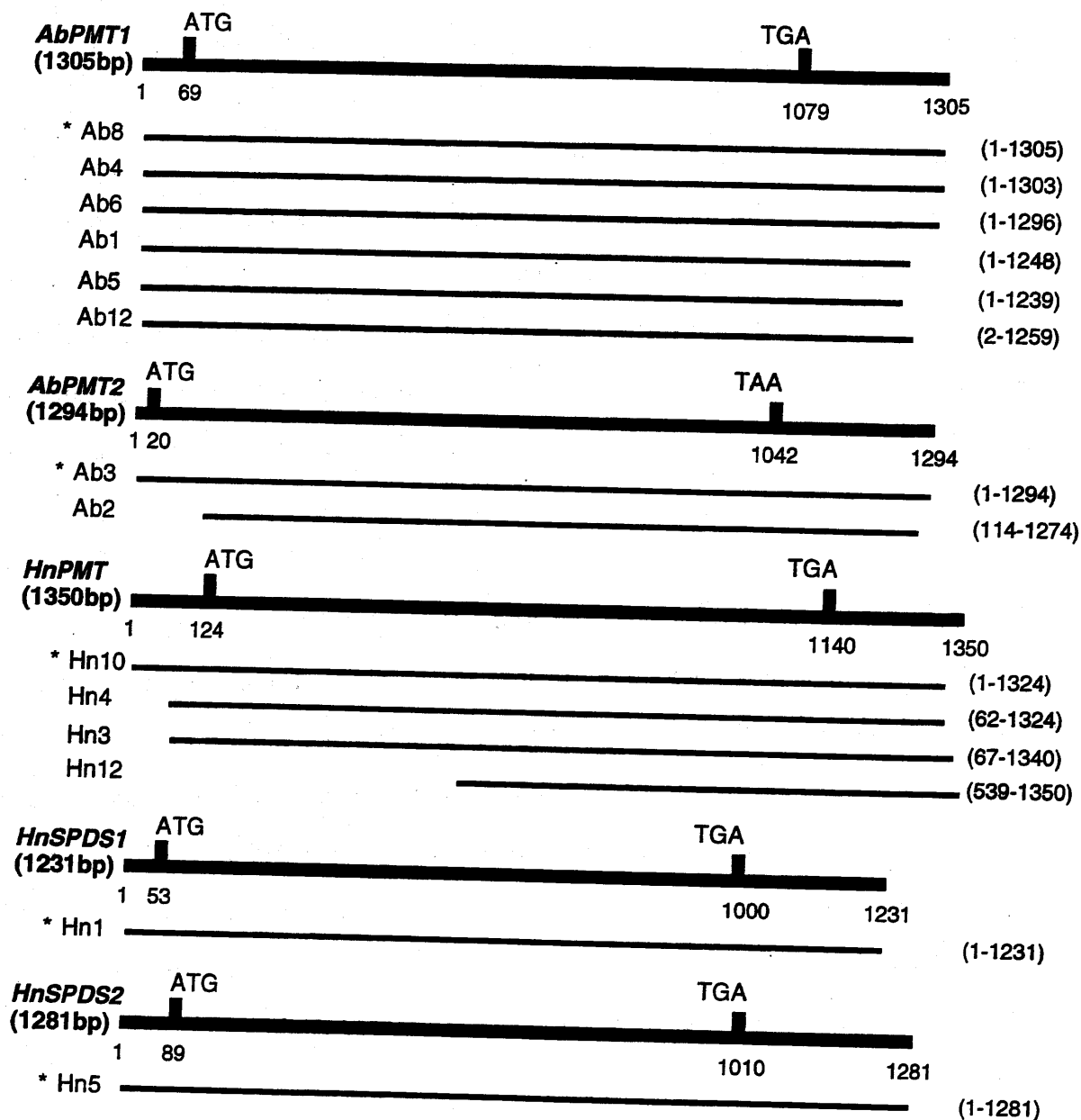
Figure 6 Classification of *NtPMT* homologue cDNAs clones isolated from root cDNA libraries of *A. belladonna* (Ab clones) and *H. niger* (Hn clones). Bold lines above each cDNA clons are designated as *AbPMT1, AbPMT2, HnPMT, HnSPDS1* and *HnSPDS2*.   Numbers of nucleotide sequence described under the cDNA structures and beside the cDNA clones, are from each 5'-end of the longest cDNA clone shown with asrerisk. Start (ATG) and stop (TGA or TAA) codons are also shown.

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A

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                                                       GCTTGTG      8
AGTGCTAAACTGCAAATTAGTTTCTTCAAATAATTGTTTTCTCTTATTTAGTTTGAAGA     68
ATGGAGGTGAACGACAACAATGGCAGCACCACCAAAATTATCCTGAAAATGGCAGTATT    128
 M  E  V  N  H  N  N  G  S  T  T  T  K  I  I  L  K  N  G  S  I    20
TGCAATGGCGATGTTAATGGCAACTCCCACACTCATAAGAAAATTGAGAATAACTTGTA    188
 C  N  G  D  V  N  G  N  S  H  T  H  K  K  I  E  N  K  L  V      40
GAGTGTACTAACTCTATCAAGCCGGTTGGTTTCTGAGTTTAGGCGACTATGGCCAGGT    248
 E  C  T  N  S  I  K  P  G  W  F  S  E  F  S  A  L  W  P  G      60
GAAGCATTTCACTTAAAATTGAAAAGTTACTCTTTCAAGGAAAGTCTGATTATCAAGAT    308
 E  A  F  S  L  K  I  E  K  L  L  F  Q  G  K  S  D  Y  Q  D      80
GTCATGCTCTTTGAGTCAGCAACTTATGGGAAGGTGTAACATTGGATGGAGCAATTCAA    368
 V  M  L  F  E  S  A  T  Y  G  K  V  L  T  L  D  G  A  I  Q     100
CATACAGAGAATGGTGGATTTCCATACACTGAGATGATTGTTCATTCCCCACTTGGTTCC    428
 H  T  E  N  G  G  F  P  Y  T  E  M  I  V  H  L  P  L  G  S     120
ATTCCATCCCCAAGAAGGTTTTAATATTCATCGGTGGAGGGATTGGTTTCACATTGTTGAG    488
 I  P  S  P  K  K  V  L  I  I  G  G  G  I  G  F  T  L  F  E     140
GTCTCTCGTTACCCAACTATCGACACAATAGACTTAGTTGAGATCGATGACGTTGGTGTA    548
 V  S  R  Y  P  T  I  D  T  I  D  I  V  E  I  D  D  V  V  V     160
GATGTAATCTAGAAAGTATTTCCGGTACTTAGCGCAGGATTCGATGATCCCAGAGTAACC    608
 D  V  S  R  K  Y  F  P  P  Y  L  A  A  G  F  D  D  P  R  V  T  180
CTTATTATTGGCGATGGACCTGCATTCGTGAAGCTGCTCAACCTGGATACATATGATGCC    668
 L  I  I  G  D  G  A  A  F  V  K  A  A  Q  P  G  Y  Y  D  A     200
ATTATTGTGGACTCTTCTGATCCTATTGGTCCAGCAAAAGACTTGTTTGAAAGGCCATTC    728
 I  I  V  D  S  S  D  P  I  G  P  A  K  D  L  F  E  R  P  F     220
TTCGAAGCAGTGGCGAAAGCGCTAAGGCCAGGAGGAGTGGTGTACACAGAGCAGAGAGC    788
 F  E  A  V  A  K  A  L  R  P  G  G  V  V  C  T  Q  A  E  S     240
ATTTGGCTCCACATGCATCTTATTAAGCAAATTATTGCTAATTGTCGCCAAGTTGTTAAG    848
 I  W  L  H  M  H  L  I  K  Q  I  I  A  N  C  R  Q  V  F  K     260
GGTTCTGTCAACTATGCATGGACTACAGTTCCTACTTACCCTACTGGTGTTATCGGTTAC    908
 G  S  V  N  Y  A  W  T  T  V  P  T  Y  P  T  G  V  I  G  Y     280
ATGCTTTGCTCTACGGAGGGACCAGAAGTTAATTTCAAGAATCCAGTTAACTCTATTGAC    968
 M  L  C  S  T  E  G  P  E  V  N  F  K  N  P  V  N  S  I  D     300
AAAGATACCAGCCATGTCAAATCCAAGGGACCTTTGAAGTTCTACAACTCTGATATTCAT   1028
 K  D  T  S  H  V  K  S  K  G  P  L  K  F  Y  N  S  D  I  H     320
AAAGCAGCTTTTATTTTGCCATCTTTTGCTAGGGATTTGGTTGAGTTTTGATCAAACAAA   1088
 K  A  A  F  I  L  P  S  F  A  R  D  L  V  E  F              336
TGATGATGATTTGATTGGTATATTATTGTTGTACCAGGTTTTGGTTGTGTATGGGGAAATTGTT   1148
GAGTTTTTCTTCAAAATTTGTATGTTTGGTTTCTGTACGACGGATGAAAATAATGAAGAG   1208
CTTAGGTCTGATCGAATTTACTTGAAAAGTTATATTGCAACAAAATAATATGGGTTATATAA   1268
ATTATATATTAATTAAAAAAAAAAAAAAAAAAAAAAAAAAAA               1305
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B

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                                                      GAGAATCCCTTTTGAAT     19
ATGGCTTTCGGCAATGGCAGCACCACCACCACCATCACTACCACTACTTAAAAATGGCGGC     79
 M  A  F  R  N  G  S  T  T  T  T  T  I  T  T  I  N  H  P  N  D      20
GCTTCAACTATCCCTAAAAATGGCACCAACATACCCACTACTCTACTTAAAAATGGCGGC    139
 A  S  T  I  P  K  N  G  T  N  I  T  T  T  L  L  K  N  G  S      40
ACTAACGAGTTCGGTTGTATTAAGCCCGGGTGGTTCTCGGAGTTTAGCCAATTCGGGGCC    199
 T  N  E  F  G  C  I  K  P  G  W  F  S  E  F  S  Q  L  W  P      60
GGCGAAGCATTCTCACTTAAAATTGAGAAGTTACTATTCCAAGGGAAGTCTGATTATCAA    259
 G  E  A  F  S  L  K  I  E  K  L  L  F  Q  G  K  S  D  Y  Q      80
GATGTTATGCTCTTTGAGTCAGCAACTTATGGGAAGGTTCTAACATTGGATGGAGCCATT    319
 D  V  M  L  F  E  S  A  T  Y  G  K  V  L  T  L  D  G  A  I     100
CAACACACAGAGAATGGTGGATTCCATATACTGAAATGATTGTTCATCTCCCACTTGGT    379
 Q  H  T  E  N  G  G  F  P  Y  T  E  M  I  V  H  L  P  L  G     120
TCCATCCCAAGCCCAAAAAAGGTGTTGATTATCGGTGGAGGAATTGGTTTTACATTATTC    439
 S  I  P  S  P  K  K  V  L  I  I  G  G  G  I  G  F  T  L  F     140
GAAGTGTTTCGTTACCCTACAATCGAAAACATAGACATAGTTGAGATCGATAACGTTGTA    499
 E  V  F  R  Y  P  T  I  E  N  I  D  I  V  E  I  D  N  V  V     160
GTCGATGTATCCAGAAAATTCTTCCCTTACCTGGCAGCTGGGTTTGATGATCCTCGTGTA    559
 V  D  V  S  R  K  F  F  P  P  Y  L  A  A  G  F  D  D  P  R  V  180
ACACTGGTTCTTGGTGATGGGCTGGCATTTGTGAAGGCTGCACAAGCAGGATATTATGAT    619
 T  L  V  L  G  D  G  A  A  F  V  K  A  A  Q  A  G  Y  Y  D     200
GCAATTATAGTGGATTCTTCTGTCCTATTGGTCCAGCAAAAGATTTGTTTGAGAGGCCA    679
 A  I  I  V  D  S  S  D  P  I  G  P  A  K  D  L  F  E  R  P     220
TTTTTTGAAGCAGTAGCCAAGGCTCTAAGGCCAGGAGGAGTTGTATGCACACAGGCTGAA    739
 F  F  E  A  V  A  K  A  L  R  P  G  G  V  V  C  T  Q  A  E     240
AGCATATGGCTTCATATGCATATTATAAAACAAATTATTGATAACTGCCGTCAAGTCTTT    799
 S  I  W  L  H  M  H  I  I  K  Q  I  I  D  N  C  R  Q  V  F     260
AAGGGTTCTGTCAACTATGCTTGGACTACAGTTCCAACCTATCCAACAGGGGTTATTGGT    859
 K  G  S  V  N  Y  A  W  T  T  V  P  T  Y  P  T  G  V  I  G     280
TATATGCTTTGCTCTACTAAAGGACCACAAGTTGACTTCAGGAATCCAGTCAATCCAATT    919
 Y  M  L  C  S  T  K  G  P  Q  V  D  F  R  N  P  V  N  P  I     300
GACAAAAAGACTTCTCATATCAAATCCAAGGACCTTGAAGTTCTACAATTCTGATATT    979
 D  K  K  T  S  H  I  K  S  K  G  P  L  K  F  Y  N  S  D  I     320
CATAAAGCAGCTTTTATTTTGCCATCGTTTGCGAGGAATTTGATGGAGTCTGAATTAGAT   1039
 H  K  A  A  F  I  L  P  S  F  A  R  N  L  M  E  S  E  L  D     340
TAAGGAAATTTTATCGATGGTCTGAAGGCGTTAAAATGGTGTTACGTGGGGAGCTATCTAG   1099
AATTCTATATTGTATATTTTTTGAGATGTCAGAAATAATTTAGTGTGGAACAACGTTTT   1159
TCTCTATGGCTTTTTAAATGAAATAAAGTTATTTATGTTGGTCAGGTCAAGCATTTG   1219
TCAATTATATGTAAATTACTAACATCCAAGAGAATAAATCAAACAATTTTGAAAAGAAA   1279
AAAAAAAAAAAAAAA                                         1294
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C

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                                                             ATA      3
TCAAACACTTCACTATAATATATGCATGAAATTCTTGAGTGAATAACCAGATAAGTGAAGA     63
AACATTTTGAGTGCTAAACTGCAAATTAGTTTCTTTAATAATTGTTCTTTTTTTAAAAGA    123
ATGGAGGTGATTAGTAACCACAACACAATGGCCAGCACCACAAAATATCCTGAAAAATGCC    183
 M  E  V  I  S  N  H  N  N  G  S  T  T  K  I  I  L  K  N  G      20
AGCATTTGCAATGGCAATGTTAATGGCAACTCCCACTCCAATGAGAAGACTGAGAATAAG    243
 S  I  C  N  G  N  V  N  G  N  S  H  S  N  E  K  T  E  N  K      40
CTTGTAGAGTGCACTAACTCTATCAAGCCCGGTGGTTTCTGAGTTTAGGCGACTCTCGG    303
 L  V  E  C  T  N  S  I  K  P  G  W  F  S  E  F  S  A  L  W      60
CCAGGTGAAGCATTTCACTTAAAATTGAAAAGTTACTATTTCAGGGAAATCTGATTAT    363
 P  G  E  A  F  S  L  K  I  E  K  L  L  F  Q  G  K  S  D  Y      80
CAAGATGTCATGCTCTTTGAGTCAGCAACTTATGGGAAGGTGTTAACTTGGATGGAGCG    423
 Q  D  V  M  L  F  E  S  A  T  Y  G  K  V  L  T  L  D  G  A     100
ATTCAACACACAGAGAATGGTGGATTCCCATACACTGAGATGATTGTTCATCTCACCACTT    483
 I  Q  H  T  E  N  G  G  F  P  Y  T  E  M  I  V  H  L  P  L     120
GGTTCTATTCCATCCCCAAGAAGGTTTTAATATTCGGTGGAGGGATTGGTTTCACATTA    543
 G  S  I  P  S  P  K  K  V  L  I  I  G  G  G  I  G  F  T  L  F  140
TTTGAGGGTTTCTCGTTACTCAACTATCGAAAAATAGATATAGTTGAGATTGATGACGTG    603
 F  E  V  S  R  Y  S  T  I  E  K  I  D  I  V  E  I  D  D  V     160
GTTATAGATGTCATCTAGAAAGTATTTCCCTACCTACAGCAGGATTCGATGATCCCAGA    663
 V  I  D  V  S  R  K  Y  F  P  P  Y  L  A  A  G  F  D  D  P  R  180
GTAACCCTTATTGTTGGCGATGGAGCTGGCATTCGTGAAGCTGCTCAACCTGGATACATT    723
 V  T  L  I  V  G  D  G  A  A  F  V  K  A  A  Q  P  G  Y  Y     200
GATGCCATTATTGTGGACTCTTCCGATCCTATTGGTCCAGCAAAGGATTTGTTTGAAAGA    783
 D  A  I  I  V  D  S  S  D  P  I  G  P  A  K  D  L  F  E  R     220
CCATTCTTCGAAGCAGTGACTAAAGCATTAAGGCCGGGAGGAGTAGTGTGTACACAAGCG    843
 P  F  F  E  A  V  T  K  A  L  R  P  G  G  V  V  C  T  Q  A     240
GAGAGTATTTGGCTTCACATGCACTTATTAAGCAAATAATTGCTAATTGTCGCCAAGTG    903
 E  S  I  W  L  H  M  H  L  I  K  Q  I  I  A  N  C  R  Q  V     260
TTTAAGGGTTCTGTCAATTATGGGTGGACTACAGTTCCTACTTACCCTACTGGTGTTATT    963
 F  K  G  S  V  N  Y  A  W  T  T  V  P  T  Y  P  T  G  V  I     280
GGTTACATGCTTTGTTCTACGGAGGGACCAGAAGTTAATTTCAAGAATCCAGTGAACTCT   1023
 G  Y  M  L  C  S  T  E  G  P  E  V  N  F  K  N  P  V  N  S     300
ATTGACAAAGATACAACTCATGTCAAATCAAAGGGACCTTTGAAGTTCTACAACTCTGAT   1083
 I  D  K  D  T  T  H  V  K  S  K  G  P  L  K  F  Y  N  S  D     320
ATTCATAAAGCAGCTTTTATTTTGCCATCTTTTGCTAGGGATTTGGTTGAGTTTGATCA   1143
 I  H  K  A  A  F  I  L  P  S  F  A  R  D  L  V  E  F  .       336
AACAAATAATGATGATCTTATTGGTATTTATTATACCACATTTGGTTATGTATGGGGGAA   1203
ATGGTTCAAAATTGTATGTTTGGTGTATCCGTGGATGATGAAATAATGAATAAGTTTGTG   1263
TTTATTGCAATAAAATTGTATGGTTATATAAAATTATATTAATTAATATCTTGTATCCAGA   1323
TGTTGAAACATAAAAAAAAAAAAAAAAAAAAAAAAAA                   1357
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D

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                           ATCCCCTCTCAAGACGCCAAGACAAGAAGGAGGAAGATAAATCTGTTGTG     52
ATGGAAGTAACCAATCAAAGTAATGGTTGCAGCAATAATGAGAAAGAGTCTCCTTATATC    112
 M  E  V  T  N  Q  S  N  G  C  S  N  N  E  K  E  S  P  Y  I      20
TCTTCTGTTCTACCTGGATGGTTCTCTGAGATTAGCCCCCTTTGGCCTGGGGAGCACAC    172
 S  S  V  L  P  G  W  F  S  E  I  S  P  L  W  P  G  E  A  H      40
TCCTTGAAGGTTGAGAAGATATTGTTTCAGGGAAAATCTGACTACCAAAATGTCATGGTT    232
 S  L  K  V  E  K  I  L  F  Q  G  K  S  D  Y  Q  N  V  M  V      60
TTCCAGTCATCAACTTATGGAAAGGTGCTTGTTCTGGATGGTGTGATCCAACTACGGAG    292
 F  Q  S  S  T  Y  G  K  V  L  V  L  D  G  V  I  Q  L  T  E      80
AGGGATGAAATGTGCTTACCAAGAGATGATCACATCACTCCCCTCTTTGTCAATTCCCAAC    352
 R  D  E  C  A  Y  Q  E  M  I  T  H  L  P  L  C  S  I  P  N     100
CCCAAAAAGTGCTGGTTATTGGAGGAGGAGATGGTGGAGTCTTGGCTGAGGTATCCGGT    412
 P  K  K  V  L  I  G  G  G  D  G  G  V  L  R  E  V  S  R       120
CATTCTTCTGTCGAACGAATCGACATTTGTGAGAGTTGACAAGATGGTAGTTGAGGTTGCT    472
 H  S  S  V  E  Q  I  D  I  C  E  I  D  K  M  V  V  E  V  A     140
AAAGAATTTTTCCCAGATGTAGCTGTTGGATATGAAGGATCCACGGTGAATCTCCACATT    532
 K  E  F  F  P  D  V  A  V  G  Y  E  D  P  R  V  N  L  H  I     160
GGTGATGGAGTGCATTTGAAAAATGTTCCTGCAGGAACTTACGATGCTGTGCATAGTG    592
 G  D  G  V  A  F  L  K  N  V  P  A  G  T  Y  D  A  V  I  V     180
GATTCATCTGACCCTATAGGTCCAGCCACAAGAATTGTTGAAAAGCCTTTCTTTGAATCT    652
 D  S  S  D  P  I  G  P  A  Q  E  L  F  E  K  P  F  F  E  S     200
ATAGCAAGAGCTCTTCGGTCCAGGAGGGGTTGTGTCTACACACAGGCTGAGAGCATTTGGCT    712
 I  A  R  A  L  R  P  G  G  V  V  S  T  Q  A  E  S  I  W  L     220
CACAGCACATAATTGAAGAAATTGTTGCTAATTGCCGGCAGATCTTCAAAGGCTCTGTC    772
 H  M  H  I  I  E  E  I  V  A  N  C  R  Q  I  F  K  G  S  V     240
AACTATGCATGGACTACTGTTCCTACTTATCCGAGTGGCATGATTGGTTTCATGCTTTGC    832
 N  Y  A  W  T  T  V  P  T  Y  P  S  G  M  I  G  F  M  L  C     260
TCTACTGAGGGACCTGCAGTGGATTTCAAGAACCCTATTAATCCCATCGATGATGAAAGC    892
 S  T  E  G  P  A  V  D  F  K  N  P  I  N  P  I  D  D  E  S     280
GGGCCCAAAACCATTGCACCTTTGAAGTTCTACAATTCTGAGATTCACCAAGCATCGTTC    952
 G  P  K  T  I  A  P  L  K  F  Y  N  S  E  I  H  Q  A  S  F     300
TGTTTGCCATCATTTGCCAAGAGGGTGATCGAATCCAAAGGAAAGTGAGGGGCACATTTT   1012
 C  L  P  S  F  A  K  R  V  I  E  S  K  G  K               315
GAAGGAGTACTTAATAAGCAGGATTAGTTGTTGTGTACAACGCCCTAATAGTTGTCGTAGTT   1072
TTAGGTTCAAGACAATGTTTTTAAGTTTACTTGGCCAAAAAAAAAGTTGGCCAAGTT   1132
CCTTTGATGGATATTAATCAGCGGTTGTTGGTTTGTACTGGGATAAAGTTTAATTTATAGC   1192
TGGTCTTTCTTGATATTCATTAAAAAAAAAAAAAAAAA                  1231
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E

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                              GGACAATCATCGCGTCTTTCCTA  23
CGCAITATCCTCAATCAGGCATIGTGACCAAGGGTGAAGAGAAGTTATCTCCATTICA  83
ATGGAGGACCAAGGCAATAATGAATCAGCCTACATTICTTCTATCTTACCTGGATGGTTC 143
  M  E  E  Q  G  N  N  E  S  A  Y  I  S  S  I  L  P  G  W  F   20
TCTGAGATTAGCCCACTTGGCCIGGGGAAGCACACTCGTTGAAAGTIGAGAAGATACTA 203
  S  E  I  S  P  L  W  P  G  E  A  H  S  L  K  V  E  K  I  L   40
TTTCAGGGGAAGTCAGTTATCAAAATGTTGTGGTTTTCAGTCATCAACTTATGGAAG 263
  F  Q  G  K  S  D  Y  Q  N  V  V  V  F  Q  S  S  T  Y  G  K   60
GTACTTGTTTGATGGTGGATGCAACTCACAGAGGGGATGAATGTGCTTACCAAGAA 323
  V  L  V  L  D  G  V  I  Q  L  T  E  R  D  E  C  A  Y  Q  E   80
ATGATCACTCATCTCCCACTTGCTGATCCAAACCCCAAAAAGGTGTTGGTTATTGGA 383
  M  I  T  H  L  P  L  C  S  I  P  N  P  K  K  V  L  V  I  G  100
GGAGGTGATGGTGGTGTCTTGCGGGAGGTGTCGGGCATCTTCGGTGAACAGATAGAC 443
  G  G  D  G  G  V  L  R  E  V  S  R  H  S  S  V  E  Q  I  D  120
ATTGTGAGATTGACAGATGGTAATTGAGTCTCTAAACAGTTTTCCCTAATGTAGCT 503
  I  C  E  I  D  K  M  V  I  D  V  S  K  Q  F  F  P  N  V  A  140
ATTGGATATGAGGATCCCGGAGTTAAACTCCAITGTTGGTGATGGAGTTGCGTTTCTGAAA 563
  I  G  Y  E  D  P  R  V  K  L  H  V  G  D  G  V  A  F  L  K  160
AATGTTCCTGAAGGAACTTATGATGCAGTTATAGTGGATTCCTCTGACCCCATAGGTCCA 623
  N  V  P  E  G  T  Y  D  A  V  I  V  D  S  S  D  P  I  G  P  180
GCCCAAGAGTTGTTCGAAAGCCTTTTTTGAATCAGTAGCAAGGGCTCTGTGTCCTGGA 683
  A  Q  E  L  F  E  K  P  F  F  E  S  V  A  R  A  L  C  P  G  200
GGGGTTGTCTGTACGCAGCGGGAGAGCATTTGCTTCACATGCACATAATTGAAGATATC 743
  G  V  V  C  T  Q  A  E  S  I  W  L  H  M  H  I  I  E  D  I  220
GTGTCAAACTGCCGCCAGATCTTCAAAGGCTCAGTCAACTATGCCTGGACCACAGTTCCT 803
  V  S  N  C  R  Q  I  F  K  G  S  V  N  Y  A  W  T  T  V  P  240
ACTTATCCAAGTGGTGTTATTGGTTCATGCTCTGCTCGACTGAGGGACCTGCTGTTGAT 863
  T  Y  P  S  G  V  I  G  F  M  L  C  S  T  E  G  P  A  V  D  260
TTCAAGAATCCAATTAACCCAATTGATGCCGATGATAGCCACACCAAAACTAGAGGACCT 923
  F  K  N  P  I  N  P  I  D  A  D  D  S  H  T  K  T  R  G  P  280
TTAAAGTTCTACAACTCTGAGATTCACTCTGCATCGTTCGTTTGCCATCTTTTGCTAAG 983
  L  K  F  Y  N  S  E  I  H  S  A  S  F  C  L  P  S  F  A  K  300
AGGGTGATCGAATCCAACCCAAAATGAGTTCTTAGAGGACCAGGATTGCTTTAGAACACC 1043
  R  V  I  E  S  N  A  K  .                                   306
TCATTCCCACCCTATACAAGTCAGGATCAITTAGAATTGGAAAATTTATGTTCAATTTGA 1103
TCCTGTGACTAGGCAGTTGGATCTGGAGAATGTATGCTTCACTTTGGTTCTTACATGCCTCC 1163
TGTTTGGTCAAGCAATCAAGATCTTGTAAIGTTAGAGAAACAATTTATCAAAATGGATGT 1223
TCACATAATTGTTTGGGACAGTGTAACAGTTTCTTTTTGACCAAAAAAAAAAAAAAAA 1281
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F

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TTTCAGGGGAAGTGGGATTATCAAAAIGTCATGGTTTTTCAGTCATCAACTTATGGAAG  60
  F  Q  G  K  W  D  Y  Q  N  V  M  V  F  Q  S  S  T  Y  G  K   20
GTGCTTATTTTGGATGGTGTGATCAACTCACAGAGCGGGATGAATGTGCTTACCAAGAA 120
  V  L  I  L  D  G  V  I  Q  L  T  E  R  D  E  C  A  Y  Q  E   40
ATGATCACTCATCTCCCACTTTGCTCAATTCCAAACCCCAAAAGGGTGTTGGTTATTGGA 180
  M  I  T  H  L  P  L  C  S  I  P  N  P  K  R  V  L  V  I  G   60
GGTGGTGATGGTGGTGTCTTGCGGGAAGTGTCTCGGGCATCTTCGTTGAGCAGATAGAC 240
  G  G  D  G  G  V  L  R  E  V  S  R  H  S  S  V  E  Q  I  D   80
ATATGTGAGATTGACAAGATGGTTATTGAGTCTCTAAACAATTTTCCCTAATGTAGCT 300
  I  C  E  I  D  K  M  V  I  D  V  S  K  Q  F  F  P  N  V  A  100
ATAGGATATGAGGATCCCGGAGTTAAACTCCAIGTTGGTGATGGAGTTGCATTTTTGAAA 360
  I  G  Y  E  D  P  R  V  K  L  H  V  G  D  G  V  A  F  L  K  120
AATGCTCCTGAAGGAACTTATGATGCAGTTATAGTGGATTCCTCTGACCCCATAGGTCCA 420
  N  A  L  E  G  T  Y  D  A  V  I  V  D  S  S  D  P  I  G  P  140
GCCCAAGAGTTGTTCGAAAAGCCTTTCTTTGAATCAGTAGCAAGGGCTCTGCGTCCCGGA 480
  A  Q  E  L  F  E  K  P  F  F  E  S  V  A  R  A  L  R  P  G  160
GGGGTTGTCTGTACACAGCGGGAGAGCATATGCTCCACATGCACATAATAAA 534
  G  V  V  C  T  Q  A  E  S  I  W  L  H  M  H  I  I  K  178
```

Figure 7 Nucleotide sequences and its deduced amino acid sequences of *PMT* and *SPDS* cDNAs. *AbPMT1* (A) and *AbPMT2* (B), and *HnPMT* (C), *HnSPDS1* (D) and *HnSPDS2* (E), were isolated from root cDNA libraries of *A. belladonna* and *H. niger*, respectively (Figure 6). *AbSPDS* cDNA (F) was amplified with a pair of degenerate PCR primers desigined based on the conserved amino acid sequences of SPDSs (Hashimoto *et al.*1998b), using reverse-transcribed total RNA from *A. belladonna* leaf.

AbSPDS cDNA fragment was 534-bp long and encoded partial AbSPDS of 178 amino acids (Figure 7F). Alignment analysis of AbSPDS with corresponding amino acid sequences of other PMTs and SPDSs indicated that AbSPDS significantly belonged to the SPDS family rather than the PMT family (data not shown).

The expected molecular weights of AbPMT1, AbPMT2, and HnPMT were all 37 kDa. The molecular weight of PMT purified from cultured roots of *Datura stramonium* was estimated to be 36 kDa by SDS-PAGE (Walton *et al.* 1994). AbPMT1 was 82% identical in amino acid sequence to AbPMT2 and 95% identical to HnPMT. *AbPMT1* cDNA was also more similar in nucleotide sequence to *HnPMT* cDNA (86% identity) than to *AbPMT2* cDNA (66% identity). Figure 8 compares the amino acid sequences of AbPMTs and HnPMT with those of tobacco
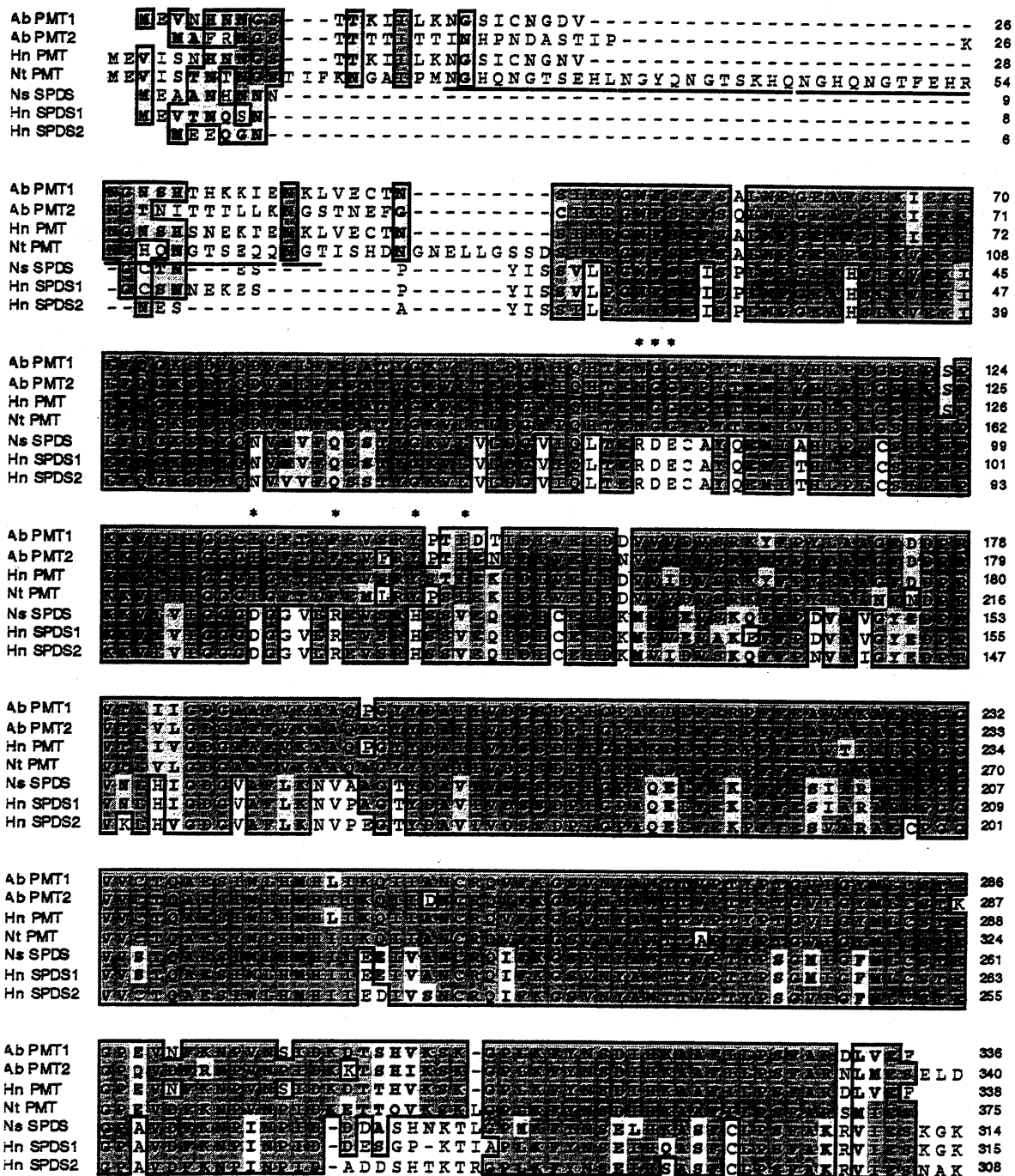
```
Ab PMT1   M E V N H H G S - - T T K I L K N G S I C N G D V - - - - - - - - - - - - - - - - - - - - - - - - - - - -   26
Ab PMT2   M A F R G S - - T T T T T T I H P N D A S T I P - - - - - - - - - - - - - - - - - - - - - - - K   26
Hn PMT    M E V I S N H H G S - - T T K I L K N G S I C N G N V - - - - - - - - - - - - - - - - - - - - - - - -   28
Nt PMT    M E V I S T T G T I F K G A P M N G H Q N G T S E H L N G Y Q N G T S K H Q N G H Q N G T F E H R   54
Ns SPDS   M E A H H N - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   9
Hn SPDS1  M E V T Q S H - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   8
Hn SPDS2  M E E Q G H - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -   6

Ab PMT1   T H K K I E K L V E C T - - - - - - - - -                                     70
Ab PMT2   N I T T T L L K G S T N E F G - - - - - - - -                                  71
Hn PMT    S N E K I E K L V E C T - - - - - - - - -                                      72
Nt PMT    H Q N G T S E Q G T I S H D N G N E L L G S S D                                108
Ns SPDS   - - E S - - - - - - - - - P - - - - - Y I S V L                                45
Hn SPDS1  N E K E S - - - - - - - - - P - - - - - - Y I S V L                            47
Hn SPDS2  E S - - - - - - - - - - - - A - - - - - - Y I S L                              39
                                          * * *

Ab PMT1                                                                              124
Ab PMT2                                                                              125
Hn PMT                                                                               126
Nt PMT                                                                               162
Ns SPDS                                                                               99
Hn SPDS1                                                                             101
Hn SPDS2                                                                              93

Ab PMT1                                                                              178
Ab PMT2                                                                              179
Hn PMT                                                                               180
Nt PMT                                                                               216
Ns SPDS                                                                              153
Hn SPDS1                                                                             155
Hn SPDS2                                                                             147

Ab PMT1                                                                              232
Ab PMT2                                                                              233
Hn PMT                                                                               234
Nt PMT                                                                               270
Ns SPDS                                                                              207
Hn SPDS1                                                                             209
Hn SPDS2                                                                             201

Ab PMT1                                                                              286
Ab PMT2                                                                             K 287
Hn PMT                                                                               288
Nt PMT                                                                               324
Ns SPDS                                                                              261
Hn SPDS1                                                                             263
Hn SPDS2                                                                             255

Ab PMT1                                                                              336
Ab PMT2                                                               E L D          340
Hn PMT                                                                               338
Nt PMT                                                                               375
Ns SPDS   - D A S H N K T L                                              K G K       314
Hn SPDS1  - E S G P - K T I A                                            K G K       315
Hn SPDS2  - A D D S H T K T R                                            N A K       308
```

Figure 8 Alignment of PMT and SPDS amino acid sequences from the solanaceaous plants. Dark-gray highlighting indicates identical amino acid residues in at least four proteins, while light-gray highlighted residues represent similarities. Underlining on NtPMT shows the tandem repeats found in *Nicotiana* PMTs (Hashimoto *et al.* 1998a). Ab, *Atropa belladonna*; Hn, *Hyoscyamus niger*; Nt, *Nicotiana tabacum*; Ns, *N. sylvestris*. Indicated above the AbPMT1 sequence by asterisks are the amino acid positions that are identical in the four PMTs shown and three NsPMTs (Hashimoto *et al.* 1998a) as well as those that are identical in the three SPDSs shown and other four SPDSs (Hashimoto *et al.* 1998b) but different between PMTs and SPDSs. Structurally similar amino acids are grouped as follows: D, E; R, H, K; A, I, L, M, F, P, W, V; and N, C, Q, G, S, T, Y.

PMT and plant SPDSs. PMTs are longer than SPDSs, N-terminal extensions in PMTs being mostly responsible for this size difference. Although the N-terminal extensions in *Nicotiana* PMTs consist of a characteristic tandem repeat array (e.g. NtPMT in Figure 8), the extensions in AbPMTs and HnPMT are not composed of any repeat elements *per se*. Except for the N-terminal extensions in PMTs, PMTs and SPDSs are considerably similar in amino acid sequences. There are, however, several amino acid residues which are only conserved among PMTs and among SPDSs but differ between PMTs and SPDSs. In these conserved amino acid residues, the seven amino acid residues (asterisks in Figure 8) which are identical in the four PMTs shown and three *N. sylvestris* PMTs (Hashimoto *et al.* 1998a) as well as those that are identical in the three SPDSs shown and other four SPDSs of *Escherichia coli*, mouse, human, and *Arabidopsis* (Hashimoto *et al.* 1998b) but different between PMTs and SPDSs, are found, and may important for the function of each enzyme. Some of these signature amino acid residues may be involved in the binding of SAM in PMT and decarboxylated SAM in SPDS.

AbPMT1 is strongly expressed in roots

To determine whether *AbPMT1* and *AbPMT2* are expressed in *A. belladonna* roots, 3'-regions of *AbPMT1* and *AbPMT2* cDNAs were used to hybridize total root RNA of mature *A. belladonna* plants in RNA gel blot analysis (Figure 9A). The hybridization signal obtained with the *AbPMT1* probe was approximately seven times stronger than the signal obtained with the *AbPMT2* probe. Because both probes hybridized to each other to some extent, I then assessed the abundance of *AbPMT* mRNAs by RT-PCR analysis (Figure 10). I designed two PCR primers which anneal to the conserved sequences between *AbPMT1* and *AbPMT2* cDNAs. The size of the expected RT-PCR fragments is 305 bp for both *AbPMT1* and *AbPMT2*, but the identity of the RT-PCR fragments can be assessed by digestion with *Cla* I and *Pvu* II: *Cla* I would cleave only the amplified fragment from *AbPMT1* mRNA into 128-bp and 177-bp fragments, while *Pvu* II would digest the *AbPMT2*-derived fragment into 127-bp and 178-bp fragments (Figure 10A). When the reverse transcripts derived from the total RNA of cultured *A. belladonna* roots were amplified with the primer set, a single band of 305 bp was obtained (Figure 10C, lane R). As calibration controls, known amounts of *AbPMT1* and *AbPMT2*

cDNAs were mixed in varying ratios and amplified as well (Figure 10B; and Figure 10C, lanes P1 to P4). When these 305-bp fragments were cleaved by *Cla* I and/or *Pvu* II, the digested fragment pattern of the root RT-PCR products was similar to the pattern of the P2 mixture, which contained ten times more *AbPMT1* cDNA than it did *AbPMT2* cDNA (Figure 10D, lanes P2 and R). Since the 305-bp PCR fragments were completely cleaved after simultaneous digestion with the two restriction enzymes, single digestions with either enzyme were expected to cleave relevant PCR fragments completely. The results of RNA gel blot and RT-PCR indicated that *AbPMT1* was expressed more strongly than *AbPMT2* in the root of *A. belladonna*.
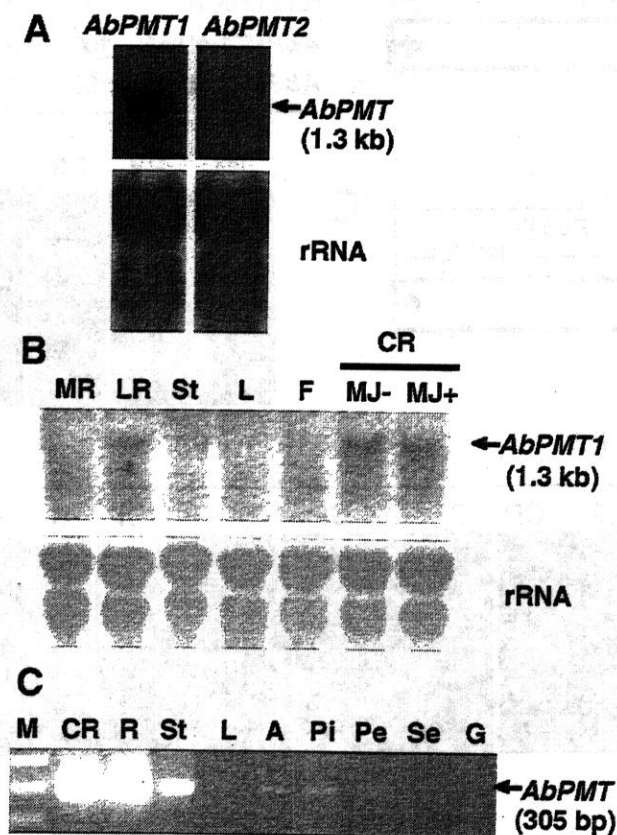
Figure 9 Analysis of *AbPMT* RNAs in *A. belladonna*. (A and B) Gel blots were made from total RNAs (10 µg) isolated from root (A) and various tissues (B) of mature *A. belladonna* plants and probed with either *AbPMT1* (A and B) or *AbPMT2* (A) cDNAs. MR: main root; LR: lateral root; St: stem; L: leaf; F: flower; CR: cultured root with treatment of methyl jasmonate (MJ+) or without treatment (MJ-). (C) RT-PCR analysis of *AbPMT* genes in various tissues (cf. Figure 10). M: 100-bp ladder marker, CR: cultured root; R: root; St: stem; L: leaf; A: anther; Pi: pistil; Pe: petal, Se: sepal; G: genomic DNA (negative control).

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To determine whether *AbPMT1* is expressed in organs other than the root, RNA gel blot and RT-PCR analyses were done in various organs of *A. belladonna*. Northern analysis indicated that *AbPMT1* RNA is present in intact lateral root and cultured root, but the hybridization signal was not found in the main root, stem, leaf, or flower (Figure 9B). RT-PCR analysis showed that expected *AbPMT1* fragments of 305 bp were amplified efficiently from the RNAs of cultured root and intact root and moderately from stem RNA (Figure 9C). These 305-bp RT-PCR products were mostly derived from *AbPMT1* RNA, since they were
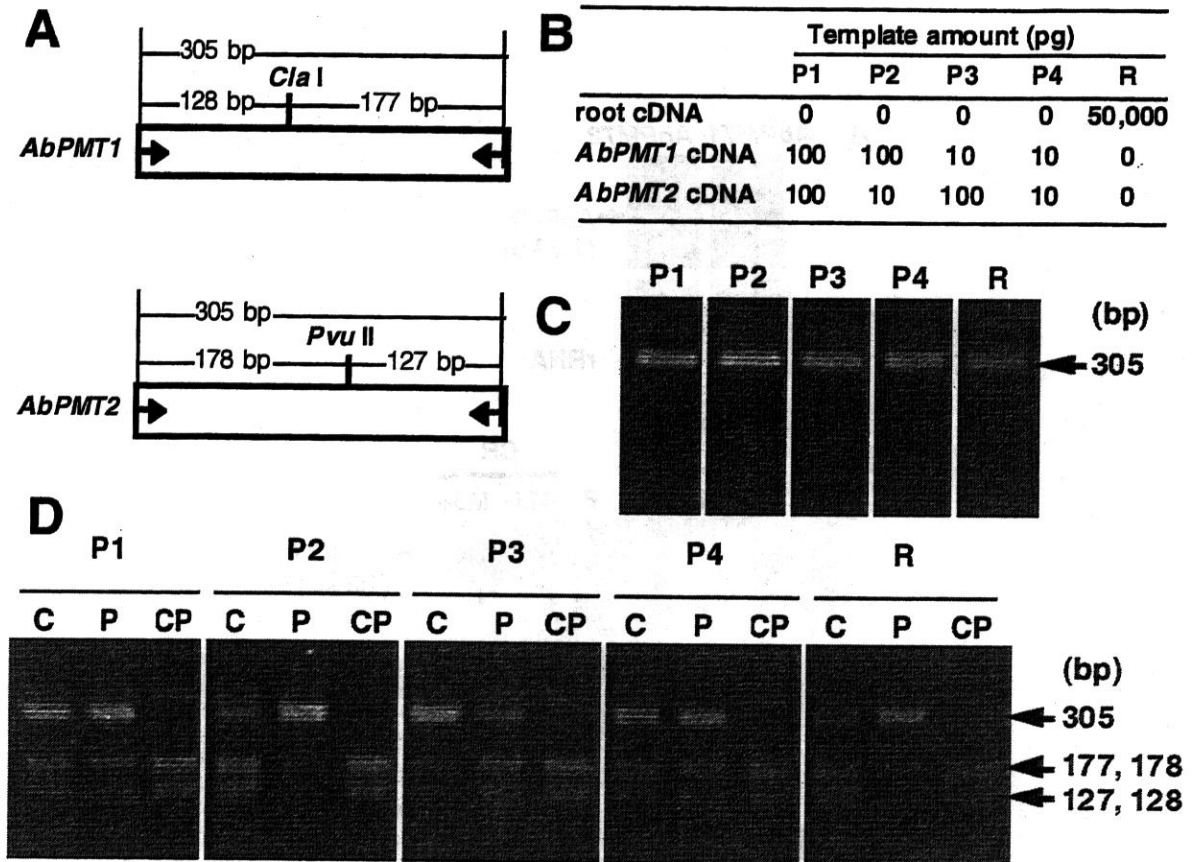
Figure 10 RT-PCR analysis of *AbPMT* genes in cultured *A. belladonna* roots. (A) RT-PCR products from *AbPMT1* and *AbPMT2* transcripts are expected to be 305-bp. *Cla* I and *Pvu* II sites, unique in either fragment, were used to determine the identity of the fragment. (B) *AbPMT1* and *AbPMT2* cDNAs mixed in different ratios (P1 to P4) and 50 ng of cDNAs reverse-transcribed from total RNA of cultured *A. belladonna* roots (R) were used as PCR templates. (C) PCR products were separated on a 2% agarose gel without enzyme digestion. (D) PCR products were first digested with *Cla* I (C), *Pvu* II (P), or *Cla* I plus *Pvu* II (CP) and then separated on a 2% agarose gel.

cleaved by *Cla I* digestion (data not shown). When cultured roots were treated with 20 μM methyl jasmonate for 4 h, RNA gel blot analysis indicated that *AbPMT1* RNA level was not significantly affected (Figure 9B lanes CR/MJ- and CR/MJ+).

Isolation of *AbPMT1* genomic clones

After screening approximately 4 x 10⁵ independent clones from the *A. belladonna* genomic library with the *AbPMT1* cDNA probe, two independent *AbPMT* clones were isolated. Restriction enzyme digestion and Southern hybridization analysis showed that these clones are overlapped and derived from one *AbPMT* gene (Figure 11). The nucleotide sequence of a 5.9-kb *Sac* I-*Sac* I fragment of the λ2 clone was determined and found to correspond to the *AbPMT1* gene (Figure 12). *AbPMT1* consisted of nine exons separated by eight introns, and the intron splice sites were consistent with the consensus splice sites found in other plant genes (Hanley *et al.* 1988). Exons 1 through 9 were 246 bp, 77 bp, 123 bp, 105 bp, 73 bp, 72 bp, 196 bp, 130 bp and 258 bp in length, respectively. They were separated by introns 1 through 8 which were 675 bp, 242 bp, 107 bp, 121 bp, 106 bp, 333 bp, 1329 bp and 79 bp in length, respectively. Comparison with three *N. sylvestris* PMT genes (*NsPMTs*; Hashimoto *et al.* 1998a) showed that intron 3 of *AbPMT1* is missing in *NsPMTs*, but the number and the position of other introns are identical, although their length and nucleotide sequences differ (data not shown).
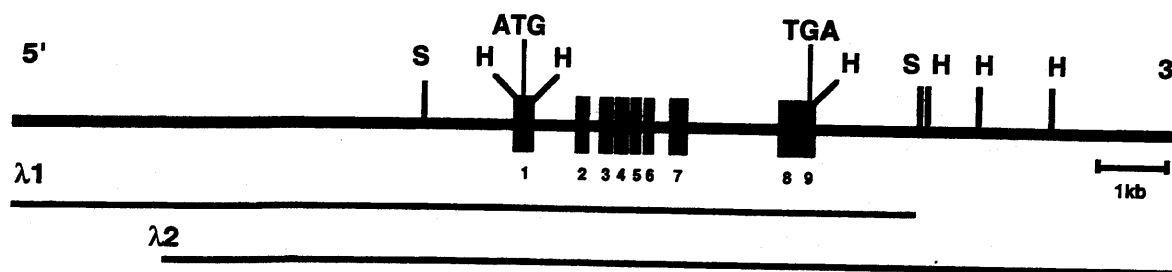
Figure 11 Structure of *AbPMT1*. Two overlapping lambda clones containing *AbPMT1* are indicated below the *AbPMT1* gene structure. Nine exons are shown in black boxes. Translational start and stop codons are shown as ATG and TGA, respectively. Restriction sites are indicated by H (*Hind* III) and S (*Sac* I).

Xho I (-1373)

gagctcgaactcagtctcgggt<u>ctcgag</u>tctgagttcggatctaggtcatgggtcatgggctatcttccagtatttacttttttgatccctgaaatattct -1297
ctatttatattttttggtcctaaaaaaaattcgaaaagtcaacagtcagccaacagtttcagacogttggagaaggactaataaagtctgattatgacgagt -1197
taaagataataatggcgagttaactcatctgatttatgacaaaattgaattcgaaataccctcattcctccctatataaatagcacagtcttgttctcttcag -1097
aacacacacaaaaattcagaagttcttctctctgaaaaagcaatttttttaatattctgttctttgccagaatcgattatatttgcttttgtattatcgatt -997
atttattgttttatcttggaagactattgcatgtttcgtaaaagtaatttacggggtaattttatcttaaagacagagagccatctctcgactcagcctg -897
tttatattgtgtttaaccttctcattgcactttttttacttacaaatttccaatgcatgaaagatgggcttcttcacatgaaaaccgaatagaaaaggaatc -797

BstX I (-748)

atttcaatttattccgagagaaatttgtacagtagtacta<u>ccaataacttgg</u>aaaacaaagtgtgttatcatgcttattataaattaatacgacttcgat -697
tatatgggtgtgatctctcgtggatttgtaggtccattttgccatataataataaacctccaagaaatgtccataacaggtttcatttaattaatatatc -597
atcaacaaccattgcactagggccoggagctatacatgtttgggagtcaattcacacctttttctagaaaaattacattgcagaaataaattaaaatttat -497
ttttatgtatatatatgtactatatattgttgaattttaacctttttcatatatttactttttttatatttaaattttcttaataaaattttatctccacc -397
actaatcgtgttacgttgctaacaaaaatatatgcttactccgtcccactttaaatggcatgattcgaagtactagggtcaaactatttaatttcgaaga -297

EcoR I (-295)

<u>gaattc</u>aacattaaattttcaaactttttgaaaataaaattaatttacatatgtaagaactacatagaacagtgacataagtctcgataaatatattaaaaa -197
aattacagtaaaagaaaattttcttgtaattctccaaatagtaacagtgtcaagacggagggagtaattccaaaatgccgaggacaacattattcacgtt -97

TATA box

gttatgtttcgagtgtaggtgtataagcatatcaaacacttcac<u>tataaat</u>atgcatgcaattcttgaggaaatagcaaggccaaagtgtgaagaaGCTT 4

+1

TGTGAGTGCTAAACTGCAAATTAGTTTCTTCAAATAATTGTTTTTCTCTTATTTAGTTTGAAGAATGGAGGTCAACCACAACAATGGCAGCACCACCAAA 104
ATTATCCTGAAAAATGGCAGTATTTGCAATGGCGATGTTAATGGCAACTCCCACACCCATAAGAAAATTGAGAATAAGCTTGTAGAGTGTACTAACTCTA 204
                       M E V N H N N G S T T K
TCAAGCCTGGTTGGTTTTCTGAGTTTAGCGCACTATGGCCAGgttagtcttaagatacatagaattttgagtttttatattctagatcttttttattaatttt 304
 I I L K N G S I C N G D V N G N S H T H K K I E N K L V E C T N S I
   K P G W F S E F S A L W P G
tttgataaaattatttacatatattaagtagatttttaaaaacataaatataagatctacgctaaagttattaggttaacatgtccttgcttactgatagtg 404
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                  E A F S L K I E K L L F Q G K S D Y Q D V M L F E
acatatactttctccaaccttatttacttgtaattttttacaaaacatagttatttaaaaatatttctcgtattaaaaaattaagatatatatatatatat 1104
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agacaactaattattgctcaaataataatatatcgacagTCAGCAACTTATGGGAAGGTGTTAACATTGGATGGGCAATTCAACATACAGAATGGTGGAT 1304
                                        S A T Y G K V L T L D G A I Q H T E N G G F

AbP-F primer →

TTCCATACACTGAGATGATTGTTCATCTCCCACTTGGTTCCATTCCATCCCCAAGAAG<u>gttaattattaattgtcctttaaattattttttctatttct</u> 1404
 P Y T E M I V H L P L G S I P S P K K
<u>tcatatattaaataaatacaaagcacacagagtatcttaattatttgtgtttttcgtgtatttcag</u>GTTTTAATCATCGGTGGAGGGATTGGTTTCACAT 1504
                                                                  V L I I G G G I G F T L

Cla I

TGTTTGAGGTCTCTCGTTACCCAACTATCGACACAATAGACATAGTTGAG<u>ATCGAT</u>GACGTGGTTGTAGATgtaagttaaattcaaattagagtactctt 1604
 F E V S R Y P T I D T I D I V E I D D V V V D
caaattatagagtacatgatccatgatagtgtaaatgtttttatcattttcaaaaactataaaatgaatataagttattttcctctcttgatagGTATCTAG 1704
                                                                                              V S R
AAAGTATTTCCCGTACTTAG<u>CAGCAG</u>GATTCGATGATCCCAGAGTAACCCTTATTATTGGCGATGgtaattaattaattaatgacacaagttatataata 1804
 K Y F P Y L A A G F D D P R V T L I I G D G
ggagtattattttttatttattgtatattctattttcctcaaccgtgttaattttttctcttcacctcgtcagGAGCTGCATTCGTGAAAGCTGCTCAACCT 1904
                                                                         A A F V K A A Q P
GGATACTATGATGCCATTATTGTGGACTCTTCTGATCCTATTG gtaaatttatttgacattcaaccaccagttatttatagaattttccttattcggtta 2004
 G Y Y D A I I V D S S D P I G
                                    ← //
                                    AbP-R primer
ccatgtaattgctttgttaattggatagtaatatatagttcctccgtcccaattttatgtgatacttttatctttatagattcaaacttaaaatagagg 2104
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ggcaaatcggtataaaaacttgaaaattcatatattattaatgtatataaaacttaaactcggggtatgtttatacag GTCCAGCAAAGACTTGTTTGAAA 2304
                                                                      // _____
                                                                   AbP-R primer
                                                                     P A K D L F E R
GGCCATTCTTCGAAGCAGTGGCGAAAGCGCTAAGGCCAGGAGGAGTAGTGTGTACACAAGCAGAGAGCATTTGGCTTCACATGCATCTTATTAAGCAAAT 2404
 P F F E A V A K A L R P G G V V C T Q A E S I W L H M H L I K Q I
TATTGCTAATTGTCGCCAAGTCGTTTAAGGGTTCTGTCAACTATGCATGGACTACAGTTCCTACTTACCCTACGtacgtaattctctttcaccatattgtt 2504
 I A N C R Q V F K G S V N Y A W T T V P T Y P T

- 24 -

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GTGGTGTTATCGGTTACATGCTTTGCTCTACGGAGGGACCAGAAGTTAATTTCAAGAATCCAGTGAACTCTATTGACAAAGATACCAGCCATGTCAAATC 3904

   G V I G Y M L C S T E G P E V N F K N P V N S I D K D T S H V K S

         PMT2F primer →

CAAGGGACCTTTGAAGTTCTACAACTCTCGATgtaattacacttctcaccacatgtttttttatttattttttgttaatttgtcaattaaccctttgctcttc 4004

   K G P L K F Y N S D

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   I H K A A F I L P S F A R D L V E F .

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gaaaaagaaaagaaaaattgctactc 4530

Figure 12 Nucleotide sequence of *AbPMT1*. The nucleotide sequence of the nine exons and its deduced amino acid sequence are shown with capital letters. The 5' end of *AbPMT1* cDNA clones was arbitrarily dubbed as +1. The vertical lines at -1373 (*Xho* I), -748 (*BstX* I), and -295 (*Eco*R I) indicate the 5' end of the *AbPMT1* upstream regions fused transcriptionally to the GUS reporter gene. A presumed TATA box is also boxed.

Expression of *AbPMT1*

Since five of the six *AbPMT1* cDNA clones had the same 5' ends, this terminal guanidine was arbitrarily designed as +1 (Figure 12), the 1373-bp 5'-upstream region from the adenosine at -1 as *AbP-1373*, and so forth. After *AbP-1373*, *AbP-748*, and *AbP-295* had been transcriptionally fused to the GUS reporter gene in a pBI 101 plant transformation vector (Figure 13F), these transgenes were introduced via *Agrobacterium rhizogenes* into *A. belladonna*. Of the 55 hairy root clones containing the *AbP-1373::GUS* transgene, 23 clones showed distinct GUS staining. In such GUS-positive roots, a few cell layers in the vascular cylinder were stained blue (Figure 13A and B). Cross sections showed that the pericycle cells were specifically stained (Figure 13E). In situ hybridization detected pericycle-specific expression of *AbPMT1* in *A. belladonna* lateral roots (data not shown), but the *AbPMT1* signal

Figure 13 GUS expression patterns in transgenic *A. belladonna* hairy roots. The hairy roots stained for GUS activity contained *AbP-1373::GUS* (A, B and E), *AbP-748::GUS* (C), or *AbP-295::GUS* (D) transgenes. (A to D) Whole-mount staining. The boxed region in A was enlarged in B. (E) Cross-section of A. Bars in A, C, and D indicate 1 mm, while bars in B and E show 100 μm. PC, pericycle; EN, endodermis; CO, cortex. (F) *AbPMT1* 5′-deleted promoter::GUS fusion constructs. Restriction sites used to make the deletion clones are indicated by B (*Bst*X I), E (*Eco*R I), H (*Hin*d III), S (*Sac* I), and X (*Xho* I).

was very weak compared to the *H6H* signal, which was also localized in the pericycle cells (see below). In some, but not all, GUS-positive clones, GUS activity was also detected at the root meristem. This GUS staining at the root tip was variable among hairy root clones and may not represent the actual localization of *AbPMT1* mRNA, as was reported for similar aberrant expressions of the *H6H* promoter at the root tip (Kanegae *et al.* 1994, see below). *A. belladonna* hairy root clones containing the *AbP-748::GUS* transgene (Figure 13C) or the *AbP-295::GUS* transgene (Figure 13D) also showed the same GUS staining in a few cell layers in the vascular cylinder as the *AbP-1373::GUS* transgene. The strength of the *AbPMT1* promoter, however, appeared to decrease in accordance with the progressive 5' truncation, since 18 out of 53 and 1 out of 35 transgenic hairy root clones, respectively containing the *AbP-748::GUS* transgene and the *AbP-295::GUS* transgene, showed clear GUS staining. When these hairy root clones were cultured in the medium containing either 0.5 or 5 μM indole-3-butyric acid for 1 h or in the medium containing 2, 20 or 200 μM methyl jasmonate for 16 h, no significant quantitative or qualitative change in GUS staining was observed (data not shown). RT-PCR analysis confirmed that expression of endogenous *AbPMT* genes in the transgenic hairy roots was not significantly affected by these plant hormones (data not shown).

In transgenic *A. belladonna* plants, 3 out of 15 lines, 2 out of 17 lines, and 2 out of 24 lines, respectively containing *AbP-1373::GUS*, *AbP-748::GUS*, and *AbP-295::GUS* transgenes, showed detectable but very weak GUS expression in the stele region of the root, but the leaf, stem and flower did not express GUS at any detectable level (data not shown). In conclusion, the 295-bp 5'-upstream region of *AbPMT1* used in this study was sufficient for the pericycle-specific expression in *A. belladonna*.

2. Molecular analysis of *H6H* genes

Isolation and characterization of *AbH6H*

After screening approximately 4 x 10⁶ independent clones from the *A. belladonna* genomic library with the *H. niger H6H* cDNA probe, six positive clones were isolated. These clones were classified into two groups according to restriction enzyme digestion and Southern hybridization analysis (Figure 14). In the first group, λ5 and λ8 clones contained a portion of the *AbH6H* gene which covers the entire cDNA sequence. In contrast, all four clones in the second group (λ10, λ11, λ12 and λ14) representing the *AbψH6H* gene lacked the 5'-flanking region and were truncated at a similar position. This genomic region might be hypersensitive to either physical force or *Sau*3 A1 digestion, thus representing a highly vulnerable site for cleavage during genomic library construction.

The nucleotide sequence of a 4.3kb *Not* I-*Xho* I *AbH6H* fragment cloned from the λ8 clone was determined (Figure 15, accession number AB017153). *AbH6H* is organized into four exons separated by three introns, and the intron splice sites were consistent with the consensus splice sites found in other plant genes (Hanlel and Schuler 1988). Exons 1 through 4 were 186 bp, 278 bp, 319 bp and 248 bp in length, respectively. They were separated by introns 1 through 3 which were 618 bp, 756 bp and 625 bp in length, respectively. Comparison with the *H. niger H6H* gene (*HnH6H*) showed that the length and nucleotide sequences of the introns differ, but their number and positions are identical. Moreover, the deduced amino acid sequence of AbH6H was 91% identical to that of HnH6H (data not shown).

The transcription initiation site was determined by 5'-RACE (Figure 15). Analysis of ten independent clones showed that five clones ended at adenine (+1), while five other clones ended at various positions downstream (+4, +4, +37, +69 and +91). The consensus for the residues adjacent to the transcription initiation site of plant genes (CTC<u>A</u>TCA, from -3 to +4; Joshi 1987) suggests that the adenine indicated in Figure 15 is the transcription initiation site. A putative TATA box (TATAAAT) was found at -33 to -27. Two perfect repeating units (TCAATTAATT) were present from -582 to -573 and from -541 to -532. A potential Myb-

binding site (TAACTG) was located in reverse orientation at -207 to -201.
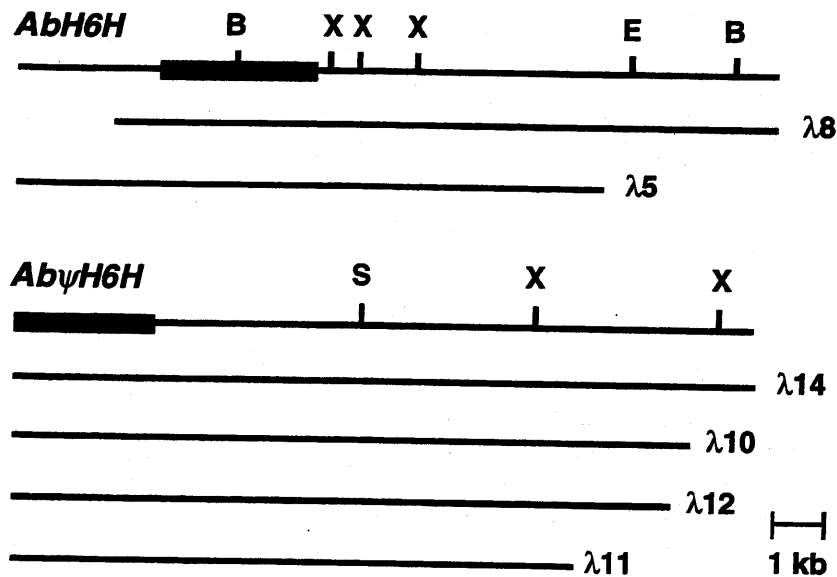
Figure 14 Lambda clones containing two *A. belladonna* *H6H*-related sequences. The regions which hybridized to the *HnH6H* cDNA probe are shown in black boxes. The lambda clones were classified into two groups, *AbH6H* and *AbψH6H*. Restriction sites are indicated by B (*Bam*H I), E (*Eco*R I), S (*Sac* I), and X (*Xho* I).

actcgatccactcataaactaaaaatgatatttaggtacgcgccttcatcgttattagggtggtaaaatttatccataaaaatatgatttctcaattaat -572
ttaagtttgagcaggtcaatgactcattcatcaattaattcaatctactttgacctgcataattcagtccacctaataattggattgatgtgtaatttaa -472
attgaccogctcatttttattaaatatgttatatagattcatattccaatcaaactcttaaaattgataaaagttggatgggttgagttatgacctattg -372
tttagcgtcttttgactcagtctatttcaactcacgtaacatttgagttgattaatgacttgttcatttattaactcaattctgatctcaaattcaaccc -272
aatctatccattggtaagagttaagcgggttaggttgtgatccattatttagcccatttttaact caqtta tttatggtaattaatattttagcaggttat -172
tgatcctttcatttgttaaatcataaaaaatcttatcaaaatatttaatacatgaatggaggggtcattaacgtgccaaatttatgtagtttgagctgat -72

TATA box   +1
tatattaaattaacctcaaggtactgggtccataacac tataaat ggcctttagctcttcatagtacatt gcaaatcttcaccatatccacaacataa 29

                                                Acc I
tttctttcttatttgagactttttactcctaaaaaacaaaaaaaaaATGGCTACTCTTGTCTCAAATGG TCTCCACA AACAATGTTTCTGAAAGTTTTGTAGC 129
                                        M A T L V S N W S T N N V S E S F V A   19
ACCATTAGAGAAAAGGG CAGAAAATGATGTTCCTTTAGGAAATGATGTGCCTATCATTGATCTCCAACAAGATCA CCTTGTTGTTGTTCAACAAATCACC 229
            5R-H3 primer   5R-H2 primer
P L E K R A E N D V P L G N D V P I I D L Q Q D H L V V V Q Q I T   52
AAAGCTTGTCAAGATTTTGGTCTCTTTCAGgttcccaaattcttcttttccaaactctttcatcttattaattaggactttttgtgtatagagtttctga 329
      5R-H1 primer
K A C Q D F G L F Q   62
tacgtgaattgatacaacaacaatataccoggtaaaatttcacaaagtggaatttttggaatacatgcattgatattagtatataaatttttttgttagtca 429

- 29 -

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tattttgttgacctattactaattcttcttaataaatatgataacttgaaaaataaagtagtaataacataaacagacgtggacgacatttttagcattg  529
tcacatttcaagaccatggcatataatgtcttctttggctcaacaagccttaagtatttatttctttgttacaccatcatttaactcaaacatgcgcatg  629
atatgtgaacttgttttatacctgataaaactcttcacttttctctttgattttaatgtgagattctcttaaaatatatatcatgtgcacccttcttcaa  729
aagtttgcctagatgtctttccgccctccgtcatgggcatcacaagcctaaatcagtaaagaaattgtggtgcttttaggaaacaaaggcacaattta  829
gatcgcgtactaccgaaattttatatatatatatttaattcttgaaacagGTGATCAACCATGGATTACCAGAAAAGCTGATGGCAGAAACAATGGATGTGT  929
                                        V  I  N  H  G  L  P  E  K  L  M  A  E  T  M  D  V  C   80
GCAAAGAATTTTTTGCACTGCCTGCTGAGGAGAAAGAAAAGCTTCAACCAAAAGGAGAGCCAGCTAAATTTGAGCTTCCACTTGAGCAGAAAGCAAAGCT 1029
    K  E  F  F  A  L  P  A  E  E  K  E  K  L  Q  P  K  G  E  P  A  K  F  E  L  P  L  E  Q  K  A  K  L  113
                                            H6H-F primer
TTATGTTGAAGGAGAACAACTCTCTGATGAAGCGTTCCTTTACTGGAAGGACACTTTGGCTCATGGTTGTCATCCTCTTGATGAAGAGTTAGTCAACTCC 1129
    Y  V  E  G  E  Q  L  S  D  E  A  F  L  Y  W  K  D  T  L  A  H  G  C  H  P  L  D  E  E  L  V  N  S   146
TGGCCTGAAAAACCAGCAACATATAGgtgaatgactaatgaataataataatctccagagatagataaagcattacaagaaataatggagtactacatgt 1229
W  P  E  K  P  A  T  Y  R                                                                            155
gatatatcaaaaaaaaaagtaatatcaaatttttttttacactaccagatattctaaatgataattatatgtgatcattcataaaaaaaaaatgagatcaataa 1329
catgaaaattatattatgcaagttatcagctatacatgttaaaaggatatgtattatgtaaaaaattcttttagattgaaagtacatatatatgacttaact 1429
cctttaaaaattaatttaagctatatacattgacatcaatgacaaagctaaagataaggaaggggggtttaactgaatcgccttcatccggatgttacaa 1529
ggtggaagatacaagataattccttaatataagggaagcttctaggtatggaatttttctattttcttgaatcccttgttaaattttcttgttgcactc 1629
tcagttatagtttaactgattatagcaggttcattactgtttacctgtcaatattatcgtatggatgtgcaactcgtaattaccttataacttataaac 1729
gtgactgtgaaattatttaaattttttgtgaatatttttagaaacaacatatctctatagttgattaaccaaaaagaaaagcaaattcagtccctaaaatt 1829
tgcatcagcattcataaataatggtacatggttgatatatttttttattttttaactttaaaattgattgtttttttttgccagAGAGGTGGTGGCTAAATA 1929
                    BamH I                                                    E  V  V  A  K  Y  161
TTCAGTGGAAGTGAGGAAGTTGACCATGAGGATCCTGGACTACATCTGTGAAGGACTTGGGCTTAAATTGGGCTACTTTGATAATGAGCTTAGCCAAATT 2029
    S  V  E  V  R  K  L  T  M  R  I  L  D  Y  I  C  E  G  L  G  L  K  L  G  Y  F  D  N  E  L  S  Q  I   194
CAGATGATGCTGACTAACTATTACCCACCATGTCCAGACCCAAGTTCAACATTGGGTTCAGGAGGACACTATGATGGTAACCTTATTACTTTGCTTCAAC 2129
                                            H6H-R primer
Q  M  M  L  T  N  Y  Y  P  P  C  P  D  P  S  S  T  L  G  S  G  H  Y  D  G  N  L  I  T  L  L  Q  Q  228
AAAACTTGCCTGGCTTGCAACAACTCATAGAGGATGCAAAATGGATTGCTGTTGAACCTATCCCTACTGCTTTTGTGGTCAATTTGGGATTGACTCTAAA 2229
    N  L  P  G  L  Q  Q  L  I  E  D  A  K  W  I  A  V  E  P  I  P  T  A  F  V  V  N  L  G  L  T  L  K   261
Ggtagttgatatagatccggtttattttttattaagattaagatatcacaaattgaatacacatgtgaatattaagatgtgtaataagattaagatatgtg 2329
aatctgaatacacatataaatattatgagatattataaaattaaggtgtgtgaatctgaatgttaagatttttgtattaagatttgaatactaaactatta 2429
ggacaatttattttctcaacatctgaaaacataaaacttttttttattacatcatcaataaaatatttttttaatatggtggttggtggaggtgaaggcga 2529
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                                            V  I  T  N  E  K  F  E  G  S  I  H  R  V  V  T   277
AATCCAACAAGAGACAGGGTTTCAATTGCAACTTTGATTGGTCCTGATTATTCTTGCACCATTGAACCTGCTAAAGAATTACTCAGCCAAGACAATCCCC 3029
N  P  T  R  D  R  V  S  I  A  T  L  I  G  P  D  Y  S  C  T  I  E  P  A  K  E  L  L  S  Q  D  N  P  P  311
CACTCTACAAACCTTATTCATATGCTGAATTTGGTGAGATTTACCTAAGTGATAAATCAGACTATGATGCTGGTGTTAAGCCATATAAAATTAATGCCTA 3129
    L  Y  K  P  Y  S  Y  A  E  F  G  E  I  Y  L  S  D  K  S  D  Y  D  A  G  V  K  P  Y  K  I  N  A  .  343
Agcaataagttaaatttattgtcagagcaaatttcctaatgtgctaaagtacaaaaatattcctatgggtattgcatgctctgtataattgattccctat 3229
atgaaggtttgtgaattatggcatgttgaattactgtctattaaataagttaaaacctcaagtcatatgtatatgaggtttctgtgtggtgttatttggt 3329
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cagttcttactcatatttaaattacacgtggaacagtcaaaatagttatattaactatcaatcatttttttcattaaagttgtcttgtgacagcgagagct 3729
tggcattgaaccctagtttgtatattaagatccatataaagtgcacacataaaggaggcagacctaaaacccaattaaaaacaat[aaatgaattggacata 3829
agtatgagacttacaaagaaataacacatccctgcctatgtcaaactgtgttatttccgaaagaattagcttgtgaacgaaatttcactcataagcaggt 3929
cacatcgtgatttagtttctatttcggagattattataccaataaagagttctattcttctccctcggttatgagtgattcaataacttgggattccttc 4029
actacattccaag                                                                                        4042
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Figure 15 Nucleotide and deduced amino acid sequences of *AbH6H*. Transcription start site was determined by 5' RACE and is shown as +1. Arrows indicate primers used for PCR experiments. Single and double underlines are conserved sequences found in the 5'-flanking regions of both *AbH6H* and *HnH6H*. The boxed *Bam*H I site at +1960 was used for expression analysis indicated in Figure 17, whereas the boxed *Acc* I site at +99 was used for translational fusion to the GUS reporter gene. The presumed TATA box is also boxed.

*Ab*ψ*H6H* is a pseudo gene

The 1.6-kb nucleotide sequence of the λ14 clone representing *Ab*ψ*H6H* was determined (Figure 16, accession number AB017154). This clone started at a position corresponding to the first intron of *AbH6H*, and lacked a putative upstream first exon. The 1.6-kb sequenced region of *Ab*ψ*H6H* was similar to the *AbH6H* sequence, and can be organized into two putative exons (exon 2 and exon 3), interrupted by a putative intron (intron 2, Figure 17). The nucleotide sequences of the putative exons 2 and 3 of *Ab*ψ*H6H* were 77% and 94% identical, respectively, to the corresponding exons of *AbH6H* (data not shown). The deduced amino acid

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tctagaaaaaatacattgtagagataggtcaatttttatgtatattatacactgaatctccttgataatgtgtgtttatgtattttcttttatatttaa   100
ctcccccttagtgaatccatcactgattatcagtgcatataaattaaattaagtcttgaaacagGTGACCAACCATGGATTTCCAGAAAGCTAATGGTA   200
                                                V  T  N  H  G  F  P  E  K  L  M  V          12
                                                V  I  N  H  G  L  P  E  K  L  M  A          74
GAGACAATGGAAGTGTGCAGAGAGTTTTTTGCACTGCCTGCTGAGGAGAAGGAAAAGCTTCAGCCAAAAGGAGAGCCAAATAAATTGAA-----------   288
E  T  M  E  V  C  R  E  F  F  A  L  P  A  E  E  K  E  K  L  Q  P  K  G  E  P  N  K  F  E  -  -  -  -   42
E  T  M  D  V  C  K  E  F  F  A  L  P  A  E  E  K  E  K  L  Q  P  K  G  E  P  A  K  F  E  L  P  L  E  108
-----------------------CAACTCTCTAAGGAG---TTCTTTTACTGGAAAGACACTTTGACTCATGGTTGTCATCCTCTGGATGA   355
-  -  -  -  -  -  -  -  -  -  Q  L  S  K  E  -  F  F  Y  W  K  D  T  L  T  H  G  C  H  P  L  D  E   64
Q  K  A  K  L  Y  V  E  G  E  Q  L  S  D  E  A  F  L  Y  W  K  D  T  L  A  H  G  C  H  P  L  D  E   141
AGAGTTAGTCAGCTCCTGGCCTGAAAAACCAGCAATATATAGgtgatcaacactataataaataataatctttctagaactattaatggagtactagtat   455
E  L  V  S  S  W  P  E  K  P  A  I  Y  R                                                     78
E  L  V  N  S  W  P  E  K  P  A  T  Y  R                                                     155
atagttctagaaaaggtttttaacttcaatacacgaacatataaaagatttttttacactattaggtcacttaaattataattaaatgtgacctttataaa   555
aagggagattaataatctggaaataatgtaggttaaactccagttattgtgtaaaaattattttacactataaatacatgtatatgagttatacacttat   655
tggaaatgaatttaaaggtaaagggaagtaagggaattcaattgaaccctttgttcggatattacactatataaataggtaagaataaatttgtaaata   755
tatatataaacagatggattcctttaacatatatataagggaagcttctaagtgtggattcttttttcattttcttaatcctcttgttaaaattcccgtctttt   855
attattgattttcaatgtaaaatttatttgcactaccaatatagtttttaacttattacagcagatttgttaacatttattaactaatattaaactacaaa   955
tgctcctattagtattaatgaaaacttaatccaagtttccaactctctcactaagattgatcttgcaaacaatgtttatcatatagatttacagcttgta  1055
attacccttataaaattgtgtaaaaattggagtatcttttatgtcagtatatacaagttaagtcattttgtatttttgaaaaacaacatatctctataact  1155
agtgaataaaaaaaaaacagattcagtccctaacattgcatgatcagctagcatgcataagtaggactagtatatatagggttaattttttattttatatt  1255
ttttgctttaaagggattatttttcttttttgggcagAGAGGTAGTGGCTAAATATTCAGTGGAAGTGAGGAAGTTGACGATGAGGATGCTGGACTACATCT  1355
                                     E  V  V  A  K  Y  S  V  E  V  R  K  L  T  M  R  M  L  D  Y  I  C   100
                                     E  V  V  A  K  Y  S  V  E  V  R  K  L  T  M  R  I  L  D  Y  I  C   177
GTGAATGACTTGGACTTAAATTGGGCTACTTTTATAATGAGCTTAGCCAAATTCAGATGATGCTGACTAACTATTACCCACCATGCCCAGACCCAAGTTC  1455
E  .  L  G  L  K  L  G  Y  F  Y  N  E  L  S  Q  I  Q  M  M  L  T  N  Y  Y  P  P  C  P  D  P  S  S  133
E  G  L  G  L  K  L  G  Y  F  D  N  E  L  S  Q  I  Q  M  M  L  T  N  Y  Y  P  P  C  P  D  P  S  S  210
AACATTGGGTTCAGGAGGACACTATGATGGTAACCTTATAGCTTTGCTTCAACAAGACTTTCCTGGCTTGCAACAACTCATTGTTAAGGATGGCAACTGG  1555
T  L  G  S  G  G  H  Y  D  G  N  L  I  A  L  L  Q  Q  D  F  P  G  L  Q  Q  L  I  V  K  D  G  N  W  166
T  L  G  S  G  G  H  Y  D  G  N  L  I  T  L  L  Q  Q  N  L  P  G  L  Q  Q  L  I  -  E  D  A  K  W  242
ATTGCTGTTGAACCTATCCCTACTGCTTTTGTGGTCAATTTGGGATTGACTCTAAAGgtacc                                       1617
I  A  V  E  P  I  P  T  A  F  V  V  N  L  G  L  T  L  K                                       185
I  A  V  E  P  I  P  T  A  F  V  V  N  L  G  L  T  L  K                                       261
```

Figure 16 Nucleotide and deduced amino acid sequences of *Ab*ψ*H6H* containing two putative exons (exon 2 and exon 3). The amino acid sequences of the corresponding exons of *AbH6H* are described under the sequence of *Ab*ψ*H6H*, respectively.

Figure 17 Expression analysis of *Atropa* H6H genes by RT-PCR. (A) Genomic structures of *AbH6H* and *AbψH6H* in the regions of exons 2 and 3. PCR primers are shown by arrows, and expected RT-PCR products are indicated below the corresponding gene structures. Restriction sites are indicated by B (*Bam*H I), Xb (*Xba* I), and K (*Kpn* I). The dotted lines in *AbψH6H* indicate regions which have yet to be sequenced. (B) Cloned genomic DNA sequences for *AbH6H* (λ8 clone) and *AbψH6H* (λ14 clone), and *A. belladonna* genomic DNA, were used as PCR templates in lanes 1, 2, and 3, respectively. M represents λ marker digested with *Bst*P I. (C) Total RNA isolated from cultured *A. belladonna* roots was amplified by RT-PCR with the primers shown in A. RT-PCR products were separated either directly (lane 1) or after *Bam*H I digestion (lane 2) on a 1.2% agarose gel, transferred onto a membrane, and hybridized with radio-labeled *HnH6H* cDNA as the probe.

sequence of this region in AbψH6H was 86% identical to the corresponding part of AbH6H (data not shown). In contrast, the nucleotide sequence of putative intron 2 was less similar to that of intron 2 from AbH6H (53% identity). The omission of 42 nucleotides from the putative exon 2 of AbψH6H would result in the deletion of 14 amino acid residues from the corresponding H6H protein (from Leu105 to Glu118 in AbH6H, Figure 16). Moreover, an amino acid codon corresponding to Gly179 in AbH6H (Figure 16) is a stop codon (TGA) in AbψH6H. Thus, AbψH6H would encode a nonfunctional, truncated H6H protein if the gene were to be expressed.

To determine the expression of AbH6H and AbψH6H in cultured A. belladonna roots, fragments of potential H6H cDNAs corresponding to exons 2 and 3 of AbH6H and AbψH6H were amplified by RT-PCR (Figure 17). The AbH6H cDNA was expected to be 270-bp long and cleavable into 125-bp and 145-bp fragments at a BamH I site, whereas the AbψH6H cDNA, if present, would also be 270-bp long yet possess no BamH I site. When A. belladonna genomic DNA was amplified by PCR, two fragments of approximately 1.0-kb and 1.2-kb, respectively corresponding to AbH6H and AbψH6H, were obtained (Figure 17B). RT-PCR amplified a 270-bp fragment from cultured root RNA. After digestion with BamH I, this fragment was completely cleaved into 125bp and 145bp fragments. These results indicate that AbH6H is expressed in cultured A. belladonna roots, but AbψH6H is not. Taken together with its presumed non-functional ORF, AbψH6H must be a pseudo gene.

AbH6H is expressed in the root pericycle and the tapetum

To determine whether AbPH6H is expressed in organs other than the root, RNA gel blot and RT-PCR analyses were done in various organs of A. belladonna. Northern analysis indicated that AbH6H RNA is present in intact lateral root and cultured root, but the hybridization signal was not found in the main root, stem, leaf, or flower (Figure 18A). Expression of AbH6H in various organs was studied by RT-PCR analysis (Figure 18B). Expected amplification products of 270-bp were obtained from RNAs of cultured root, intact root, and anther from A. belladonna. These DNA fragments were confirmed to be derived from AbH6H RNA by digestion with BamH I (data not shown). AbH6H was not expressed in stem,

leaf, pistil, petal, and sepal tissues. When cultured roots were treated with 20 μM methyl jasmonate for 4 h, RNA gel blot analysis indicated that *AbPH6H* RNA level was not significantly affected (Figure 18A lanes CR/MJ- and CR/MJ+).
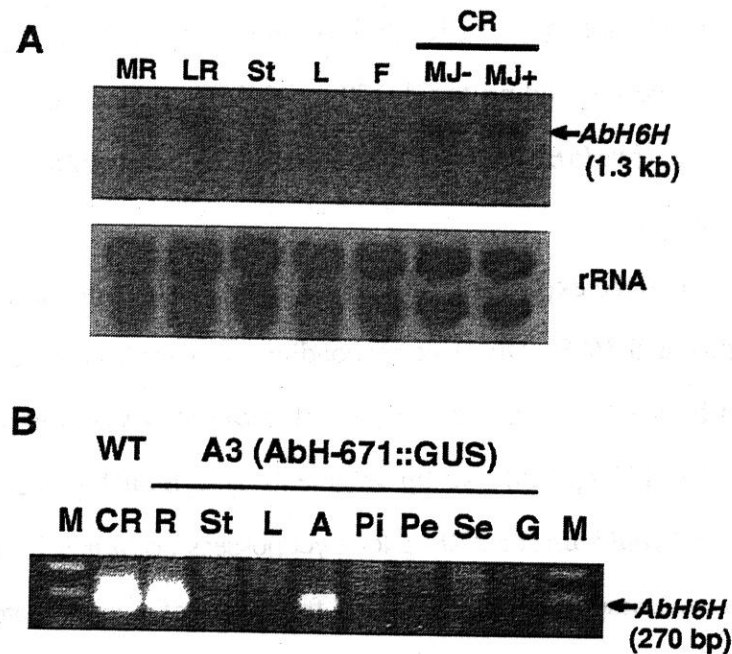
Figure 18 Analysis of *AbH6H* RNA in *A. belladonna*. (A) Gel blots were made from total RNAs (10 μg) isolated from root various tissues of mature *A. belladonna* plants and probed with either *AbH6H* cDNA. MR: main root; LR: lateral root; St: stem; L: leaf; F: flower; CR: cultured root with treatment of methyl jasmonate (MJ+) or without treatment (MJ-). (B) Total RNA was isolated from wild-type cultured roots and the transgenic plants (A3 line) containing the *AbH-671::GUS* transgene, and amplified by RT-PCR with H6H-F and H6H-R primers. M, 100-bp ladder marker; CR, cultured root; R, root; St: stem; L, leaf; A, anther; Pi, pistil; Pe, petal; Se, sepal; G, genomic DNA (negative control).

In situ hybridization was used to localize the cell types in which *AbH6H* is expressed (Figure 19). For the root samples, cultured roots of *H. niger* were also included because they express a higher level of *H6H* RNA in the root (Kanegae *et al.* 1994). Longitudinal and cross sections of cultured *H. niger* roots (Figure 19A, B) showed that *H6H* mRNA was detected only in pericycle cells. No signal was observed in lateral root primordia, which develop from pericycle cells adjacent to primary xylem poles. The pericycle cells near the xylem poles often

stained more strongly than other pericycle cells. The *H6H* signal in pericycle cells was much

stronger in the differentiation zone, where xylem vessels are formed and lateral roots develop,

than in the developmentally younger region including the elongation zone (data not shown).
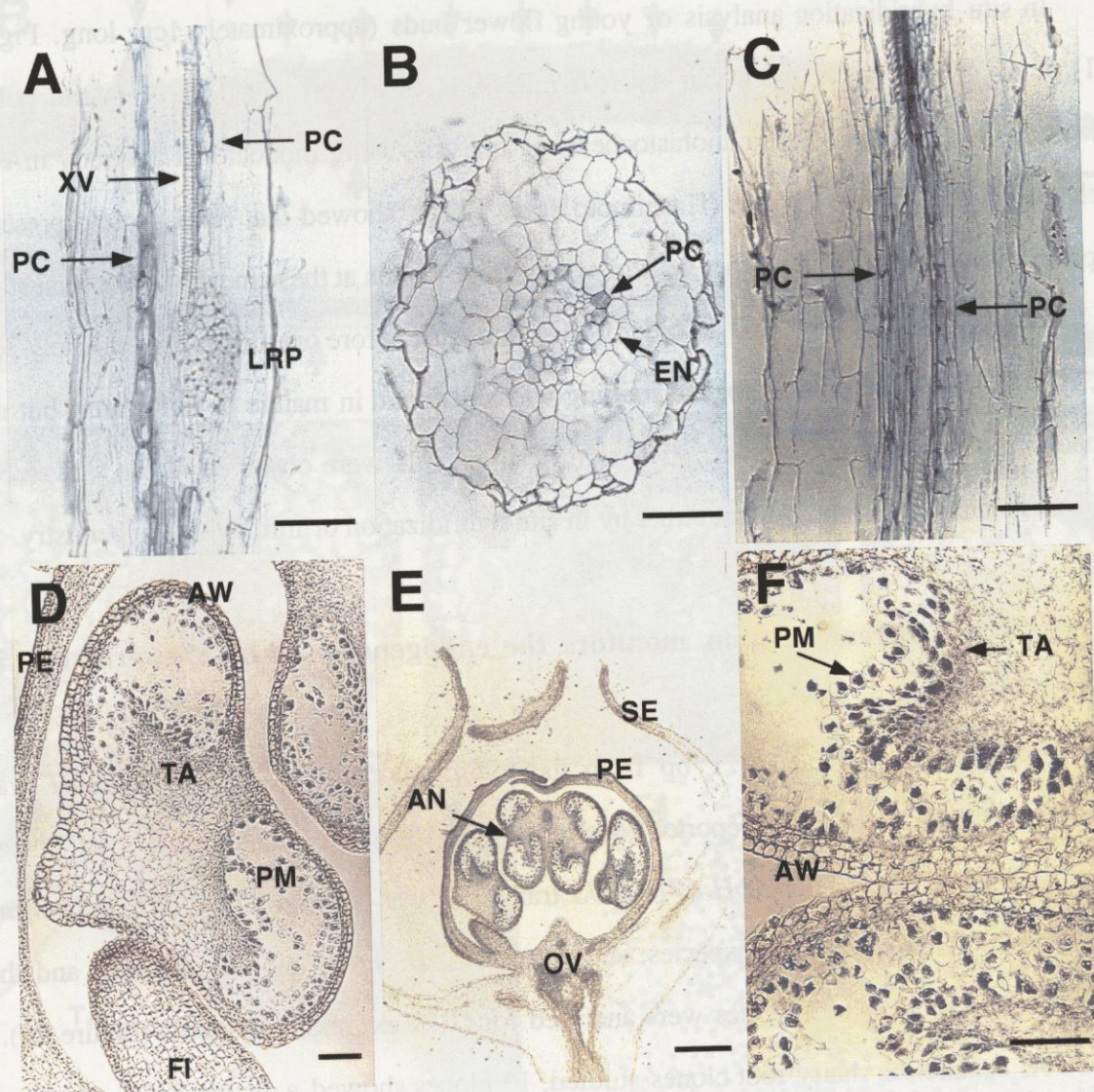
Figure 19 Localization of *AbH6H* RNA and protein. (A-D) In situ hybridization.
Longitudinal (A) and cross (B) sections of *H. niger* cultured roots, and longitudinal
sections of *A. belladonna* cultured roots (C) and young flower bud (D) were
hybridized with an *AbH6H* antisense probe. (E-F) Immunohistochemistry.
Longitudinal sections of *A. belladonna* flower bud were probed with an anti-H6H
monoclonal antibody. Bars in A to D, and F indicate 100 μm, while a bar in E shows
1mm. AN, anther; AW, anther wall; EN, endodermis; FI, filament; LRP, lateral root
primordia; OV, ovary; PC, pericycle; PE, petal; PM, pollen mother cells; SE, sepal;
TA, tapetum; and XV, xylem vessel.

Longitudinal sections of cultured *A. belladonna* roots showed that pericycle cells were moderately stained (Figure 19C). The signals were weaker than those found in *H. niger* roots. The root apices of *H. niger* and *A. belladonna* gave a brownish background color with both antisense and sense probes, and no clear *H6H* signals were observed in this region (data not shown).

In situ hybridization analysis of young flower buds (approximately 1cm long, Figure 3B-1) of *A. belladonna* revealed that *AbH6H* mRNA is clearly present in tapetum and pollen mother cells (Figure 19D). Immunohistochemical analysis, using monoclonal antibody mAb5, which recognizes AbH6H protein (Hashimoto *et al.* 1991), showed that H6H protein presents in tapetum tissues and pollen mother cells of young flower buds at the same developmental stage as in Figure 19D (Figure 19E, F). In older flower buds just before opening (approximately 3cm long, Figure 4B-3), the immunological staining was still found in mature pollen grains, but not in tapetum tissues (data not shown). No significant signals were observed in the flower cell types other than tapetum and pollen, either by in situ hybridization or immunohistochemistry.

An *AbH6H* 5'-upstream region monitors the endogenous *AbH6H* expression in transgenic tissues

A 5'-upstream region (-671 bp from the transcriptional start site) of *AbH6H* was translationally fused to the GUS reporter gene in the pBI 101 plant transformation vector (see Material and Methods). This *AbH-671::GUS* transgene was introduced via *Agrobacterium rhizogenes* into three solanaceous species; *A. belladonna*, *H. niger* and *N. tabacum*, and the resulting transgenic hairy root clones were analyzed for GUS expression patterns (Figure 20). Of the 36 *A. belladonna* hairy root clones studied, 13 clones showed a distinct GUS staining. In such GUS-positive roots, a few cell layers in the vascular cylinder were stained blue in patches, and interdigitated with non-staining regions (Figure 20A). In most of the non-staining regions which were flanked by GUS stained cells, lateral root primordia were emerging (arrowheads in Figure 20). Cross sections showed that the pericycle cells adjacent to the xylem poles were specifically stained (Figure 20D). Some hairy root clones showed no GUS-staining in the root apex and in the elongation zone (e.g. Figure 20A), while others showed blue-staining
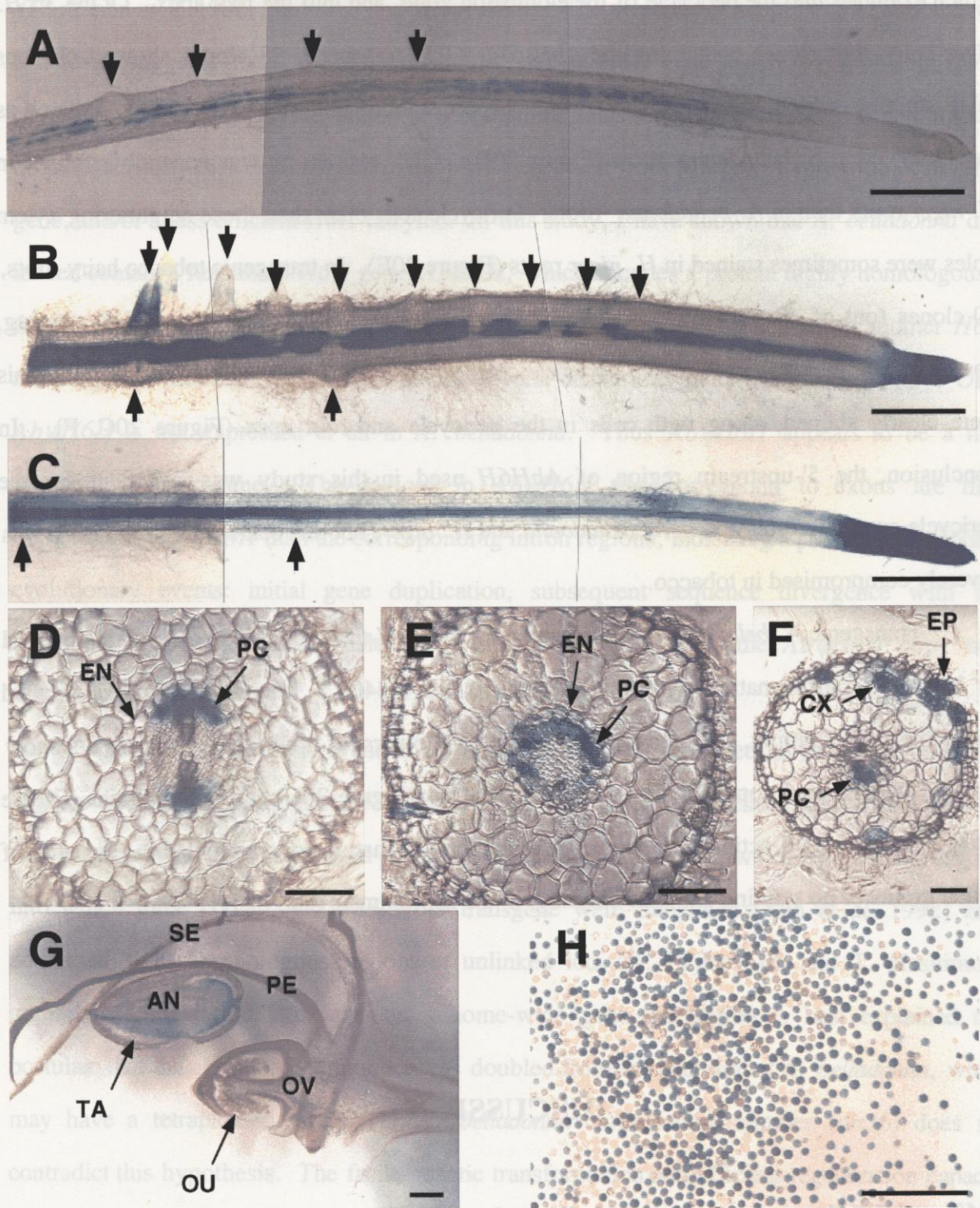
Figure 20 GUS expression in transgenic hairy roots and transgenic plants. X-Gluc staining of transgenic hairy roots containing the *AbH-671::GUS* transgene, of *Atropa belladonna* (A and D), *Hyoscyamus niger* (B and E), and *Nicotiana tabacum* SR1 (C and F). (A to C) Whole-mount staining. (D to F) Cross sections. GUS reporter expression in tapetum (G) and pollen grains (H) in a mature flower bud of a transgenic *A. belladonna* plant containing the *AbH-671::GUS* transgene. Bars in A to C, and G indicate 1 mm, while bars in D to F, and H show 100 µm. CX, cortex; EP, epidermis; OU, ovule. For other abbreviations, see Figure 19. Arrows in A-C indicate lateral root primordia.

which extended into the pericycle of the elongation zone, and into the root apex. Of the 35 *H. niger* hairy root clones containing the *AbH-671::GUS* transgene, 28 clones showed obvious GUS staining, which, with regard to the cell-specific staining pattern, was basically the same as that observed in *A. belladonna* roots (Figure 20B). GUS expression was generally stronger in *H. niger* roots than in *A. belladonna* roots. Pericycle cells other than those neighboring xylem poles were sometimes stained in *H. niger* roots (Figure 20E). In transgenic tobacco hairy roots, 20 clones (out of 43 clones examined) showed detectable GUS expression. GUS staining, however, was not restricted to the pericycle; some, but not all, cells in cortex and epidermis were clearly stained, along with cells in the pericycle and root apex (Figure 20C, F). In conclusion, the 5'-upstream region of *AbH6H* used in this study was sufficient for the pericycle-specific expression in *A. belladonna* and *H. niger*, but this cell-specific regulation was severely compromised in tobacco.

Transgenic *A. belladonna* plants containing the *AbH-671::GUS* transgene were obtained by leaf disc transformation with *A. tumefaciens* LBA 4404. Mature plant roots showed negligible GUS staining. The aerial parts, except for flowers, of transgenic plants did not show any GUS staining. In opened mature flowers, tapetum (Figure 20G) and pollen grains (Figure 20H) expressed GUS activity. It should be noted that the mature pollen population consisted of pollen grains of no staining and blue staining, in the approximate ratio of 1:1.

DISCUSSION

PMT and *H6H* genes might be duplicated

Atropa belladonna belongs to the subtribe Lyciinae of the Solanaceae family, according to the classification of Wettstein. Most members of the Lyciinae, which includes 14 genera, produce alkaloids of the hyoscyamine type (Evans 1979). Earlier extensive phytochemical research on *A. belladonna* has shown that this species contains hyoscyamine as the principal alkaloid throughout the entire plant, and small amounts of scopolamine in young plants

(Hartmann *et al.* 1986, Harborne and Khan 1993). The presence of scopolamine, although at a low level, suggests that *A. belladonna* should have an *H6H* gene which is responsible for the two-step conversion of hyoscyamine to scopolamine (Hashimoto *et al.* 1993). Small amounts of scopolamine which accumulated in the plant may result from low level expression of the *H6H* gene, and/or a less efficient H6H enzyme. In this study, I have shown that *A. belladonna* does indeed contain a functional *H6H* gene, *AbH6H*, which encodes a protein highly homologous to the *H. niger* H6H amino acid sequence. *AbψH6H* was also determined as an another *H6H*-related gene. Interestingly, besides having several deleterious mutations in the putative ORF, *AbψH6H* is not expressed at all in *A. belladonna*. Thus *AbψH6H* appears to be a non-functional pseudo-gene. The regions in *AbψH6H* that correspond to exons are more homologous to *AbH6H* than the corresponding intron regions, indicating a potential sequence of evolutionary events: initial gene duplication, subsequent sequence divergence with two functional genes, and finally deleterious mutations which made the *AbψH6H* copy non-functional.

The gene duplication event in *A. belladonna* is not restricted to the *H6H* gene, but appears to be widespread: in *A. belladonna*, there are two closely related genes for PMT, the first enzyme in the tropane alkaloid pathway (Figure 8) , and at least two of the genomic regions into which three copies of a *35S::H6H* transgene were integrated (Yun *et al.* 1992) have additional highly homologous regions at unlinked loci (K. Fujibayashi and T. Hashimoto, unpublished results). This apparent genome-wide gene duplication is best explained if I postulate that the chromosome number was doubled from the ancestor of *A. belladonna*, which may have a tetraploid nature. The *A. belladonna* chromosome number (n=36) does not contradict this hypothesis. The facile genetic transformation and efficient regeneration capacity from tissue culture make *A. belladonna* a model medicinal plant for the study of tropane alkaloid biosynthesis (Bajaj and Simola 1991), but the widespread occurrence of gene duplication may hinder gene isolation and characterization in this species to some extent.

N-terminal repeat array is not necessary to PMT function

PMT catalyzes the first committed step for the biosynthesis of various alkaloids which

originate by N-methylation of putrescine. Thus, it may represent a potential regulatory point which controls the metabolite flux into polyamines and putrescine-derived alkaloids. The structures and hormonal regulation of *Nicotiana* PMT genes involved in nicotine biosynthesis have been previously reported (Hibi *et al.* 1994, Hashimoto *et al.* 1998a, Imanishi *et al.* 1998). Analogous studies are reported here for the PMTs involved in tropane alkaloid biosynthesis. Nicotine and tropane alkaloids are synthesized in related plant species of the Solanaceae, but their ecological functions are probably distinct (Wink 1998). Therefore, not only similarities but also differences between PMTs involved in these two different types of alkaloids are expected.

Nicotiana PMTs have two notable structural features: N-terminal tandem repeats and a remaining catalytic domain highly similar to SPDSs. The N-terminal repeats are dispensable for the enzymatic activity of PMT, and the number of repeat elements is highly variable among *Nicotiana* PMTs (Hashimoto *et al.* 1998a). As the genomic DNA blot previously suggested (Hashimoto *et al.* 1998a) and the molecular cloning of *PMT* cDNAs from *A. belladonna* and *H. niger* in this study clearly showed, such tandem repeats may be specific to the PMTs of the *Nicotiana* genus. However, it should be noted that although *A. belladonna* and *H. niger* PMTs lack characteristic tandem repeats at their N-termini, they do contain N-terminal extensions of 20-30 amino acid residues which are not present in the structurally related SPDSs, and the extensions in AbPMT1 and HnPMT are hydrophilic, as are those in *Nicotiana* PMTs. These N-terminal regions in PMTs may be just relics of enzyme evolution, or their possible function might be sought in the putative physical interaction with other enzymes in alkaloid pathways.

PMTs probably originated from SPDSs

The overall primary structure of *A. belladonna* and *H. niger* PMTs is highly similar to that of various SPDSs, as in the case of *Nicotiana* PMTs (Hibi *et al.* 1994, Hashimoto *et al.* 1998b). We previously pointed out that 11 amino acid residues are strictly conserved among all SPDS sequences but different in a tobacco PMT sequence (Hashimoto *et al.* 1998b). A total of 7 PMT amino acid sequences is now available from *A. belladonna*, *H. niger*, *N. sylvestris*, and *N. tabacum*. When these PMT sequences are compared with all available SPDS sequences, 7 amino acid residues are found to be strictly conserved among PMTs and among SPDSs,

differing, however, between PMTs and SPDSs: these are Asn104, Gly105, Gly106, Ile134, Phe139, Tyr144, and Ile147 in AbPMT1. These residues in PMTs and the corresponding residues in SPDSs may differentiate the selective binding of the co-factor (SAM or decarboxylated SAM) and the transferred moiety from the co-factor (methyl group or aminopropyl group).

Tropane alkaloids are produced at the root pericycle and translocated from roots to aerial parts through the xylem

PMT enzyme activity has been detected in the root, but not in the leaf, stem, flower, or cultured cells of three tropane alkaloid-producing species: *A. belladonna*, *H. niger*, and *Datura stramonium* (Hibi *et al.* 1992). RNA gel blot and RT-PCR analyses (Figure 9) of the *AbPMT* expression have confirmed the root as the main organ of *PMT* expression, and thus as a primary site of alkaloid biosynthesis (Hashimoto and Yamada 1992). RT-PCR amplification of the *AbPMT1* cDNA from the stem RNA of *A. belladonna* indicates that the modest expression of PMT in the stem may have been missed in previous studies, requiring future confirmation in the same as well as in other plant species. The *AbPMT1* promoter::GUS transgene expression in *A. belladonna* hairy roots suggested that *AbPMT1* is expressed in the pericycle cells in the root (Figure 13). The pericycle is present in the central cylinder of developmentally young roots without secondary growth, such as young lateral roots and cultured roots (Fahn 1990). Higher abundance of *AbPMT1* mRNA in the lateral root than in the main root (Figure 9B) may reflect this pericycle-specific expression.

In situ hybridization (Figure 19) and promoter analysis (Figure 20) showed that *AbH6H* is expressed in pericycle cells adjacent to xylem poles in young roots. We also noted that mature plant roots contained considerably less *AbH6H* mRNA than cultured roots (Kanegae *et al.* 1994), and showed negligible GUS staining in mature roots of *AbH-671::GUS* transgenic plants. *AbH6H* expression in young roots reflects the moderately high scopolamine content of young *A. belladonna* plants.

The localized expression of *AbH6H* and *AbPMT1* in particular pericycle cells indicates that scopolamine is synthesized mainly in these cells. Since the first and the last enzymes in the

biosynthetic pathway of scopolamine are now found to be expressed in the pericycle, other enzymes in this pathway may well be localized there. Since tropane alkaloids are translocated from roots to aerial parts through the xylem (Luckner 1990), alkaloids synthesized in cells next to the xylem pole should be efficiently transported into the translocation stream (Figure 21). The pericycle-specific expression of H6H has also been demonstrated in *Hyoscyamus* and *Duboisia* roots (Hashimoto *et al.* 1991, Kanegae *et al.* 1994).
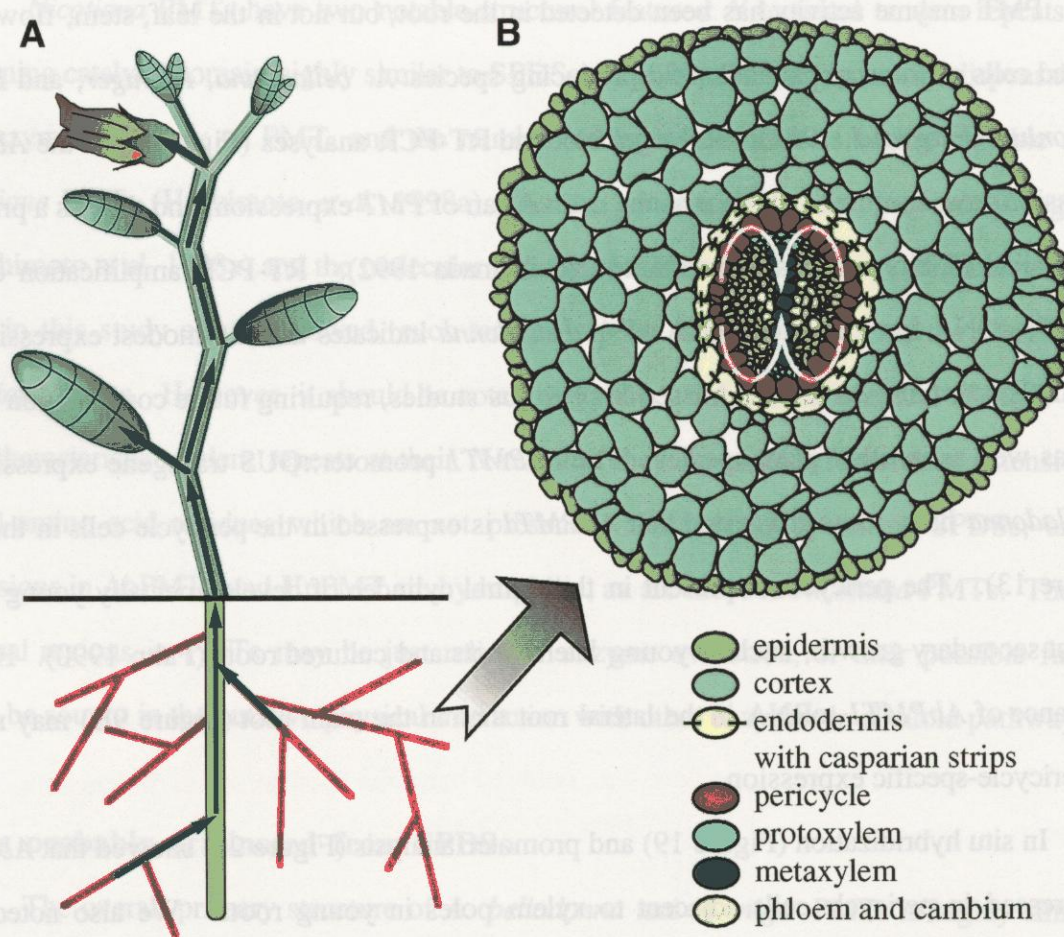
Figure 21 Model of the production and translocation of tropane alkaloids in *A. belladonna*. Tropane alkakoids are mainly produced in the pericycle cells and translocated to the protoxylem cells (B, white arrows) of the lateral roots (A, red), and then translocated to the stem, leaf, and flower organs through the xylem stream (A, blue arrows).

Low expression of *H6H* gene in *A. belladonna* roots may be because of differences of trans factors among the host plants

In the previous study of the *H. niger H6H* promoter in transgenic roots, an *HnH6H::GUS* transgene was specifically expressed in pericycle cells of *H. niger* and *A. belladonna*, but not in tobacco pericycle cells (Kanegae *et al.* 1994). GUS expression levels of the transgene were stronger in *H. niger* roots than in *A. belladonna* roots. Again in this study, I observed basically the same GUS expression patterns in transgenic roots of the three plant species using the *AbH6H::GUS* transgene (Figure 20). These results suggest that tobacco roots lack a critical cellular factor(s) relating to the activation/repression of *HnH6H* and *AbH6H* promoter regions. The low expression level of endogenous *AbH6H* in *A. belladonna* may not be a result of a property of its promoter (e.g. absence of enhancer sequences). It may be caused by a low level or the inefficient properties of a regulatory factor(s) which drives expression through the *H6H* promoter. At least some of such putative *trans*-acting factors are expected to be specific to the genes for scopolamine biosynthesis, and therefore to the species that produce scopolamine.

The *cis*-acting element(s) for the pericycle expression may be too loosely conserved, or different *cis*-elements may be used to drive expression of alkaloid-synthesis genes in the pericycle

Similar pericycle-specific expression patterns driven by the 5'-upstream sequences of *HnH6H* and *AbH6H* suggest that some of the *cis*-acting elements required for the cell-specific regulation may be shared by the two promoters. The promoter region 827-bp upstream from the transcriptional start site of *HnH6H* was previously used to demonstrate pericycle-specific expression (Kanegae *et al.* 1994). This *HnH6H* promoter region also expressed the downstream GUS gene in pollen grains from a transgenic *A. belladonna* plant (H. Kajiya and K. Suzuki, unpublished results). This -827 bp promoter region includes an AT-rich region (Kanegae *et al.* 1994), which is not present in the *AbH6H* promoter. A computer-assisted comparison between the two *H6H* promoters reveals only one significantly conserved region. The sequence ATATTAAATTAACCTCAAGGTACT in *AbH6H* (from -70bp to -47bp; single

underlined in Figure 15) is very similar to the sequence ATATTATA<u>TTAACCT</u>CAAGATACT in *HnH6H* (from -126bp to -103bp), and the core sequence GGTTAA in reverse orientation (underlined) has been reported as the transcription factor GT-1 binding site (Green *et al.* 1988), also known as a GT element. GT elements and their binding factors modulate cell type-specific transcription (Villain *et al.* 1996). The 52/56 box in the pollen-specific promoter of tomato *LAT 52* is a GT element and may bind a member of the GT-1 family (Eyal *et al.* 1995). Further, a PLACE database search (Higo *et al.* 1998) identified TAACTG motifs in both the *HnH6H* promoter (from -657bp to -652bp and from -77bp to -72bp) and the *AbH6H* promoter (in reverse orientation, from -207bp to -202bp; double underlined in Figure 15). The TAACTG motif conforms to a potential binding site for some R2R3-MYB proteins (Romero *et al.* 1998). Although a synthetic promoter consisting of a trimer of this TAACTG motif fused to the -46 CaMV35S TATA-box did not drive detectable expression of a downstream GUS gene in *A. belladonna* transgenic hairy roots (unpublished results), combinations of a conserved motif and a divergent partner element might be used for the cell-type specific and developmental control of plant promoters, as reported for nuclear-encoded photosynthetic genes (Puente *et al.* 1996).

Since the 5'-upstream regions of *H. niger* and *A. belladonna H6H* genes and of *AbPMT1* all drive expression of the downstream reporter GUS at the pericycle cells in scopolamine-producing hairy roots, DNA sequences conserved among the three promoters were also searched by computer. Although a few relatively short DNA sequences are conserved between the *H6H* promoters of *H. niger* and *A. belladonna*, these elements are not found in the *AbPMT1* promoter (data not shown). The *cis*-acting element(s) for the pericycle expression may be too loosely conserved to be detected by computer search, or different *cis*-elements may be used to drive expression of alkaloid-synthesis genes in the pericycle.

The regulation of tropane alkaloid biosynthesis is differ from nicotine biosynthesis

Mechanical leaf wounding of *N. sylvestris* results in the systemic increase of jasmonic acid in the root and the concomitant increase of nicotine synthesis in the root (Baldwin *et al.* 1994, Zhang and Baldwin 1997). Increased nicotine accumulation in wounded plants may

function as part of the defense response. Exogenous application of methyl jasmonate also induces PMT expression in *N. sylvestris* plants (Baldwin *et al.* 1994) and tobacco cell cultures (Imanishi *et al.* 1998). Induction of biosynthetic genes by jasmonic acid or derivatives of the octadecanoic pathway has also been reported for several other plant alkaloids (Gundlach *et al.* 1992, Blechert *et al.* 1995). However, similar treatment of *A. belladonna* hairy roots with methyl jasmonate did not increase the *AbPMT* and *AbH6H* RNA levels or the expression of the *AbPMT1* promoter. Although the ecological functions of hyoscyamine and scopolamine are not yet known, these tropane alkaloids may not function as defense compounds in producing plants, as their synthesis may not respond to wounding signals as with jasmonic acid.

What is a possible function of *H6H* in the male reproductive tissues?

AbH6H was also expressed in tapetum and pollen mother cells (Figures 19 and 20). H6H protein could be detected immunohistochemically in mature pollen grains (data not shown). Possible functions of H6H in these male reproductive tissues are a matter of debate. For its enzymatic catalysis, H6H requires hyoscyamine and the co-factors, dioxygen, ferrous ion, 2-oxoglutarate, and ascorbate (Hashimoto *et al.* 1986). It is not known whether substrate and co-factors are present in tapetum and pollen cells, although I detected hyoscyamine in mature pollen grains and in the whole anther tissues (Detzel and Wink 1993, unpublished results). If scopolamine is indeed produced in the male reproductive cells, it may have some elusive role(s) in the proper functioning of these reproductive cells. Flavonols, a specific subclass of flavonoids, are required for efficient pollen grain germination, and thus are essential for full male fertility in maize (Mo *et al.* 1992) and petunia (Ylstra *et al.* 1994), but not in Arabidopsis (Burbulis *et al.* 1996). Such species-specific functions might also be discovered for other subclasses of secondary metabolites. Alternatively, scopolamine may serve as a chemical defense compound and may deter bees from feeding on nectar and pollen (Detzel and Wink 1993).

Summary

Hyoscyamine and scopolamine are the two most common tropane alkaloids found in the Solanaceae, which include *Atropa belladonna* and *Hyoscyamus niger*, and plants containing these alkaloids have been used for their medicinal, hallucinogenic, and poisonous properties. Putrescine *N*-methyltransferase (PMT) catalyzes the *S*-adenosylmethionine-dependent *N*-methylation of putrescine at the first committed step in the biosynthetic pathways of tropane alkaloids. Hyoscyamine 6ß-hydroxylase (H6H) is the last enzyme in the biosynthesis pathway of scopolamine and converts hyoscyamine to scopolamine.

The cDNAs encoding PMT were isolated from *A. belladonna* and *H. niger*. These PMTs, however, lacked the *N*-terminal tandem repeat arrays previously found in *Nicotiana* PMTs. Two cDNAs, *AbPMT1* and *AbPMT2*, and one genomic clone of *AbPMT1* were isolated from *A. belladonna*. *AbPMT1* transcript was much more abundant in the root of *A. belladonna* than was *AbPMT2* transcript. The 5'-flanking region of the *AbPMT1* gene was fused to the ß-glucuronidase (GUS) reporter gene and transferred to *A. belladonna*. Histochemical analysis showed that GUS is expressed specifically in root pericycle cells and that the 295-bp 5'-upstream region was sufficient for pericycle-specific expression.

The *AbH6H* gene for H6H was isolated from *A. belladonna*. This plant also possessed a related sequence, *AbψH6H*, which appears to be a non-functional pseudo-gene. *AbH6H* transcript was detected in cultured root, native root and anther, but not in stem, leaf, pistil, petal, and sepal tissues. In situ hybridization, immunohistochemistry and promoter::GUS transgene analysis showed that *AbH6H* is expressed specifically in root pericycle cells, and in tapetum and pollen mother cells. A 671-bp 5'-upstream region from *AbH6H* was sufficient for pericycle-specific expression in hairy roots of *A. belladonna* and *H. niger*, which both produce scopolamine. But this cell-specific regulation was severely compromised in tobacco hairy roots, which do not produce scopolamine.

The localized expression of *AbH6H* and *AbPMT1* in particular pericycle cells indicates that scopolamine is synthesized mainly in these cells. Since the first and the last enzymes in the

biosynthetic pathway of scopolamine are now found to be expressed in the pericycle, other enzymes in this pathway may well be localized there. Since tropane alkaloids are translocated from roots to aerial parts through the xylem, alkaloids synthesized in cells next to the xylem pole should be efficiently transported into the translocation stream. Treatment of *A. belladonna* roots with methyl jasmonate did not up-regulate the expression of *AbPMT* and *AbH6H* genes. The regulation of tropane alkaloid biosynthesis is discussed and compared with that of nicotine biosynthesis.

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