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Expression of *Atropa belladonna* Putrescine *N*-Methyltransferase Gene in Root Pericycle

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The cDNAs encoding putrescine *N*-methyltransferase (PMT), which catalyzes the *S*-adenosylmethionine-dependent *N*-methylation of putrescine at the first committed step in the biosynthetic pathways of tropane alkaloids, were isolated from *Atropa belladonna* and *Hyoscyamus niger*. These PMTs, however, lacked the *N*-terminal tandem repeat arrays previously found in *Nicotiana* PMTs. *AbPMT1* RNA was much more abundant in the root of *A. belladonna* than was *AbPMT2* RNA. The 5′-flanking region of the *AbPMT1* gene was fused to the β-glucuronidase (GUS) reporter gene and transferred to *A. belladonna*. Histochemical analysis showed that GUS is expressed specifically in root pericycle cells and that the 0.3-kb 5′-upstream region was sufficient for pericycle-specific expression. Treatment of *A. belladonna* roots with methyl jasmonate did not up-regulate the expression of GUS or endogenous *AbPMT* genes. The regulation of tropane alkaloid biosynthesis is discussed and compared with that of nicotine biosynthesis.

Key words: *Atropa belladonna* — Pericycle — Putrescine *N*-methyltransferase (EC 2.1.1.53) — Tropane alkaloids.

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). Tropane alkaloids, nicotine, and polyamines are all synthesized from putrescine, which is formed from either ornithine or arginine (Hashimoto and Yamada 1994). 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 (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).

*Nicotiana PMT*s, which encode a protein with a distinct sequence similarity to plant and animal SPDSs, probably originated from SPDSs during diversification of the Solanaceae (Hashimoto et al. 1998a, b). 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), but it is not known whether *A. belladonna* and *H. niger* PMTs have any *N*-terminal repeat array.

Hyoscyamine 6β-hydroxylase (H6H; EC 1.14.11.11) belongs to the 2-oxoglutarate-dependent dioxygenases (Hashimoto and Yamada 1986) and catalyzes two consecutive oxidation reactions from hyoscyamine to scopolamine by way of 6β-hydroxyhyoscyamine (Hashimoto et al. 1993). Immunohistochemistry and promoter::GUS transgene analysis showed that *H. niger H6H* is expressed specifically in root pericycle cells (Hashimoto et al. 1991, Kanegae et al. 1994). It should be of considerable interest to examine whether other structural genes involved in tropane alkaloid biosynthesis, including *PMT*, show the same cell-type specificity.

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. In this report, we isolated *PMT* cDNAs from *A. belladonna* and *H. niger*

Abbreviations: AbPMT, *Atropa belladonna* putrescine *N*-methyltransferase; GUS, β-glucuronidase; H6H, hyoscyamine 6β-hydroxylase; HnPMT, *Hyoscyamus niger* putrescine *N*-methyltransferase; PMT, putrescine *N*-methyltransferase; SAM, *S*-adenosylmethionine; SPDS, spermidine synthase.

The nucleotide sequences reported in this paper have been submitted to GenBank under the accession numbers AB018570 (*AbPMT1* cDNA), AB018571 (*AbPMT2* cDNA) AB018572 (*HnPMT* cDNA), and AB018573 (*AbPMT1* genomic clone).

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and a *PMT* gene from *A. belladonna* and examined whether these PMTs have any *N*-terminal tandem repeat array. We then compared the expression of *A. belladonna PMT* with tobacco *PMT* and *H. niger H6H* with respect to cell-specificity and hormonal regulation.

Materials and Methods

Plant materials—Atropa belladonna L. and *Hyoscyamus niger* L. are maintained in our laboratory for studies on alkaloid biosynthesis, being grown in a greenhouse. Shoot (Mathis and Hinchee 1994) and root (Hashimoto and Yamada 1986) cultures were grown as reported.

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, 2× 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 0.1× 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. Screening of an *A. belladonna* genomic DNA library (reported elsewhere) was performed as described above by using the full-length *AbPMT1* cDNA as a probe. 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 2×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.

RT-PCR—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′-TCTTTTGCTGGACCAATAGG) and the following protocol: amplification for 3 min at 94°C, followed by 35 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. As quantification controls, *AbPMT1* and *AbPMT2* cDNAs were also amplified in different concentration combinations (see Fig. 3B). The amplification products were digested with *Cla*I and/or *Pvu*II and subsequently electrophoresed on a 2% agarose gel.

Construction of the promoter::GUS fusion vectors and plant transformation—The *Hin*dIII 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*HI site by *Hin*dIII digestion, filling-in by a Klenow fragment, *Bam*HI-linker ligation, and self ligation. The resultant plasmid was cut with *Xho*I, filled in by a Klenow fragment, and then digested with *Bam*HI to generate an *AbPMT1* fragment containing a 1,373-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*RV and *Bam*HI sites of pBluescript II SK$^-$. The *AbP-748* fragment and the *AbP-295* fragment were similarly obtained by using the *Bst*XI and *Bam*HI sites and the *Eco*RI and *Bam*HI sites, respectively, except for the addition of a step of blunt ending after *Bst*XI 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*RV and *Bam*HI sites of pBluescript II SK$^-$. Subsequently, *AbP-1373, AbP-748*, and *AbP-295* were subcloned between the *Hin*dIII and *Bam*HI sites in pBI 101 (Clontech). 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 performed according to Kanegae et al. (1994). Transgenic hairy roots were selected for kanamycin-resistance (250 mg liter^{-1}) and subcultured on a solid culture medium every 4 weeks. Transgenic *A. belladonna* plants were regenerated from leaf discs and grown in a greenhouse according to Yun et al. (1992).

Histochemical analysis of β-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-β-D-glucuronide, 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 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 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

Isolation and characterization of PMT *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. 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 was 1,305-bp long and encoded AbPMT1 of 336 amino acids, whereas the longest

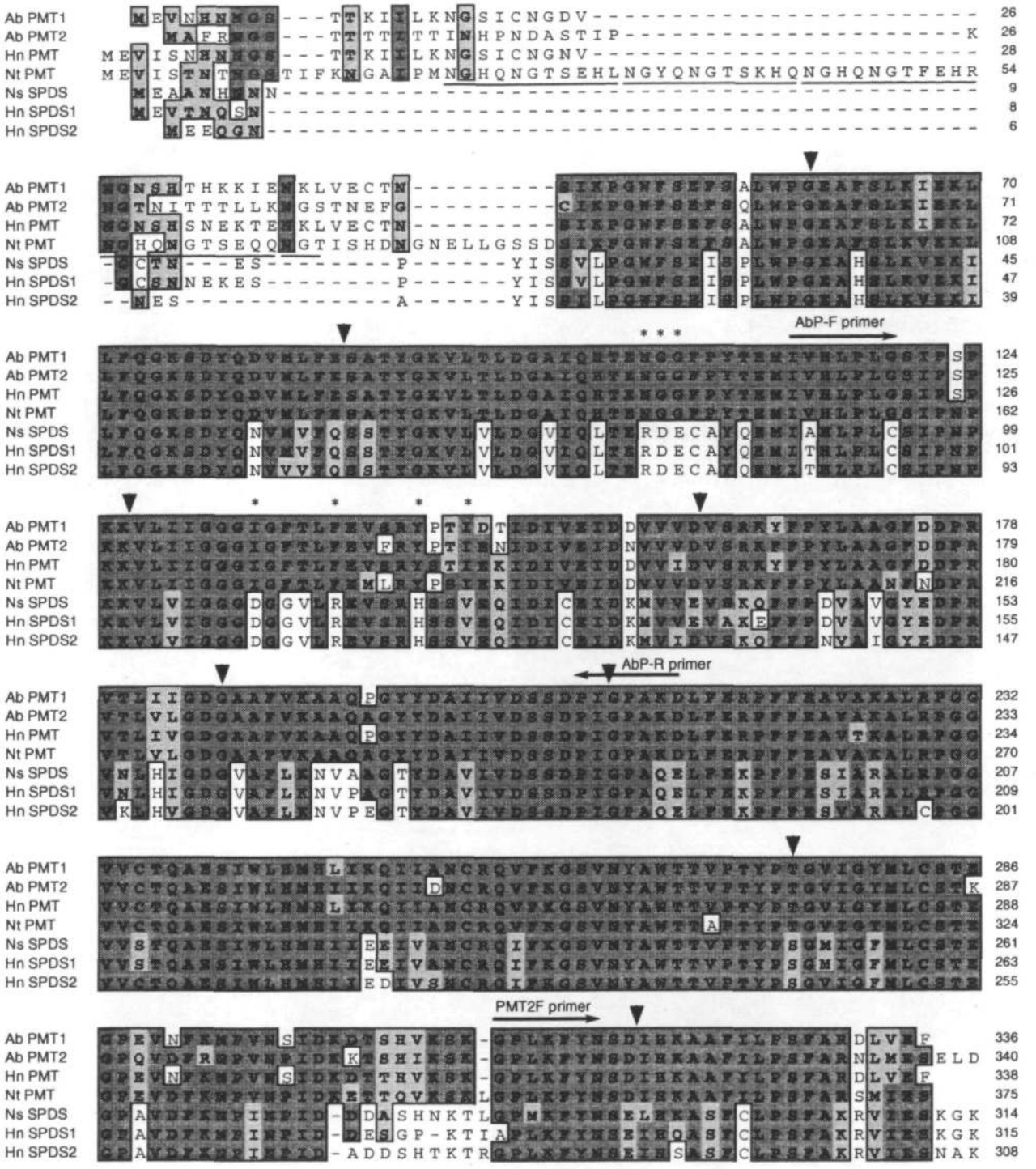

Fig. 1 Alignment of PMT and SPDS amino acid sequences from the solanaceaous plants. Dark-gray highlighting indicates identical amino acid residues in at least four proteins, while light-gray highlighted residues represent similarities. Underlining on NtPMT shows the tandem repeats found in *Nicotiana* 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. Arrows indicate primers used for PCR experiments in Fig. 2 and 3. Splicing junction sites of *AbPMT1* are marked by arrowheads.

AbPMT2 cDNA was 1,294-bp long and encoded AbPMT2 of 340 amino acids. From a cultured root cDNA library of *H. niger*, one *PMT* cDNA (*HnPMT*) and two *SPDS* cDNAs (Hashimoto et al. 1998b) were isolated. The *HnPMT* cDNA was 1,350-bp long and encoded HnPMT of 338 amino acids. 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 1 compares the amino acid sequences of AbPMTs and HnPMT with those of tobacco 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 Fig. 1), 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 (asterisks in Fig. 1). 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 (Fig. 2A). 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, we then assessed the abundance of *AbPMT* mRNAs by RT-PCR analysis (Fig. 3). We designed two PCR primers which an-

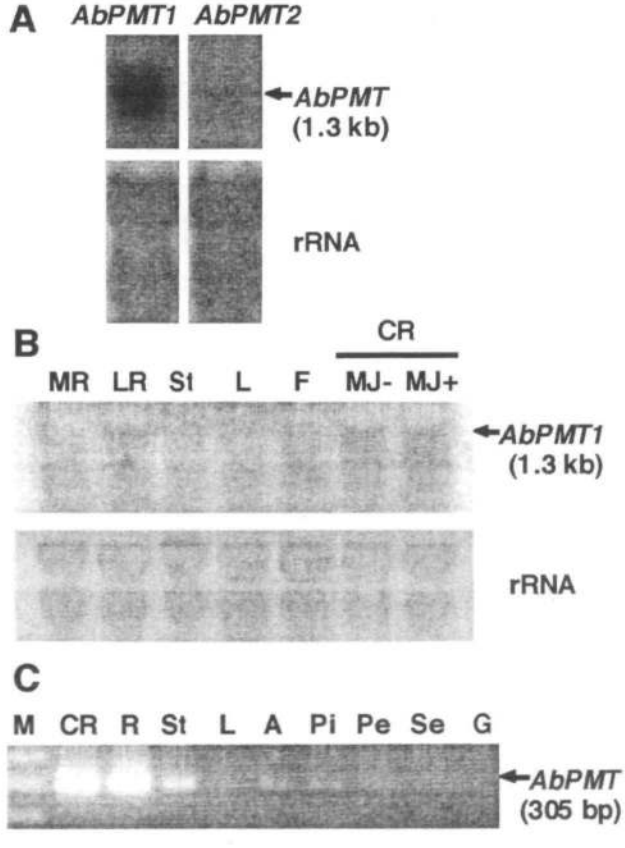

Fig. 2 Analysis of *AbPMT* RNAs in *A. belladonna*. (A and B) Gel blots were made from total RNAs (10 μg) isolated from root (A) and various tissues (B) of mature *A. belladonna* plants and probed with either *AbPMT1* (A and B) or *AbPMT2* (A) cDNAs. MR, main root; LR, lateral root; St, stem; L, leaf; 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. Fig. 3). 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).

neal 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 (Fig. 3A). 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 (Fig. 3C, lane R). As calibration controls, known amounts of *AbPMT1* and *AbPMT2* cDNAs were mixed in varying ratios and amplified as well (Fig. 3B; and Fig. 3C, 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 (Fig. 3D, 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*.

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 (Fig. 2B). 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 (Fig. 2C). These 305-bp RT-PCR products were mostly derived from *AbPMT1* RNA, since they were 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 (Fig. 2B lanes CR/MJ− and CR/MJ+).

Isolation of AbPMT1 *genomic clones*—After screening approximately 4×10^5 independent clones from the *A. belladonna* genomic library with the *AbPMT1* cDNA probe, two independent *AbPMT* clones were isolated.

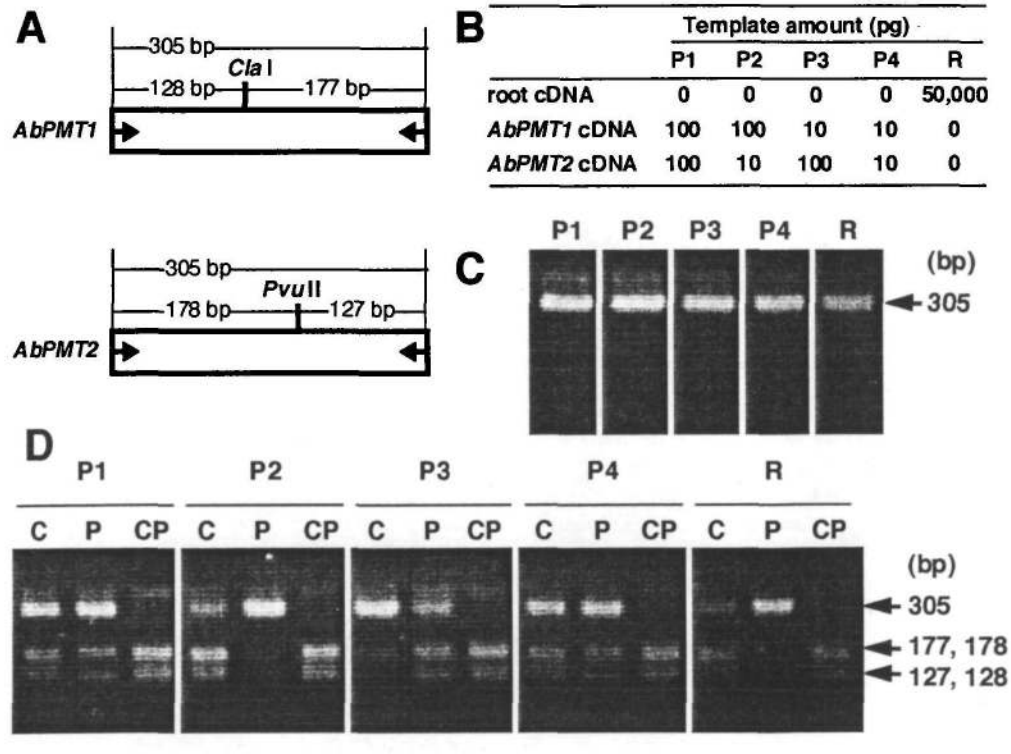

Fig. 3 RT-PCR analysis of *AbPMT* genes in cultured *A. belladonna* roots. (A) RT-PCR products from *AbPMT1* and *AbPMT2* transcripts are expected to be 305-bp. *Cla*I and *Pvu*II sites, unique in either fragment, were used to determine the identity of the fragment. (B) *AbPMT1* and *AbPMT2* cDNAs mixed 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.

Restriction enzyme digestion and Southern hybridization analysis showed that these clones are overlapped and derived from one *AbPMT* gene (Fig. 4A). 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 (Fig. 4). *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, 1,329 bp and 79 bp in length, respectively. Comparison with three *N. sylvestris PMT* genes (*NsPMT*s; Hashimoto et al. 1998a) showed that intron 3 of *AbPMT1* is missing in *NsPMT*s, but the number and the position of other introns are identical, although their length and nucleotide sequences differ (data not shown).

Expression of AbPMT1—Since five of the six *AbPMT1* cDNA clones had the same 5′ ends, this terminal guanidine was arbitrarily designed as +1 (Fig. 4B), the 1,373-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, 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 (Fig. 5A, B). Cross sections showed that the pericycle cells were specifically stained (Fig. 5E). In situ hybridization detected pericycle-specific expression of *AbPMT1* in *A. belladonna* lateral roots (data not shown), but the *AbPMT1* signal was very weak compared to the H6H signal, which was also localized in the pericycle cells (Suzuki et al. 1999). 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

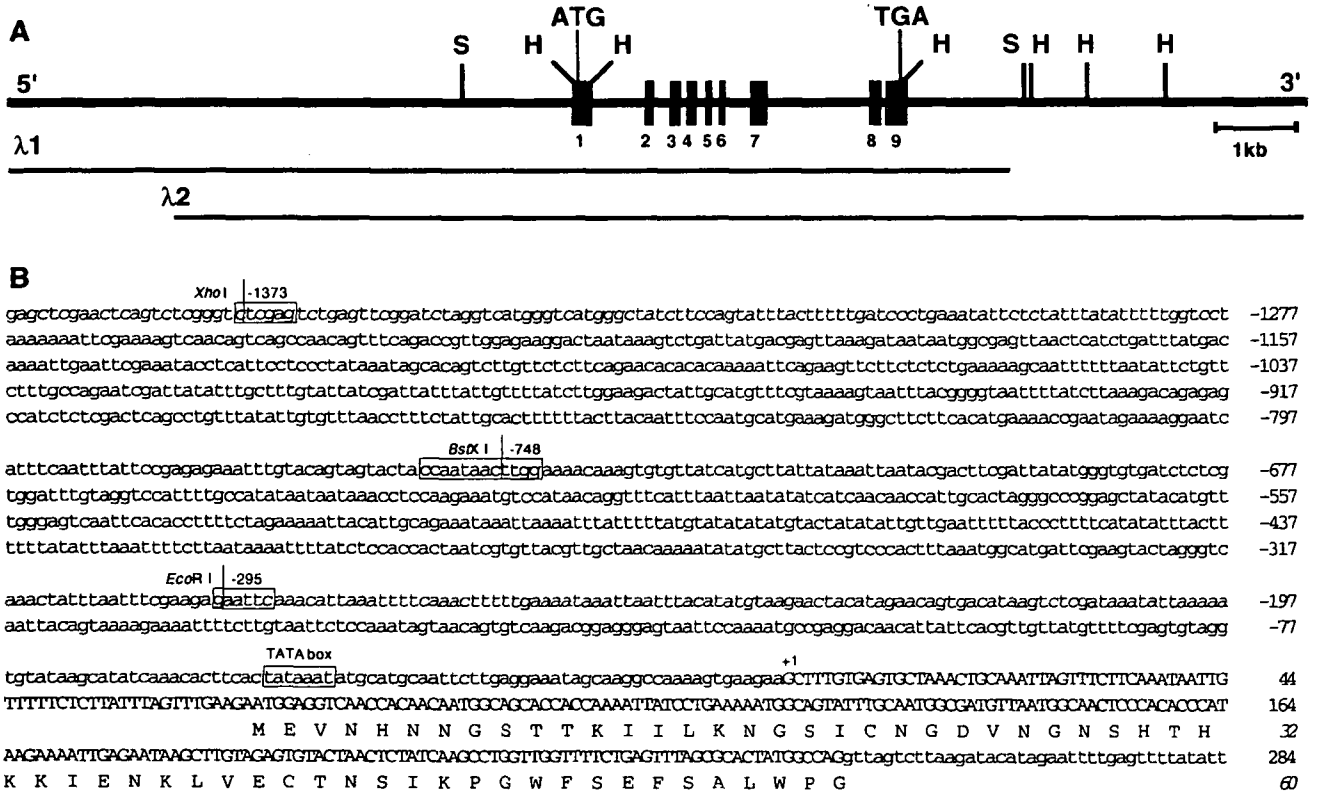

Fig. 4 (A) Structure of *AbPMT1*. Two overlapping lambda clones containing *AbPMT1* are indicated below the *AbPMT1* gene structure. Nine exons are shown in black boxes. Translational start and stop codons are shown as ATG and TGA, respectively. Restriction sites are indicated by H (*Hind*III) and S (*Sac*I). (B) Nucleotide sequence of a 5′-upstream region of *AbPMT1*. The nucleotide sequence of the first exon 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 −1,373 (*Xho*I), −748 (*Bst*XI), and −295 (*Eco*RI) indicate the 5′ end of the *AbPMT1* upstream regions fused transcriptionally to the GUS reporter gene. A presumed TATA box is also boxed.

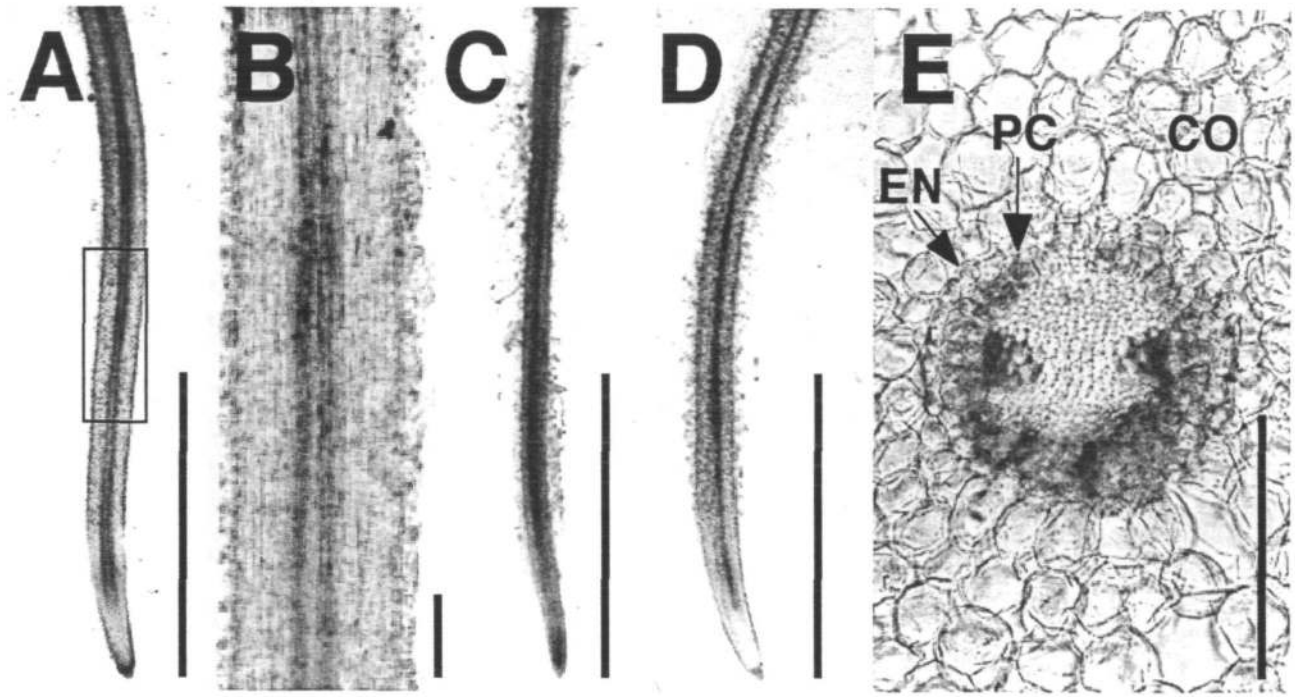

Fig. 5 GUS expression patterns in transgenic *A. belladonna* hairy roots. The hairy roots stained for GUS activity contained *AbP-1373::GUS* (A, B and E), *AbP-748::GUS* (C), or *AbP-295::GUS* (D) transgenes. (A to D) Whole-mount staining. The boxed region in A was enlarged in B. (E) Cross-section of 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.

tip (Kanegae et al. 1994, Suzuki et al. 1999). *A. belladonna* hairy root clones containing the *AbP-748::GUS* transgene (Fig. 5C) or the *AbP-295::GUS* transgene (Fig. 5D) also showed similar GUS staining in a few cell layers in the vascular cylinder. 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*.

Discussion

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 cDNA*s 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.

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).

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 (Fig. 2) 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 (Fig. 5). Much higher abundance of *AbPMT1* mRNA in the lateral root than in the main root (Fig. 2B) may reflect this pericycle-specific expression. The pericycle-specific expression of enzymes in the tropane alkaloid pathway has been well documented for H6H, the last enzyme in the scopolamine biosynthesis (Hashimoto et al. 1991, Kanegae et al. 1994, Suzuki et al. 1999). 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. Biosynthesis of tropane alkaloids in the pericycle has been proposed to be advantageous for the translocation of the root-originating alkaloids to the aerial parts (Hashimoto et al. 1991).

Since the 5′-upstream regions of *H. niger* and *A. belladonna H6H* genes (Kanegae et al. 1994, Suzuki et al. 1999) 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 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.

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* RNA level or the expression of the *AbPMT1* promoter. Although the ecological functions of hyoscyamine and scopolamine are yet to be known, these tropane alkaloids may not function as defense compounds in producing plants, and therefore their synthesis may not respond to wounding signals as with jasmonic acid.

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