Plant Physiol. (1994) 105: 483–490

Species-Dependent Expression of the Hyoscyamine 6β-Hydroxylase Gene in the Pericycle[1]

Takeshi Kanegae, Hiromi Kajiya, Yasuhiro Amano[2], Takashi Hashimoto*, and Yasuyuki Yamada

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kyoto 606-01, Japan

The tropane alkaloid scopolamine is synthesized in the pericycle of branch roots in certain species of the Solanaceae. The enzyme responsible for the synthesis of scopolamine from hyoscyamine is hyoscyamine 6β-hydroxylase (H6H). The gene for H6H was isolated from *Hyoscyamus niger*. It has an exon/intron organization very similar to those for ethylene-forming enzymes, suggesting a common evolutionary origin. The 827-bp 5′ flanking region of the H6H gene was fused to the β-glucuronidase (GUS) reporter gene and transferred to three solanaceous species by *Agrobacterium*-mediated transformation systems: *H. niger* and belladonna (*Atropa belladonna*), which have high and low levels, respectively, of H6H mRNA in the root, and tobacco (*Nicotiana tabacum*), which has no endogenous H6H gene. Histochemical analysis showed that GUS expression occurred in the pericycle and at the root meristem of transgenic *H. niger* hairy roots, but only at the root meristem of hairy roots and plants of transgenic tobacco. In transgenic hairy roots and regenerated plants of belladonna, the root meristem was stained with GUS activity, except for a few transformants in which the vascular cylinder was also stained. These studies indicate that the cell-specific expression of the H6H gene is controlled by some genetic regulation specific to scopolamine-producing plants.

Secondary metabolites with diverse chemical structures are usually synthesized in some plant tissues at certain developmental stages. The rates of metabolite formation are often greatly influenced by internal hormone balances and external stimuli, such as light and pathogen attack. Our knowledge of how the expression of structural genes encoding biosynthetic enzymes involved in secondary metabolism is controlled comes primarily from genetic and molecular studies of the biosynthesis of flavonoids, typically anthocyanin pigments (Dooner et al., 1991). These studies have shown that the distribution of anthocyanins in plant tissues is determined by the expression patterns of, and the interactions among, specific transcriptional activators.

In the absence of similar genetic and molecular studies of other secondary metabolites, it is not certain whether, and, if so, how much, the basic regulatory mechanisms that have been identified in the anthocyanin pathway can be extended to other groups of secondary metabolites in plants. Flavonoids are atypical among secondary products in that all flowering plants synthesize some subgroups of flavonoids, indicating very early evolution of the flavonoid pathway in land plants (Stafford, 1991). Usually, any given secondary products are limited to certain, often phylogenetically related, species (Hegnauer, 1986). Among secondary products, alkaloids constitute the largest group of secondary products in terms of chemical diversity and have been studied extensively by chemists and biochemists because they frequently exhibit pharmacological activity. Several genes encoding biosynthetic enzymes in independent alkaloid pathways have recently been cloned (Kutchan et al., 1988; De Luca et al., 1989; Dittrich and Kutchan, 1991; Matsuda et al., 1991; Nakajima et al., 1993) and the molecular analysis of the control of these alkaloid pathways is forthcoming.

Scopolamine is a tropane alkaloid with anticholinergic properties. It is synthesized from hyoscyamine via 6β-hydroxyhyoscyamine in select species of the Solanaceae (Hashimoto and Yamada, 1992). The 2-oxoglutarate-dependent dioxygenase H6H (EC 1.14.11.11) catalyzes the two-step epoxidation of hyoscyamine to scopolamine (Hashimoto et al., 1993a) and is especially active in cultured roots (Hashimoto and Yamada, 1986). H6H cDNA cloned from cultured roots of *Hyoscyamus niger*, a typical scopolamine-rich plant, has been used to demonstrate that H6H mRNA is abundant in cultured roots, is present in plant roots, but is absent in stems, leaves, and cultured cells (Matsuda et al., 1991). Immunohistochemical analysis has further localized the H6H protein to the pericycle in the root, and the pericycle-specific expression of H6H has been proposed to be important for the root-to-shoot translocation of tropane alkaloids through xylem (Hashimoto et al., 1991). To understand the molecular mechanism behind this highly cell-specific biosynthesis of scopolamine, the regulatory components that control the expression of the H6H gene in scopolamine-producing plants must first be identified. We report here that the 5′ flanking region of the *H. niger* H6H gene controls cell-specific gene expression in roots in a species-dependent manner.

MATERIALS AND METHODS

Plant Materials

Cultured roots of *Hyoscyamus niger* and *Atropa belladonna* were grown at 25°C in the dark on a rotary shaker (100 rpm) in Gamborg B5 medium containing 30 g/L Suc and 1 μM

[1] This work was supported in part by a Grant-in-Aid for Scientific Research on Priority Areas (No. 4257208) from The Ministry of Education, Science and Culture to T.H.

[2] Present address: Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Machida, Tokyo 194, Japan.

* Corresponding author; fax 81-7437-2-5489.

Abbreviations: EFE, ethylene-forming enzyme; GUS, β-glucuronidase; H6H, hyoscyamine 6β-hydroxylase.

indole-3-butyric acid (Hashimoto et al., 1986). *H. niger, Hyoscyamus muticus, A. belladonna, Nicotiana tabacum* cv SR1, and *Petunia hybrida* plants were grown either in a greenhouse or under sterile conditions.

DNA Preparation and Analysis

DNA was prepared as described by Doyle and Doyle (1987). For gel-blot analysis, DNA was digested with restriction endonucleases, fractionated on a 0.7% agarose gel, and transferred to a nylon membrane (Biodyne A, Pall, Glen Cove, NY) by the conventional capillary method. Hybridization was performed at 65°C in 5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhardt's solution (1× Denhardt's solution = 0.02% [w/v] Ficoll, 0.02% [w/v] BSA, 0.02% [w/v] PVP), 0.1% (w/v) SDS, and 100 μg/mL denatured salmon testis DNA. A hybridization probe was prepared from an *Xho*I fragment of pBHH1 containing *H. niger* H6H cDNA (Matsuda et al., 1991) using a random primer labeling kit (Pharmacia). The membrane was washed twice in 2× SSC, 0.1% (w/v) SDS at room temperature and then in 0.1× SSC, 0.1% (w/v) SDS at 42°C.

RNA Preparation and Analysis

RNA was isolated by phenol/SDS extraction, followed by sodium acetate precipitation (Ausubel et al., 1987). Total RNA was separated on formaldehyde-containing agarose gel and blotted onto a nylon membrane (Biodyne A, Pall). The membrane was hybridized at 42°C in 50% (v/v) formamide, 5× SSPE (1× SSPE is 0.18 M NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.7), 5× Denhardt's solution, 0.1% (w/v) SDS, and 100 μg/mL denatured salmon testis DNA. The hybridization probe was prepared as described above. The membrane was washed twice in 2× SSC, 0.1% (w/v) SDS at room temperature, and then in 0.2× SSC, 0.1% (w/v) SDS at 65°C.

Construction and Screening of the Genomic Library and Nucleotide Sequencing

Genomic DNA isolated from cultured roots of *H. niger* was partially digested with *Sau*3AI and size-fractionated by Suc gradient ultracentrifugation. Size fractions of 10 to 23 kb were ligated into either λ EMBL3 or λ DASHII that had been digested with *Bam*HI. The ligates were packaged using Gigapack Gold (Stratagene) and then infected to *Escherichia coli* PLK17 and ER1647. The library of approximately 1.4×10^6 recombinants was screened by plaque hybridization with an H6H cDNA probe prepared as described above. After plaque purification and isolation, positive clones were mapped by Southern analysis of restriction fragments and subcloned into pBluescript II KS− or pBluescript II SK−. Nested-deletion clones were made using a kilo-sequence deletion kit (Takara Shuzo, Kyoto, Japan) and DNA sequences were determined by the dideoxy chain-termination method (Sanger et al., 1977) using a Sequenase kit (United States Biochemical).

Primer Extension

A 36-mer oligonucleotide with a sequence of 5′-GACCAGTTCGACACAAAAGTAGCCATCAAATGTCTC-3′, which is complementary to the 5′ region of the H6H mRNA (see Fig. 3B), was labeled at the 5′ end using T4 polynucleotide kinase and [γ-^{32}P]ATP. Primer extension reaction was carried out according to Ausubel et al. (1987) and the extension product was electrophoresed on an 8% sequencing gel with sequencing reactions of the HN3 clone (see Fig. 3A) using the same primer.

Plasmid Construction

A 5′ flanking region of the H6H gene was isolated as a *Hin*dIII fragment from −827 to +108 bp relative to the transcription initiation site, cloned into pBluescript II KS−, and designated as pHN26. The pHN26 was digested with *Sal*I and *Bam*HI in the polylinker of the pBluescript II and fused in frame with a GUS cassette from pBI101.2 (Jefferson, 1987) that had been digested similarly. The resulting plasmid was confirmed by direct double-stranded nucleotide sequencing with appropriate primers.

Plant Transformation

Binary vector was transferred into *Agrobacterium rhizogenes* strain 15834 and *Agrobacterium tumefaciens* strain LBA4404 by electroporation as described by Nagel et al. (1990). Sterile leaf discs of *H. niger, A. belladonna,* or *N. tabacum* were briefly dipped in a culture of *A. rhizogenes* that carried the desired plasmid and then placed onto hormone-free Gamborg B5 medium, which had been solidified with 3 g/L Gellan Gum and supplemented with 30 g/L Suc. After 2 d, discs were transferred to selection plates consisting of the same medium supplemented with 250 μg/mL kanamycin, 250 μg/mL cefotaxim, and 500 μg/mL carbenicillin. Well-grown hairy roots emerging from leaf discs were excised, grown once on the same selection plates, and then transferred to the liquid medium without antibiotics. To regenerate shoots of transgenic belladonna hairy roots, transformed roots were placed onto solidified Murashige and Skoog medium containing 4 μg/mL kinetin and 0.1 μg/mL naphthaleneacetic acid. For tobacco, the leaf disc infection and plant regeneration were carried out according to Horsch et al. (1985), and some of the regenerants were grown to maturity and selfed.

Histochemical Analysis

Histochemical staining for GUS activity was performed according to Jefferson et al. (1987) with some modifications recommended by Kosugi et al. (1990). Cross-sections of fresh root material were obtained using a microtome (model MT-2, Nihon Ika-kiki, Osaka, Japan). Sections 30 to 50 μm thick and whole plant material were incubated in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 50 mM sodium phosphate buffer (pH 7.0), 0.1% (v/v) Tween 20, 20% (v/v) methanol, and 5 mM DTT at 37°C for periods ranging from 4 h to overnight. For aerial material, pigments were removed by successive extraction in 70 to 100% (v/v) ethanol (Jefferson and Wilson, 1991). A fixation step was not included.

RESULTS

Distribution and Expression of the H6H Gene

H6H is part of the scopolamine biosynthetic pathway in certain species of the Solanaceae. To determine whether

phylogenetic distributions of the H6H gene and scopolamine are correlated, we hybridized an H6H cDNA probe from *H. niger* with a gel blot containing genomic DNAs from several solanaceous species. *H. niger*, *H. muticus*, and *A. belladonna* produce scopolamine, whereas tobacco and petunia do not. Figure 1 shows that at the washing conditions of relatively low stringency, the H6H probe reacted with one to three DNA fragments (digested with *Hin*dIII or *Eco*RV) from *H. niger*, *H. muticus*, and belladonna, but not with DNAs from tobacco and petunia. Thus, H6H genes and closely related DNA sequences seem to occur only in scopolamine-producing plants.

To study the pattern and the level of H6H gene expression in belladonna, RNA was isolated from cultured roots, roots, stems, and leaves of belladonna and from cultured roots of *H. niger*. RNA blot analysis using the H6H cDNA probe (Fig. 2) revealed that H6H mRNA is present in cultured roots, but either occurs at low levels or is absent in plant roots, leaves, and stems of belladonna. This high abundance of belladonna H6H mRNA in cultured roots is consistent with the distribution of *H. niger* H6H mRNA (Matsuda et al., 1991). Furthermore, comparison of the hybridization signals of cultured roots from belladonna and *H. niger* indicates that the level of expression of the belladonna H6H gene is much lower than

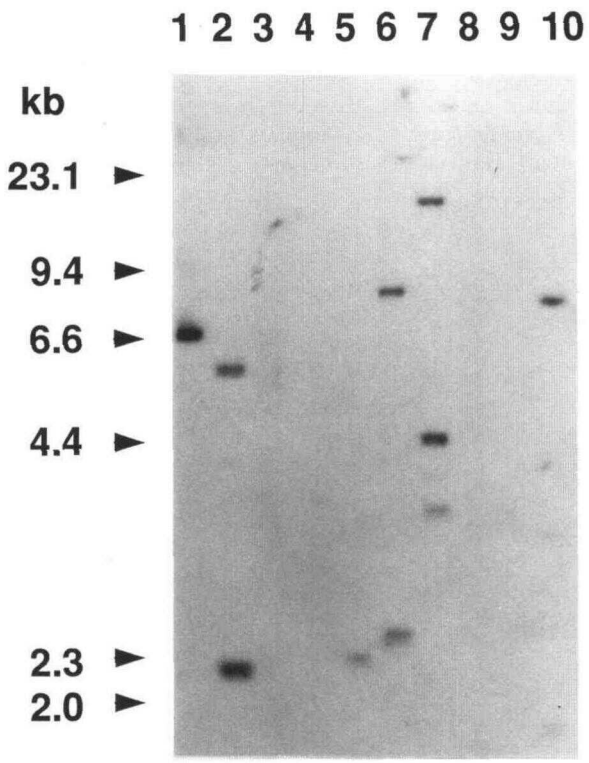

Figure 1. Genomic DNA gel-blot analysis of the H6H gene. Genomic DNAs were isolated from *H. niger* (lanes 1 and 6), *A. belladonna* (lanes 2 and 7), *N. tabacum* (lanes 3 and 8), *P. hybrida* (lanes 4 and 9), and *H. muticus* (lanes 5 and 10). Genomic DNA (10 μg) digested with *Hin*dIII or *Eco*RV were run in lanes 1 to 5 and lanes 6 to 10, respectively, and probed with a radiolabeled H6H cDNA sequence. The positions of the molecular size markers are indicated on the left.

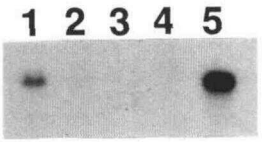

Figure 2. RNA gel-blot analysis of belladonna H6H mRNA. Total cellular RNA from cultured roots (lane 1), plant roots (lane 2), stems (lane 3), and leaves (lane 4) of belladonna, and from cultured *H. niger* roots (lane 5) were subjected to electrophoresis, transferred to a nylon membrane, and hybridized to a radiolabeled H6H cDNA probe. Lanes 1 to 4 contained 20 μg of total RNA; lane 5 contained 2 μg of total RNA.

that of the *H. niger* gene. Detectable hybridization signals were not observed in any tobacco tissues (data not shown).

Isolation and Structural Features of the H6H Gene

Libraries constructed from *H. niger* genomic DNA were screened using an *H. niger* H6H cDNA probe, and three positive clones, designated HN1, HN3, and HN4, were obtained. Analysis by restriction enzyme digestion and Southern blot hybridization showed that these three clones overlap, and that the longest clone, HN3, contains the 5′ portion of the H6H gene (Fig. 3A). Several restriction fragments from HN3 were subcloned into pBluescript II KS− for sequence analysis. The sequence of 4297 nucleotides, which spans the first *Hin*dIII site and the *Sac*II site, was determined from overlapping sequential deletions and has been submitted to DDBJ. Only the sequence of about 0.9 kb from the 5′ flanking region is shown in Figure 3B. The sequences of predicted exons perfectly match the H6H cDNA sequence (Matsuda et al., 1991). The H6H gene of *H. niger* contains three introns of 966, 379, and 334 bp. The positions of the introns are well conserved among the genes for H6H, EFE, and EFE's homolog E8 (Fig. 4), although the E8 gene lacks one intron that corresponds to the first intron of the H6H and EFE genes. It has been pointed out (Matsuda et al., 1991) that the amino acid sequences for these proteins show considerable homology. Our data further suggest that the genes for H6H, EFE, and E8 have evolved from a common ancestor.

The transcription initiation site was determined by a primer extension experiment using a synthetic oligonucleotide primer at positions +84 to +49 of the genomic clone HN3 (Fig. 5). The primer extension products were electrophoresed in parallel with a sequencing reaction of the HN3 clone using the same primer. The major primer extension product corresponded to the T residue (nucleotide +1, Fig. 3B) in the sequence ACACTACAAA. A TATA-box-like sequence (TATAAAT) occurred at −33 to −27. Three repeating units were present in the 5′ flanking region. Two 10-bp perfect repeats at −740 to −749 and −706 to −697 included a TGACG motif that is recognized by a class of plant basic/Leu zipper transcription factors (Katagiri and Chua, 1992). Two 31-bp highly conserved repeats were found at −824 to −794 and −357 to −327. Another repeating unit consisting of five perfect copies of a nonameric sequence (TGTTCCGTA) was found at region −740 to −581. The region −318 to −215 showed a remarkably high A+T content (90.4%).

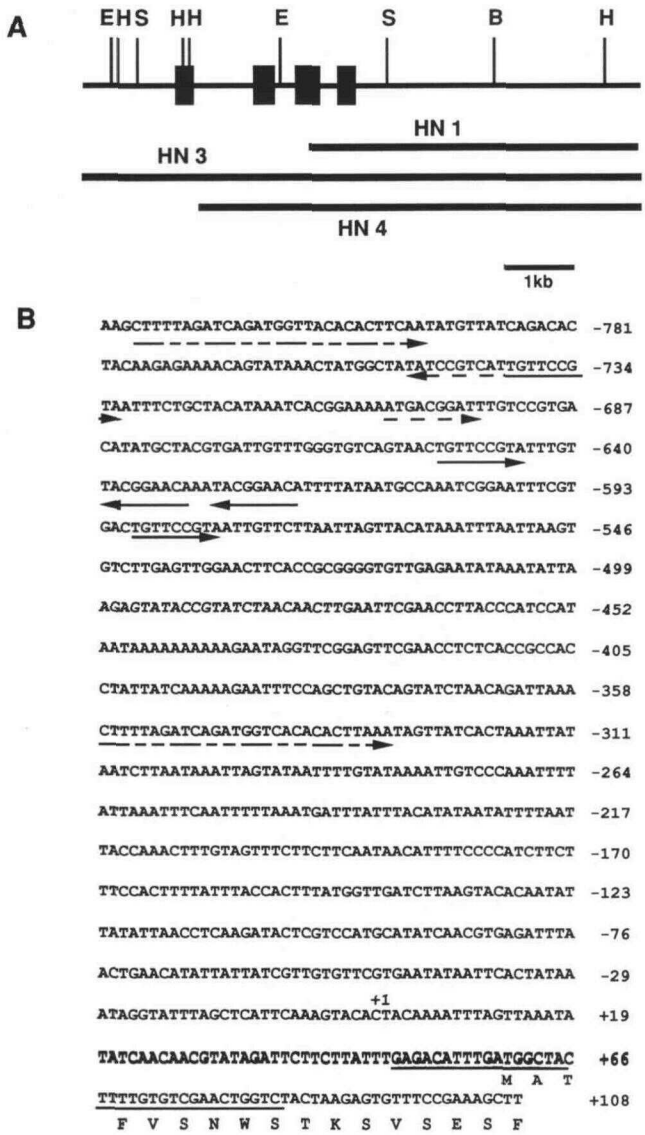

Figure 3. The structure of the H6H gene and the nucleotide sequence of the H6H gene promoter. A, Schematic representation of gene structure with several restriction endonuclease sites indicated. Solid boxes indicate a protein coding region, and isolated genomic clones are shown by solid bars. E, *Eco*RV; H, *Hin*dIII; S, *Sac*II; B, *Bam*HI. B, The sequence from −827 to +108 (derived from *Hin*dIII digestion), in relation to the transcription initiation site of the H6H gene, is shown. The oligonucleotide used for primer extension was complementary to the region underlined. Three repeating units are indicated by the solid and broken arrows.

The 5′ Flanking Region Confers Species-Dependent Expression Patterns in the Root

To study the function of the 5′ flanking region of the H6H gene, the sequence from −827 to +108 was translationally fused to the GUS reporter gene in pBI101 and introduced into three solanaceous plants for histochemical GUS assays. *A. rhizogenes* was used to transfer the GUS construct to the hairy roots of all these species. In addition, transgenic plants were regenerated from some of the belladonna hairy root clones and were regenerated directly from tobacco leaf discs after infection with *A. tumefaciens* LBA4404.

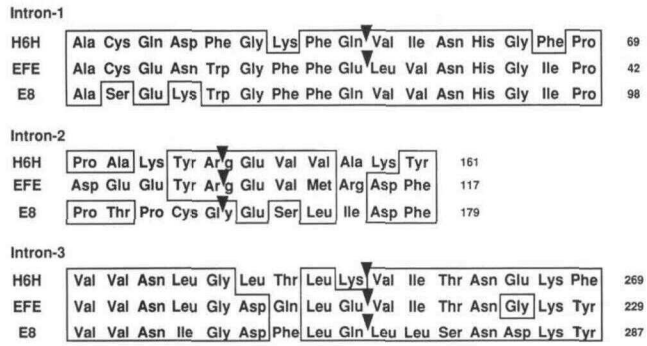

Figure 4. Comparison of intron insertion positions in H6H and related genes. The deduced amino acid sequences and the intron positions of the EFE gene and the tomato E8 gene are from Holdsworth et al. (1987a, 1987b) and Deikman and Fischer (1988), respectively. Numbers at the ends of sequences indicate the positions of amino acid residues. The splice junction sites are marked by arrowheads. Identical or similar amino acids are boxed according to Dayhoff et al. (1978).

In transgenic *Hyoscyamus* hairy roots, GUS activities were detected near the vascular cylinder (Fig. 6A) or at both the vascular cylinder and the root meristematic region (Fig. 6B). GUS activity was also observed early at the site of lateral root initiation, which is a region of meristematic activity (Fig. 6C). Similar spatial patterns of GUS expression were observed when *H. muticus* was used as a transgenic host (data not shown). Transverse sections were examined to locate cell

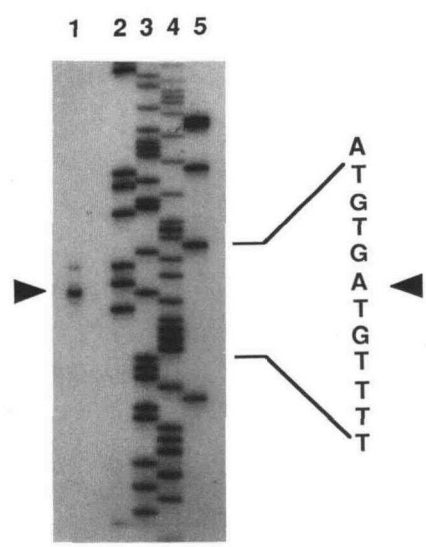

Figure 5. Identification of the transcription initiation site. Primer extension analysis of the RNA from cultured roots of *H. niger*. Lane 1, The primer extension product with root RNA; lanes 2 to 5, sequencing reactions (GATC) of the HN3 clone using the same primer. The primer used is indicated in Figure 3B. The arrowhead on the left shows the 5′ end signal for the H6H mRNA, which corresponds to the A residue on the noncoding strand (right arrowhead).

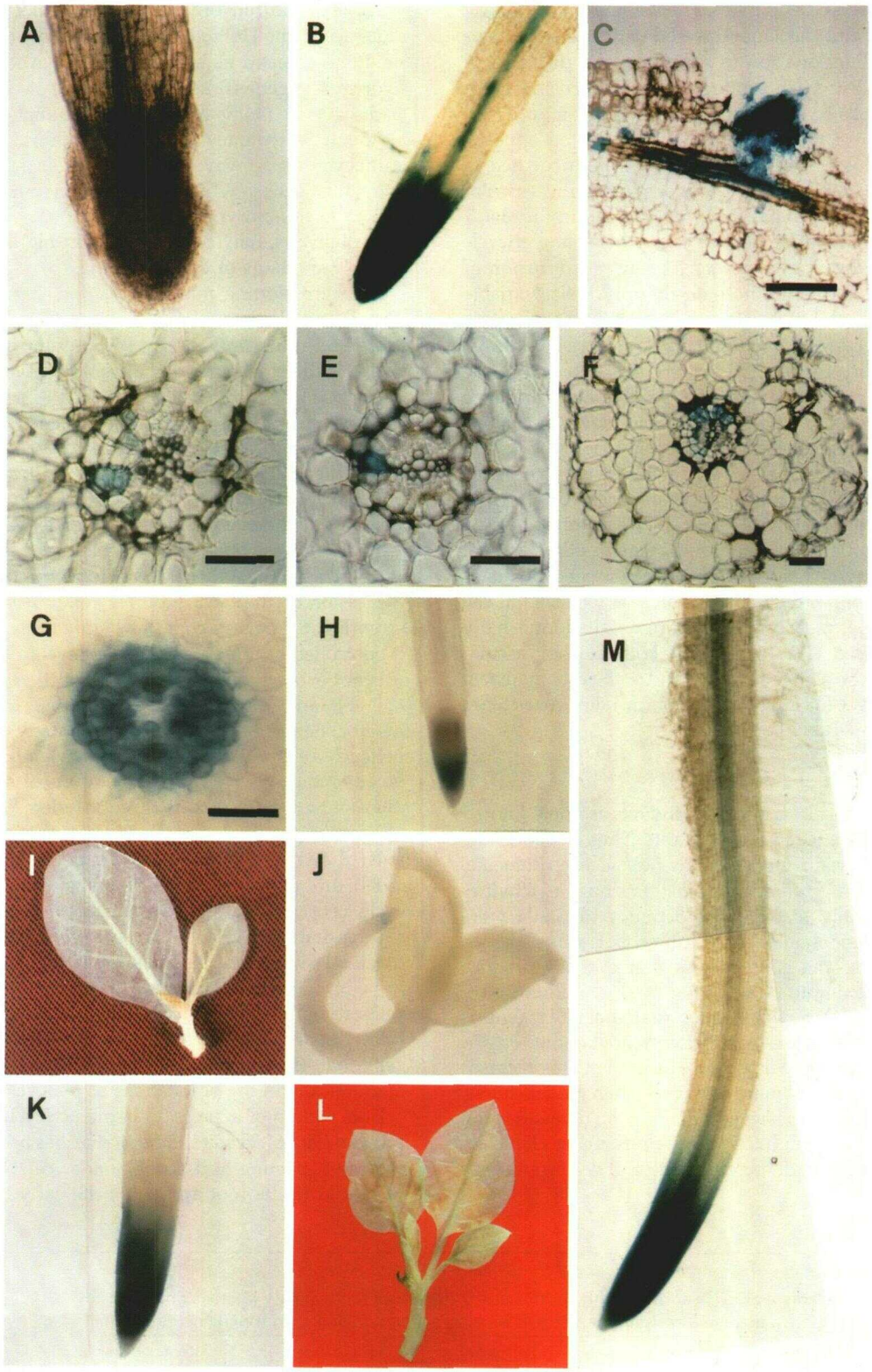

Figure 6. Histochemical localization of GUS activity in transgenic tissues. Transformants contained the GUS reporter gene that had been translationally fused to the H6H gene promoter region (−827 to +108). A and B, Two representative transformants of *H. niger* hairy roots. C, Longitudinal section of transgenic *H. niger* hairy roots. Bar = 0.5 mm. D to G, Cross-sections of transgenic *H. niger* hairy roots. Root sections were understained (D–F) or overstained (G). Bars = 0.1 mm. H, Hairy roots of transgenic tobacco. I, Transgenic tobacco plant. J, Four-day seedling of transgenic tobacco. K to M, Hairy roots and regenerated plants of transgenic belladonna.

types that expressed GUS activity. The young root of *H. niger* is mostly diarch, and the single-layered pericycle sometimes underwent periclinal division to form two to four layers of pericycle cells. Clear GUS staining was observed in one or more pericycle cells in each transverse section (Fig. 6, D–F). Many but not all of the stained cells were located opposite primary xylem poles. When the sections were incubated in the GUS reaction mixture for a longer period, the vascular cylinder, excluding the metaxylem, was stained in the shape of a ring (Fig. 6G). Staining of stele cells other than pericycle in the overstained root sections may be due to diffusion of GUS reaction products into these neighboring cells. Variable GUS expression in the pericycle and/or at the root meristem was often observed in individual hairy root clones. In transgenic tobacco hairy roots, GUS activity was detected at the root meristem (Fig. 6H). Similarly, GUS activity was localized at the root meristem in transgenic tobacco plants but not in aerial parts (including the shoot meristem) (Fig. 6I). When surface-sterilized seeds from selfed transgenic tobacco plants were germinated, GUS activity was observed at the root meristem in 4-d-old seedlings (Fig. 6J). GUS expression was not observed at cotyledons, shoot meristem, or other parts of seedlings. In many of hairy roots and regenerated plants of transgenic belladonna, GUS expression was localized at the root meristem (Fig. 6, K and L), but few belladonna hairy root clones showed GUS expression both at the vascular cylinder distal to the elongation zone and at the root meristem (Fig. 6M). The data for GUS expression in hairy root clones are summarized in Table I.

DISCUSSION

A feature of typical secondary products is their limited phylogenetic occurrence in higher plants. Thus, scopolamine is found only in certain species of the Solanaceae (Romeike, 1978). Our previous studies showed that enzyme activities and immunologically reactive polypeptides for H6H were present in eight scopolamine-producing solanaceous species, but not in four solanaceous species that did not produce scopolamine (Hashimoto et al., 1986, 1991). Southern blotting analysis (Fig. 1) now indicates that the H6H gene is limited to plant species that synthesize scopolamine. Phylogenetic distribution of hyoscyamine, the immediate precursor of scopolamine, in the Solanaceae is wider than, and includes, that of scopolamine. This suggests that during diversification of the Solanaceae, certain hyoscyamine-producing plants acquired the H6H gene and, therefore, the ability to synthesize scopolamine.

The exon/intron organization and the predicted protein sequence of the H6H gene are remarkably similar to those of the EFE gene and its homolog E8 (Fig. 4). The flavanone 3-hydroxylase gene (Britsch et al., 1992) also shows an amino acid sequence that is highly similar to that of the H6H gene, although the genomic organization of this hydroxylase in the anthocyanin pathway has not yet been reported. Such homologous genes, which encode nonheme oxygenases involved in the biosynthesis of plant hormones and anthocyanin pigments, may be ubiquitous in higher plants, and are possible ancestors of the H6H gene.

Northern blotting analysis of various organs of belladonna (Fig. 2) and *H. niger* (Matsuda et al., 1991) has demonstrated that H6H transcripts are mostly present in the root tissue, which is the site of tropane alkaloid biosynthesis. An abundance of H6H transcripts in cultured roots, as compared to the levels in the plant root, is consistent with earlier immunohistochemical studies (Hashimoto et al., 1991) that detected the H6H protein specifically in the pericycle of scopolamine-producing plants. The pericycle is present in the stele of developmentally young roots without secondary growth, such as young branch roots and cultured roots (Fahn, 1990). The fact that the *H. niger* H6H promoter region confers expression of the GUS reporter gene in the pericycle of *H. niger* (Fig. 6) suggests that this highly cell-specific expression is controlled at the transcriptional level. The H6H promoter was not expressed uniformly in all of the pericycle cells (Fig. 6, D–F), indicating that the pericycle cells are physiologically heterogeneous. Such expression was frequently observed in pericycle cells adjacent to primary xylem poles, which may be advantageous for the translocation of scopolamine from the root to the aerial parts through xylem.

There is good anatomical evidence that the pericycle consists of two cell populations. In onion, pea (Lloret et al., 1989), and radish (I. Sussex, personal communication), pericycle cells located opposite xylem poles are significantly shorter than cells located opposite phloem poles and give rise to lateral root primordia. In carrot, lateral root primordia arise in pericycle cells located next to the phloem poles, and such cells are significantly shorter than those opposite xylem poles (Lloret et al., 1989). It remains to be seen whether pericycle cells that express the H6H promoter can be distinguished anatomically in the populations of pericycle cells.

This pattern of expression in the pericycle is not present in tobacco (Fig. 6), despite some similarities between the biosynthesis of nicotine and scopolamine, i.e. both alkaloids are produced in the root and share the early biosynthetic steps

Table I. *Histochemical analysis of transgenic hairy roots containing H6H-GUS construct*

Three solanaceous plants were transformed with *A. rhizogenes* that harbored a binary vector containing the NPT-II gene and the H6H promoter-GUS fusion gene. Kanamycin-resistant hairy roots were screened and analyzed for GUS expression.

Host Plant	Total No. of Clones Analyzed	Pericycle Only	Pericycle plus Root Meristem	Root Meristem Only	No Expression[a]
H. niger	20	7	3	0	10
A. belladonna	30	0	3	16	11
N. tabacum	23	0	0	18	5

[a] GUS activity was not detected in any tissues of hairy roots. These clones were not tested for presence of the transgene, thus they might include escapes.

leading from putrescine to the 1-methyl-Δ^1-pyrrolinium cation (Hashimoto and Yamada, 1993). Transgenes are often regulated similarly in different plants, but with several notable exceptions. For example, the expression patterns conferred by specific combinations of cauliflower mosaic virus 35S promoter subdomains differ in tobacco and petunia (Benfey and Chua, 1990). Promoter regions of several plant genes (Benfey and Chua, 1989; Budelier et al., 1990; Schmid et al., 1990; Stanford et al., 1990; Miao and Verma, 1993) do not show tissue-specific or correctly regulated expression patterns of the donor species in the heterologous host tobacco. These studies indicate that *trans*-acting proteins that interact with particular promoters may differ quantitatively, qualitatively, or in their distribution patterns in different species. The present study suggests the interesting possibility that expression of the H6H gene might be controlled by regulatory proteins specific to scopolamine biosynthesis. Although such proteins have not yet been identified biochemically or genetically, regulatory proteins and regulatory genes that act on the structural genes of a particular secondary metabolism are well known in the biosynthesis of anthocyanin pigments (Dooner et al., 1991).

H. niger and belladonna are close relatives, both belonging to the same Solaneae tribe of the Solanaceae. Although both species possess the H6H gene (Fig. 1) and synthesize scopolamine, the steady-state level of the H6H transcript in the cultured root is much lower in belladonna than in *H. niger* (Fig. 2). Presumably, this contributes to the large accumulation of hyoscyamine and comparatively low amounts of scopolamine in belladonna plants. There are marked differences between different scopolamine-producing plants with regard to H6H enzyme activities and, correspondingly, to the ratios of the levels of scopolamine to hyoscyamine (Hashimoto and Yamada, 1986). These differences between scopolamine high producers and low producers are presumably caused by varying expression of the H6H gene in different plants. Mutations in the *cis*-regulatory enhancer regions of the H6H promoter may increase or decrease the level of H6H expression while maintaining original tissue specificity.

Alternatively, the "activity" of the putative *trans*-acting regulator for scopolamine biosynthesis may be low in belladonna. The difference in activity could be due to either low expression or deleterious structural changes of the regulator. Low expression of the H6H promoter at the pericycle of belladonna (Table I) supports the latter *trans* regulator model but does not exclude simultaneous occurrence of the former *cis* model as well. It further implies that the regulatory gene for scopolamine biosynthesis, if such a gene exists, would control primarily the last two steps of scopolamine biosynthesis, i.e. the two-step epoxidation of hyoscyamine to scopolamine via 6β-hydroxyhyoscyamine, both steps of which are catalyzed by the bifunctional enzyme H6H (Hashimoto and Yamada, 1992). Regulatory genes that act on a subset of structural genes in a given biosynthetic pathway of secondary products have been reported. The structural genes of the latter part of the flavonoid pathway are coordinately regulated by the regulatory genes *Delila* and *Eluta* in *Antirrhinum* (Martin et al., 1991) and by several *An* genes in petunia (Gerats et al., 1984). In future studies, the H6H promoter of belladonna should be tested in both *H. niger* and belladonna.

Our previous immunohistochemical studies demonstrated that H6H polypeptides were localized in the pericycle of several cultured roots. Longitudinal sections of young roots of scopolamine-producing *Duboisia myoporoides* showed clear immunoreactive signals in the pericycle layer, but scarcely any signals at the root meristem (Hashimoto et al., 1991). However, GUS expression driven by the 827-bp 5′ flanking region of the *H. niger* H6H gene was clearly detectable at the root meristem region of *H. niger*, as well as at the root meristem region of tobacco and belladonna (Fig. 6). We suggest three possibilities to account for this apparent discrepancy. Endogenous H6H gene expression at the root meristem of *H. niger* might be suppressed by negative *cis* elements, which are absent from the promoter region used in this study. Negative *cis* elements that control spatial expression patterns were found in upstream regions of several plant genes (Burow et al., 1992; van der Meer et al., 1992), and several *cis*-acting elements have been reported to be present outside the 0.8-kb 5′ upstream region of the plant genes (Benfey et al., 1990; Douglas et al., 1991; Dietrich et al., 1992). The second possibility is that there might be some interspecies variation on the expression of H6H at the root meristem of scopolamine-producing plants, and that the endogenous H6H gene of *H. niger* might be expressed at the root meristem. The final possibility is that the H6H transcript or protein might be much more unstable in the root meristematic cells than in the pericycle cells. For example, the stability of pea *Fed-1* mRNA has been shown to be regulated by light (Dickey et al., 1992). Analysis of the H6H transcript and protein in transgenic belladonna plants (Yun et al., 1992) or cultured roots (Hashimoto et al., 1993b) that express *H. niger* H6H cDNA constitutively should address this possibility. Clearly, we need in situ localization of the H6H transcript in the roots of several scopolamine-producing species. In future studies, expression of the H6H gene at the root meristem will be further examined.

ACKNOWLEDGMENTS

We wish to thank Asuka Hayashi-Itoh for technical assistance in DNA sequencing, and Professor Ian M. Sussex (University of California, Berkeley) for discussions on anatomical features of pericycle cells.

Received October 15, 1993; accepted February 24, 1994.

The GSDB, DDBJ, EMBL, and NCBI accession number for the nucleotide sequence reported in this article is D26583.

LITERATURE CITED

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1987) Current Protocols in Molecular Biology. Green Publishing/Wiley Interscience, New York

Benfey PN, Chua N-H (1989) Regulated genes in transgenic plants. Science **244:** 174–181

Benfey PN, Chua N-H (1990) The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. Science **250:** 959–966

Benfey PN, Takatsuji H, Ren L, Shah DM, Chua N-H (1990) Sequence requirements of the 5-enolpyruvylshikimate-3-phosphate synthase 5′-upstream region for tissue-specific expression in flowers and seedlings. Plant Cell **2:** 849–856

Britsch L, Ruhnau-Brich B, Forkmann G (1992) Molecular cloning, sequence analysis, and in vitro expression of flavanone 3β-hydroxylase from *Petunia hybrida*. J Biol Chem **267:** 5380–5387

Budelier KA, Smith AG, Gasser CS (1990) Regulation of a stylar transmitting tissue-specific gene in wild-type and transgenic tomato and tobacco. Mol Gen Genet **224:** 183–192

Burow MD, Sen P, Chlan CA, Murai N (1992) Developmental control of the β-phaseolin gene requires positive, negative, and temporal seed-specific transcriptional regulatory elements and a negative element for stem and root expression. Plant J **2:** 537–548

Dayhoff MO, Schwartz RM, Orcutt BC (1978) *In* MO Dayhoff, ed, Atlas of Protein Sequences and Structure, Vol 5, Suppl 3. National Biomedical Research Foundation, Silver Spring, MD, pp 345–352

Deikman J, Fischer RL (1988) Interaction of a DNA binding factor with the 5′-flanking region of an ethylene-responsive fruit ripening gene from tomato. EMBO J **7:** 3315–3320

De Luca V, Marineau C, Brisson N (1989) Molecular cloning and analysis of cDNA encoding a plant tryptophan decarboxylase: comparison with animal dopa decarboxylases. Proc Natl Acad Sci USA **86:** 2582–2586

Dickey LF, Gallo-Meagher M, Thompson WF (1992) Light regulatory sequences are located within the 5′ portion of the *Fed-1* message sequence. EMBO J **11:** 2311–2317

Dietrich RA, Radke SE, Harada JJ (1992) Downstream DNA sequences are required to activate a gene expressed in the root cortex of embryos and seedlings. Plant Cell **4:** 1371–1382

Dittrich H, Kutchan TM (1991) Molecular cloning, expression, and induction of berberine bridge enzyme, an enzyme essential to the formation of benzophenanthridine alkaloids in the response of plants to pathogenic attack. Proc Natl Acad Sci USA **88:** 9969–9973

Dooner HK, Robbins TP, Jorgensen RA (1991) Genetic and developmental control of anthocyanin biosynthesis. Annu Rev Genet **25:** 173–199

Douglas CJ, Hauffe KD, Ites-Morales M-E, Ellard M, Paszkowski U, Hahlbrock K, Dangl JL (1991) Exonic sequences are required for elicitor and light activation of a plant defense gene, but promoter sequences are sufficient for tissue specific expression. EMBO J **10:** 1767–1775

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull **19:** 11–15

Fahn A (1990) Plant Anatomy, Ed 4. Pergamon Press, Oxford, UK, pp 270–307

Gerats AGM, Farcy E, Wallroth M, Groot SPC, Schram A (1984) Control of anthocyanin synthesis in *Petunia hybrida* by multiple allelic series of the genes *An1* and *An2*. Genetics **106:** 501–508

Hashimoto T, Hayashi A, Amano Y, Kohno J, Iwanari H, Usuda S, Yamada Y (1991) Hyoscyamine 6β-hydroxylase, an enzyme involved in tropane alkaloid biosynthesis, is localized at the pericycle of the root. J Biol Chem **266:** 4648–4653

Hashimoto T, Matsuda J, Yamada Y (1993a) Two-step epoxidation of hyoscyamine to scopolamine is catalyzed by bifunctional hyoscyamine 6β-hydroxylase. FEBS Lett **329:** 35–39

Hashimoto T, Yamada Y (1986) Hyoscyamine 6β-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, in alkaloid-producing root cultures. Plant Physiol **81:** 619–625

Hashimoto T, Yamada Y (1992) Tropane alkaloid biosynthesis: regulation and application. *In* B Singh, H Flores, J Shannon, eds, Biosynthesis and Molecular Regulation of Amino Acids in Plants. American Society of Plant Physiologists, Rockville, MD, pp 122–134

Hashimoto T, Yamada Y (1993) Nicotine and tropane alkaloids. *In* PJ Lea, ed, Methods in Plant Biochemistry, Vol 9. Academic Press, London, pp 369–379

Hashimoto T, Yukimune Y, Yamada Y (1986) Tropane alkaloid production in *Hyoscyamus* root cultures. J Plant Physiol **124:** 61–75

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

Hegnauer R (1986) Phytochemistry and plant taxonomy—an essay on the chemotaxonomy of higher plants. Phytochemistry **25:** 1519–1535

Holdsworth MJ, Bird CR, Ray J, Schuch W, Grierson D (1987a) Structure and expression of an ethylene-related mRNA from tomato. Nucleic Acids Res **15:** 731–739

Holdsworth MJ, Schuch W, Grierson D (1987b) Nucleotide sequence of an ethylene-related gene from tomato. Nucleic Acids Res **15:** 10600

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. Science **227:** 1229–1231

Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol Biol Rep **5:** 387–405

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907

Jefferson RA, Wilson KJ (1991) The GUS gene fusion system. Plant Mol Biol Manual **B14:** 1–33

Katagiri F, Chua N-H (1992) Plant transcription factors: present knowledge and future challenges. Trends Genet **8:** 22–27

Kosugi S, Ohashi Y, Nakajima K, Arai Y (1990) An improved assay for β-glucuronidase in transformed cells: methanol almost completely suppresses a putative endogenous β-glucuronidase activity. Plant Sci **70:** 133–140

Kutchan TM, Hampp N, Lottspeich F, Beyreuther K, Zenk MH (1988) The cDNA clone for strictosidine synthase from *Rauvolfia serpentina*: DNA sequence determination and expression in *Escherichia coli*. FEBS Lett **237:** 40–44

Lloret PG, Casero PJ, Pulgarín A, Navascués J (1989) The behaviour of two cell populations in the pericycle of *Allium cepa, Pisum sativum*, and *Daucus carota* during early lateral root development. Ann Bot **63:** 465–475

Martin C, Prescott A, Mackay S, Bartlett J, Vrijlandt E (1991) Control of anthocyanin biosynthesis in flowers of *Antirrhinum majus*. Plant J **1:** 37–49

Matsuda J, Okabe S, Hashimoto T, Yamada Y (1991) Molecular cloning of hyoscyamine 6β-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, from cultured roots of *Hyoscyamus niger*. J Biol Chem **266:** 9460–9464

Miao G-H, Verma DP (1993) Soybean nodulin-26 gene encoding a channel protein is expressed only in the infected cells of nodules and is regulated differently in roots of homologous and heterologous plants. Plant Cell **5:** 781–794

Nagel R, Elliott A, Masel A, Birch RG, Manners JM (1990) Electroporation of binary Ti plasmid vector into *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*. FEMS Microbiol Lett **67:** 325–328

Nakajima K, Hashimoto T, Yamada Y (1993) Two tropinone reductases with different stereospecificities are short-chain dehydrogenases evolved from a common ancestor. Proc Natl Acad Sci USA **90:** 9591–9595

Romeike A (1978) Tropane alkaloids—occurrence and systematic importance in angiosperms. Bot Not **131:** 85–96

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA **74:** 5463–5467

Schmid J, Doerner PW, Clouse SD, Dixon RA, Lamb CJ (1990) Developmental and environmental regulation of a bean chalcone synthase promoter in transgenic tobacco. Plant Cell **2:** 619–631

Stafford HA (1991) Flavonoid evolution. An enzymic approach. Plant Physiol **96:** 680–685

Stanford AC, Northcote DH, Bevan MW (1990) Spatial and temporal patterns of transcription of a wound-induced gene in potato. EMBO J **9:** 593–603

van der Meer IM, Brouwer M, Spelt CE, Mol JNM, Stuitje AR (1992) The TACPyAT repeats in the chalcone synthase promoter of *Petunia hybrida* act as a dominant negative *cis*-acting module in the control of organ specific expression. Plant J **2:** 525–535

Yun DJ, Hashimoto T, Yamada Y (1992) Metabolic engineering of medicinal plants: transgenic *Atropa belladonna* with an improved alkaloid composition. Proc Natl Acad Sci USA **89:** 11799–11803