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Two Tropinone Reductases, That Catalyze Opposite Stereospecific Reductions in Tropane Alkaloid Biosynthesis, Are Localized in Plant Root with Different Cell-Specific Patterns

Keiji Nakajima and Takashi Hashimoto [1]

Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara, 630-0101 Japan

In the plant species that produce tropane alkaloids, two tropinone reductases (TRs) catalyze the stereospecific reductions of the 3-carbonyl group of tropinone. This reduction is a key branch point that determines the metabolite flow into the separate alkaloid groups, each with different stereospecific configurations. In this study, a specific antibody was prepared for each of the TRs by immunizing mice with recombinant TR protein and subsequent immuno-affinity purification of the antiserum. Immunoblot analyses revealed that accumulation of both TRs was highest in the lateral roots of *Hyoscyamus niger* throughout its development. In cultured roots, TR proteins were accumulated in a basal region but not in root apex. These patterns were similar to that of hyoscyamine 6β-hydroxylase (H6H), an enzyme that catalyzes a downstream step in the same biosynthetic pathway. However, an immunohistochemical analysis revealed that the two TRs and H6H were accumulated with different cell-specific patterns in the cultured root, suggesting transportation of the alkaloid intermediate(s) across the different cell layers.

Key words: *Hyoscyamus niger* — Immunohistochemistry — Tropane alkaloids — Tropinone reductases (EC 1.1.1.206 and EC 1.1.1.236) — Stereospecificity.

Tropane alkaloids are a class of plant secondary products found mostly in several solanaceous plant species (Leete 1990). The alkaloid family includes several medicinally important compounds that act on mammalian nervous systems, such as L-hyoscyamine (its racemic form termed atropine) and L-scopolamine. Due to the complicated structures and stereoisomerism of the alkaloids, major commercial sources even at present are plants in the genera, *Duboisia, Scopolia*, and *Datura*. Therefore, regulation of tropane alkaloid biosynthesis in planta has attracted interest not only in view of plant physiology, but also for its applications in the pharmaceutical industry.

Abbreviations: DTT, dithiothreitol; GUS, β-glucuronidase; H6H, hyoscyamine 6β-hydroxylase; IPTG, Isopropyl β-D-thiogalactoside; PMT, putrescine *N*-methyltransferase; TR, tropinone reductase; ψ-tropine, pseudotropine.

[1] To whom correspondence should be addressed. E-mail: hasimoto @bs.aist-nara.ac.jp

Localized tropane alkaloid production in the plant root was first predicted from a grafting experiment, where a rootstock from a tropane alkaloid-producing species caused alkaloid accumulation in a scion from a non-producing species (cited in Waller and Nowacki 1978). This prediction has been corroborated more recently by measuring the enzyme activities of putrescine *N*-methyltransferase (PMT) and hyoscyamine 6β-hydroxylase (H6H), the enzymes that catalyze the first committed step of the tropane ring production and the last step of scopolamine biosynthesis, respectively (Fig. 1) (Hashimoto and Yamada 1986, Hashimoto et al. 1989). An immunohistochemical study using highly specific monoclonal antibody against H6H revealed this enzyme to be localized in the pericycle tissue of several scopolamine-producing species. Subsequent analysis of the hairy roots that had been transformed with the H6H promoter region from *Hyoscyamus niger* fused to the β-glucuronidase (GUS) reporter gene demonstrated that the pericycle-specific expression is controlled at the level of gene transcription (Kanegae et al. 1994). The promoter region of the PMT gene from *Atropa belladonna* conferred similar reporter gene expression pattern in the roots of the same species (Suzuki et al. 1999).

In the tropane alkaloid biosynthetic pathway, there is a key branch point which involves the conversion of tropinone to either tropine or pseudotropine (ψ-tropine). Each of these molecules is then incorporated into separate alkaloid group, each having opposite stereochemistry at their 3-hydroxyl groups. Two distinct NADPH-dependent tropinone reductases (TRs) are responsible for these stereospecific reactions: TR-I (EC 1.1.1.206) reduces the 3-carbonyl group of tropinone to the 3α-hydroxy group of tropine, whereas TR-II (EC 1.1.1.236) reduce the same carbonyl group to the 3β-hydroxy group of ψ-tropine (Fig. 1). All the tropane alkaloid-producing species so far examined have both TR activities, though their activity ratios vary considerably among the species. Since tropine and ψ-tropine do not interconvert in vivo, localization and activity of the two TR enzymes are the primary determinant of the metabolite flow into the branching pathways (Yamada et al. 1990). The two TR enzymes have been purified from the cultured roots of *Hyoscyamus niger*, and characterized in detail (Hashimoto et al. 1992). Subsequent cloning of the TR cDNAs from *Datura stramonium* revealed that TRs from this species are 64% identical at the

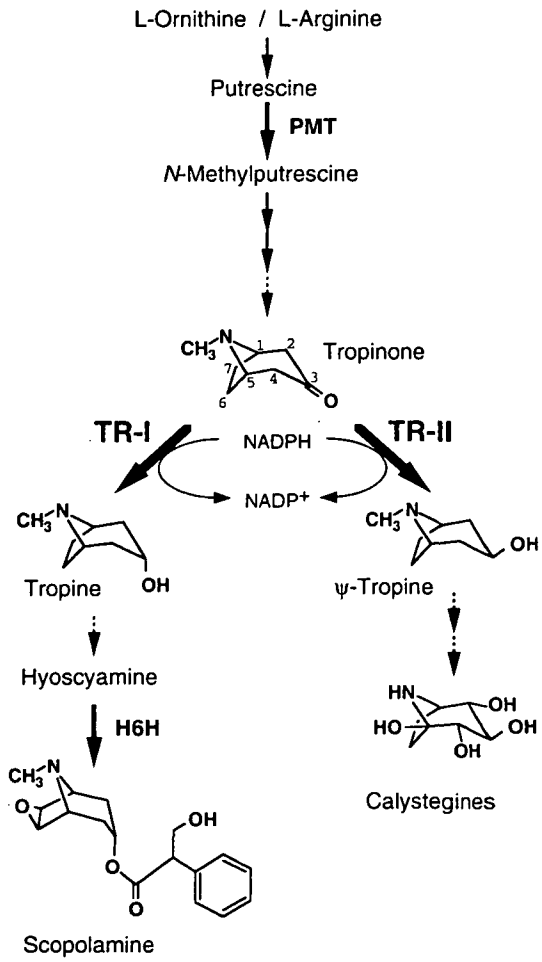

Fig. 1 Metabolic pathway of tropane alkaloids in *H. niger*. The tropane ring system arises from ornithine and/or arginine by way of putrescine. Putrescine is methylated by PMT and converted to tropinone, through partly unknown reactions. Tropine formed by TR-I is esterified with a phenylalanine derivative to give hyoscyamine, which is then converted to the end product scopolamine by H6H. ψ-Tropine, the product of TR-II, is thought to be converted to calystegines, but little is known about the reactions involved. At least three types of calystegines (A, B1, and B2) have been found in *H. niger*, but only the structure of calystegine B2, the most abundant form, is shown for simplicity. Carbon atoms of the tropane ring are numbered in the structure of tropinone. Dashed arrows indicate the steps whose chemical reactions are not completely known.

amino acid level, and hence have a common evolutionary origin (Nakajima et al. 1993b). In this study, highly specific antibodies were prepared for each TR, and used to analyze the localization of the TR proteins in *H. niger* plant and cultured root. Comparison of the accumulation patterns between TRs and H6H revealed possible translocation of alkaloid intermediates across different tissue layers in the root.

Materials and Methods

Plant materials—Plants of *H. niger* L. were maintained in a growth chamber set at 23°C under a short-day condition (8-h light/16-h dark). For the transition to reproductive growth, plants were placed under a long-day condition (16-h light/8-h dark). Root cultures of *H. niger* (line Hn) were maintained in a liquid Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 3% (w/v) sucrose on a rotary shaker (90 rpm) in the dark.

Construction of E. coli expression plasmids—Because both TR-I and TR-II cDNAs were obtained as separate 5′ and 3′ clones (Nakajima et al. 1993a, 1999a), they were first assembled to reconstitute a cDNA that covers an intact coding region.

After sequence confirmation, the assembled cDNA inserts were transferred to the *E. coli* expression vector pET21d (Novagen) using *Nco*I and another downstream restriction site to give pETTR1H and pETTR2H, which respectively express the TR-I and TR-II protein in native form without any tag peptide.

Protein purification and antibody production—pETTR1H and pETTR2H were introduced into *E. coli* strain BL21 (DE3) (Novagen). Bacteria were grown in 5 liters of LB medium supplemented with 200 μg ml^{-1} of ampicillin with vigorous shaking at 37°C. After the OD600 of the culture reached 1.0, isopropyl β-D-thiogalactoside (IPTG) was added at a concentration of 0.1 mM. The culture was gently agitated (100 rpm) at 25°C for 16 h to induce protein expression. Bacteria were harvested and lysed in 100 mM K-phosphate buffer pH 7.0, containing 3 mM dithiothreitol (DTT), 0.1% (v/v) Triton X-100, and 10 μg ml^{-1} lysozyme, by three rounds of a freeze-thaw cycle followed by sonication on ice. The lysate was centrifuged and the supernatant was fractionated with ammonium sulfate (45–75% saturation). TR-I was purified by three successive chromatographic steps on Butyl-Sepharose Fast Flow (Amersham Pharmacia Biotech), Red-Toyopearl 650ML (Toso, Japan), and MonoQ (Amersham Pharmacia Biotech). Purification of TR-II was done similarly by four successive chromatographic steps on Butyl-Sepharose Fast Flow, Q-Sepharose Fast Flow, Phenyl-Superose, and MonoQ (All the four gels from Amersham Pharmacia Biotech). Anti-TR antisera were produced in mice by a custom antibody production service (Takara Shuzo Co.). Purified TR-I (1.15 mg) and TR-II proteins (2.60 mg) were used to immunize three and four mice, respectively.

Purification of antibodies—Purified TR-I or TR-II protein (1 mg) was immobilized on a Hi-Trap NHS-activated column (1 ml of gel bed, Pharmacia), according to the manufacturer's instruction. The gel was then removed from the column, and treated with 0.5% (v/v) glutaraldehyde in TBS buffer (20 mM Tris-HCl pH7.5, 150 mM NaCl) to cross-link the TR subunits. The gel was washed with GBST buffer (50 mM Glycine-HCl pH2.5, 150 mM NaCl, 0.05% (v/v) Tween-20), and then equilibrated with TBST buffer (TBS containing 0.05% (v/v) Tween-20). Purification was done in two steps. In the first step, each antiserum was diluted by ten-fold with TBST (total 200 μl) and mixed with the gel (approx. 100 μl bed) that had been immobilized with the same antigen which was used to raise each antiserum. After gentle agitation at 4°C for 1 h, the gel was recovered from the mixture by centrifugation and then successively washed with TBST and CBST (50 mM Na-citrate pH 4.2, 150 mM NaCl, 0.05% (v/v) Tween-20) buffers. Bound antibodies were recovered from the gel by washing with GBST buffer, and immediately neutralized by adding 1/10 volume of 1 M Tris-HCl pH7.5. In the second step, antibodies from the first step were mixed with the gel that had been immobilized with the different TR protein from that used to raise each antiserum, i.e.,

TR-II-immobilized gel for anti-TR-I antiserum, and vice versa. After gentle agitation at 4°C for 1 h, gel was removed by centrifugation and the supernatant aliquoted and stored at −20°C until use.

Immunoblot analysis—Proteins were separated by an SDS-PAGE (12.5%T), and transferred to an Immobilon filter (Millipore) with Transblot SD (Bio-Rad). The filter was then immersed in a blocking solution (1% (w/v) bovine serum albumin (Nacalai Tesque, Japan) in TBS) for 1 h. Incubation with primary and secondary antibodies was carried out in TBST buffer containing 1% (w/v) bovine serum albumin. Signal detection was done with either the ECL or ECL-plus Western Blotting System (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Immunohistochemisty—Root samples were cut into 1-cm pieces and immersed in a fixation solution composed of 33 mM Na, K-phosphate buffer pH 7.4 and 2.7% (w/v) parafolmaldehyde (TAAB, U.K.) for 16 h at room temperature. Roots were washed three times in 66 mM Na, K-phosphate buffer pH 7.4, and then embedded in a 5% (w/v) agar solution containing 50 mM K-phosphate pH 7.0 and 5 mM DTT. After cooling at 4°C, roots in the gel block were sliced into 75-μm sections by a microslicer (Dosaka EM, Japan). Root sections were separated from the surrounding gel and washed once with TBST buffer. The root sections were then successively incubated in blocking solution (the same solution as used for the above immunoblot) and then the same solution containing the purified anti-TR antibodies. Each incubation was 1 h. The sections were washed three times with TBST buffer and then incubated with the blocking solution containing the goat anti-mouse IgG (H+L) antibody labeled with 1-nm gold particles (British BioCell, U.K.). Sections were washed three times with TBST buffer and then twice with H_2O. The signal of the secondary antibodies on the root sections was enhanced using a Silver Enhancing Kit (British BioCell). For a control experiment, sections from the same root sample were incubated with 5.5 μg ml^{-1} of anti-H6H monoclonal antibody (clone mAb5) (Hashimoto et al. 1991).

Results

Preparation of the specific antibodies—In order to prepare sufficient amount of proteins needed for the antibody preparation, TR-I and TR-II proteins were expressed in *E. coli* from their respective cDNAs using the T7 expression system (Studier et al. 1990). After three and four chromatographic steps for TR-I and TR-II, respectively, both TR proteins were purified to apparent homogeneity. The recombinant TR proteins were enzymatically active and showed the same strict stereospecificity observed for the enzymes purified from the cultured roots (data not shown) (Hashimoto et al. 1992). Purified recombinant proteins were used to raise murine polyclonal antibodies. Among the three and four antisera produced for TR-I and TR-II, respectively, the one with highest titer was chosen for each TR, and was used in the subsequent experiments.

Because TR-I and TR-II proteins are highly homologous to each other, it was predicted that the antisera would have some degree of cross-reactivity. This was tested by using a filter dot-blotted with the known amounts of the antigens. For both anti-TR-I and anti-TR-II antisera, crude

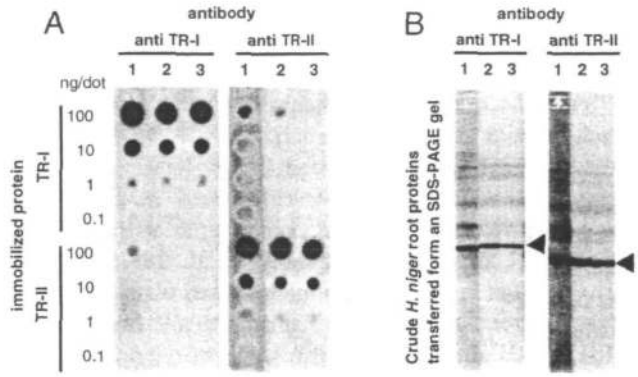

Fig. 2 Specificity of crude anti-TR antisera and their purification products by immuno-affinity purification. Cross-reactivity to the different TR and other root proteins was tested, with filter strips dot-blotted with known amounts of the purified TRs (A) and western-blot strips of root extract from *H. niger* cultured roots (B). Numbers on top of the strips indicate the antibodies from the following purification steps; 1, crude antiserum before purification; 2, antibodies selectively eluted from the antigen-immobilized gel; 3, the same antibodies as 2, but after the absorption by the gel linked to the different TR. In (B), each lane contained 5 μg of protein.

mouse serum cross-reacted to the different TR (Fig. 2A). Although cross-reacting signal was only about 5% of that for the true TR antigen, this may have caused a problem in analyzing specific TR accumulation, especially in the immunohistochemical analysis, where TR-I and TR-II can not be distinguished by their different molecular weight. We therefore removed the cross-reacting activities with a two-step immunoaffinity purification procedure. In the first step, antibodies that bind to the true antigen were selectively captured by an antigen-immobilized matrix at a neutral pH and then released by an acidic buffer. After neutralization of this fraction, the cross-reacting antibodies were removed by absorption to the matrix attached with the different TR proteins. Antibodies from each purification step were tested for their cross-reactivities by using the above dot-blot filters (Fig. 2A), as well as the western blot strips of an *H. niger* root extract (Fig 2B). For the anti-TR-I antiserum, the first purification step alone was sufficient to remove the antibodies which cross-reacted with TR-II. In contrast, this step failed to remove the cross-reactivity of the TR-II antiserum completely, (though it was effective in removing non-specific binding to the filter or to bovine serum albumin used for the blocking), and the specific antibodies were obtained only after the second purification step. Because the amount of the antisera was limited, this purification procedure could not be scaled up to provide sufficient antibodies for the subsequent immunoblot analysis. We therefore used crude anti-TR antisera for the immunoblot analysis, where cross-reactions

can be recognized from the electrophoretic migration, while the purified antibodies were used in the immunohistochemical studies of the root sections.

Accumulation of TRs in an H. niger plant at various developmental stages—Accumulation of TRs was analyzed first by immunoblot using the protein extracts from *H. niger* plants at various developmental stages (Fig. 3). The replicated blots were also incubated with anti-H6H monoclonal antibody (Hashimoto et al. 1991) for comparison. High levels of accumulation was observed in root extracts after 6 d post germination for all three enzymes (TR-I, TR-II and H6H). At this stage, cotyledons had just fully expanded, and the root extended 2–3 cm without apparent branching. Although weak signal was also detected in the aerial part, this was likely due to incomplete separation of the roots from the rest of the plant. This pattern was maintained 16 d post germination, when first two true leaves expanded and the root started to branch. At 25 d post germination, four true leaves expanded and the mature part of the primary root (the first root emerging from a seed) showed radial swelling. At this stage, TR-I and TR-II showed slightly different distribution between primary root and lateral roots. While TR-I was detected at a higher level in lateral roots than in primary root, TR-II protein was more abundant in primary root than in lateral roots. Accumulation of H6H at this stage was similar to that of TR-I. At 48 d post germination, the plants underwent rapid vegetative growth and the primary root became swelled and stiff, typical for the main root. At this time the accumulation of all three enzymes was prominent in the lateral roots.

After this stage, some of the plants were transferred to a long-day condition. This treatment caused the plant to start bolting and initiate reproductive organ differentiation at the shoot apex. After 28 d in the long-day condition (76 d post germination), the first apical flowers had fully opened. In this growth phase, the lateral root specific ac-

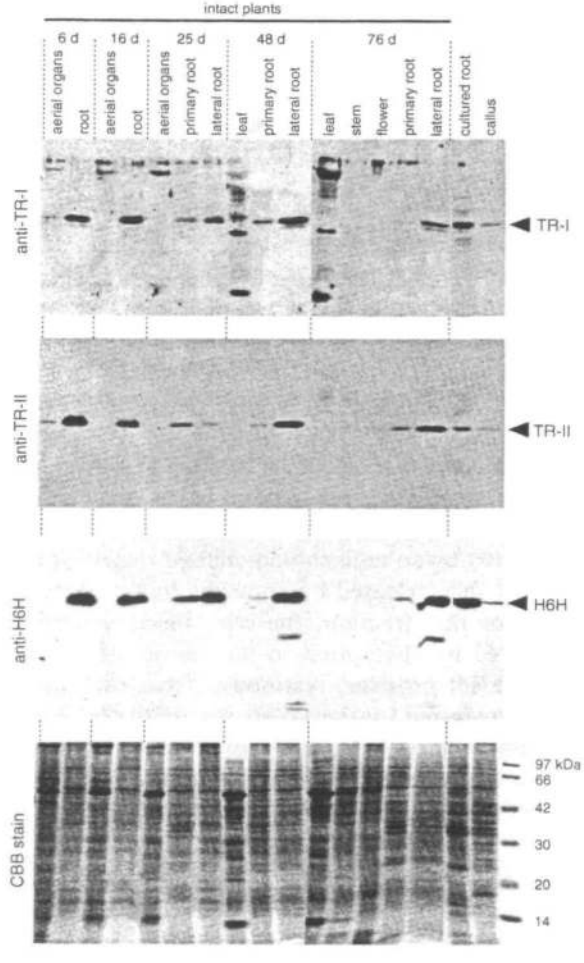

Fig. 3 Immunoblot analyses of the protein extracts from intact *H. niger* plants at various developmental stages, cultured roots and callus. Each lane contained 10 μg of protein. For 6, 16, and 25-day plants, the protein sample 'aerial organs' was extracted from a mixture of leaves and very small hypocotyl region. The TR-I antiserum showed non-specific binding to some abundant proteins (mostly to the Rubisco subunits) in the leaf extracts.

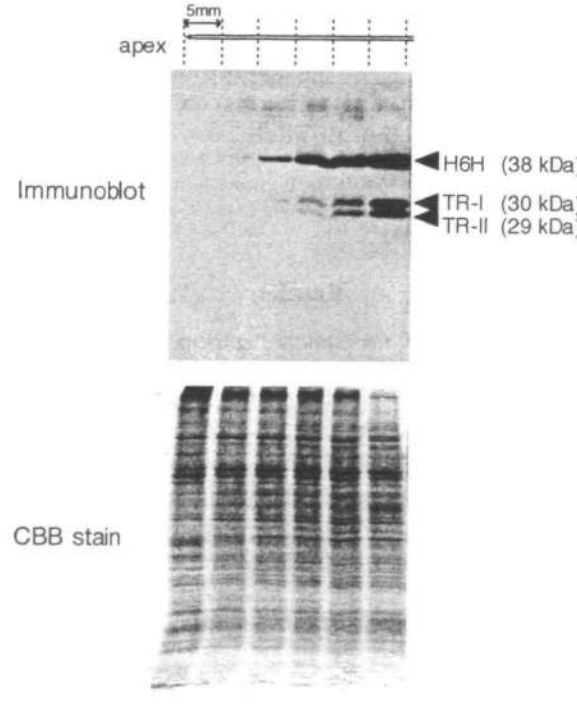

Fig. 4 Immunoblot analysis of the proteins extracted from the consecutive 5-mm segments of *H. niger* cultured roots. Antibodies for the three enzymes (TR-I, TR-II and H6H) were incubated with a single blot. All three enzymes were absent in the apical 10-mm region. Note that signal intensity can be compared within one protein species, but not across different enzymes, because of different detection limit of the three antibody preparations. Each lane contained 10 μg of protein.

cumulation of all three enzymes was still maintained. In general, *H. niger* plants constitutively accumulated the three enzymes in relatively young roots throughout the plant's development.

Accumulation of the alkaloid biosynthetic enzymes was also analyzed using both cultured roots and callus of *H. niger*. In cultured roots, accumulation of the three enzymes was similar to that in the plant lateral roots as compared with that in the plant main roots (Fig. 3). This observation is consistent with a previous study in which

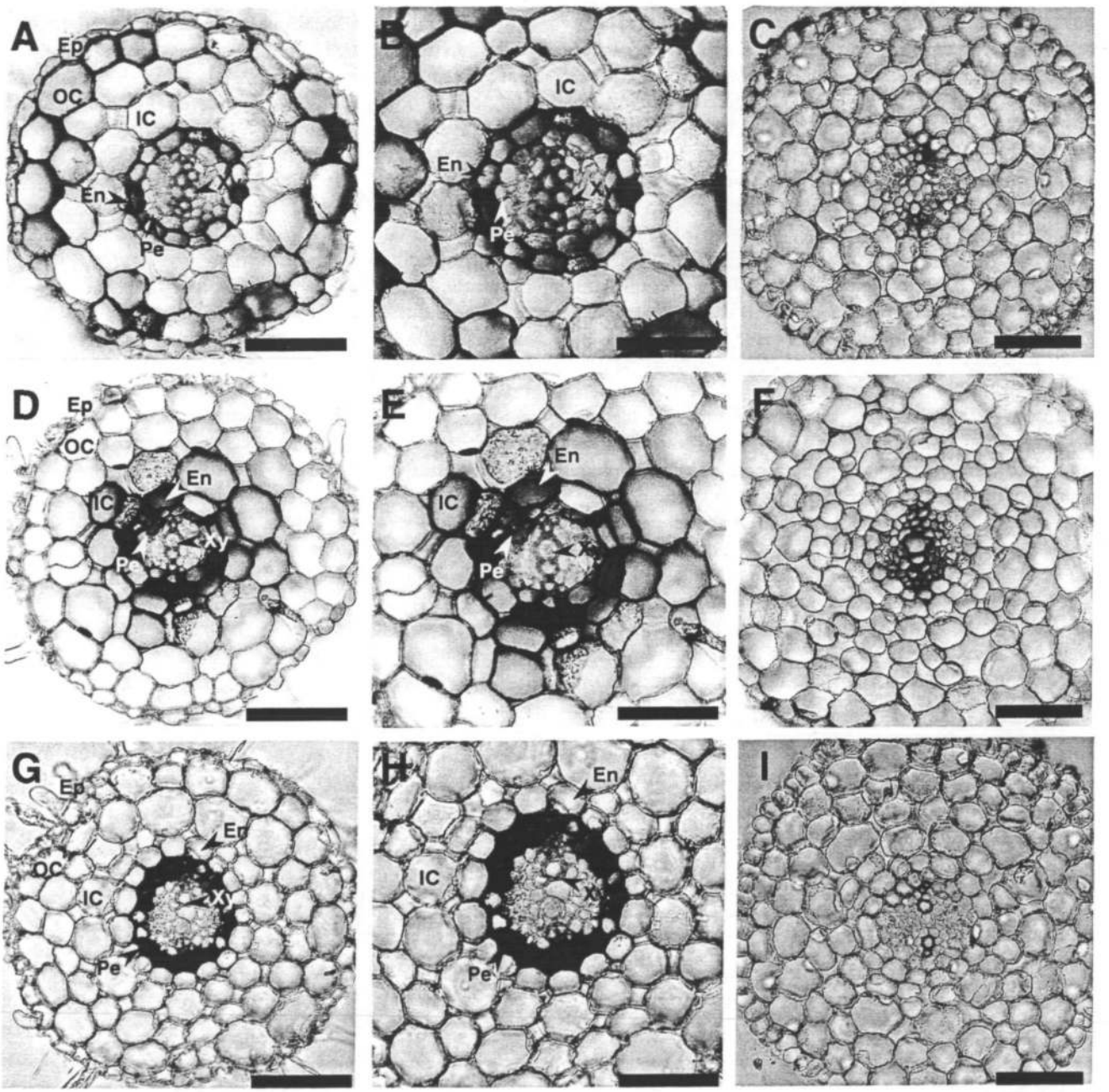

Fig. 5 Immunohistochemical analyses of the root cross sections for TR-I, TR-II and H6H. (A) Root cross section of *H. niger* treated with anti-TR-I antibodies. (B) Magnification of (A). (C) Root cross section of *N. tabacum treated* with anti-TR-I antibodies. (D) Root cross section of *H. niger* treated with anti-TR-II antibodies. (E) Magnification of (D). (F) Root cross section of *N. tabacum treated* with anti-TR-II antibodies. (G) Root cross section of *H. niger* treated with anti-H6H antibodies. (H) Magnification of (G). (I) Root cross section of *N. tabacum treated* with anti-H6H antibodies. Ep, epidermis; OC, outer cortex; IC, inner cortex; En, endodermis; Pe, pericycle; Xy, xylem. Bar = 0.1 mm.

plant lateral roots and cultured roots had similar TR activities (Hashimoto et al. 1992). In callus, only weak accumulation was detected for any of the three enzymes (Fig. 3).

Localization patterns of TRs in H. niger roots—Accumulation of the TR enzymes in *H. niger* root was studied in detail. In these experiments, accumulation of H6H was again analyzed in parallel for comparison. Cultured roots were chosen, because they could be handled more easily than roots in soil, and had similar levels of the biosynthetic enzymes to the plant lateral root. In the first experiment, about 200 cultured roots were cut into 5 mm-pieces, and segments pooled according to their distance from the root apex. Proteins were extracted from each pool, and subjected to an immunoblot analysis. A single blot was simultaneously incubated with the three antisera (or antibody), because limited amounts of the protein extracts were available. As seen in Fig. 4, the three proteins migrated as clearly separated bands. All the three enzymes were absent in the apical 10-mm region of the root and began to accumulate in the more basal portions of the roots.

In the next series of experiments, protein localization was analyzed at the tissue level, using the silver-enhanced immunogold detection method. Based on the result from the above immunoblot, the region at about 2 cm from the root apex was used to prepare sections. As seen in Fig. 5A, for instance, *H. niger* root has a typical radial tissue pattern (Esau 1977). In the center is a stele that usually consists of two xylem ridges and a surrounding pericycle layer. The stele is surrounded by one cell layer of endodermis, three layers of cortex, and one epidermal layer. As a negative control, roots of *N. tabacum* that does not possess neither of the three enzyme genes (Nakajima et al. 1993b, Kanegae et al. 1994), were treated in parallel (Fig. 5; panels C, F and I).

Strong signal for TR-I protein was found in the endodermis and cortex (Fig. 5; panels A and B). In the cortex, TR-I protein was preferentially localized in the outer cell layers. Epidermis, pericycle and stele parenchyma cells did not give any signal above the background. TR-II, however, showed a different localization pattern (Fig. 5; panels D and E). The protein was accumulated in the pericycle, endodermis and inner cortex layers, but not in stele parenchyma, the outermost cortical layer or the epidermis. The highly specific localization of H6H protein in pericycle, as shown in a previous study (Hashimoto et al. 1991), was reproduced regardless of the different experimental conditions adopted in this study (Fig. 5; panels G and H).

Differentiated cells in some root tissue have the potential to redifferentiate into different cell types. The most dramatic event that occurs in normal root development may be the formation of a lateral root from a pericycle cell. Because TR-II and H6H were accumulated in the pericyclic cells, this indicates that the gene expression for these two proteins may cease early during the formation of lateral roots. In order to analyze TR accumulation in this process, we prepared section from the region of lateral root initiation, and analyzed for the protein accumulation. As seen in Fig. 6, no signal was detected in the cells of lateral root primordia for either of the three biosynthetic enzymes. Interestingly, the endodermal cells surrounding the primordia, which themselves are not incorporated into the nascent lateral root, also ceased to express the two TR proteins. For TR-I protein, signal was detected at the basal region of the primordia in a tube-shaped zone surrounding a putative vascular cylinder of the emerging lateral root (asterisks in Fig. 6A). Judging from their position in

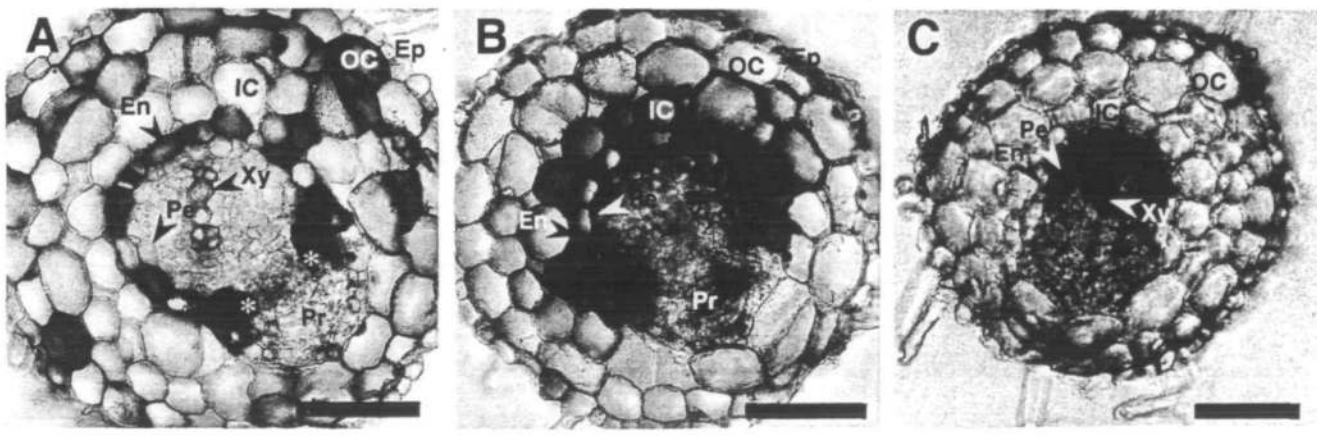

Fig. 6 Immunohistochemical analyses of root cross sections prepared form *H. niger* cultured roots with a nascent lateral root primordia. Antibodies used were anti-TR-I (A), anti-TR-II (B), and anti-H6H (C). Asterisks in *A* mark the primordial cells that gave a signal for the TR-I protein accumulation. Ep, epidermis; OC, outer cortex; IC, inner cortex; En, endodermis; Pe, pericycle; Xy, xylem; Pr, lateral root primordia. Bar=0.1 mm.

the primordia, these cells likely correspond to the newly differentiated endodermis (Malamy and Benfey 1997).

Discussion

Production of specific antibodies for homologous enzymes—Two TRs from *H. niger* share 64% identity at the amino acid level. In order to prepare specific antibodies for each of these homologous proteins, several strategies can be used. Since the C-terminal half of TRs is less conserved than the N-terminal half, heterologously expressed C-terminal polypeptides might be used as antigens. However, the C-terminal regions still share considerable homology, and do not constitute a defined structural domain in the TR subunits (Nakajima et al. 1998). Use of synthetic oligopeptides as an antigen does not always produce the desired antibodies with sufficient specificity. A close inspection of the TR crystallographic structures revealed that the non-homologous amino acids were often located at the protein's surface, raising the possibility that the TR proteins in their native form can serve as specific antigens. Based on this prediction, each TR protein was expressed in *E. coli* as in its native form, and purified by column chromatography. Crude murine antisera raised against these purified proteins showed remarkable specificities to each TR (Fig. 2). A weak cross-reactivity was still observed in both antisera, but this cross-reactivity was removed by an immunoaffinity purification method; the resultant specific antibodies were successfully used for immunohistochemistry. The method adopted in this study is relatively simple and straightforward, and could be used in other cases where accumulation of homologous protein is to be analyzed.

Constitutive expression of the alkaloid synthetic enzymes in plant root—Tropane alkaloid production has been postulated to be localized to the plant root, because grafting roots of an alkaloid-producing plant to a scion of a non-producing plant, but not vice versa, resulted in alkaloid accumulation in the scion (Waller and Nowacki 1978). After identification of several enzymes in the biosynthetic pathway, localization of these enzymes in specific tissues became the subject of biochemical studies. Activities of PMT and H6H, that catalyze the first committed step of the tropane ring formation and the last two steps of scopolamine biosynthesis respectively, were both high in root cultures but negligible or no activity was detected in shoot cultures and callus (Hashimoto and Yamada 1986, Hashimoto et al. 1989). In this study, distribution of the TR proteins in *H. niger* was studied using specific antibodies. The results clearly indicate that the two TR enzymes were localized in developmentally young roots, i.e. plant lateral roots and cultured roots (Fig. 3). By an RNA gel blot using *D. stramonium*, TR transcripts have been shown to accumulate in root organs (our unpublished results). The root-specific accumulation of TRs is not surprising, considering the first and the last step of the scopolamine biosynthesis (PMT and H6H) are also localized in young roots. Taken together, these observations suggest that all the other enzymes of the tropane alkaloid biosynthesis, including those not yet identified, are localized in the root of *H. niger*, and that the tropane alkaloids are produced entirely in the plant root.

Immunoblot analysis using anti-TR and anti-H6H antibodies revealed that all three enzymes accumulate at a constant level throughout the plant development. The roots of 6-d old seedlings had accumulated similar amounts of their proteins as compared with amounts accumulated at later stages. In a parallel quantification of alkaloids in these plant organs, leaves of the 6-d old seedlings were found to accumulate similar amount of scopolamine to that found in the plants at the later stages (data not shown). These observations are reminiscent of the postulated ecochemical role of tropane alkaloids, where the toxic nature of these metabolites may act in repelling herbivores (Wink 1998). Unlike attack by fungal or bacterial pathogens, attack by herbivores, especially by vertebrates, destroys large parts of the plant instantaneously. Therefore, constitutive accumulation of toxic compounds may reduce the chance of herbivory, and may enhance the survival rate of a plant population at a certain location over long time.

Different tissue-specific localization patterns of TRs and H6H in H. niger root—The two TR proteins showed a different cell-specific localization pattern, not only from each other, but also from H6H. While TR-I was preferentially localized in the endodermis and the outer cortical layer, TR-II was found in the pericycle, endodermis and inner cortical layers, as shown schematically in Fig. 7. The most striking feature of the TR localization pattern may be

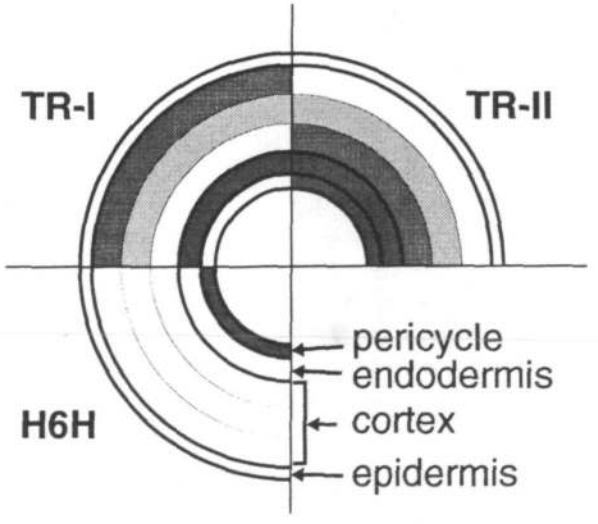

Fig. 7 Schematic representation of the tissue-specific accumulation patterns of TR-I, TR-II and H6H.

the absence of TR-I in the pericycle layer, because the downstream enzyme, H6H, has been found exclusively in this layer (Hashimoto et al. 1991). The different compartmentation of TR-I and H6H in root tissue layers indicates that at least one of the two intermediates between the TR-I and H6H reactions (tropine and hyoscyamine) must move across the root tissue layers. Although little is known about cell to cell movement of plant secondary metabolites, different compartmentation of biosynthetic enzymes has also been reported recently for monoterpenoid indole alkaloid metabolism of *Cathranthus roseus* (St-Pierre et al. 1999).

Because the endodermal cell wall possesses a casparian strip that physically blocks apoplastic metabolite flow across this tissue layer (Esau 1977), only the tropane intermediate(s) that come out on the inner side of the endodermal cells can be incorporated into scopolamine. Taking these into account, it may be important to note that the endodermis is the only tissue where both TR-I and TR-II co-exist and hence compete for the common substrate, tropinone. Because both TR-I and TR-II are expressed at a relatively constant level, as seen in the immunoblot analysis (Fig. 3), endodermis can be considered to be a tissue that constitutively supplies constant amounts of tropine and ψ-tropine. The two tropane alcohols (or their derivatives) may be transported from the endodermis to the adjacent cell layers (pericycle and inner cortex), with those reaching the pericycle possibly being converted to scopolamine, which is then translocated to aerial organs through the adjacent xylem tissue (cited in Wink and Roberts 1998).

Absence of TR-I protein in the pericycle layer also raised a question on its relation to earlier steps of the tropane alkaloid biosynthesis, since promoter region of PMT gene from *A. belladonna* conferred expression of GUS reporter gene in root pericycle (Suzuki et al. 1999). Substrate of PMT is produced by decarboxylation of amino acids, ornithine and arginine. Because amino acids can be translocated in plant through vascular tissue, it may be reasonable to assume that PMT protein also is localized in the pericycle, where amino acids flowing in vascular tissue are unloaded. In this hypothesis, methylputrescine produced by PMT (or its derivative) may then be transported to outer tissue layers, where downstream enzymes (including TRs) convert them to various alkaloid intermediates (see discussion below). Alternatively, PMT protein may be distributed in the cell layers other than pericycle, since tentatively assigned promoter regions confer different expression patterns to reporter genes in some cases. Analysis of PMT protein localization is necessary to distinguish between these two possibilities. In this respect, it is noteworthy that localization of GUS gene product driven by excised TR promoters did not completely match the TR protein localization revealed in this study (Nakajima et al. 1999b).

Without a detailed description of the biosynthetic enzymes involved in the formation of tropane alkaloids other than scopolamine, it is difficult to correlate the presented TR accumulation patterns to the possible metabolic regulation at the branch point. TR-I protein was also found to be accumulated in the outer cortex layer, but it is unlikely that TR-I in this layer is directly involved in the scopolamine formation. This is so because this layer is quite distant from the pericycle. One possibility is that tropine formed in the outer cortex layer is incorporated into a minor tropane ester(s), such as 3α-tigloyloxytropane (Romeike 1978). As compared with tropine, much less is known about the metabolism of ψ-tropine. Recent identification of calystegines (polyhydroxylated norpseudotropines) in both leaf and root of *H. niger* plants, however, suggested that most of the ψ-tropine produced in root is converted to this alkaloid group (Dräger et al. 1995). Since calystegines have a glycosidase-inhibiting activity, accumulation of these alkaloids in aerial organs may also be effective in protecting plants against herbivorous animals. Calystegines are in fact accumulated in the aerial organs (Molyneux et al. 1993, Dräger et al. 1995, Asano et al. 1997). Localized accumulation of TR-II in the inner root cell layers may reflect the roles of these ψ-tropine derivatives in the aerial organs. The identification of calystegine biosynthetic enzymes in the future should be interesting, especially the analysis of the localization of these enzymes compared with those of TRs.

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