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Two tropinone reductases with different stereospecificities are short-chain dehydrogenases evolved from a common ancestor

(stereospecificity/protein evolution/tropane alkaloids)

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ABSTRACT **In the biosynthetic pathway of tropane alkaloids, tropinone reductase (EC 1.1.1.236) (TR)-I and TR-II, respectively, reduce a common substrate, tropinone, stereospecifically to the stereoisomeric alkamines tropine and pseudotropine (ψ-tropine). cDNA clones coding for TR-I and TR-II, as well as a structurally related cDNA clone with an unknown function, were isolated from the solanaceous plant *Datura stramonium*. The cDNA clones for TR-I and TR-II encode polypeptides containing 273 and 260 amino acids, respectively, and when these clones were expressed in *Escherichia coli*, the recombinant TRs showed the same strict stereospecificity as that observed for the native TRs that had been isolated from plants. The deduced amino acid sequences of the two clones showed an overall identity of 64% in 260-amino acid residues and also shared significant similarities with enzymes in the short-chain, nonmetal dehydrogenase family. Genomic DNA-blot analysis detected the TR-encoding genes in three tropane alkaloid-producing solanaceous species but did not detect them in tobacco. We discuss how the two TRs may have evolved to catalyze the opposite stereospecific reductions.**

Two stereospecific reductases constitute a branching point in the biosynthesis of tropane alkaloids (1) (Fig. 1). Tropinone reductase (EC 1.1.1.236) I (TR-I) converts the 3-keto group of tropinone to the 3α-hydroxyl of tropine (3α-hydroxytropane), whereas tropinone reductase II (TR-II) reduces the same keto group to the 3β-hydroxyl of pseudotropine (ψ-tropine) (3β-hydroxytropane). Various tropane esters, such as hyoscyamine and scopolamine, are derived from tropine. Although the metabolism of ψ-tropine is not well understood, calystegins and tigloidine are possible metabolites of this stereoisomer. Cocaine found in *Erythroxylum* is a well-known tropane ester of the ψ-tropine type. Both TR-I and TR-II are present together in any given tropane alkaloid-producing species and are absent altogether in nonproducing species (2). The ratios of the two TR activities vary considerably, depending on the species, and may determine the flux of metabolites at the branching point because no interconversion between tropine and ψ-tropine has been observed *in vivo* (3).

Characterization of these two highly purified TRs from the cultured roots of *Hyoscyamus niger* revealed that they had both common and different properties (2). Both TRs are probably homotetramers with 29- to 30-kDa subunits, and belong to the NADPH-dependent, class B oxidoreductases, which transfer the pro-*S* hydrogen of NAD(P)H to their substrates. The affinity for NADPH is similar in both enzymes, and the inability of EDTA to inhibit either TR indicated that neither contains zinc. In contrast, there are several notable differences between TR-I and TR-II with regard to the reversibility of their reactions and their reactivity toward several ketones. Molecular characterization is necessary to further establish the relationship between the two TRs. We report here the primary structures of TR-I and TR-II and discuss how these enzymes may have evolved to catalyze opposite stereospecific reductions.†

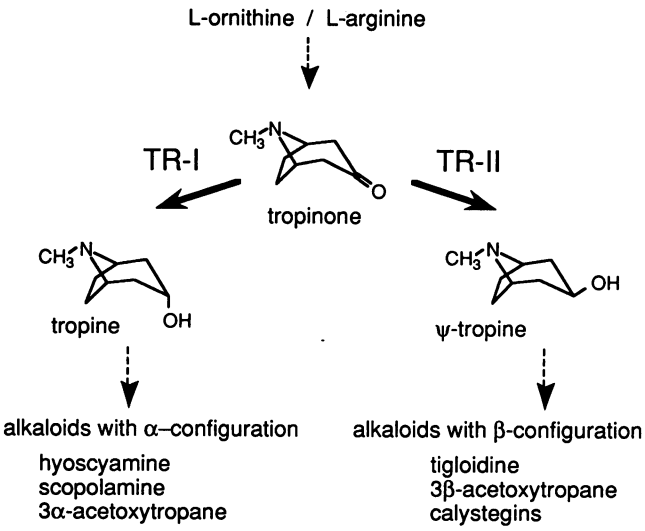

FIG. 1. Metabolism of tropinone. Tropinone is reduced stereospecifically either to tropine by TR-I or to ψ-tropine by TR-II. Tropine and ψ-tropine are then esterified with various organic acids to give, for example, hyoscyamine, scopolamine, and 3-acetoxytropanes. Further hydroxylations at other tropane carbons may give calystegins.

MATERIALS AND METHODS

Plant Materials. A hairy root clone (DS1) of *Datura stramonium* L. was induced by the infection of *Agrobacterium rhizogenes* (strain 15834) at our laboratory and grown at 25°C in the dark on a gyratory shaker (90 rpm) in Gamborg B5 medium/3% (wt/vol) sucrose/1 μM indole-3-butyric acid. Cultured roots of *H. niger* L. were grown as described (2). Plants of *D. stramonium*, *H. niger*, *Atropa belladonna* L. and *Nicotiana tabacum* L. were grown in a growth chamber at 20°C and 80% relative humidity and harvested at the flowering stage.

Protein Sequencing. TR-I and TR-II were purified to homogeneity from cultured roots of *D. stramonium* and *H. niger*, respectively, by a series of chromatographic steps, essentially as described (2). Purification was followed by preparative electrophoresis using model 491 Prep Cell (Bio-

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Abbreviations: TR, tropinone reductase; SDH, short-chain dehydrogenase; ψ-tropine, pseudotropine.

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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. L20473, L20474, and L20475).

Rad). Because no sequence was obtained from the direct sequencing of the amino termini of these purified proteins, probably due to the amino-terminal blockage, the internal amino acid sequences were analyzed. The purified proteins were digested by lysylendopeptidase (EC 3.4.21.50; Wako Pure Chemical, Osaka), and the peptide fragments were separated by reversed-phase HPLC using YMC packed column AP-303 S-5 300 Å octadecylsilyl silica (YMC, Kyoto), with a linear 20–70% gradient of acetonitrile in 0.1% (vol/vol) trifluoroacetic acid. The amino-terminal sequences of several peptide fragments were determined with a 477A/120A gas-phase protein sequencer (Applied Biosystems).

Construction and Screening of the cDNA Library. Manipulations of DNA and RNA were done as described (4). The cDNA library was constructed from poly(A)$^+$ RNA isolated from the hairy roots of *D. stramonium* by using the Librarian II cDNA library construction system (Invitrogen) with pcDNAII (Invitrogen) as a cloning vector.

An oligonucleotide probe of the sequence 5′-AAYTTYG-ARGCIGCITAYCA-3′ (Y, T or C; R, A or G) represents the internal amino acid sequence Asn-Phe-Glu-Ala-Ala-Tyr-His commonly found in both TR-I and TR-II. The probe was end-labeled with [α-^{32}P]ATP by T4 polynucleotide kinase and used to screen 1.5×10^5 clones of the cDNA library.

Fourteen clones were isolated and classified into three groups (groups I, II, and III), according to the nucleotide sequences of both ends of the cDNA inserts. The complete nucleotide sequence of the clone that contained the longest cDNA insert of each group was determined on both strands by using a set of exonuclease III-deleted clones. The clones from groups I and II encoded the internal amino acid sequences corresponding to TR-I and TR-II, respectively, but they lacked the amino-terminal regions. The group III clones contained a full open reading frame that coded for a protein homologous to TRs. The polypeptide encoded on the group III cDNAs is subsequently referred to as P29X.

An additional 5×10^4 clones of the cDNA library were screened with the newly synthesized oligonucleotide probe with the sequence 5′-AGAGCCACCAGTAACAAGGGC-3′, which corresponds to a nucleotide sequence found in the 5′-region of the cDNAs of both groups I and II. Clones that comprised the entire open reading frames were isolated. The longest clones from groups I, II, and III were named pDTR1, pDTR2, and pDX, respectively. pDTR1 and pDTR2 gave the same restriction map as those of the previously isolated shorter clones in each group, and the nucleotide sequences also matched except for 1 nt in pDTR2. Because the other parts of the sequences matched completely, it is believed that the mismatch was introduced either at the reverse transcription step or during other procedures of the cDNA library construction, and that all of the group II cDNAs were derived from one gene.

Expression of the Cloned cDNAs in *Escherichia coli*. A cDNA truncated by several amino acid residues at the amino terminus was selected from each group, connected in frame with the β-galactosidase-coding region of pcDNAII and expressed as a fusion protein in *E. coli* strain NM522 under the control of *lac* promoter. Fusion proteins were designed to have the 38-amino-terminal residues of β-galactosidase and the polypeptide of either TR-I, TR-II, or peptide P29X minus 16-, 4-, or 2-amino-terminal residues, respectively.

TR activities in the *E. coli* extracts were measured by using gas/liquid chromatography with a described method (2).

Genomic DNA-Blot Analysis. Genomic DNAs isolated from mature leaves were digested with either *Bam*HI, *Eco*RI, or *Kpn* I, separated by electrophoresis through a 0.8% agarose gel, and transferred to GeneScreen*Plus* (DuPont). The filter was hybridized with a DNA probe ^{32}P-labeled by the random-primed DNA labeling kit (Boehringer Mannheim) in a hybridization solution [2× sodium chloride/sodium citrate (SSC)/0.1% (wt/vol) SDS/10% (wt/vol) dextran sulfate/50% (vol/vol) formamide/denatured salmon sperm DNA at 100 μg/ml] at 42°C and washed in 0.1× SSC containing 0.1% (wt/vol) SDS at 55°C. To reprobe the filter, the hybridized probe was removed by washing the filter in 0.1× SSC/0.1% (wt/vol) SDS at 100°C, and removal of the probe was confirmed by autoradiography.

RESULTS

Isolation of cDNAs Encoding TRs. TR-I and TR-II were purified to homogeneity from cultured roots of *D. stramonium* and *H. niger*, respectively, and amino acid sequences of several internal peptide fragments were obtained from the purified enzymes. An amino acid sequence of seven residues, found in peptide fragments from both TR-I and TR-II, was chosen for designing an oligonucleotide probe, which was then used to screen 1.5×10^5 clones from a cDNA library derived from the cultured roots of *D. stramonium*. Fourteen hybridization-positive clones were obtained and classified into three closely related but distinct groups, on the basis of their nucleotide sequences. Another oligonucleotide probe was synthesized according to the DNA sequence commonly found in the 5′ ends of the clones of the two groups, and the cDNA library of 5×10^4 clones was screened again. These two rounds of screening gave pDTR1, pDTR2, and pDX, which, respectively, represent the clones with the longest cDNA insert in groups I, II, and III.

Prokaryotic expression plasmids pDTR1E, pDTR2E, and pDXE were constructed so that the polypeptides encoded by pDTR1, pDTR2, and pDX were expressed in *E. coli* under the control of *lac* promoter as proteins fused to β-galactosidase. After induction by 1 mM isopropyl β-D-thiogalactoside at 15°C for 14 hr, soluble proteins were extracted from the bacteria, and proteins of the expected molecular weights were detected by SDS/PAGE analysis. Each expression plasmid produced a similar amount of the fusion protein (data not shown). TR activities in the bacterial extracts were measured by using gas/liquid chromatography (Fig. 2). The protein extracts prepared from bacteria transformed with pDTR1E and pDTR2E, respectively, catalyzed NADPH-dependent reduction of tropinone to tropine and ψ-tropine. The reduction was highly specific: only one of the two stereoisomers was produced in each assay. The proteins extracted from bacteria transformed with the vector alone or with pDXE showed no TR activities. Therefore, pDTR1 and pDTR2 encoded TR-I and TR-II, respectively, whereas pDX encoded neither.

Sequence Analysis. pDTR1, pDTR2, and pDX, respectively, contain open reading frames of 822 bp, 783 bp, and 807 bp, which encode polypeptides composed of 273-, 260-, and 268-amino acid residues (Fig. 3). The calculated molecular weights of these polypeptides are 29,615, 28,310, and 28,587. The subunits of both TR-I and TR-II have been determined to be 29 kDa by SDS/PAGE analysis of TR-I and TR-II purified from the cultured roots of *D. stramonium* (unpublished results) and *H. niger* (2), respectively. The deduced amino acid sequence of TR-I (encoded by pDTR1; Fig. 3*A*) contains all seven of the peptide sequences determined for purified TR-I.

pDTR2 has three well-conserved direct repeats that extend from the carboxyl terminus to the 3′-noncoding region (Fig. 3*B*). These repetitive sequences were found in all of the independent clones of *D. stramonium* that encoded TR-II polypeptide. However, these repeats are absent in the TR-II cDNA of *H. niger* (unpublished results) and are therefore not a common feature of the TR-II gene.

The deduced amino acid sequences of TR-I, TR-II, and peptide P29X show a high degree of homology to each other. Of the 260-amino acid residues that overlap the sequences of

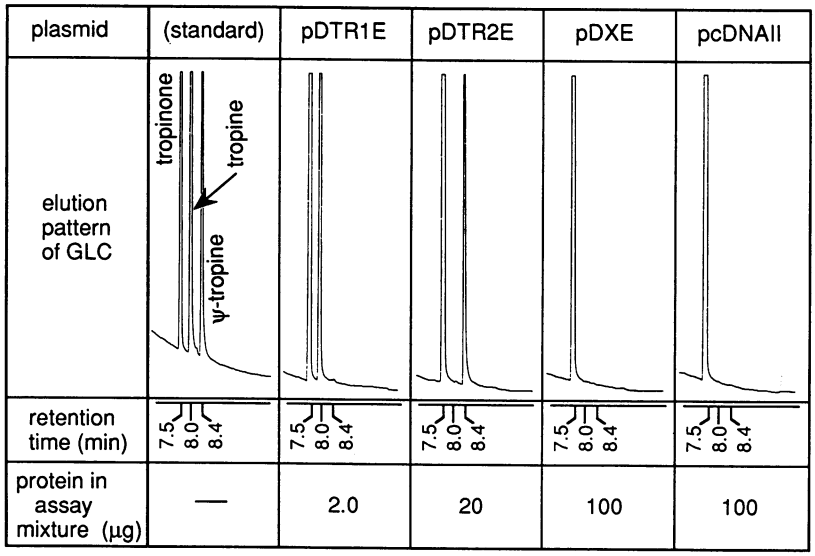

FIG. 2. TR activities in *E. coli* that have been transformed with expression plasmids. An elution pattern of authentic alkaloids on gas/liquid chromatography (GLC) is shown at left. pcDNAII is a vector with no cDNA insert. Note that different amounts of protein were used for enzyme assays.

TR-I and TR-II, 167 residues (64%) are identical, whereas pair-wise comparisons of P29X/TR-I and P29X/TR-II show the somewhat reduced identities of 61% (164/268) and 58% (152/260), respectively. These results indicate that these three proteins have evolved from a common ancestor. The amino acid homology between TR-I and TR-II was plotted with GeneWorks software (IntelliGenetics) (data not shown). The plot indicated that the amino-terminal halves of TR-I and TR-II share a higher homology (72%, 91/126) than the carboxyl-terminal halves (57%, 76/134).

The protein data base (National Biomedical Research Foundation) was searched for proteins homologous to TR-I. A considerable degree of homologies (29–33%) was found in several short-chain dehydrogenases (SDHs), including glucose dehydrogenase of *Bacillus megaterium* (5), 20β-hydroxysteroid dehydrogenase of *Streptomyces hydrogenans* (6), and 15-hydroxyprostaglandin dehydrogenase in humans (7). The members of this dehydrogenase family use NAD(H) or NADP(H) as cofactors, have subunits of ≈250-amino acid residues, and do not require zinc for catalysis (see ref. 8 for a review).

Six amino acid residues of possible functional importance are strictly conserved in all 20 of the SDHs characterized so far (8). These residues are also conserved in TR-I, TR-II, and peptide P29X, except for position 147 in P29X (Ala-147), where glycine is found in all other SDHs (Fig. 4): Gly-28 and Gly-34 (in the numbering of TR-I) form a tight turn at the nucleotide-binding region (11), Asp-78 has been suggested to participate in hydrogen bonding to the coenzyme (8), and the hydroxy group of Tyr-171 may participate in the hydride-

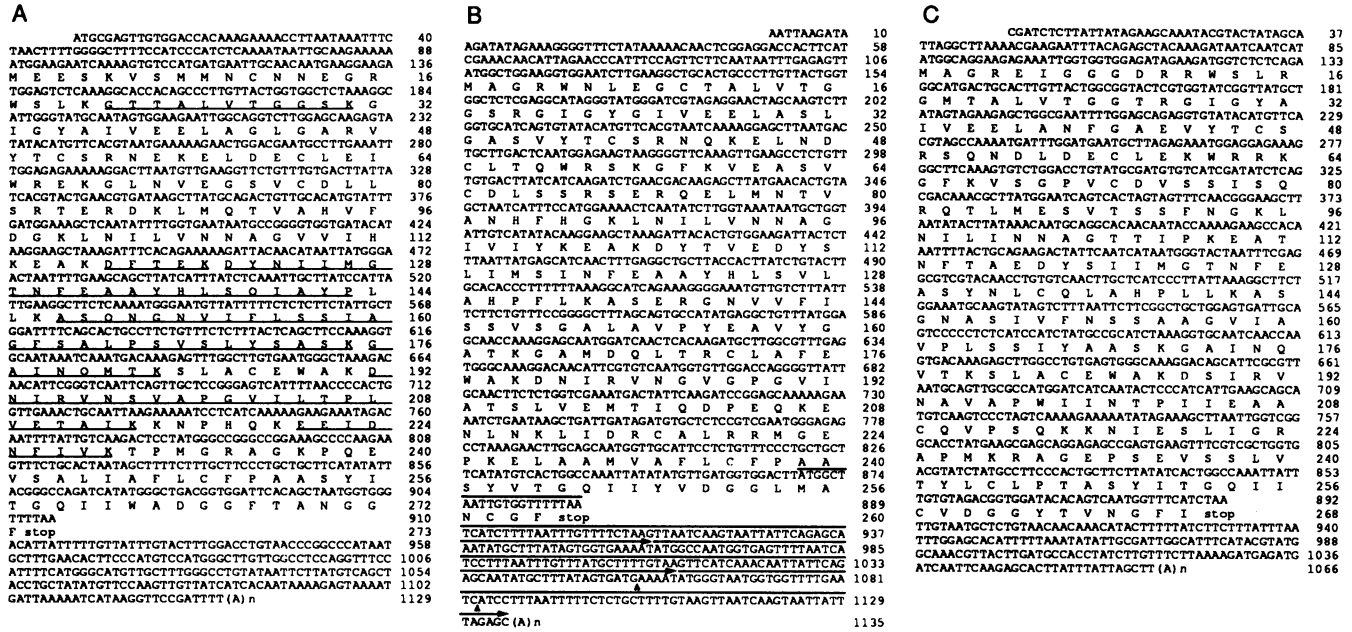

FIG. 3. Nucleotide and deduced amino acid sequences of TR-I (*A*), TR-II (*B*), and P29X (*C*) cDNAs of *D. stramonium*. Amino acid sequences are shown below the nucleotide sequences in one-letter codes. The underlined amino acid sequences (*A*) were found in proteolytic peptide fragments of TR-I that had been purified from the cultured roots of *D. stramonium*. Arrows above the TR-II nucleotide sequence and closed arrowheads below the sequence (*B*) indicate three well-conserved direct repeats and alternative poly(A) start sites, respectively.

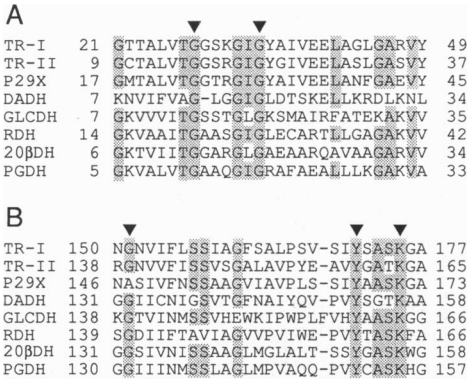

FIG. 4. Comparison of the protein sequences of the two TRs, peptide P29X, and representative enzymes of the SDH family at the amino-terminal cofactor binding fold (*A*) and at the mid-chain region surrounding the conserved tyrosine residue (*B*). Amino acids are shown in one-letter codes. Residues conserved in more than six proteins are hatched. Arrowheads indicate 5 of the 6-amino acid residues reported to be strictly conserved among the 20 SDHs characterized so far (8). DADH, alcohol dehydrogenase of *Drosophila melanogaster* (9); GLCDH, glucose dehydrogenase of *B. megaterium* (5); RDH, ribitol dehydrogenase of *Enterobacter aerogenes* (10); 20βDH, 20β-hydroxysteroid dehydrogenase of *S. hydrogenans* (6); PGDH, 15-hydroxyprostaglandin dehydrogenase in humans (7).

transfer process (12). These results indicate that the TRs and peptide P29X are members of the SDH family and, to our knowledge, SDHs from plants have not before been reported.

DNA Gel-Blot Analysis. Three tropane alkaloid-producing solanaceous species, *D. stramonium*, *H. niger*, and *A. belladonna*, and a nonproducing Solanaceae, *N. tabacum*, were analyzed for the presence of the genes for TR-I, TR-II, and P29X by genomic Southern hybridization. DNA fragments corresponding to the carboxyl-terminal half region of each cDNA were used as hybridization probes to avoid cross-hybridization among the three genes (for the precise cDNA regions used for the probes, see the legend for Fig. 5). Both TR-I and TR-II genes were detected in the three tropane alkaloid-producing species but were not found in tobacco, whereas the P29X-encoding gene was detected only in *D. stramonium* and *A. belladonna* (Fig. 5).

DISCUSSION

The deduced primary structures of TR-I and TR-II show that both enzymes belong to the SDH family. The nucleotide-binding region in SDH is localized at the amino terminus, which is more conserved than the carboxyl terminus in both TRs. The fact that the two TRs show similar affinities for NADPH and transfer the pro-*S* hydrogen of NADPH (2) also supports the idea that both enzymes bind NADPH similarly at the amino-terminal region. The carboxyl-terminal region appears to contribute most binding sites for tropinone, which differ considerably in the two enzymes. The most plausible model for the substrate binding in TRs is that when tropinone is bound at the active site of TR-I, the pro-*S* hydrogen of NADPH is located in the *si* face (the same side as the tropane nitrogen atom) of the 3-keto group, whereas tropinone is bound in the upside-down orientation at the active site of TR-II, so that the pro-*S* hydrogen is transferred from the *re* face of the 3-keto group.

When the DNA sequences of TR-I and TR-II are aligned, homology extends throughout the cDNA sequences (data not shown). Our previous study showed that the solanaceous species that produce tropane alkaloids have both enzyme activities and that these activities occur in various ratios (2). One possible scenario for the evolution of TRs is that a prototype of the TR gene evolved from an SDH gene during the early stage of diversification of the Solanaceae. This prototype TR protein did not necessarily have distinct stereospecificity for the reduction of tropinone. Soon after evolution of the prototype TR gene, gene duplication occurred, followed by accumulating mutations in the coding regions (especially in the tropinone-binding region) of each duplicated gene, to the point that each TR enzyme acquired very strict stereospecificity. Absence of TR genes in tobacco means either that the prototype TR gene evolved after the Datureae tribe and the Solaneae tribe (to which *Hyoscyamus* and *Atropa* belong) diverged from the Cestreae tribe (to which tobacco belongs) or that the Cestreae tribe, in which tropane alkaloids have not been found, lost the prototype TR gene, together with the ability to synthesize tropane alkaloids, very early during formation of the tribe.

The enzymatic reaction catalyzed by peptide P29X has not been identified. Judging from the high homology to the two TRs, P29X appears to be an SDH that has diverged from the putative prototype TR. P29X might catalyze the reduction of ketones in the biosynthetic pathway of tropane alkaloids with chemical structures related to tropinone. Therefore, we examined whether the recombinant P29X fusion protein (Fig. 2) can reduce hygrine, a proposed precursor of tropinone (13) and 2-carbomethoxy-3-tropinone, a putative intermediate in the biosynthesis of cocaine (13), in the presence of either NADPH or NADH as a cofactor. However, we could not detect enzyme activity toward these substrates (our unpublished results). The P29X gene is present in *Datura* and

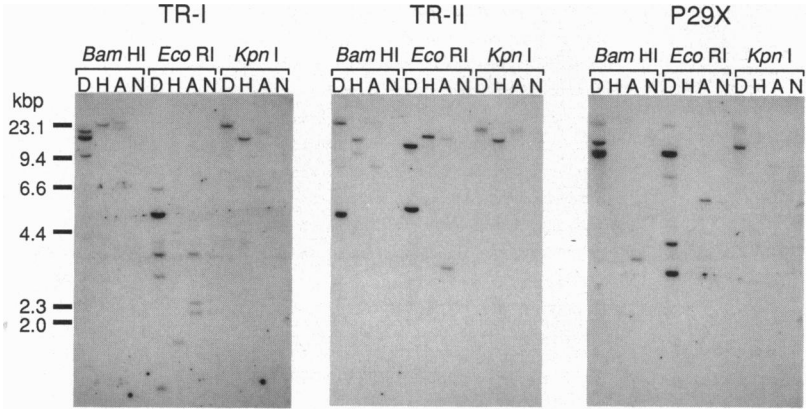

FIG. 5. Detection of TR and P29X genes in solanaceous plants by genomic DNA-blot analysis. DNAs (20 μg) from *D. stramonium* (lanes D), *H. niger* (lanes H), *A. belladonna* (lanes A), and *N. tabacum* (lanes N) were digested with the enzymes shown and loaded on each lane. The filter was successively hybridized with three cDNA probes: TR-I, 307-bp *Ear* I–*Nde* I fragment of pDTR1 (nt 563–869); TR-II, 261-bp *Nde* I fragment of pDTR2 (nt 569–829); or P29X, 425-bp *Pst* I–*Acc* I fragment of pDX (nt 434–858). Positions of size markers are shown at left.

Atropa, but is not present in *Hyoscyamus* and tobacco. Furthermore, this gene is expressed relatively strongly in the root and the stem of *D. stramonium* but is expressed very little in the plant of *A. belladonna* (our unpublished results). Therefore, the natural catalytic products of P29X might be secondary metabolites that are present in *Datura* (and possibly in *Atropa* at low levels) but are absent in *Hyoscyamus* and tobacco, although we cannot suggest suitable candidate metabolites on the basis of the current phytochemical information available regarding these species.

In view of the relatively recent evolution of flowering plants during the last 150 to 200 million years, the chemical diversity of secondary metabolites is the consequence of continuing evolution of various enzymes with distinctive specificities. Studies of enzymes from various sources suggest that only relatively small changes in amino acid sequences are needed to alter substrate or positional specificities of some enzymes. For example, only 1-amino acid substitution in lactate dehydrogenase of *Bacillus stearothermophilus* is sufficient to convert the enzyme to malate dehydrogenase (14). The positional specificity of oxygenation in mammalian lipoxygenases is determined by a small number of amino acid residues that position the substrate in the active site (15). Similar primary structures of two plant flavonol sulfotransferases suggest that a new positional specificity for sulfation may have evolved in *Flaveria* and related species (16). Our present study suggests that stereospecificity of enzymes may also be altered during diversification of species. It should be noted, however, that catalytically related enzymes with different stereospecificities do not always evolve from common ancestors. D-Lactate dehydrogenase of *Lactobacillus plantarum*, for example, belongs to the D-isomer-specific dehydrogenase family and shows no significant homology to L-lactate dehydrogenase of the same species (17). Molecular characterization of many other enzymes involved in secondary metabolism is expected to provide further examples of the fascinating process of enzyme evolution.

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