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

(stereospecificity/protein evolution/tropane alkaloids)

Keiji Nakajima, Takashi Hashimoto*, and Yasuyuki Yamada

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

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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 DNAblot 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) (3 β -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 wellknown tropane ester of the ψ -tropine type. Both TR-I and TR-II are present together in any given tropane alkaloidproducing 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 reac-



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.

tivity 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.[†]

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 μ M 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.

^{*}To whom reprint requests should be addressed.

[†]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 $[\alpha^{-32}P]$ 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 ³²P-labeled by the randomprimed DNA labeling kit (Boehringer Mannheim) in a hybridization solution $[2 \times \text{ sodium chloride/sodium citrate}]$ (SSC)/0.1% (wt/vol) SDS/10% (wt/vol) dextran sulfate/ 50% (vol/vol) formamide/denatured salmon sperm DNA at 100 μ g/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 NADPHdependent 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. 3A) 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. 3B). 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

plasmid	(standard)	pDTR1E	pDTR2E	pDXE	pcDNAII	
elution pattern of GLC	tropinone w-tropine tropine					
retention time (min)	8.0	8.0	7.5 8.0 8.4	7.5 8.0 8.4	7.5 8.0 8.4	
protein in assay mixture (μg)		2.0	20	100	100	

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 assavs.

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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-

Α		В	
	40	AATTAAGATA	10
TANCTTTTCCCCCTTTTCCATCCCATCTCAAAAAACCTTAATAA	88	AGATATAGAAAGGGGTTTCTATAAAAACAACTCGGAGGACCACTTCAT	58
ATCCARCANTCARANCTCCCATCATCANTCARCANCANTCARCANCANCA	136	CGAAACAACATTAGAACCCATTTCCAGTTCTTCAATAATTTGAGAGTT	106
	16	ATGGCTGGAAGGTGGAATCTTGAAGGCTGCACTGCCCTTGTTACTGGT	154
TOCACTOTCAAACCCACCACCACCTOTTCTTACTCCTCCTCCTCTAAACCC	184	MAGRWNLEGCTALVTG	16
W S L K G T T A L V T G G S K G	32	GGCTCTCGAGGCATAGGGTATGGGATCGTAGAGGAACTAGCAAGTCTT	202
ATTGGGTATGCAATAGTGGAAGAATTGGCAGGTCTTGGAGCAAGAGTA	232	G S R G I G Y G I V E E L A S L	32
T G Y A T V E E L A G L G A R V	48	GGTGCATCAGTGTATACATGTTCACGTAATCAAAAGGAGCTTAATGAC	250
TATACATGTTCACGTAATGAAAAAGAACTGGACGAATGCCTTGAAATT	280	G A S V Y T C S R N Q K E L N D	48
YTCSBNEKELDECLEI	64	TGCTTGACTCAATGGAGAAGTAAGGGGTTCAAAGTTGAAGCCTCTGTT	2 98
TGGAGAGAAAAAGGACTTAATGTTGAAGGTTCTGTTTGTGACTTATTA	328	C L T Q W R S K G F K V E A S V	64
WREKGLNVEGSVCDLL	80	TGTGACTTATCATCAAGATCTGAACGACAAGAGCTTATGAACACTGTA	346
TCACGTACTGAACGTGATAAGCTTATGCAGACTGTTGCACATGTATTT	376	C D L S S R S E R Q E L M N T V	80
SRTERDKLMQTVAHVF	96	GCTAATCATTTCCATGGAAAACTCAATATCTTGGTAAATAATGCTGGT	394
GATGGAAAGCTCAATATTTTGGTGAATAATGCCGGGGTGGTGATACAT	424	A N H F H G K L N I L V N N A G	96
DGKLNILVNNAGVVIH	112	ATTGTCATATACAAGGAAGCTAAAGATTACACTGTGGAAGATTACTCT	442
AAGGAAGCTAAAGATTTCACAGAAAAAGATTACAACATAATTATGGGA	472	I V I Y K E A K D Y T V E D Y S	112
KEAKDFTEKDYNIIMG	128	TTAATTATGAGCATCAACTTTGAGGCTGCTTACCACTTATCTGTACTT	4 90
ACTAATTTTGAAGCAGCTTATCATTTATCTCAAATTGCTTATCCATTA	520	LIMSINFEAAYHLSVL	128
TNFEAAYHLSOIAYPL	144	GCACACCCTTTTTTTAAAGGCATCAGAAAGGGGGAAATGTTGTCTTTATT	538
TTGAAGGCTTCTCAAAATGGGAATGTTATTTTTCTCTCTC	568	A H P F L K A S E R G N V V F I	144
LKASONGNVIFLSSIA	160	TCTTCTGTTTCCGGGGGCTTTAGCAGTGCCATATGAGGCTGTTTATGGA	586
GGATTTTCAGCACTGCCTTCTGTTTCTCTTTACTCAGCTTCCAAAGGT	616	S S V S G A L A V P Y E A V Y G	160
<u>G.E.S.A.L.P.S.V.S.L.Y.S.A.S.K.G</u>	176	GCAACCAAAGGAGCAATGGATCAACTCACAAGATGCTTGGCGTTTGAG	634
GCAATAAATCAAATGACAAAGAGTTTGGCTTGTGAATGGGCTAAAGAC	664	A T K G A M D Q L T R C L A F E	176
<u>AINOMTK</u> SLACEWAK <u>D</u>	192	TGGGCAAAGGACAACATTCGTGTCAATGGTGTTGGACCAGGGGTTATT	682
AACATTCGGGTCAATTCAGTTGCTCCGGGAGTCATTTTAACCCCACTG	712	W A K D N I K V N G V G P G V I	192
N.I.R.V.N.S.V.A.P.G.V.I.L.T.P.L.	208	GCAACTTCTCTGGTCGAAATGACTATTCAAGATCCGGAGCAAAAAGAA	730
GTTGAAACTGCAATTAAGAAAAATCCTCATCAAAAAGAAGAAAAAAGAA	760	A T S L V E M T I Q D P E Q K E	208
<u>VETAIKKNPHQKEEID</u>	224	AATCTGAATAAGCTGATTGATAGATGTGCTCTCCGTCGAATGGGAGAG	778
AATTTTATTGTCAAGACTCCTATGGGCCGGGCCGGAAAGCCCCCAAGAA	808	N L N K L I D R C A L R R M G E	224
<u>N F I V K</u> T P M G R A G K P Q E	240	CUTAAAGAACTIGUAGUAATGGTTGUATTUUTUTGTTTUUUTGGTGUTGUT	826
GTTTCTGCACTAATAGCTTTTCTTTGCTTCCCTGCTGCTTCATATATT	856	P K E L A A M V A F L C F P A A	240
V S A L I A F L C F P A A S Y I	256	TCATATGTCACTGGCCAAATTATATATGTTGATGGTGGACTTATGGCT	8/4
ACGGGCCAGATCATATGGGCTGACGGTGGATTCACAGCTAATGGTGGG	904	SIVIGULIIVDGGLMA	256
T G Q I I W A D G G F T A N G G	272	AATTGTGGTTTTTAA	889
TTTTAA	910	N C G F stop	260
F stop	273	TCATCTTTTAATTTGTTTTCTAAGTTAATCAAGTAATTATTCAGAGCA	937
ACATTATTTTGTTATTTGTACTTTGGACCTGTAACCCGGCCCATAAT	958	AATATGCTTTATAGTGGTGAAAATATGGCCAATGGTGAGTTTTAATCA	985
GCTTTGAACACTTCCCATGTCCATGGGCTTGTTGGCCTCCAGGTTTCC	1006	TCCTTTAATTTGTTTATGCTTTTGTAAGTTCATCAAACAATTATTCAG	1033
ATTTTCATGGGCATGTTGCTTTGGGCCTGTATAATTCTTATGTCAGCT	1054	AGCANTATGCTTTATAGTGATGAAAATATGGGTAATGGTGGTTTTGAA	1081
ACCTGCTATATGTTCCAAGTTGTTATCATCACAATAAAAGAGTAAAAT	102	A	
GATTAAAAATCATAAGGTTCCGATTTT (A) n	1129	TCATCCTTTAATTTTTCTCTCTGCTTTTGTAAGTTAATCAAGTAATTATT	1129
		TAGAGC (A) n	1135

C)															
				CGI	TCI	CTT	TAT:	FAT/	GA	AGC/	-	AC	TAC	TAT	AGCA	. 3
тт	AGG	CTT	AA	ACO	AAG	AAT	TTT/	ACAC	AG	CTAC	CAN	GA	CAA1	CAA	TCAT	. 8
AT	GGC	AGG	AAG	GAG	AAT	TGO	TGO	TGC	AG	TAC	SAAC	ATO	GTO	TCT	CAGA	13
м	A	G	R	E	I	G	G	G	D	R	R	W	s	L	R	1
GG	CAT	GAC	TGC	ACT	TGT	TAC	TGC	SCGC	TAC	CTCG	TGO	TAT	rcgo	TT	TGCT	18
G	м	T	λ	L	v	т	G	G	т	R	G	I	G	Y	A	3
AT	AGT	AGA	AGI	\GC1	GGC	GA	TTT	TGG	AG	CAGA	GGT	GT	TAC	ATG	TTCA	22
I	v	E	E	L	A	N	F	G	A	E	v	Y	т	с	s	4
CG	TAG	CCA	AAA	TG/	TTT	GGA	TG/	ATC	CTT	raga	GA	ATC	GAG	GAG	AAAG	27
R	S	Q	N	D	L	D	E	с	L	E	ĸ	W	R	R	ĸ	6
GG	CTT	CAA	AGT	GTC	TGG	ACC	TG	ATG	CG1	TGT	GTC	ATC	GAI	ATC	TCAG	32
G	F	к	v	s	G	P	v	с	D	v	s	S	I	s	Q	8
CG	ACA	AAC	GCI	TAT	GGA	ATC	AGT	CAC	TAC	TAG	TTT	CA	CGG	GAA	GCTT	37
R	Q	т	L	м	E	s	v	т	s	s	F	N	G	ĸ	L	9
AA	TAT	ACT	TAT	. YYY	CAA	TGC	AGG	CAC	AAC	CAAT	ACC		AGA	AGC	CACA	42
N	I	L	I	N	N	A	G	т	т	I	Р	ĸ	E	A	т	11
AA	TTT	TAC	TGC	AGA	AGA	CTA	TTC	AAT	CAT	AAT	GGG	TAC	TAA	TTT	CGAG	46
N	F	т	A	E	D	Y	s	I	I	м	G	т	N	F	E	12
GC	GTC	GTA	CAA	CCT	GTG	TCA	ACT	TGC	TCA	TCC	CTT	ATT	AAA	GGC	TTCT	51
A	s	Y	N	L	с	Q	L	A	н	P	L	L	ĸ	A	s	14
GG	AAA	TGC	AAG	TAT	AGT	CTT	TAA	TTC	TTC	GGC	TGC	TGG	AGT	GAT	TGCA	56
G	N	A	s	I	v	F	N	S	s	A	λ	G	v	I	A	16
GT	ccc	сст	CTC	ATC	CAT	СТА	TGC	CGC	ATC	TAA	AGG	TGC	AAT	CAA	CCAA	61
v	P	L	s	S	I	Y	A	A	s	ĸ	G	A	I	N	Q	17
GT	GAC	AAA	GAG	CTT	GGC	CTG	TGA	GTG	GGC	AAA	GGA	CAG	CAT	TCG	CGTT	66
v	т	ĸ	s	L	A	с	E	W	A	ĸ	D	s	I	R	v	19
λA	TGC	AGT	TGC	GCC	ATG	GAT	CAT	CAA	TAC	TCC	CAT	CAT	TGA	AGC	AGCA	70
N	λ	۷	A	P	W	I	I	N	т	Р	I	I	£	A	A	20
ΤG	TCA	AGT	ccc	TAG	TCA	AAA	GAA	AAA	TAT	AGA	AAG	CTT	AAT	TGG	TCGG	75
С	Q	۷	P	S	Q	ĸ	ĸ	N	I	E	s	L	I	G	R	22
GC.	ACC	TAT	GAA	GCG	AGC.	AGG	AGA	GCC	GAG	TGA	AGT	TTC	GTC	GCT	GGTG	80
A	P	м	ĸ	R	A	G	E	P	s	Е	v	s	s	L	v	24
¥C)	GTA	TCT.	ATG	CCT	TCC	CAC	TGC	TTC	TTA	TAT	CAC	TGG	CCA	AAT	TATT	85
т	Y	L	с	L	P	т	A	s	Y	1	т	G	Q	I	I	25
ΤG	TGT.	AGA	CGG	TGG	ATA	CAC	AGT	CAA	TGG	TTT	CAT	СТА	A			8 93
с	v	D	G	G	Y	т	v	N	G	F	I	st	ор			26
TT	GTA	ATG	стс	TGT	AAC	AAC	AAA	CAT	ACT	TTT	TAT	CTT	CTT	TAT	TTAA	940
TT	TGG	AGC.	ACA	TTT	TTA	AAT	ATA	TTG	CGA	TTG	GCA	ттт	CAT	ACG	TATG	988
GC.	AAA	CGT	TAC	TTG	ATG	CCA	ССТ	ATC	TTG	TTT	CTT	AAA	AGA	TGA	GATG	103
AT	CAN	TTC.	AAG	AGC	ACT	FAT	TTA	TTA	GCT	T (A) л					106

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.

A		* *	
TR-I	21	GTTALVTGGSKGIGYAIVEELAGLGARVY	49
TR-II	9	GCTALVTGGSRGIGYGIVEELASLGASVY	37
P29X	17	GMTALVTGGTRGIGYAIVEELANFGAEVY	45
DADH	7	KNVIFVAG-LGGIGLDTSKELLKRDLKNL	34
GLCDH	- 7	GKVVVITGSSTGLGKSMAIRFATEKAKVV	35
RDH	14	GKVAAITGAASGIGLECARTLLGAGAKVV	42
20BDH	6	GKTVIITGGARGLGAEAARQAVAAGARVV	34
PGDH	5	GKVALVTGAAQGIGRAFAEALLLKGAKVA	33
В		• • •	
TR-I	150	NGNVIFLSSIAGFSALPSV-SIYSASKGA	177
TR-II	138	RGNVVFISSVSGALAVPYE-AVYGATKGA	165
P29X	146	NASIVFNSSAAGVIAVPLS-SIYAASKGA	173
DADH	131	GGIICNIGSVTGFNAIYQV-PVYSGTKAA	158
GLCDH	138	KGTVINMSSVHEWKIPWPLFVHYAASKGG	166
RDH	139	SGDIIFTAVIAGVVPVIWE-PVYTASKFA	166
20BDH	131	GGSIVNISSAAGLMGLALT-SSYGASKWG	158
PGDH	130	GGIIINMSSLAGLMPVA00-PVYCASKHG	157

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); 20 β DH, 20 β -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 nucleotidebinding 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 $(20 \ \mu g)$ 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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