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Review

Tropinone reductases, enzymes at the branch point of tropane alkaloid metabolism

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In Memoriam Professor Martin Luckner 1935–2004

Abstract

Two stereospecific oxidoreductases constitute a branch point in tropane alkaloid metabolism. Products of tropane metabolism are the alkaloids hyoscyamine, scopolamine, cocaine, and polyhydroxylated nortropane alkaloids, the calystegines. Both tropinone reductases reduce the precursor tropinone to yield either tropine or pseudotropine. In Solanaceae, tropine is incorporated into hyoscyamine and scopolamine; pseudotropine is the first specific metabolite on the way to the calystegines. Isolation, cloning and heterologous expression of both tropinone reductases enabled kinetic characterisation, protein crystallisation, and structure elucidation. Stereospecificity of reduction is achieved by binding tropinone in the respective enzyme active centret in opposite orientation. Immunolocalisation of both enzyme proteins in cultured roots revealed a tissue-specific protein accumulation. Metabolite flux through both arms of the tropane alkaloid pathway appears to be regulated by the activity of both enzymes and by their access to the precursor tropinone. Both tropinone reductases are NADPH-dependent short-chain dehydrogenases with amino acid sequence similarity of more than 50% suggesting their descent from a common ancestor. Putative tropinone reductase sequences annotated in plant genomes other that Solanaceae await functional characterisation.
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Keywords: Solanaceae; Tropane alkaloid; Hyoscyamine; Scopolamine; Calystegine; Tropinone reductase; Short-chain dehydrogenase

Contents

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1. Tropane alkaloids

Tropane alkaloids have been established drugs for medical treatment since many years. They are obtained from Solanaceae, e.g., *Atropa belladonna*, *Hyoscyamus niger*, and *Datura stramonium*. After alkaloids were detected as chemical constituents of plants and named as such (Meissner, 1819), efforts to provide pure compounds for medication yielded atropine from *Atropa belladonna* L., deadly nightshade (Mein, 1833), and hyoscyamine from *Hyoscyamus niger* L., black henbane (Geiger and Hesse, 1833). Later "atropine" was defined as the racemic mixture of the enantiomer (*S*)-hyoscyamine, which occurs naturally in plants and possesses high pharmacological activity, and of (*R*)-hyoscyamine, which forms during plant extraction and which is pharmacologically mostly inactive. The alkaloid scopolamine was detected only 1892 from *Scopolia atropoides* (Schmidt, 1892) and proved later to be identical with a compound named hyoscine that was separated from hyoscyamine in extracts from *Hyoscyamus muticus* (Ladenburg, 1881). Nowadays, therapeutic tropane alkaloids of Solanaceae are extracted from *Duboisia* cultivars, mostly hybrids of *D. leichhardtii* and *D. myoporoides*, native to Australia. *Duboisia* species grow as perennial trees, and cultivated hybrids may contain more than 4% alkaloids (dry mass) in their leaves, scopolamine being a major alkaloid (Lean and Ralph, 1944; Hills et al., 1954). World consumption of scopolamine is several fold higher than that of hyoscyamine (Bruhn, 1989) mainly due to the fact that scopolamine is used as starting material for the semi-synthesis of several important drugs.

Atropine is applied as injection for premedication for surgery, against colic in the digestive system, and as antidote against intoxication with organophosphorous insecticides. Scopolamine is used against motion disease in the form of an adhesive tape fixed behind the ear (Scopoderm TTS®, Novartis), from which it is liberated over several hours. Scopolamine as *N*-butyl hydrobromide derivative for oral application acts against spasm of the bladder, of the intestine, or of the gall bladder, as it carries the advantage of having no side effects in the central nervous system due to the quarterny ammonium salt not being transported through the brain blood barrier. Further derivatives of scopolamine are anticholinergic drugs inhaled against asthma, such as ipratropiumbromid. Research on tropane derivatives recently produced a novel compound, tiotropiumbromide (Spiriva® Boehringer Ingelheim). The compound is another semi-synthetic derivative of scopolamine with an exceptional long half-life time of several days and was specifically designed to treat chronic obstructive pulmonary disease (COPD).

Tropane alkaloid containing Solanaceae, however well-known they seemed to be, revealed a new group of nortropane alkaloids only 15 years ago. Calystegines carry three to five hydroxyl groups in various positions, thereby are water-soluble and escape in typical alkaloid extraction schemes (Fig. 1). The compounds were initially detected in *Calystegia sepium* (L.) R. Br., Convolvulaceae (Goldmann et al., 1990). Based on the nortropane bicyclic ring it was assumed that calystegines arise from the tropane alkaloid biosynthetic pathway, and they were found in tropane alkaloid containing Solanaceae (Dräger et al., 1994, 1995). Specific extraction and purification schemes had to be developed for the polyhydroxy alkaloids; chromatography also demanded adapted techniques (Dräger, 1995, 2002). Calystegines resemble monosaccharides in structure, and it is not surprising that they have shown to be strong glycosidase inhibitors (Asano et al., 2000). Intoxication of cattle by feed plants containing alkaloidal glycosidase inhibitors is known for several Fabaceae, e.g., *Swainsonia*, *Oxytropis* and *Astragalus* species, the indolizidine derivative swainsonine being one of the strongest toxins. Similar intoxications symptoms after ingestion of *Ipomoea* species (Convolvulaceae) prompted phytochemical investigation and yielded several calystegines in *I. aff. calobra* (Molyneux et al., 1995) and in *I. carnea* (Haraguchi et al., 2003). Swainsonine, however, was found in addition, and toxicity of calystegines for cattle was questioned (Ikeda et al., 2003).

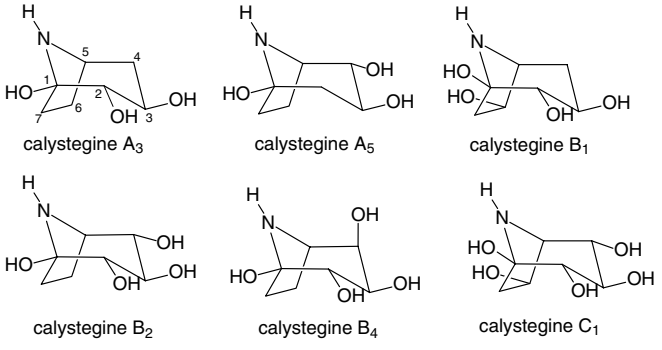

Fig. 1. Examples for calystegine structures. The basic skeleton is 8-azabicyclo[3.2.1]octane-3β-ol with two to four further hydroxyl groups in various positions and orientations.

2. Two separate tropinone reductases

The tropine esters hyoscyamine and scopolamine were taken as the major products of the tropane pathway in Solanaceae, as was the methylecgonine ester cocaine in some *Erythroxylum* species. Martin Luckner in his renowned book on Secondary Metabolism in Microorganisms, Plants, and Animals wrote in 1990 "In most of the alkaloids the tropane moiety at position 3 is ester-bound to an acid" (Luckner, 1990). It was thought accordingly that tropinone should be reduced stereospecifically to tropine (3α-tropanol) in Solanaceae (Fig. 2), and not to the isomeric pseudotropine (3β-tropanol). Measurement of tropinone reducing enzyme activities in *Datura stramonium* protein extracts confirmed this view: tropine only was found as reduction product, pseudotropine was not detected (Koelen and Gross, 1982). The first tropinone

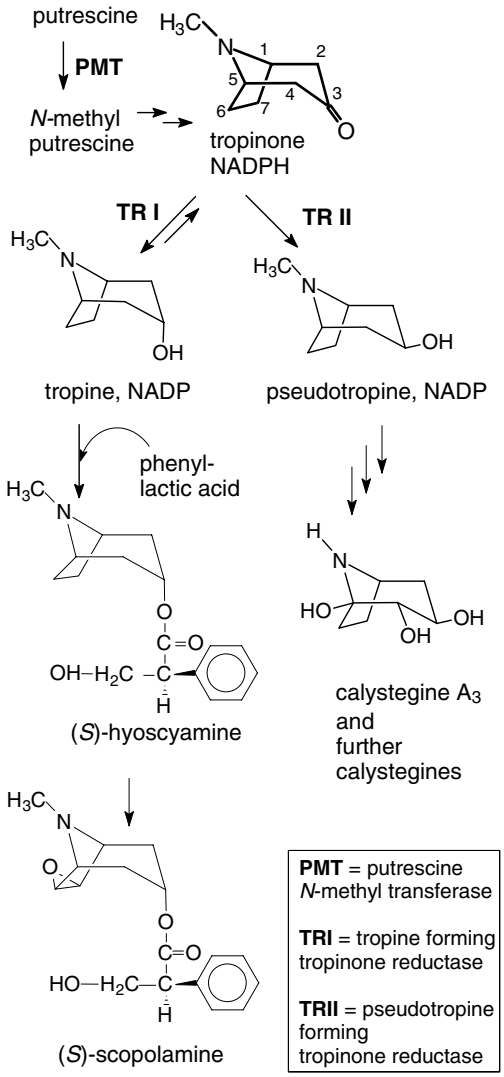

Fig. 2. Biosynthesis of tropane alkaloids. Tropinone reductases form a branch point in the pathway leading to hyoscyamine and scopolamine (TRI) and to calystegines (TRII).

reductase purification from *Hyoscyamus niger*, however, yielded an enzyme specific for pseudotropine formation (Dräger et al., 1988). In addition, pseudotropine was proved not to be isomerised into tropine in plant tissues (Yamada et al., 1990). As this enzyme was not responsible for tropine formation, another tropine-specific reductase was postulated. Two separate tropinone reductases were purified from *H. niger* root cultures (Hashimoto et al., 1992) and also from *D. stramonium* roots (Portsteffen et al., 1992). *Atropa belladonna* also contained two specific enzymes (Dräger and Schaal, 1994). It became customary to abbreviate the tropine-forming enzyme with TRI (EC 1.1.1.206) and the pseudotropine-forming enzyme with TRII (EC 1.1.1.236). TRII activity was found to be strong in many Solanaceae tissues, e.g., shortly after application of tropinone, pseudotropine accumulated faster than tropine (Dräger and Schaal, 1994). Esters of pseudotropine, e.g., with acetic acid or tiglic acid, were identified only as minor alkaloids in those plants, and the metabolic role of TRII and the destination of pseudotropine formed were enigmatic.

3. Pseudotropine forming tropinone reductase and calystegines

The molecular structure of calystegines shows an equatorial hydroxyl group (referring to the six-membered chair-shaped ring) in position 3, the typical feature of pseudotropine (Fig. 1). It has been hypothesised since 1994 that TRII and the reduction product pseudotropine were the first molecules on the way to the formation of calystegines from tropinone (Dräger et al., 1994). Calystegines were subsequently detected in many Solanaceae, also in species that were before assumed not to possess the tropane alkaloid pathway such as *Solanum tuberosum*, potato (Dräger et al., 1995). Further species and genera of Convolvulaceae other than *Calystegia sepium* were screened for calystegines, and the compounds were found in many genera and considered as a typical chemical constituent of Convolvulaceae (Schimming et al., 1998, 2005). In *Calystegia sepium* root cultures, calystegines were identified as the only products of the tropane alkaloid pathway, and this gave the chance for a targeted and efficient incorporation of labelled tropane precursors. ^{15}N-labelled tropinone was administered to root cultures, and the label was traced in calystegines by GC–MS and by NMR (Fig. 3). Six days after treatment almost total calystegine A_3 and about half of the calystegines B_1 and B_2 were labelled by ^{15}N. This was the first experimental evidence for the decent of calystegines from the tropane pathway (Scholl et al., 2001, 2003). In potato, the surprising finding of calystegines in sprouting tubers was confirmed by a detailed analysis of plant tissues of many developmental stages (Keiner and Dräger, 2000). Biosynthesis was assumed to proceed by the tropane pathway in potato as well, and the identification of potato genes and enzymes specific to tropane alkaloid metabolism proved the concept. A typical TRII was isolated and characterised from potato (Keiner et al., 2002), and recently putrescine *N*-methyltransferase, the first enzyme of tropane alkaloid metabolism was cloned and characterised from potato (Stenzel et al., 2006).

4. Tropinone reductase protein structure and function

Purification of both tropinone reductases from *D. stramonium* (Portsteffen et al., 1992, 1994) and from *H. niger* (Hashimoto et al., 1992) yielded enzymes with similar protein properties, but with different catalytic and kinetic behaviour. Molecular mass of the protein subunits was determined to be between 28,000 and 30,000 Dalton. Sequencing of cDNA coding for *D. stramonium* TRs confirmed the protein subunits to consist of 273 (TRI) and 260 (TRII) amino acids and to have a molecular mass of

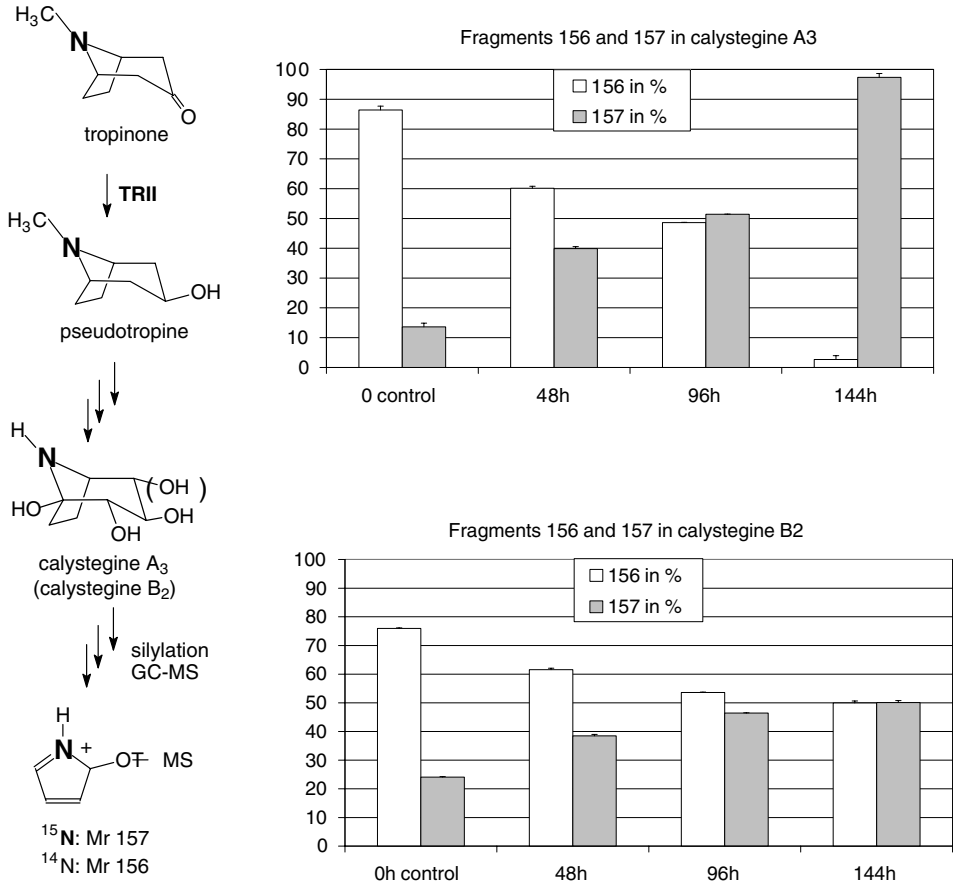

Fig. 3. Calystegine biosynthesis by the tropane alkaloid pathway. ^{15}N-labelled tropinone applied to root cultures is incorporated into calystegines. Percentage of label in calystegines is detected by GC–MS. The pyrrolidine ring fragment mass (156) is enhanced +1 (157) after ^{15}N-tropinone application in a time-dependent manner, as shown for calystegines A_3 and B_2 (data adapted from Scholl et al., 2001).

29,615 and 28,310 Dalton, respectively (Nakajima et al., 1993). Amino acid sequence homology (167 identical amino acid residues, 64%) and comparison of conserved amino acid motifs grouped both tropinone reductases into the family of short-chain dehydrogenases.

The similarity in protein type of both reductases rendered the apparent difference in reaction stereospecificity even more intriguing. Differences in tropinone acceptance and fixation were suspected to be responsible for the selective formation of tropine and pseudotropine, because reaction velocity, substrate affinity, and pH optima for TRI and TRII were conspicuously different (Table 1). TRI catalysed reduction of tropinone and oxidation of tropine, whereas TRII was not, or only very slightly, active in catalysis of the oxidation reaction (Hashimoto et al., 1992; Portsteffen et al., 1994). In particular, pH dependency of turnover velocities and K_M values were different for both enzymes. The catalytic constant of TRI of *D. stramonium* was 11-fold higher than that of TRII at pH 7.0. TRI showed the maximal reduction activity at acidic pH with a very high K_M value only. Together, this was taken as an indication that acidic pH favoured turnover, but uncharged tropinone was fixed better in the active centre of the enzyme (Portsteffen et al., 1994). Another strong indication for differential substrate handling of both TRs came from incubations with substrate analogues (Table 2). Molecules with variations in charge and shape were accepted differentially by TRI and TRII. Quinuclidinone and TBON (8-thiabicylo[3.2.1]octan-3-one, the sulfur analogue of tropinone), for example are good substrates for all TRI enzymes, but are not accepted by TRIIs.

After heterologous expression in *E. coli*, sufficient enzyme protein was available for crystallisation and protein structure elucidation of TRI and TRII (Nakajima et al., 1998). Modelling of the tropinone binding site of TRI and TRII suggested two different ways of substrate fixation (Fig. 4). In TRII tropinone is attached by ionic interaction of the tropinone nitrogen to the side chain of glutamic acid (Glu156) in the active centre. In TRI, the nitrogen is repulsed by a histidine (His112) in the same position and fixed in the opposite orientation by hydrophobic interactions. This causes uncharged tropinone to be fixed more easily, and it may also fix TBON, which is not charged. Selective and differential fixation of the substrate tropinone in the active centre (with NADPH always occupying the same position) explains how both enzymes succeed in stereospecific product formation. The concept was supported by site-directed mutagenesis at those residues

Table 1
Tropinone reductases from Solanaceae

Enzyme	K_M tropinone (mM)	K_M NADPH (μM)	K_{cat} (s^{-1})	pH optimum reduct./oxid.	K_M tropine or ψ-tropine (mM)	K_M NADP+ (μM)	References
D. stramonium TRI	0.775 (pH 5.9)	58	25.6 (pH 7.0)	6.4/9.9	0.18	105	Nakajima et al. (1994) Portsteffen et al. (1994) Nakajima et al. (1999a)
H. niger TRI	1.01 (pH 5.9)	11.3	n. d.	6.1/7.6	2.6	41.5	Hashimoto et al. (1992)
D. stramonium TRII	0.033 (pH 5.95)	16	2.73 (pH 7.0)	6.25 broad/–	–	–	Nakajima et al. (1994) Portsteffen et al. (1994) Nakajima et al. (1999a)
H. niger TRII	0.034 (pH 5.9)	6.1	n.d.	5.8 broad/–	0.687 (1.3% of act. with tropinone)	251	Dräger et al. (1988) Hashimoto et al. (1992)
A. belladonna TRII	0.090 (pH 6.25)	21	n.d.	6.25 broad/–	–	–	Dräger and Schaal (1994)
S. tuberosum TRII	0.033 (pH 6.4)	20	n. d.	5.0 broad/–	–	–	Keiner et al. (2002)

TRI and TRII enzymes can be grouped by typical differences in kinetic performance.
n.d., not determined; –, no activity.

assumed to be responsible for substrate fixation. Some of the enzymes with exchanged amino acids formed both products (Nakajima et al., 1994, 1999a). The higher reaction velocity of TRI was explained by the relatively low substrate – and product – fixation caused by the charged nitrogen at the hydrophobic protein active site. Results of reduction of the sulfur analogue TBON confirmed this idea. TBON was reduced slowly by TRI (35% of maximal catalytic velocity), probably because it is non-charged and bound more strongly. Reduction yielded both products, 80% α-TBOL (8-thiabicylo[3.2.1]octan-3α-ol) with an axial hydroxyl group like tropine and 20% β-TBOL with equatorial hydroxyl group like pseudotropine, demonstrating that the substrate TBON was fixed less stereospecifically in TRI

Table 2
Substrates for tropinone reductases

Substrate	*D. stramonium* TRI	*H. niger* TRI	*D. stramonium* TRII	*H. niger* TRII	*A. belladonna* TRII	*S. tuberosum* TRII
Tropinone V_{max}	100%	100%	100%	100%	100%	100%
K_M	1.30 mM, pH 6.4	1.01 mM	0.033 mM	0.034 mM	0.090 mM	0.033 mM
N-Methyl-4-pieridinone V_{max}	180%	13%	28%	512%	313%	140%
K_M	1.40 mM	0.231 mM	20 mM	0.770 mM	0.650 mM	n.d.
N-Propyl-4-pieridinone V_{max}	78%	–	–	530%	129%	84%
K_M	–	–	–	0.265 mM	n.d.	n.d.
4-Methyl-cyclohexanone V_{max}	39%	64%	22%	113%	n.d.	17.2%
K_M	0.030 mM	0.012 mM	2.8 mM	2.03 mM	n.d.	n.d.
3-Methyl-cyclohexanone V_{max}	n.d.	85%	n.d.	172%	n.d.	13.4%
K_M	n.d.	0.041 mM	n.d.	7.580 mM	n.d.	n.d.
4-Ethyl-cyclohexanone V_{max}	41%	107%	58%	172%	n.d.	n.d.
K_M	0.045 mM	0.030 mM	n.d.	0.534 mM	n.d.	n.d.
Quinuclidinone V_{max}	80%	136%	–	–	–	–
K_M	2.2 mM	1.810 mM	–	–	–	–
TBON[a] V_{max}	35%	40%	–	–	–	–
K_M	0.033 mM	0.033	–	–	–	–
4-Tetrahydro-thiopyranone V_{max}	83%	n.d.	115%	n.d.	71%	n.d.
K_M	0.030 mM	n.d.	2.0 mM	n.d.	0.380 mM	n.d.
References	Portsteffen et al. (1994)	Hashimoto et al. (1992)	Portsteffen et al. (1994)	Hashimoto et al. (1992)	Dräger and Schaal (1994)	Keiner et al. (2002)

TRI and TRII enzymes show differential preferences for substrate analogues.
n.d., not determined; –, no activity.
[a] 8-Thiabicylo[3.2.1]octan-3-one.

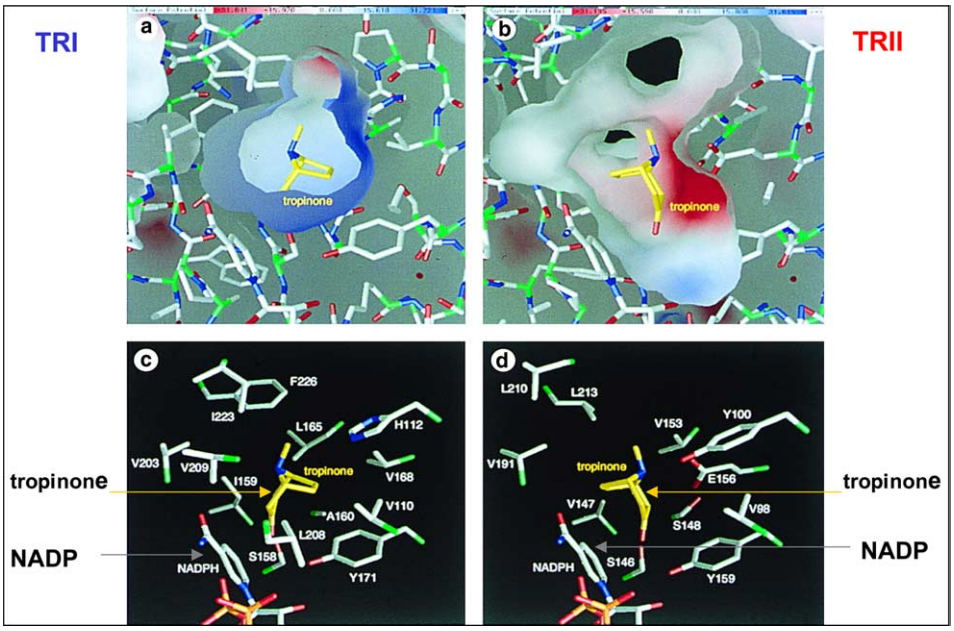

Fig. 4. Protein models of *D. stramonium* TRI and TRII. Blue colour on the protein surface indicates positive charge; red colour indicates negative charge. Stereospecific fixation of the substrate tropinone is influenced in TRI by repulsion of the alkaloid nitrogen on a positively protein surface and by hydrophobic interaction. In TRII, ionic attraction of the nitrogen by a negatively charged glutamic acid side chain holds tropinone in place. The hydrogen from NADPH is transferred to yield an equatorial or axial position of the resulting hydroxyl, respectively. Molecule models from Nakajima et al. (1998), PNAS 95, 4876–4881, adapted with kind permission of the authors. Copyright 1998 National Academy of Sciences, USA.

(Portsteffen et al., 1994). Detailed elucidation of the catalytic mechanism of TRII was achieved by crystal saturation with the substrate tropinone and multiwavelength Laue X-ray diffraction (Yamashita et al., 2003). Transient structures were captured, and it could be demonstrated that a slight rotation of the product pseudotropine upon formation from the substrate was responsible for efficient catalysis. In conclusion, positioning of the substrate in an optimal angle for hydride transfer from NADPH is considered an important prerequisite for efficient catalysis, and the enzyme protein must be able to adjust to each transition state of the reaction.

5. Flux regulation of the tropane pathway by tropinone reductases

The distribution of the substrate tropinone on the two arms of the diverged tropane alkaloid metabolism appears to be determined by the activity of the tropinone reductases. First evidence for this view was given by addition of the tropinone sulfur analogue TBON (Table 2) to root cultures of *D. stramonium*. TBON perturbed the alkaloid metabolism considerably, leading to more pseudotropine but decreased levels of tropine, acetyltropine and hyoscyamine (Parr et al., 1991). TBON is accepted as substrate by TRI only and in competition with tropinone acts as inhibitor of TRI; TRII is not affected. The reduction products α-TBOL and β-TBOL are also strong inhibitors of tropinone reduction by TRI (Dräger et al., 1992). Application of 1 or 2 mM TBON or β-TBOL to root cultures of *D. stramonium* shifted the ratio of TRII products to TRI products from 0.04 to 0.31. In combination with 2.5 mM tropinone, the effect was even stronger yielding twice as much pseudotropine-derived products than tropine-derived products. In *A. belladonna* root cultures, both hyoscyamine and calystegine A_3 were measured after TBON application. After 10 days, hyoscyamine had decreased considerably while the concentration of calystegine A_3 was double of that in control roots (Dräger et al., 1994). Further analogues of tropinone with different alkylation on the nitrogen or with enlarged ring (pseudopelletierine derivatives) were accepted as substrates by TRI and TRII to various degrees (Boswell et al., 1999a,b). Application of these analogues to root cultures again shifted the ratio of TRI to TRII-derived products. These data suggest that even if TR activities are higher than those of other tropane alkaloid pathway enzymes, and usually not limiting for the total flux through the pathway (Hashimoto et al., 1992), the ratio of products is influenced by the activity of both enzymes.

Availability of TR coding sequences enabled overexpression of TRI and TRII in tropane alkaloid producing plant tissues. The first successful transformation with TRI cDNA inserted behind a strong 35S promoter was achieved in *Nicotiana tabacum*. Leaves, after tropinone application, produced tropine and acetyltropine (Rocha et al., 2002). Shortly thereafter, *A. belladonna* was transformed with either TRI or TRII cDNA (Richter et al., 2005). Numerous root clones of both transformations experiments showed very different enzyme activities and

alkaloid patterns. Wild type roots contained two times more TRII products than TRI products. The transformation effect on the alkaloid pattern was stronger with TRI overexpression than with TRII (Fig. 5). These results confirm that in vivo activities of TRI and TRII both affect the products of the tropane alkaloid biosynthesis. They do not, however, prove that both enzymes compete for their substrate tropinone within the same tissues or cells.

Similar bifurcations involving specific and stereoselective reduction and oxidation steps are known from other secondary product biosynthesis pathways. In *Mentha piperita* plants (Lamiaceae), the major constituent of the essential oil is (−)-menthol, a monoterpene. In the course of the biosynthesis, the menthol precursor (+)-pulegone is reduced to give either (−)-menthone by NADPH dependent double bond reduction or (+)-menthofuran by P450 dependent monooxidation. At this branch point of the pathway, the monooxygenase menthofuran synthase was proven to exert control over the pulegone reductase and to regulate the total flux into menthol biosynthesis (Mahmoud and Croteau, 2003). In flavonoid biosynthesis, a diversion of the pathway is located at the metabolite dihydroflavanol, in which the 3-ketofunction is stereospecifically reduced by NADPH dependent dihydrokaempferol reductase (EC 1.1.1.219) leading finally to coloured anthocyanins. Alternatively, dihydroflavanols are oxidised by flavonol synthase introducing a double bond in 2–3-position leading to white or yellow flavonols. Flavonol synthase is, dependent on the plant species, a soluble dioxygenase or a P450 monooxygenase. The flux regulation at this branch point again is species dependent and mostly performed by enzyme expression in a development- and tissue-specific manner (Winkel-Shirley, 2001a,b). Combinations of *cis*-acting elements and DNA-binding factors have recently been shown to be responsible for differential expression of flavonoid pathway enzymes in *Arabidopsis thaliana* (Hartmann et al., 2005). Cardiac glycoside biosynthesis, as well, involves stereospecific reduction of a double bond at a branch point of the pathway. An instructive and colourful overview on the biology and biochemistry of cardiac glycoside was written by Professor Martin Luckner together with his colleague and friend Professor Max Wichtl (Luckner and Wichtl, 2000). In the course of the formation of the cardenolide skeleton, progesterone is reduced to either 5β-pregnan-3,20-dione or to 5α-pregnan-3,20-dione, the latter being a metabolite in the formation of brassinosteroids, ubiquitous phytohormones in plants (Nomura et al., 2004). Progesterone 5β-reductase is specific for cardenolide forming plant tissues and was isolated and purified from *Digitalis purpurea* (Gaertner et al., 1990, 1994). The molecular structure of the gene and of the protein forming this key enzyme of cardenolide biosynthesis will be most interesting in order to clarify the question how the flux of steroid skeletons into the cardenolide pathway is controlled.

The substrate tropinone is hardly ever seen to accumulate in any tropane alkaloid producing plant tissue. Besides specific reduction activities the availability of the substrate tropinone may be regulating the metabolite flow into either arm of the tropane alkaloid biosynthesis. Transcripts and proteins of both enzymes were localised in root cultures of *H. niger*. Reporter gene fusion to promoter regions of both TRs suggested expression in mature roots rather than in young roots (Nakajima et al., 1999b). Within the root cross sections not much difference between TRI and TRII promoter controlled expression was seen. Endodermis,

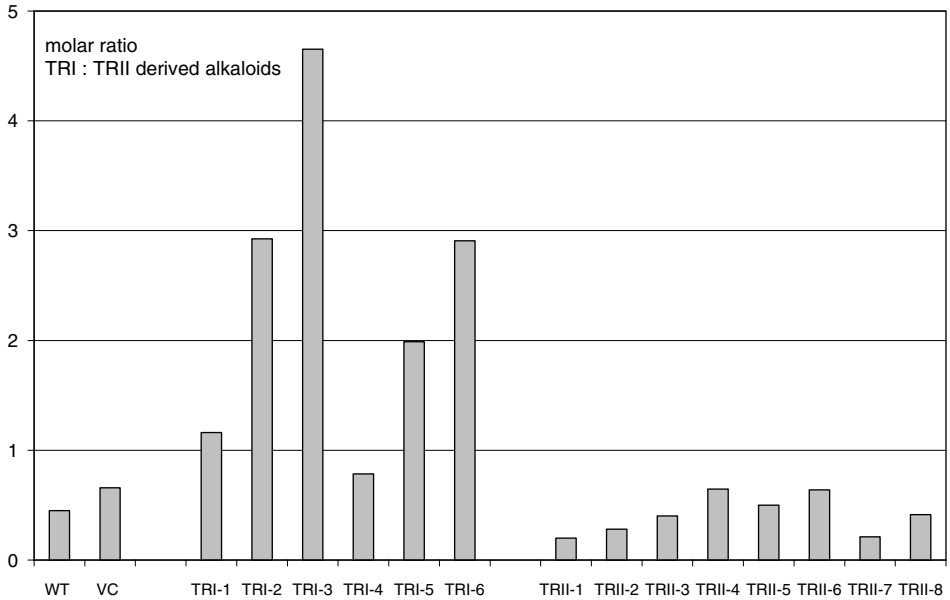

Fig. 5. Molar alkaloid ratio in root cultures of *A. belladonna* after overexpression of TRI and TRII cDNA. Several clones of each transformation experiment are shown. WT, wild type; VC, vector control; TRI-1–TRI-6, TRI overexpressing clones; TRII-1–TRII-8, TRII overexpressing clones.

pericycle, and some cortex cells were stained by expression of the reporter gene. Run-on transcription assays and Northern blots showed similar transcription rates for both enzymes. Actual in situ activity of enzymes however, is better demonstrated by visualisation of the individual enzyme proteins. Immunostaining of TRI and TRII proved partial different localisation of TRI and TRII within the root diameter (Nakajima and Hashimoto, 1999). TRI protein signal was strong in the endodermis and in some cortex cells, while TRII protein concentration was highest in the pericycle. Two further enzyme proteins participating in tropane alkaloid formation were localised before in the pericycle of roots: the first specific enzyme of the alkaloid pathway, putrescine *N*-methyltransferase (EC 2.1.1.53) in *A. belladonna* (Suzuki et al., 1999) and hyoscyamine 6β-hydroxylase (EC 1.14.11.11, forming scopolamine) in *H. niger* (Hashimoto et al., 1991). It is therefore striking that TRI responsible for hyoscyamine formation was absent from the pericycle. The results require the concept that there is transport of metabolites between tissue layers in order to complete tropane alkaloid biosynthesis. As a consequence, transport as well as enzyme activity may be limiting and regulating alkaloid biosynthetic activity.

In *Solanum tuberosum*, transcripts for TRII involved in calystegine formation were found in tuber sprouts, which are stem tissues (Keiner et al., 2002). Before, tropane alkaloid formation was thought to be generally restricted to roots, and it will be interesting to see in detail the localisation of tropane alkaloid metabolism and the enzymes involved in potato.

6. Evolution of tropinone reductases

In the rice and in the *Arabidopsis* genomes, putative tropinone reductases were annotated, similarly in EST collections of apple (*Malus* × *domestica*, Rosaceae), of *Medicago truncatula* and soy bean, *Glycine max* (both Fabaceae), and of tomato (*Lycopersicon esculentum*, Solanaceae). In tomato, calystegines have been detected (Asano et al., 1997). For the other plants with putative TR sequences tropane alkaloids were never described, and phytochemical investigations of some of these species did not reveal any tropine, pseudotropine or derived products (Dräger, unpublished). Tropane alkaloid accumulation is known as typical feature of Solanaceae; further sporadic occurrence was reported in several mostly unrelated taxa. Surveys on occurrence of tropane alkaloids in general (Lounasmaa and Tamminen, 1993; Griffin and Lin, 2000) and of calystegines in particular (Dräger, 2004) encompass eight plant families. They appear scattered within the large clade of core eudicots (Fig. 6). *Erythroxylum* species are known for cocaine and related alkaloid structures, and recently calystegines were identified in many *Erythroxylum* species (Brock et al., 2005). Proteaceae contain tropane alkaloids with an extraordinary pyranone structure linked to the tropane bicyclus. *Morus* species like Convolvulaceae contain calystegines. *Cochlearia arctica* (Brassicaceae) was reported to synthesise cochlearine, which is the 3-hydroxybenzoic acid ester of tropine. The widespread occurrence of tropane alkaloids in higher plants raises the question for their evolutionary development. A repeated and independent (polyphyletic) emergence of the tropane biosynthetic steps in distant plant families appears to be the most rational explanation. Sequencing and comparison of tropinone reductase genes and of further enzymes involved in tropane alkaloid formation is required for definite conclusions. Up to now, DNA sequences of tropinone reductases with enzymatic characterisation have only been isolated from Solanaceae. A cDNA coding for a tropinone reductase from *Calystegia sepium* (EMBL accession AJ540305) with 55% amino acid identity to *H. niger* TRI was sequenced, but enzyme activity of the protein encoded was not yet described. It is conceivable that this enzyme is involved in tropane alkaloid formations as tropine derivatives occur in Convolvulaceae, and Solanaceae and Convolvulaceae are closely related in the same order Solanales. The DNA sequences that are annotated as ''putative tropinone reductases'' in fully sequenced genome share about 50% identity to TRs suggesting that they are related more closely and possibly possess a common ancestor like TRI and TRII (Nakajima et al., 1993).

Tropinone reductases belong to the enzyme family of short-chain dehydrogenases/reductases (SDR), which is large and of old origin. SDRs are present in all living organisms. About 3000 different sequences of SDR enzymes were annotated in the databases in 2003. In the human genome for example, 63 SDR genes were identified (Oppermann et al., 2003). The proteins are characterised by a length of approximately 250 amino acids and a NADP or NADPH binding domain at the N-terminus. The three-dimensional structure of SDRs contains a Rossman fold typical for nucleotide binding proteins and comprised of a sheet of six to seven parallel β-strands flanked by mostly four α-helices (Kallberg et al., 2002). Hydride transfer is catalysed by a conserved triad of amino acids Ser, Tyr, and Lys. Mutagenesis and structure determination on 3β/17β-hydroxysteroid dehydrogenase identified an additional Asp as crucial residue for catalysis that was found in most SDRs (Filling et al., 2002). In spite of a highly conserved protein structure, DNA sequences coding for SDR enzymes share low residue identity; 10–30% are mostly found. The DNA sequences that are annotated as ''putative tropinone reductases'' in fully sequenced genome share about 50% identity to TRs suggesting that they are related more closely and possibly possess a common ancestor like TRI and TRII (Nakajima et al., 1993). Clustering of SDR with the aim of reconstruction of evolutionary origins has been difficult because of the low sequence residue similarity. Therefore, a protein structure-based phylogenetic analysis for SDR with a focus on 17β-hydroxysteroid dehydrogenases was undertaken (Breitling et al., 2001). This approach proved successful for detecting two structurally independent subgroups of 17β-hydroxysteroid dehy-

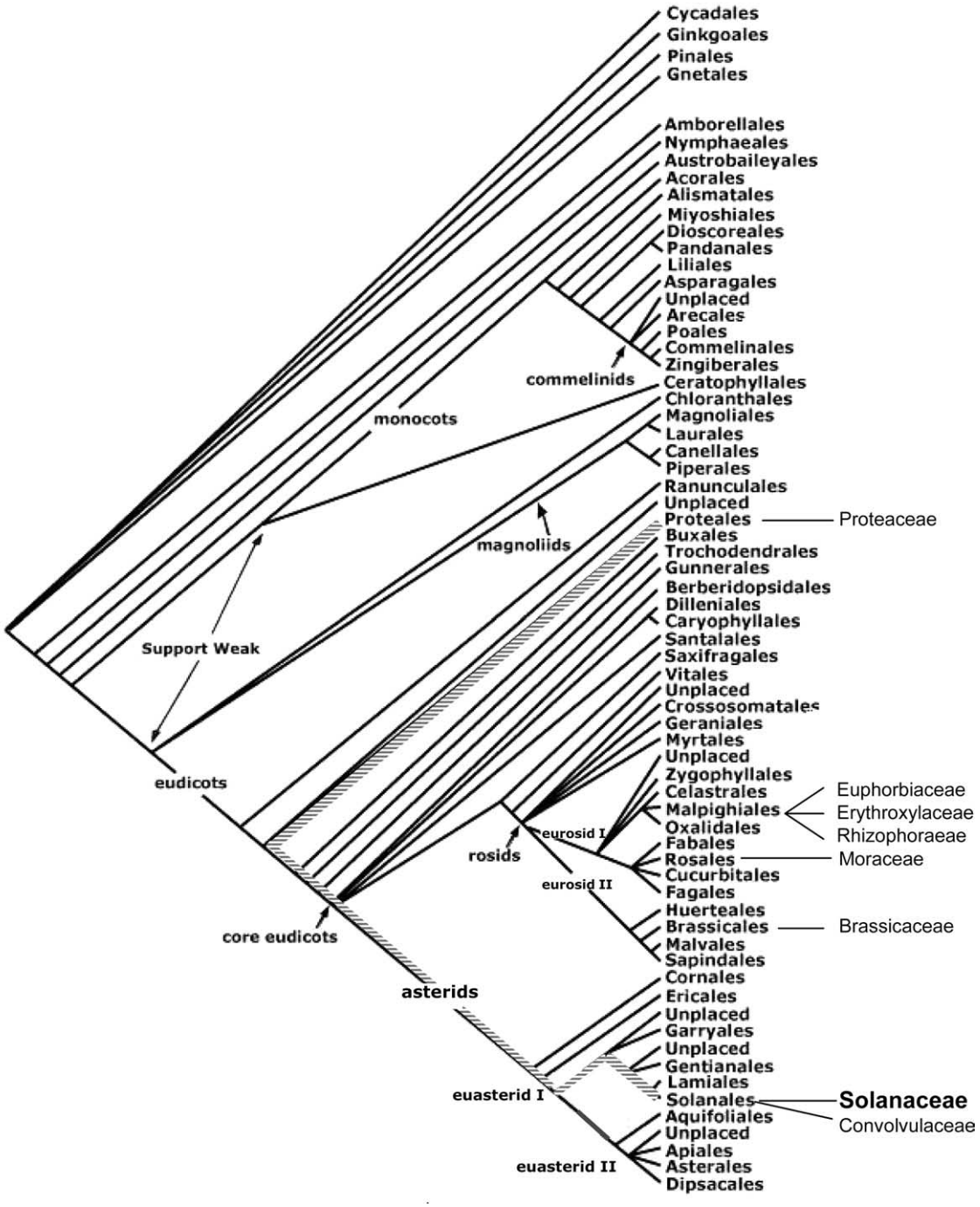

Fig. 6. Occurrence of tropane alkaloids in the angiosperms. Tropane alkaloids were reported within a section of the eudicots. Hatched lines indicate this section. The tree diagram was taken from the angiosperm phylogeny website, Missouri Botanical Garden (http://www.mobot.org/MOBOT/Research/APweb/welcome.html) with kind permission of the author Dr. Peter Stevens. The original tree on the website is linked on all terminal taxa to pages with detailed taxon characterisation.

drogenases. TRs were included in this analysis, but depending on the clustering algorithm, they were placed into different neighbourhoods together with, e.g., carbonyl or steroid reducing enzymes from different fungal, mammalian, or bacterial origin suggesting that more TR and related SDR protein structures must be included to provide a better clustering basis for this method.

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