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Plant Biotechnology of Tropane Alkaloids

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1. Historical review of tropane alkaloid-containing plants

Tropane alkaloids occur in many genera of Solanaceae, especially in species of *Mandragora, Atropa, Hyoscyamus, Datura, Scopolia,* and *Duboisia,* which have provided important materials for medicine all over the world, as well as for magic and witchcraft. Crude drugs and alkaloids prepared from these poisonous plants are used as an antispasmodic, sedative, anesthetic, mydriatic, *etc.* in therapies. It is well known that their remarkable physiological properties are principally due to the tropane alkaloids *l*-hyoscyamine and *l*-scopolamine (=*l*-hyoscine). Although the medicinal use of these plants probably began in prehistoric times, Dioscorides (A.D. 40-90), who served as an army surgeon under the Emperor Nero, first gave a detailed description of *Mandragora* and *Hyoscyamus* in his great Herbal "De Materia Medica", which is also known as the "Greek Herbal" [1].

1. 1 Mandragora (*Mandrake, Devil's Apple*)

The representative species *Mandragora officinarum* L. is a perennial herb native to southern Europe and the Mediterranean areas. Dioscorides explained that the fresh juice or a wine extract of the mandrake root is used against sleeplessness and pain due to cut or cauterization, warning that overdrinking is fatal to the patient. During the Middle Ages, however, mandrake was commonly believed to be a magic herb with a large bifurcated root bearing a resemblance to the human form. It was said that whoever uprooted a mandrake plant which uttered a terrible shriek of pain would go mad and die, consequently plants were pulled out by a dog which was tied to the neck of the root by a rope [2-4]. Roots taken in this manner then were dried and kept in the house as a talisman to bring luck, wealth, and magical protection [4]. For medicinal purposes, mandrake was used by ancient and medieval Europeans as an anodyne and soporific for continued pain, melancholy, *etc.* [2]. In Act I of "Antony and Cleopatra" by Shakespeare, Cleopatra asks her attendant for a drink of mandragora, so that "I might sleep out this gap of time my Antony is away." Mandrake contains *l*-hyoscyamine, atropine, *l*-scopolamine and various minor bases (total content: 0.3-0.4%) [6], but is no longer used as an official drug in modern life.

1. 2 Atropa belladonna *L.* (*Belladonna, Deadly Nightshade*)

Belladonna (beautiful lady) is a perennial plant distributed over central and southern Europe; the name probably derives from a traditional cosmetic use of its juice among Italian and Spanish ladies who applied drops into their eyes giving them greater brilliance by dilating the pupils [5]. Because of its power to dilate the pupil, belladonna extracts had been utilized as a mydriatic essential for performing operations on the eye, until it was replaced by its active principle, atropine. In addition, various preparations of belladonna roots and leaves have been applied externally for neuralgia, gout, rheumatism, and sciatica to lessen pain, and internally to relax spasms of the stomach and to ease asthma [6]. Belladonna contains *l*-hyoscyamine (main alkaloid), atropine, *l*-scopolamine, and apoatropine (total content: 0.45-0.85%) [6].

1. 3 Hyoscyamus niger *L.* (*Henbane*)

Hyoscyamus niger is a biennial herb widely distributed throughout Europe, North America, and Asia. It is as poisonous as belladonna, containing *l*-hyoscyamine (leaf: 0.06-0.17%, root: 0.08%, seeds: 0.05-0.3%) as the main alkaloid and small amounts of atropine, scopolamine, apoatropine, *etc.* [6]. Dioscorides [1] described that the juice of seeds, leaves or stalks, as well as a hot water extract of crushed seeds or leaves, eases pains from rheumatism and gout and inflammation of eyes, ears and feet. In Europe, *Hyoscyamus* preparations from leaves have been used in relief of painful spasmodic affections of the smooth muscles of the digestive system and against asthma [2].

In many villages in Turkey, the seeds of *Hyoscyamus* are still used for relieving toothaches, and eye pains which are believed to be caused by a worm

which lays its eggs on the mucous membranes of the mouth or on the cornea of the eye. When a sufferer either inhales or exposes his eyes to the fumes emitted from crushed *Hyoscyamus* seeds scattered on either embers or a hot iron plate, it is said that the tiny pain-causing worms drop from his mouth or eye onto a piece of cloth held under his face [7].

The oldest Chinese Herbal "Shen-nong Ben-tsao Jing", which was compiled by Tao Hong-jing (456-536) [8], describes the use of *Hyoscyamus niger* seeds for the relief of toothaches and paralysis and spasm of muscles, but that overeating will cause one to run around in a crazed fashion. *Hyoscyamus* was not used in Japan where none of its species grows wild.

1. 4 Datura *spp.*

Of about 10 species of *Datura* distributed throughout the world, *D. stramonium* L. (thornapple, Jimsonweed) and *D. metel* L. were the most frequently used medicinal plants by Europeans and Asians, respectively. On the other hand, American Indians from Mexico and South America employed mainly *D. meteloides* and *D. sanguinea* in the treatment of various diseases and wounds, as well as for ceremonial drinks used especially by medicine men, to communicate with the spirits [9].

The common name of *D. stramonium*, "Jimsonweed", probably originated from an incident that occurred in Jamestown, Viginia in 1676. British soldiers stationed in the town ate a salad prepared from the boiled plant, whereupon they behaved foolishly for several days. After 11 days they returned to normal, but remembered nothing which had happened [9]. *D. stramonium* is more or less similar to belladonna in its properties as an antispasmodic, anodyne, and hypnotic. It is used as the chief ingredient in "asthma cigarettes". Stramonium leaf usually contains 0.2-0.45% alkaloids, which consist mainly of hyoscyamine and scopolamine [10]. *D. tatula* L. is distinguished from white-flowered *D. stramonium* by its purple flower, which is due to a dominant gene. *D. tatula* L. should be considered a variety of the latter, since the results of breeding experiments, as well as of chemical analyses indicate no significant differences in both morphological characteristics and alkaloids between the two except in a few quantitative traits [11].

Datura metel L. which is a native to tropical Asia, was introduced into China in the 13th century, where its flowers and seeds have been used both externally and internally to allay the severe pains associated with complicated bone fractures. *D. metel* var. *alba* (=*D. alba* Nees) was also cultivated in Japan from the 17th century onwards largely as an exotic horticultural plant, 'mandarage', by plant lovers and partly for medicinal use [12], but it has rarely been employed by Japanese physicians of the Kanpõ school of medicine.

However, Seishu Hanaoka (1760-1835), a great creative surgeon of the Edo period, devised a powerful anesthetic, "Mafutsu-Tõ (="Tsûsen-San"), which is a concoction prepared from a mixture of *D. metel* seeds or leaves at the chief component, along with four other types of crude drugs; *Aconitum japonicum* root, *Angelica dahurica* root, *Angelica acutiloba* root, and *Cnidium officinale* rhizome. All of which have been shown to have analgesic and sedative activities in modern studies [13, 14]. On October 13th, 1805, he anesthetized a 60 year old woman with Mafutsu-Tõ to perform an operation for breast cancer with remarkable success [15, 16]. It is recorded by his student Honma [17] that oral administration of the anesthetic caused the patient to display a rise in body temperature, high pulse, thirst, dilation of the pupils, senseless speech, delirium and general anesthesia within 1-2 hours, and that the operation was performed 4 hours after drug administration. The operation performed by Hanaoka with the aid of the anesthetic, Mafutsu-Tõ, preceded the anesthetic use of chloroform (1842) and ethyl ether (1853) by about 40 years.

In *D. metel* seeds, the content of scopolamine (0.24%) is higher than that of *l*-hyoscyamine (0.02%) or of atropine (0.0025%) [13]. The total alkaloid content of the leaf is 0.55% and scopolamine (*ca.* 0.16%) is the main alkaloid [6]. Thus, the seeds of *D. metel* were used for preparing scopolamine hydrobromide.

1. 5 Scopolia japonica *Maxim.* (*Hashiridokoro*)

Scopolia japonica is a wild perennial herb native to Japan, growing on the shady slopes of forest brooks. It is named Hashiridokoro, because eating its rhizome will cause one to run around in a crazed fashion [18]. *Scopolia* had never been regarded as a drug in Japan until the German doctor Philipp Franz von Siebold [19], who visited Edo in 1826, taught an eye doctor of the Shogunate, Genseki Habu, that *Scopolia* could substitute for belladonna as a mydriatic [20, 21].

In Japan today, an extract of *Scopolia* rhizome and root is used as an antispasmodic for the relief of stomachache.

The rhizome of *S. japonica* contains tropane alkaloids (0.2-0.4%), consisting chiefly of *l*-hyoscyamine and scopolamine. Similar alkaloids are also found in other species of *Scopolia*: *S. parviflora* in Korea, *S. sinensis* and *S. tangutica* in China, *S. lurida* in the Himalayas, and *S. carniolica* in Europe, in spite of the differences in chromosome number and alkaloid content found between these species [22, 23].

1. 6 Duboisia *spp.*

Duboisia is an evergreen shrub or small tree endemic to Australia. Three species of *Duboisia* con-

tain a variety of tropane alkaloids; the main alkaloid in the leaf of *D. myoporoides* R. Br. (corkwood tree) is scopolamine (*ca.* 2%), whereas that of *D. leichardtii* F. Muell. is hyoscyamine [6, 10]. Interestingly, the third species *D. hopwoodii* F. Muell. contains the tobacco alkaloids nicotine, nornicotine, and several minor bases, although tropane alkaloids do occur in the roots. The natives of Australia chew and smoke the leaf of *D. hopwoodii* for its stimulating effects which can reduce fatigue and hunger.

The amount of total alkaloids in the dried leaves of *Duboisia* is variable up to 5.0% [10] and the leaves are exported to foreign countries for the extraction of scopolamine and *l*-hyoscyamine.

2. Pharmacology of tropane alkaloids

The principal tropane alkaloids of medicinal interest are *l*-hyoscyamine (tropic acid ester with tropine), its racemate atropine (*d, l*-hyoscyamine), and *l*-scopolamine (tropic acid ester with scopine), although a number of minor alkaloids including apoatropine, aposcopolamine, belladonine, norhyosyamine, meteloidine, valeroidine, and atroscine (=*d, l*-scopolamine), have been isolated from various species of *Mandragora, Atropa, Hyoscyamus, Datura, Scopolia,* and *Duboisia* [6, 24]. Atropine was first isolated from *Atropa belladonna* by Mein in 1833, which was immediately followed by the isolation of its optical isomer *l*-hyoscyamine by Geiger & Hesse in the same year, Scopolamine was isolated from *Scopolia japonica* by Schmidt in 1850 [24].

Atropine and scopolamine are anticholinergic agents (=parasympatholytic agents) which block the parasympathetic system by interfering with the binding of acetylcholine to its receptor in the nerve ending in a competitive manner [25]. A large dose of atropine causes blurred vision, suppressed salivation, vasodilation, hyperpyrexia, excitement, agitation, and delirium [26]. Therapeutically, a small dose of atropine sulfate (0.5-1.0 mg/day) is administered orally to the patient as an antispasmodic or smooth muscle relaxant to suppress the movement of the digestive system and the secretion of gastric juices during hyperacidity, stomachache, gastric cramp, and peptic ulcer. It is also administered externally as a mydriatic to relax the sphincter of the pupil (dilation). Atropine sulfate is also injected as an antidote to poisoning by organophosphorous compounds, such as insecticides and poison gas [27, 28].

Scopolamine hydrobromide has been used as a sedative, a preanesthetic, and a preventive against seasickness. Scopolamine shows stronger parasympatholytic activity than atropine does, but also depresses the central nervous system acting as a sedative, unlike atropine [25].

3. Alkaloid production by *Datura* and *Scopolia* tissue cultures

Callus and cell suspension cultures of various species of *Datura* and *Scopolia* were initiated by several workers to investigate alkaloid-producing ability, effects of chemical factors, and the relationship between organogenesis and alkaloid production. These studies have shown that undifferentiated cultures of *Datura* and *Scopolia* produce tropane alkaloids, but that their alkaloid contents are generally much lower than those of the original plants.

In 1965, Staba and Chan [29] reported that phenylalanine stimulated the production of alkaloids (chiefly scopolamine) when added to *Datura stramonium* leaf suspension cultures. Netien and Combet [30] observed that callus cultures of *D. metel* showed an noteworthy decrease in scopolamine. Remeike and Koblitz [31], however, detected neither scopolamine nor hyoscyamine in *Datura* tissue cultures. Tabata *et al.* [32] reported for callus cultures of various species of *Datura* (*stramonium, tatula, inermis,* and *innoxia*) and *Scopolia* (*japonica* and *parviflora*) that their alkaloid contents gradually decreased with time to a low level (0.005-0.01%) during the period of successive subculturing, irrespective of origin, although the spectrum of alkaloids showed a close resemblance to that in the original plants.

In concordance with a report by Stohs [33] that C^{14}-tropic acid (the acidic moiety of the ester alkaloid hyoscyamine) administered to *D. stramonium* tissue suspension cultures was incorporated into scopolamine and hyoscyamine, the addition of tropic acid to *Datura* and *Scopolia* cultures markedly increased the alkaloid content up to 0.12% from the initial low level (less than 0.01%) [32]. In contrast, phenylalanine (the precursor of tropic acid), ornithine (the precursor of tropine), and tropine were ineffective in increasing alkaloids. Furthermore, tropine administered to *D. innoxia* cell suspension cultures was not converted into hyoscyamine but into an abnormal metabolite, acetyltropine, presumably by a tropine-specific esterase [34]. These results suggested that the biosynthetic process for converting phenylalanine or phenylpyruvic acid to tropic acid, possibly the reaction step involving the rearrangement of the carboxyl group in phenyl-pyruvate as proposed by Leete [35], is strongly repressed with the 'de-differentiation' which occurs in cultured cells. It appears therefore that the regulation of tropic acid biosynthesis is of vital importance in the production of tropane alkaloids. However, the enzyme system involved in the biosynthesis of tropic acid still remains to be clarified.

It has been demonstrated that alkaloid-producing

activity was restored to a great extent when callus cultures of *Atropa belladonna* [36] and *Scopolia parviflora* [37] initiated roots, suggesting the operation of self-regulation for alkaloid biosynthesis. The promotion of alkaloid production associated with organogenesis was also confirmed by investigating the change in the pattern and content of tropane alkaloids at consecutive stages of development from callus tissue to mature plant through the artificial induction of shoot and root formation in *D. innoxia* [38]. Although hyoscyamine was detected throughout the course of development, scopolamine was not detected in the shoot-forming callus but appeared first at the stage of root formation and eventually surpassed hyoscyamine in quantity to become the main alkaloid in mature plants, 82% of which were normal diploids ($2n=24$). This suggests that the site of scopolamine biosynthesis is probably localized in the root. The total alkaloid content, which was as low as 0.01% of dry weight in callus tissues, increased with organization and plant growth until it reached a maximum (0.33% on the average) in the leaves of mature plants before flowering. High scopolamine production was also shown in redifferentiated roots of *Hyoscyamus niger* by Hashimoto and Yamada [39].

It is considered therefore that the genetic potentiality for synthesizing tropane alkaloids has generally been retained by cultured cells. This means that it would not be impossible to produce large amounts of useful tropane alkaloids not only by root cultures, but also by undifferentiated cell cultures, if the regulatory mechanism for alkaloid biosynthesis can be fully elucidated on the molecular level. Such biochemical and biotechnological approaches which have recently been made towards tropane alkaloid production in cell and hairy root cultures of *Hyoscyamus* and *Duboisia* will be reviewed in the following section.

Recently, mass production of *l*-scopolamine by root cultures of *Duboisia* species was reported by two Japanese industrial companies [40-44].

4. Biochemistry and molecular biology of the biosynthesis of tropane alkaloids

Tropane alkaloids contain the tropane ring, a bicyclic amine which is derived from pyrrolidine and piperidine rings, and have more than 150 members, including several well-known compounds of pharmacological importance, such as cocaine, scopolamine and atropine. *l*-Hyoscyamine and *l*-scopolamine are esters formed from tropic acid and derivatives of 3α-hydroxytropane (the hydroxyl group at C-3 is trans to the *N*-methyl bridge). Atropine is a racemic mixture of *d*- and *l*-hyoscyamine. Only the *l*-isomer is active pharmacologically. The Solanaceae species, *Atropa, Datura, Duboisia, Hyoscyamus* and *Scopolia,* are rich sources of *l*-hyoscyamine and *l*-scopolamine.

The tropane ring system originates from the basic amino acid, ornithine or arginine, by way of putrescine. This diamine is methylated at one of its amino group by putrescine *N*-methyltransferase, then oxidatively deaminated by diamine oxidase, producing 4-aminobutanal, which spontaneously cyclizes to 1-methyl-delta-1-pyrrolinium cation. In tobacco, this cation is condensed with nicotinic acid or its derivative to give nicotine. In coca, it is converted to cocaine. For tropane alkaloid biosynthesis, this compound is converted to tropinone. Tropine is formed from this ketone by tropinone reductase-I, and tropine and a tropic acid derivative, which is derived from phenylalanine, are condensed to give *l*-hyoscyamine. *l*-hyoscyamine is converted to *l*-scopolamine in two successive oxidation steps, both of which are catalyzed by hyoscyamine 6β-hydroxylase. **(Fig. 1)**

4. 1 Early pathways for alkaloids and polyamines

The first enzyme in the alkaloid pathway is putrescine *N*-methyltransferase. Previously, it was assumed that tropane alkaloids were synthesized by way of δ-*N*-methylornithine [45]. However, studies using high alkaloid producing root cultures established that *N*-methylputrescine is formed by way of the symmetrical diamine, putrescine. First, labelled ornithine and arginine administered to *Hyoscyamus* root cultures was first incorporated into putrescine and *N*-methylputrescine, then into *l*-hyoscyamine [46]. Second, similarly, the labelled putrescine was incorporated first into *N*-methylputrescine and then into *l*-hyoscyamine [47]. Third, a specific suicide inhibitor of ornithine decarboxylase significantly decreased the incorporation of the labelled ornithine into putrescine and *l*-hyoscyamine. Fourth, when the ornithine labelled at C-5 was fed to *Hyoscyamus* root cultures, the label was incorporated equally into C-1 and C-5 of the tropane moiety of *l*-hyoscyamine [48, 49]. Putrescine *N*-methyltransferase activity was detected always (and only) in the tissues and plant species which produce tropane and related alkaloids. PMT activity was present in the main root, and cultured root of *Hyoscyamus niger, Datura innoxia* and *Atropa belladonna,* but was absent in other plant organs tested.

When increasing concentrations of the potent PMT inhibitor, *n*-butylamine, are added exogenously the concentrations of *l*-hyoscyamine and its biosynthetic intermediates were dramatically decreased, while growth of the root culture was not drastically affected. The *n*-butylamine treatment caused a large increase in the cellular putrescine pool, which was large enough to account for the decrease in the total alkaloid pool size. Similar results were also obtained

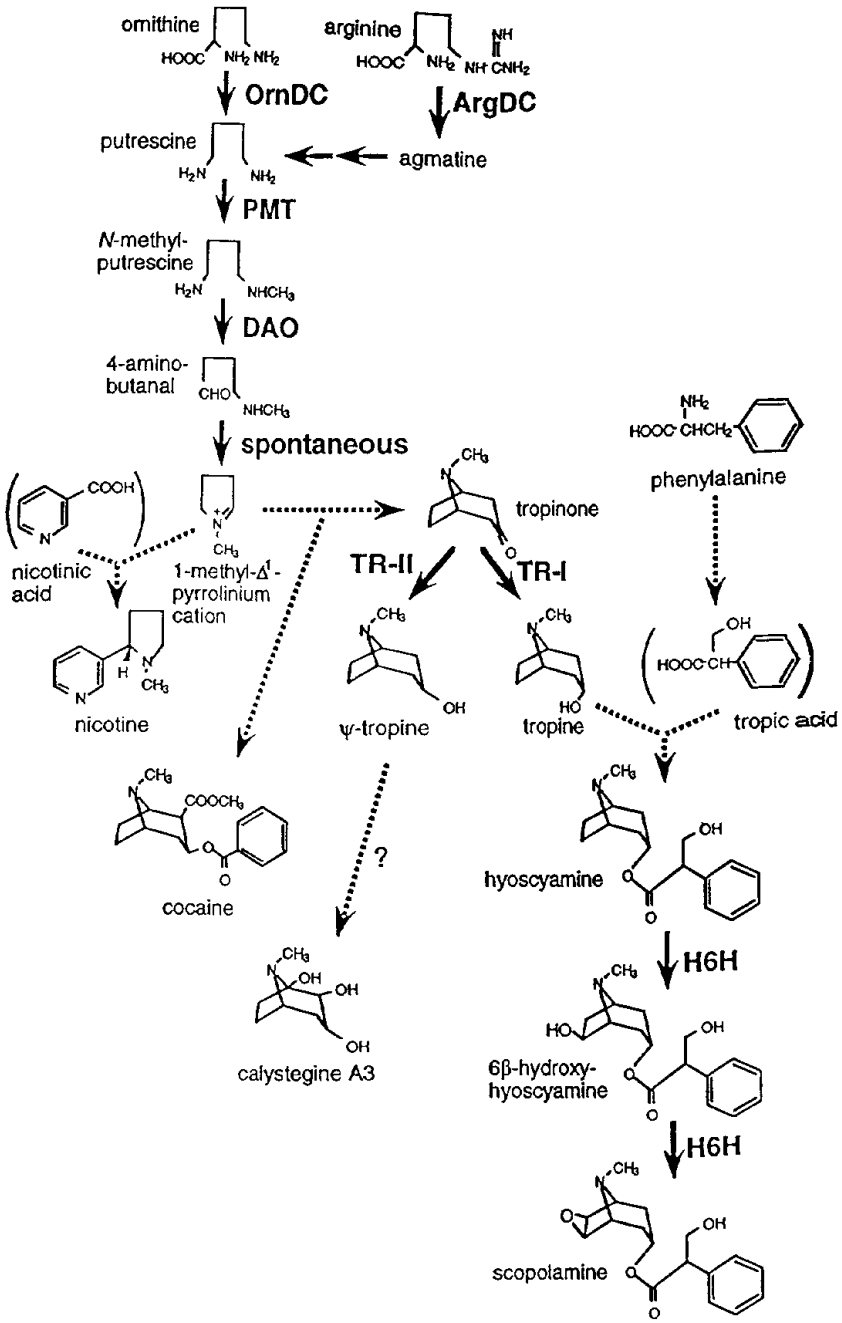

Fig. 1 The metabolic pathway of tropan alkaloids.
Solid lines indicate that Yamada's group has resolved the enzyme reactions biochemically and/or molecular biologically.

in root cultures of *Datura stramonium* [50]. These studies further support the role of PMT as the first committed enzyme specific to alkaloid biosynthesis. However, it is very difficult to purify PMT by conventional chromatographic procedures. Therefore, to clone the PMT gene, a more direct, molecular genetic approach, was used. Thorough genetic studies a very low-alkaloid tobacco mutant have demonstrated that the nicotine level is controlled by two non-linked loci, Nicl and Nic2. A cDNA library was constructed from wild-type cultured root of tobacco, and screened with both a subtracted cDNA probe and a non-subtracted cDNA probe from nicl nic2 roots. After analysis by cross-hybridization, clones were classified into two groups, in which A411 and A622 were the longest cDNAs [51, 52].

The protein encoded by A411 is homologous to mammalian spermidine synthases, whereas the protein encoded by A622 is homologous to plant isoflavone reductases.

Because isoflavone reductase is specifically involved in the biosynthesis of isoflavonoid phytoalexins in the Leguminosae, and because tobacco apparently does not use isoflavonoid phytoalexins in its response to fungal attack, tobacco is not expected to have an isoflavone reductase gene. The A622 gene may be an enzyme in the biosynthetic pathway of nicotine or related alkaloids [52].

Spermidine synthase transfers the aminopropyl moiety of decarboxylated SAM to putrescine, where-

as PMT transfers the methyl moiety of SAM to putrescine. In addition, studies on substrate specificity and inhibition kinetics by amines have predicted that spermidine synthase and PMT may have similar active sites for enzyme catalysis.

However, when the A411 cDNA was expressed in an *E. coli* mutant which lacks the spermidine synthase gene, only PMT activity was detected. Therefore, A411 encodes PMT, not spermidine synthase. Except at its N-terminus, PMT has very high homology to spermidine synthases from human, mouse and *E. coli*. The unique N-terminus of PMT consists of a highly hydrophilic peptide of about 80 amino acids, with four repeats of a heptapeptide, and a total of six potential N-glycosylation sites. Amino acid sequences similar to the proposed binding motifs for SAM were found in PMT, as well as in the spermidine synthases. The phylogenetic tree indicates that mammalian spermidine synthase is more closely related to PMT than to *E. coli* spermidine synthase, and that PMT and SPDS form one particular subfamily, distinct from other methyltransferases. It is likely that the PMT gene has evolved from the spermidine synthase gene in tobacco during the diversification of the Solanaceae.

4. 2 Diamine oxidase

Diamine oxidase catalyzes the oxidative deamination of *N*-methylputrescine. Diamine oxidases are widespread in nature, and a symmetric diamine like putrescine is a good substrate for most diamine oxidases. However, the diamine oxidases in alkaloid-producing plants have a strong preference for *N*-methylated diamines over symmetric diamines. Putresine is probally metabolized predominantly to methylputrescine and then to the 1-methyl-Δ^1-pyrrolinium cation, rather than being deaminated directly by diamine oxidase. The 1-methyl-Δ^1-pyrrolinium cation is a reactive compound. Its imino bond condenses, at least *in vitro*, by Mannich reaction with acetoacetic acid to give hygrine, a putative precursor of the tropane ring, whereas its coupling with nicotinic acid is presumed to form nicotine [54].

4. 3 Stereospecific reactions of tropinone reductases

Two tropinone reductases form a branching point in the biosynthesis of tropane alkaloids. One, TR-I, catalyzes the reduction of the 3-keto group of tropinone to the 3α-hydroxy group of tropine. The other, TR-II, converts the same keto group to the 3β-hydroxy of pseudotropine [55]. Various tropane esters are derived from tropine, and cocaine is a well-known ester of the ψ-tropine type. Plants that produce tropane alkaloids have both TR enzymes, but the ratios of their activities differ with species. The different activity ratios may determine metabolite flow at this branching point. The enzymes have been characterized at the enzymological and molecular biological levels, in order to determine the structures and the evolution of this highly stereospecific pair of reductases. TR activities in the root tissues of three tropane alkaloid-producing species are strongest in developmentally young branch roots and cultured roots. Activities are also present in the stems of some of these plant species. This localization of TR activities is consistent with the localization of activities of other biosynthetic enzymes, PMT and hyoscyamine 6β-hydroxylase, and supports the belief that alkaloids are synthesized in root tissues [56].

The two TR enzymes were purified from cultured roots of *Hyoscymus niger* to apparent homogeneity by several chromatographic steps. The two TR enzymes have similar subunit sizes of 29 to 30 kilodaltons. In the comparison of the characteristics of the two TRs, similarities include optimal reaction pH, cofactor requirements and affinities, subunit size, and inhibition by metal ions. The stereospecificities for the two hydrogens of NADPH are also the same, with both TRs transferring the pro-S hydrogen of NADPH to carbon-3 of tropinone. Differences are found in the reversibility of their reactions, affinities for tropinone, effects of residue-specific modification reagents, substrate-specificities, and competitive inhibition by analogues of tropinone. In general, the greatest similarity is in the binding of cofactor, and the greatest difference is in the binding of ketone substrates [57].

The respective subunits of TR-I and TR-II consist of 273 and 260 amino acids, of which 168 residues are identical; that is, 65% of the total amino acids. The homology is greater at the amino-terminal halves than at the carboxy-terminal halves. A protein data bank search for proteins homologous to TR-I, turned up several dehydrogenases that have considerable homology. These dehydrogenases have been classified as members of a short-chain dehydrogenase family. Alcohol dehydrogenases and related enzymes have been classified in one of two families; a long-chain dehydrogenase family that has subunits of about 350 amino acid residues and zinc atoms essential for activity, and a short-chain dehydrogenase family that has subunits of about 250 residues and no metal ion requirement. The substrate- and cofactor-binding domains are organized differently in the two families. The two TRs belong to the short-chain dehydrogenase family, and have the NADPH-binding site in the amino-terminal end and tropinone-binding sites in the carboxy-terminal end. Short-chain dehydrogenases have several strictly conserved residues. Glycine residues in the amino-terminal region are thought to form tight turns, called Rosmann folds, in

the cofactor-binding domain. The carboxy-terminal region contains the postulated reaction centers, with a tyrosine and a lysine residue which are considered to be important functionally [58].

To investigate the structural basis for the stereospecificities of the TR enzymes, several chimeric enzymes were synthesized. The stereospecificities and substrate preferences of the chimeric enzymes were closely correlated, and both were determined by the carboxyl-terminal regions of the TR polypeptides. This means that these regions determine the substrate-binding sites of the TRs, and that opposite TR stereospecificities are derived from the structural differences in these regions. Of the 120 amino acid residues that make up the carboxyl terminal regions in TR-I and TR-II, 53 differ. This number includes conserved residues. TR stereospecificity, however, may be determined by fewer residues than this.

In the schematic structures of TRs, the amino-terminal regions form NADPH-binding domains, consisting of Rosmann folds, which are well conserved in these enzymes. The carboxy-terminal regions are the tropinone-binding sites, but have very different structures. Tropinone may bind in opposite orientations at these differently structured sites, with hydrides being transferred from opposing sides of the tropane ring. The ionic and hydrophobic properties of the tropinone-binding sites are predicted from the substrate preferences of the TR enzymes. The crystallized TRs for X-ray analysis will show the actual structures and how the stereoisomers are produced [59].

4. 4 The pathway from l-*hyoscyamine to* l-*scopolamine*

Early feeding studies with labeled putative precursors showed that *l*-scopolamine is derived from *l*-hyoscyamine. Because labeled *l*-hyoscyamine is also incorporated into 6β-hydroxyhyoscyamine, this hydroxylated alkaloid was postulated to be an intermediate between *l*-hyoscyamine and *l*-scopolamine. One feeding study showed that chemically synthesized 6, 7-dehydrohyoscyamine is converted to *l*-scopolamine when fed to alkaloid-producing plants. No natural unsaturated 6, 7-dehydrohyoscyamine, however, has ever been isolated from plants. Yamada's group discovered a novel enzyme in cell free extracts from alkaloid-producing root cultures which converts *l*-hyoscyamine to 6β-hydroxyhyoscyamine. This enzyme, hyoscyamine 6β-hyodroxylase, or H6H, is a typical 2-oxoglutarate-dependent dioxygenase [60]. It incorporates one atom of molecular oxygen into the 6β-position of *l*-hyoscyamine. Simultaneously, it uses the other oxygen atom to decarboxylate 2-oxoglutarate into carbon dioxide and succinate. When 2-oxoglutarate labeled at this carbon is used, labeled carbon dioxide is released. When the amounts of carbon dioxide released and the alkaloid product 6β-hydroxyhyoscyamine are plotted against each other, there is a one to one stoichiometry [61].

Alkaloid contents and H6H activity were measured in various root cultures. Tobacco root cultures do not produce *l*-scopolamine and do not have H6H activity. Root cultures of *Datura stramonium* and *Datura leichhardtii* produce *l*-hyoscyamine but not *l*-scopolamine, and there is no H6H activity. In contrast, root cultures of *Duboisia leichhardtii* produce more *l*-scopolamine than *l*-hyoscyamine, and there is H6H activity. When the auxin concentration of the culture medium was low, both alkaloid contents and H6H activity were increased. H6H was purified to apparent homogeneity from root cultures of *Hyoscyamus niger*.

An H6H cDNA clone from a cDNA library of *H. niger* root cultures was isolated in full length. H6H cDNA was expressed in *E. coli* as a fusion protein to maltose-binding protein. The H 6 H protein showed hyoscyamine 6β-hydroxylase and, furthermore, 6β-hydroxyhyoscyamine epoxidase activities. H6H not only hydroxylates *l*-hyoscyamine and related alkaloids, it converts the hydroxylated products to their epoxides. The unsaturated alkaloid also is efficiently converted to *l*-scopolamine by H6H. Interstingly, H6H catalyzes three reactions; hydroxylation and two types of epoxidation [62].

To determine which of the two epoxidase pathways is involved in *l*-scopolamine biosynthesis *in vivo*, 6β-hydroxyhyoscyamine labeled at the 6β-hydroxyloxygen with oxygen-18 was fed to cultured *Duboisia* shoots whereupon, it was converted to *l*-scopolamine. If 6, 7-dehydrohyoscyamine is a true intermediate of *l*-scopolamine, the oxygen-18 should be lost during formation of the double bond, and *l*-scopolamine labeled with oxygen-16 produced. In contrast, a direct dehydrogenation/epoxidation reaction would produce *l*-scopolamine labeled with oxygen-18 at the epoxide bridge. As a result of these experiments, the *Duboisia* shoots showed that the isolated *l*-scopolamine had oxygen-18 at the epoxide bridge. In plants, *l*-hyoscyamine is first converted to 6β-hydroxyhyoscyamine, then to *l*-scopolamine by dehydrogenation, with both of these oxidative reactions catalyzed by H6H. Althought the second epoxidase activity of H6H is weak, it may not be a limiting factor in normal plants. The semi-synthetic 6, 7-dehydrohyoscyamine is also converted by H6H to *l*-scopolamine when fed to plants, but this is not the normal biosynthetic pathway [63, 64].

4. 5 Reaction Mechanisms of H6H

These hydroxylation, epoxidation and saturation reactions are all catalyzed by H6H. First, a highly

reactive ferryl enzyme species is formed when H6H oxidatively decarboxylates 2-oxoglutarate. This oxidizing species is capable of cleaving a carbon-hydrogen bond homolytically, as well as inserting ferryl oxygen directly into a double bond. Second, with hyoscyamine as the substrate, the enzyme preferentially cleaves the C6-Hβ bond, with the subsequent insertion of the ferryl oxygen. The hydroxylation at C-6 would occur as a result. The carbon radical produced from the homolytic cleavage of the C7-Hβ bond of 6β-hydroxyhyoscyamine would be attacked by the adjacent 6β-hydroxyl oxygen giving the epoxide, *l*-scopolamine. Cleavage of the C7-Hβ bond would take place slowly because the ferryl oxygen of H6H must be positioned much closer to the C-6 than to the C-7 of the substrate. The epoxide formation would also be achieved by the direct insertion of the ferryl oxygen into the 6, 7-double of 6, 7-dehydrohyoscyamine, which would also produce *l*-scopolamine [65, 66].

The amino acid sequence of H6H is homologous not only to the sequences of other 2-oxoglutarate-depen dent dioxygenases, but also to sequences of such iron-requiring oxygenases as ethylene-forming enzyme and isopenicillin N synthase. The homology to these proteins is not very high, but there are three strictly conserved amino acid residues among these homologous sequences. When any of two histidines or an aspartic acid residue in H6H are mutated to other amino acids, the enzyme activity of the resulting H6H proteins is completely lost, indicating that these invariant amino acids are required for the functioning of the 2-oxoglutarate-dependent dioxygenases and related enzymes. Proteins homologous to H6H include enzymes which are involved in the biosynthesis of antibiotics in microorganisms: isopenicillin N synthase, deacetoxycephalosporin C synthase, and deacetylcephalosporin C synthase. In the anthocyanin biosynthetic parhway, flavone synthase type 1, flavanone 3-hydroxylase, flavonol synthase, and probably anthocyamidin synthase are homologous to H6H. A subgroup of non-requiring 2-oxoglutarate, iron-dependent oxygenases of the same evolutionary origin exists, in addition to the group of 2-oxoglutarate-dependent dioxygenases of the related primary sequences. Interestingly, many of the 2-oxoglutarate-dependent dioxygenases found in animals and some microorganisms share no significant homology to any of these oxygenases nor to each other. All of these iron-dependent oxygenases have two conserved histidines and one aspartate or glutamate juxtaposed to one of the histidines in their amino acid sequences. These three amino acid residues may serve as ligand to the active site iron. The postulated ferryl enzyme intermediate of these oxygenases would be a strong oxidative agent that catalyzes several reactions: hydroxylation, desaturation, ring expansion, oxidation and epoxidation.

The tissue-specific expression of the H6H gene in *Hyoscyamus niger* has become clear. Of the tissues tested, H6H mRNA is most abundant in cultured roots. It is also present in the plant's roots, but is undetectable in cultured cells and in stem and leaves. To learn in which type of root cell the H6H protein is located, monoclonal antibody Mab5, which specifically recognizes H6H proteins of various species, was used. The H6H protein was localized specifically to the pericycle cells of young root. This cell-specific expression may be advantageous, in that tropane alkaloids synthesized in the pericycle cells are easily transferred to the adjacent xylem for translocation through the stem to aerial plant parts where alkaloids usually accumulate [67].

l-Hyoscyamine and *l*-scopolamine are both anticholinergic agents, but their actions on the central nervous system differ. *l*-Hyoscyamine first stimulates then depresses the CNS, whereas *l*-scopolamine depresses it. Because of this, scopolamine is preferred in clinical applications, and the world-wide market for it is about ten times that for *l*-hyoscyamine. Many plants produce *l*-hyoscyamine, but *l*-scopolamine-rich plants are relatively few. Attempts to increase the *l*-scopolamine contents in these medicinal plants by conventional breeding methods have met with no success. The use of H6H cDNA to increase *l*-scopolamine contents in normally *l*-hyoscymaine-rich *Atropa belladonna* has been successful [68-70]. Transgenic belladonna plants show no obvious differences in growth and development when compared with control and wild-type plants. The leaf, stem, main root, and branch roots of *A. belladonna* were analyzed by immunoblotting and enzyme assays. The selfed progeny, show strong expression of H6H protein and H6H enzyme activity in all parts analyzed. Wild-type *A. belladonna,* however, shows weak expression of H6H protein and weak enzyme activity only in the branch root [71].

The alkaloids present in mature plants after seed formation were analyzed in detail. During this stage, *l*-hyoscyamine constituted more than 92% of the total alkaloid contents of the leaves, stems, and main roots of the wild-type and control plants. The branch roots of these plants contained relatively high amounts of *l*-scopolamine, a reflection of the endogenous H6H activity in this organ. The alkaloid contents in the aerial parts of the primary T0 tranformant and five randomly selected T 1 progeny consisted almost exclusively of *l*-scopolamine. This almost exclusive presence of *l*-scopolamine in medicinal plants cannot be achieved by conventional breeding methods. In the main roots of some T1 progeny, and in the branch

roots of the T0 and T1 transformants, *l*-hyoscyamine conversion was not as efficient as in the aerial parts. Efficient conversion of *l*-hyoscyamine to *l*-scopolamine in transformants may occur during the translocation and storage of alkaloids [72]. The results of this research represent fundamental aspects of enzymology and molecular biology in plants. From this basic research, extremely useful applications can be developed.

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