Alkaloid Biosynthesis—The Basis for Metabolic Engineering of Medicinal Plants

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INTRODUCTION

Alkaloids—the term is linguistically derived from the Arabic word *al-qali*, the plant from which soda was first obtained—are nitrogenous compounds that constitute the pharmacologically active "basic principles" of predominantly, although not exclusively, flowering plants. Since the identification of the first alkaloid, morphine, from the opium poppy, *Papaver somniferum*, by Sertürner in 1806, ~10,000 alkaloids have been isolated and their structures elucidated (Southon and Buckingham, 1989). Historically, the use of alkaloid-containing plant extracts as potions, medicines, and poisons can be traced back almost to the start of civilization. Famed examples include Socrates' death in 399 B.C. by consumption of conine-containing hemlock (*Conium maculatum*) and Cleopatra's use during the last century B.C. of atropine-containing extracts of Egyptian henbane (*Hyoscyamus muticus*) to dilate her pupils and thereby appear more alluring. Medieval European women utilized extracts of deadly nightshade, *Atropa belladonna*, for the same purpose, hence the name *belladonna*. Although conine is too toxic to find therapeutic use today outside of homeopathy, tropicamide, an anticholinergic that is a synthetic derivative of atropine, is routinely used in eye examinations to dilate the pupil. Tropicamide has also recently shown promise as an early diagnostic tool in the detection of Alzheimer's disease (Scinto et al., 1994). A tonic prepared from the bark of *Cinchona officinalis* that contains the antimalarial drug quinine greatly facilitated European exploration and inhabitation of the tropics during the past two centuries.

In total, ~13,000 plant species are known to have been used as drugs throughout the world (Tyler, 1994). Approximately 25% of contemporary materia medica is derived from plants and used either as pure compounds (such as the narcotic analgesic morphine, the analgesic and antitussive codeine, and the chemotherapeutic agents vincristine and vinblastine, Figure 1) or as teas and extracts. Plant constituents have also served as models for modern synthetic drugs, such as atropine for tropicamide, quinine for chloroquine, and cocaine for procaaine and tetracaine. In fact, active plant extract screening programs continue to result in new drug discoveries. The most recent examples of anticancer alkaloids are taxol (clinically available since 1994) from the western yew, *Taxus brevifolia*, and camptothecin (and derivatives currently in clinical trials) from the Chinese "happy tree," *xi shu* (*Camptotheca acuminata*), both of which were originally isolated and assayed for biological activity in the 1960s in the laboratory of M.E. Wall. In other areas, there are intense searches for novel antivirals and antimalarials.

We also encounter alkaloids as the stimulants caffeine in coffee and tea and nicotine in cigarettes. Although a wealth of information is available on the pharmacological effects of these compounds, surprisingly little is known about how plants synthesize these substances, and almost nothing is known about how this synthesis is regulated. This is due, in part, to the complex chemical structures of many alkaloids, which contain multiple asymmetric centers. For example, although nicotine (one asymmetric center) was discovered in 1828, its structure was not known until it was synthesized in 1904, and the structure of morphine (five asymmetric centers) was not unequivocally elucidated until 1952, 146 years after its isolation. Beginning in the late 1950s, radiolabeled precursors were fed to plants and the resultant radioactive alkaloids were chemically degraded to identify the position of the label. This opened the field of alkaloid biosynthesis to experimentation. As analytical instrumentation became more sophisticated, precursors labeled with stable isotopes were fed to plants and the products analyzed by nuclear magnetic resonance spectroscopy. No real progress was made in identifying alkaloid biosynthetic enzymes until the use of plant cell cultures as experimental systems was introduced in the 1970s. Since then, on the order of 80 new enzymes that catalyze steps in the biosynthesis of the indole, isoquinoline, tropane, pyrrolizidine, and purine classes of alkaloids have been discovered and partially characterized.

Alkaloids belong to the broad category of secondary metabolites. This class of molecule has historically been defined as naturally occurring substances that are not vital to the organism that produces them. Alkaloids have traditionally been of interest only due to their pronounced and various physiological activities in animals and humans. A picture has now begun to emerge that alkaloids do have important ecochemical functions in the defense of the plant against pathogenic organisms and herbivores or, as in the case of pyrrolizidine alkaloids, as pro-toxins for insects, which further modify the alkaloids and then incorporate them into their own defense secretions (reviewed in Hartmann, 1991). Alkaloids have now
Figure 1. Some Physiologically Active Alkaloids and Plants That Produce Them.
been isolated from such diverse organisms as frogs, ants (pheromones), butterflies (defense), marine bacteria, sponges, fungi, spiders (venom neurotoxin), beetles (defense), and mammals, although it is not yet clear whether de novo alkaloid biosynthesis occurs in each organism.

With the introduction of molecular biology into the plant alkaloid field, induction of alkaloid biosynthesis in response to exposure to wounding or to elicitors can be analyzed at the level of gene activation, and gene expression patterns in the plant can be determined and interpreted as a first indication of possible function. We also now have the capability to alter the pattern of alkaloid accumulation in plants for the purpose of studying the biological function of alkaloids, for engineering tailor-made plants that accumulate increased quantities of desired pharmaceuticals, or for producing foodstuffs plants with lower alkaloid content (for example, coffee without caffeine). Plants are some of nature’s very best chemists, and sophisticated structures such as codeine, vinblastine, taxol, and camptothecin remain well beyond the reach of commercially feasible total chemical syntheses. With the ability to express alkaloid biosynthetic enzymes heterologously in organisms with better fermentation characteristics than plants, we can achieve unlimited quantities of these “biocatalysts” for use in syntheses of important drugs. In this review, I discuss the progress that has been made in these areas as a result of the fusion of alkaloid chemistry, enzymology, and molecular biology, as well as some perspectives for future developments in this field.

MONOTERPENOID INDOLE ALKALOIDS

The monoterpenoid indole alkaloids comprise a large family of alkaloids, with over 1800 members of rich structural diversity. Many of these natural products are physiologically active in mammals. Among the monoterpenoid indole alkaloid pharmaceuticals that are still commercially isolated from plant material are the antimalarial drug quinine from C. officinalis, the antineoplastic drug camptothecin from C. acuminate, the rat poison and homeopathic drug strychnine from Strychnos nux vomica, and the antineoplastic chemotherapeutic agents vincristine and vinblastine from Catharantus roseus (periwinkle) (Figure 1). Total chemical syntheses of these complex alkaloids would be of academic interest but due to low yields are not likely to be applied commercially. To develop novel sources of these drugs, two options are available. The cDNAs for enzymes that catalyze those biosynthetic steps that are difficult to achieve by chemical means can be isolated and heterologously expressed for use in biomimetic syntheses. Alternatively, instead of single transformation steps, microorganisms could be engineered to express short pathways, thus producing an end-product alkaloid of interest. Alkaloid biosynthetic pathways that are too long to be introduced into a single microorganism could be modified in the parent plant using antisense or co-suppression technologies such that a desired alkaloid can be accumulated by blocking side pathways or catabolic steps (schematically depicted in Figure 2).

All of these approaches require thorough knowledge of the alkaloid biosynthetic pathway and the enzymes that catalyze the individual transformation steps. Progress toward identifying the enzymes of monoterpenoid indole alkaloid biosynthesis has been made primarily in the laboratory of J. Stöckigt using Rauvolfia serpentina cell suspension cultures in studies of the biosynthesis of the antiarhythmic drug ajmaline and in the laboratory of V. De Luca using C. roseus cell suspension cultures and plants to study the biosynthesis of vindoline, a precursor to the antineoplastics vincristine and vinblastine (reviewed in Herbert, 1994; Kutchan, 1994). The first successful cDNA cloning experiments in the alkaloid field were achieved with two cDNAs encoding enzymes that catalyze early steps in the biosynthetic pathway that leads to all monoterpenoid indole alkaloids, tryptophan decarboxylase (De Luca et al., 1989) and strictosidine synthase (Kutchan et al., 1988) (Figure 3).

Tryptophan Decarboxylase

Tryptophan decarboxylase (aromatic L-amino-acid decarboxylase; EC 4.1.1.28) catalyzes the decarboxylation of the amino acid L-tryptophan to the protoalkaloid tryptamine. Tryptamine can then serve as substrate for the enzyme strictosidine synthase (Stöckigt and Zenk, 1977), which catalyzes the first committed step in monoterpenoid indole alkaloid biosynthesis. The cDNA clone encoding tryptophan decarboxylase was
Plant alkaloid genes can be functionally expressed in microorganisms to produce either single biotransformation steps or short biosynthetic pathways. Likewise, using overexpression or antisense or cosuppression technologies, medicinal plants can be tailored to produce important pharmaceutical alkaloids by introducing side pathways, eliminating side pathways, or accumulating biosynthetic intermediates. For example, a known plant biosynthetic pathway contains an enzyme encoded by gene \( G_3 \) that catalyzes a transformation step, of compound C to alkaloid D, which is difficult to achieve by chemical synthesis. The \( G_3 \) gene can be heterologously expressed in a microorganism and the gene product used in a biomimetic synthesis of alkaloid D (that is, the microorganism is supplied with compound C and produces alkaloid D). Likewise, a short pathway consisting of enzymes encoded by genes \( G_1, G_2, G_3, \) and \( G_4 \), could be expressed in a microorganism to produce alkaloid E directly from precursor A. Many alkaloid biosynthetic pathways involve 20 to 30 enzymes. Our current knowledge of these pathways indicates that the genes encoding the biosynthetic enzymes are neither clustered nor coordinately controlled by one operon. Expression of an entire, long alkaloid pathway in a single microorganism is currently beyond our technical capability. In this case, it may be possible to alter the pathway in the plant cell and produce the desired alkaloid either in culture or in the field. For example, to accumulate alkaloid Z, which is not normally produced in a particular plant species, a transgene (from another plant or a microorganism) can be introduced. To accumulate alkaloid E, a side pathway that also uses precursor D may have to be blocked. If intermediate alkaloid C is the target for accumulation, catabolism to D could be interrupted.

isolated from \textit{C. roseus} by antibody screening of a cDNA expression library prepared from poly(A)+ RNA of developing seedlings (De Luca et al., 1989). The tryptophan decarboxylase amino acid sequence shows similarities with an aromatic L-amino acid decarboxylase from \textit{Drosophila melanogaster} and with L-amino acid decarboxylases from diverse animal origins. The tryptophan decarboxylase transcript was shown to accumulate in \textit{C. roseus} cell suspension cultures exposed to a variety of biotic elicitors (Pasquali et al., 1992; Roewer et al., 1992). Auxin reduces transcription of the gene, as demonstrated by run-off transcription experiments with \textit{C. roseus} nuclei (Goddijn et al., 1992).

A main point of interest in \textit{C. roseus}—and the likely reason for the recent increase in the number of researchers working on this plant—is that it produces the dimeric alkaloids vinblastine and vincristine. It has been well established that cell cultures of \textit{C. roseus} do not produce these commercially interesting and valuable alkaloids because the cultures lack vindoline, one component of both dimers. Induction of the last two enzymes of vindoline biosynthesis in \textit{C. roseus} seedlings, 2-oxoglutarate-N(1)-methyl-16-methoxy-2,3-dihydro-3-hydroxytaibersonine 4-hydroxylase and 17-O-deacetylvinindoline O-acetyltransferase, appears to be regulated by phytochrome (Aerts and De Luca, 1992), but red light-induced accumulation of at least the acetyl transferase has not been observed in cell cultures. The biotechnologically most important experiments, those that may potentially result in the production of the anti-neoplastic dimeric alkaloids in cell culture, thus await the isolation of these final genes of vindoline biosynthesis.

The tryptophan decarboxylase cDNA from \textit{C. roseus} has
been heterologously expressed in tobacco plants (Songstad et al., 1990, 1991), and it increased their levels of tryptamine and tyramine, the product of L-tyrosine decarboxylation. A fine example of what metabolic engineering can achieve in secondary metabolism has been provided by the transformation of *Brassica napus* with the *C. roseus* tryptophan decarboxylase cDNA (Chavadej et al., 1994). The seed of this oil-producing crop has limited use as animal feed due in part to the presence of indole glucosinolates, which make the protein meal less palatable. The introduced cDNA for tryptophan decarboxylase redirects tryptophan pools away from indole glucosinolate production and into tryptamine. The mature seed of the transgenic *B. napus* plants contain reduced levels of indole glucosinolates but no tryptamine, achieving a potentially economically useful product.

**Strictosidine Synthase**

Strictosidine synthase (EC 4.3.3.2) catalyzes the stereospecific condensation of the primary amino group of tryptamine and the aldehyde moiety of the iridoid glucoside secolaginin to form the first monoterpenoid indole alkaloid, 3a(S)-strictosidine. Strictosidine synthase is of biotechnological interest in the biomimetic syntheses of monoterpenoid indole alkaloids because, although the condensation of tryptamine and secolaginin can be achieved chemically, the reaction product is a mixture of the diastereomers vincoside and strictosidine. Only strictosidine with the 3a(S) configuration, the exclusive product of the enzymatic reaction, can serve as a precursor to the monoterpenoid indole alkaloids (Stöckigt and Zenk, 1977). The enzyme is stable, requires no cofactor addition, and is readily immobilized (Pfitzner and Zenk, 1987) for producing strictosidine.

The cDNA encoding strictosidine synthase was first isolated from *R. serpentine* (Kutchan et al., 1988), a plant used today as the commercial source of antihypertensive indole alkaloids such as ajmaline and ajmalicine; a strictosidine synthase cDNA was subsequently isolated from *C. roseus* (McKnight et al., 1990). These two enzymes show 80% amino acid homology, which probably reflects the fact that both plants are members of the Apocynaceae. DNA gel blot analysis using the *R. serpentine* cDNA as a probe shows that not all species that contain the enzyme contain a similar gene, possibly due to differences in codon usage (Kutchan, 1993a). Strictosidine synthase from *R. serpentine* has been functionally expressed in *Escherichia coli* and *Saccharomyces cerevisiae*, and in insect cells using a baculovirus vector (Kutchan, 1989; Kutchan et al., 1994). The *C. roseus* enzyme has been expressed in tobacco and *E. coli* (McKnight et al., 1991; Roessner et al., 1992). A comparison of these as production systems has been reviewed by Kutchan (1994).
The gene for strictosidine synthase, strf, has been isolated from R. serpentina (Bracher and Kutchan, 1992). A single gene appears to code for the enzyme in both R. serpentina and C. roseus (Pasquali et al., 1992). In both species, the transcript accumulates predominantly in the roots and leaves of mature plants, although it can also be detected in flowers and stems. Levels of C. roseus strictosidine synthase transcript can be increased in cell culture (Pasquali et al., 1992; Roewer et al., 1992) and during a very narrow time period in developing seedlings (Aerts et al., 1994) by treatment with various elicitors. However, the high levels of transcript present in mature plants suggest that strf is not one of the regulating genes of indole alkaloid biosynthesis.

TROPANE AND NICOTINE ALKALOIDS

The tropane class of alkaloids, which are found mainly in the Solanaceae, contains the anticholinergic drugs hyoscyamine (the racemate of which is called atropine) and scopolamine. Solanaceous plants have been used traditionally for their medicinal, hallucinogenic, and poisonous properties, which are due to their tropane alkaloids. The narcotic topical anesthetic and central nervous system stimulant cocaine is a tropane alkaloid found outside of the Solanaceae in Erythroxylon coca. What is a useful medicinal in small, controlled doses is potentially lethal when abused. Hence, scopolamine isolated from Duboisia is commonly used today in the form of a transdermal patch to combat motion sickness, but Datura leaves are smoked for the hallucinogenic effects of this alkaloid. Although cocaine has served as a "lead," a starting structure that medicinal chemists modify to design an optimized drug, for the development of synthetic topical anesthetics, this alkaloid is illicitly applied to mucus membranes for its addictive stimulatory effects. Metabolic engineering of the plants that serve as commercial sources of nicotine or scopolamine could enhance classic breeding in the effort to develop plants with optimal alkaloid patterns, that is, that serve as improved sources of pharmaceuticals.

A study of the biosynthesis of tropane alkaloids implies a study of nicotine biosynthesis also, because the N-methyl-Δ1-pyrrolinium cation is a precursor to both classes of alkaloids. Analyses of nicotine biosynthesis in tobacco and cocaine biosynthesis in coca were pioneered in the laboratory of E. Leete. The enzymeology and molecular biology of scopolamine biosynthesis in Hyoscyamus have been studied principally by T. Hashimoto and Y. Yamada. The cDNAs encoding hyoscyamine N-methyltransferase (EC 2.1.1.53) were used to isolate a cDNA for an enzyme of nicotine biosynthesis, putrescine N-methyltransferase (EC 2.1.1.68). This enzyme catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to an amino group of putrescine, which is the first committed step in the biosynthesis of nicotine and tropane alkaloids. The availability of the near-isogenic Burley strains made it possible to use subtractive hybridization to isolate a cDNA encoding this enzyme (Hibi et al., 1994). The deduced amino acid sequence of one of the clones obtained by subtractive hybridization is homologous to spermidine synthase from human (73% identical), mouse (70% identical), and E. coli (58% identical). Heterologous expression of the tobacco cDNA in an E. coli deletion mutant lacking the spermidine synthase gene resulted in accumulation of N-methyl putrescine, confirming the identity of the cDNA. Transcripts for putrescine N-methyltransferase accumulate predominantly, if not exclusively, in root tissue of the wild-type tobacco plant, suggesting that this organ is the major site of nicotine biosynthesis. This corresponds to the site of tropane alkaloid biosynthesis in other members of the Solanaceae (Hashimoto et al., 1991). The logical progression of this work is to isolate a putrescine N-methyltransferase gene from a plant that produces tropane alkaloids.

Tropinone Reductase

Along the biosynthetic pathway that leads specifically to the tropane alkaloid scopalamine, tropinone reductase I (EC 1.1.1.236) converts the 3-keto group of tropinone to the 3α-hydroxyl of tropine. The cDNA encoding this enzyme was isolated from Datura stramonium (jimsonweed) by screening a cDNA library prepared from hairy roots with oligonucleotides based on peptide amino acid sequences from purified native tropinone reductase I (Nakajima et al., 1993). The cDNA was expressed as a β-galactosidase fusion protein in E. coli and found to have tropinone reductase I activity. DNA gel blot analysis identified homologous DNA fragments in the nuclear DNA of the tropane alkaloid-producing species H. niger and A. belladonna, but not in tobacco, a species in which tropane alkaloids are not biosynthesized.

A cDNA for a second reductase, tropinone reductase II, was also isolated in these experiments. Tropinone reductase II reduces tropinone with a stereospecificity opposite to that of tropinone reductase I. Hence, the 3-keto group of tropinone is converted to the 3β-hydroxyl of pseudotropine. The deduced amino acid sequences of the two cDNAs are 64% identical. In a series of elegant experiments, various domains of these
two reductases were exchanged and the chimeric enzymes were expressed in E. coli to identify the domain that confers the stereospecificity of the reaction (Nakajima et al., 1994). In this manner, it was demonstrated that a 120-amino acid residue peptide at the C terminus of each reductase determines the stereospecificity of the reaction it catalyzes.

Hyoscyamine 6β-Hydroxylase

The final two steps in the biosynthetic pathway leading from hyoscyamine to the medicinally important alkaloid scopolamine is catalyzed by a 2-oxoglutarate–dependent enzyme, hyoscyamine 6β-hydroxylase (hyoscyamine [6S]-dioxygenase; EC 1.14.11.11). This dioxygenase first hydroxylates hyoscyamine in the 6β position and subsequently catalyzes epoxide formation. Molecular cloning and heterologous expression of hyoscyamine 6β-hydroxylase have demonstrated that this enzyme catalyzes both the hydroxylation reaction and the intramolecular epoxidation reaction (Matsuda et al., 1991). RNA gel blot analysis of the hyoscyamine 6β-hydroxylase gene has corroborated the results obtained by immunohistochemical localization of the enzyme (Hashimoto et al., 1991), which showed that hyoscyamine 6β-hydroxylase is localized to the root pericycle in scopoline-producing species of Hyoscyamus, Atropa, and Duboisia. Similarly, the hyoscyamine 6β-hydroxylase transcript is localized to roots and cultured roots of H. niger but is not found in stem, leaves, or cultured cells of this same species. These results explain why it has not been possible to produce substantial quantities of tropane alkaloids in cell culture. The gene encoding hyoscyamine 6β-hydroxylase should help with the elucidation of the underlying mechanisms of tissue- and cell-specific expression of tropane alkaloids in the Solanaceae.

The current commercial source of scopolamine is Duboisia, which was cultivated originally in Australia. Certain tropane alkaloid–producing species, such as Atropa, accumulate hyoscyamine instead of scopamine, as the major alkaloid. To ask whether expression of a transgene in a medicinal plant could alter the alkaloid pattern such that more of the pharmacologically useful alkaloid, scopamine, could be produced, the cDNA encoding hyoscyamine 6β-hydroxylase from H. niger was introduced into A. belladonna using either Agrobacterium tumefaciens– or Agrobacterium rhizogenes–mediated transformation. The resultant transgenic plants (Yun et al., 1992) and hairy roots (Hashimoto et al., 1993) each contained elevated levels of scopamine.

Each of these two successful transformation experiments has a distinct implication for the future of metabolic engineering of medicinal plants. First, the transgenic A. belladonna plants, although not necessarily commercially useful, provide the first example of how medicinal plants can be successfully altered using molecular genetic techniques to produce increased quantities of a medicinally important alkaloid. The future of this field is completely dependent upon a thorough knowledge of the alkaloid biosynthetic pathway at the enzyme level so that meaningful transformation experiments can be designed. It is also limited by our ability to transform and regenerate medicinal plants. To date, expertise in this important area lags well behind that for tobacco, petunia, and cereal crops, among others. For example, in the area of tropane alkaloids, transformation and regeneration of Duboisia, a plant for which plantation, harvesting, and purification techniques have already been commercially established, will have to be developed before any potential commercialization. The second implication is that metabolic engineering may make it possible to produce alkaloids in cultured cells. The production of alkaloids in tissue and cell culture, such as in hairy roots, is an area that received much attention in the past because it promised an alternative source of pharmaceuticals that would be independent of weather, blight, and politics. It has become clear with time, however, that many important compounds, such as vincristine, vinblastine, plicarcarine, morphine, and codeine, are not synthesized to any appreciable extent in culture. The reason is thought to be tissue-specific expression of alkaloid biosynthetic genes, because in some cases, plants regenerated from nonproducing callus cells contain the same alkaloid profile as the parent plant. For certain costly alkaloids (for example, taxol), cell culture production at a commercial level would be worthwhile establishing. The increased accumulation of scopoline in hairy roots that contain an hyoscyamine 6β-hydroxylase transgene is an elegant demonstration that this approach remains a viable one.

**BENZYLISOQUINOLINE ALKALOIDS**

The benzylisoquinoline alkaloids are a very large and diverse class of alkaloids. This family contains such varied physiologically active members as emetine (an antiamoebic), colchicine (a microtubule disrupter and gout suppressant), berberine (an antimicrobial against eye and intestinal infections), morphine (a narcotic analgesic), codeine (a narcotic analgesic and antitussive), and sanguinarine (an antimicrobial used in oral hygiene). As with the pharmacologically active members of the indole and tropane classes of alkaloids, those of pharmacetical interest from the benzylisoquinoline class are largely still isolated from plants, again due to the complexity of their structures. One notable exception to this is the benzylisoquinoline alkaloid berberine, which is isolated in Japan from cell suspension cultures of Coptis japonica (summarized in Zenk et al., 1988).

Benzo(c)phenanthridine alkaloids, a subclass of the benzylisoquinolines, are produced in a number of species within the Papaveraceae, including _Sanguinaria canadensis_ (bloodroot), _P. somniferum_ (opium poppy), and _Eschscholtzia californica_ (California poppy, used as a sedative by Native Americans). Extracts of bloodroot that are rich in the intensely red benzophenanthridine alkaloid sanguinarine are currently added
to toothpastes and oral rinses in the United States because of this alkaloid's antiplaque properties. Benzophenanthridine alkaloids have been shown to accumulate in cell cultures of *E. californica* in response to fungal attack and to treatment with numerous elicitor substances (Schumacher et al., 1987). This phenomenon, when exploited in cell suspension cultures, provided the main experimental system with which the entire biosynthetic pathway leading from two molecules of L-tyrosine to the most highly oxidized benzophenanthridine alkaloid, macarpine, was elucidated in the laboratory of M.H. Zenk. This pathway, encompassing 21 conversions, 20 of which are enzymatic and one of which is a spontaneous skeletal rearrangement, is the single longest secondary metabolic pathway to have been completely elucidated at the enzyme level (summarized in Kutchan and Zenk, 1993; Kammerer et al., 1994). A cDNA encoding one of seven inducible enzymes from this pathway, the berberine bridge enzyme (Figure 3), has been isolated (Dittrich and Kutchan, 1991).

**Berberine Bridge Enzyme**

The berberine bridge enzyme ([S]-reticuline:oxygen oxidoreductase [methylene bridge forming]; EC 1.5.3.9) catalyzes the stereospecific conversion of the N-methyl group of (S)-reticuline into the berberine bridge carbon, C-8, of (S)-scoulerine. There are two main reasons why this particular enzyme is of interest. First, the reaction catalyzed by the berberine bridge enzyme is very elegant from the chemist's point of view. The direct conversion of the N-methyl group into a methylene bridge moiety is not presently achievable by synthetic chemical methods and exists nowhere else in nature. It is of biochemical interest to know how an enzyme catalyzes a reaction that we as chemists cannot. The second point of interest is that the *E. californica* berberine bridge enzyme is elicitor inducible. This implies that regulation of berberine bridge enzyme transcript accumulation may regulate benzophenanthridine alkaloid accumulation. From an analysis of the promoter of the *bbe1* gene, details can therefore be learned about how alkaloid biosynthesis is regulated in response to pathogen attack.

The cDNA encoding the berberine bridge enzyme has been isolated from a cDNA bank prepared from elicited cell suspension cultures of *E. californica* using oligonucleotides based on peptide amino acid sequences of the purified native enzyme (Dittrich and Kutchan, 1991). Translation of the nucleotide sequence confirmed the presence of a signal peptide (by comparison with the experimentally determined sequence of the mature protein N terminus) that directs the enzyme into the endoplasmic reticulum and then into the smooth vesicles in which it accumulates (Amann et al., 1986). Heterologous expression of this cDNA in insect cell culture using a baculovirus expression vector resulted in the production of sufficient quantities (4 mg of homogeneous enzyme per liter of insect cell culture medium) for a biochemical analysis of the berberine bridge enzyme (Kutchan et al., 1994). A series of spectral determinations and modified-substrate conversion-rate measurements has demonstrated that the berberine bridge enzyme is covalently flavinylated and that closure of the ring system to form (S)-scoulerine during the course of the enzymatic reaction proceeds via an ionic mechanism with the methylene iminium ion as a reaction intermediate (T.M. Kutchan, unpublished data).

An analysis of the time course of the elicitation process using the cDNA clone as a hybridization probe in RNA gel blot experiments revealed that the berberine bridge enzyme transcript reaches maximal levels within 6 hr after addition of a yeast elicitor to *E. californica* cultures. Enzyme activity increases until 17 to 22 hr after elicitation, and total benzophenanthridine alkaloids continue to accumulate for several days after elicitation (Dittrich and Kutchan, 1991). This scenario is indicative of de novo transcription of phytoalexin biosynthetic genes, which also occurs during stress-induced phytoalexin metabolism (see Dixon and Paiva, 1995, this issue). Methyl jasmonate induces the accumulation of low molecular weight compounds in a large number of plant species in culture (Gundlach et al., 1992), and both methyl jasmonate and the jasmonic acid biosynthetic precursor 12-oxo-phytodienoic acid induce transcription of the single *bbe1* gene in *E. californica* cell cultures (Kutchan, 1993b; Kutchan and Zenk, 1993). It has recently been shown that the *Pseudomonas syringae* von Hall phytopotoxin, coronatine, mimics 12-oxo-phytodienoic acid in its ability to induce a series of physiological responses such as tendril coiling, secondary metabolite accumulation in cell culture, and *bbe1* transcription in *E. californica* (Weiler et al., 1994). Unlike various abiotic and biotic elicitors (Gundlach et al., 1992; Mueller et al., 1993), coronatine affects gene transcription without inducing endogenous 12-oxo-phytodienoic acid and jasmonic acid accumulation. Induction of the berberine bridge enzyme transcript with trihomo-jasmonate has demonstrated that β-oxidation is not necessary for gene activation (Blechert et al., 1995). Analysis of the *cis* and *trans* elements necessary for elicitor-induced transcription of *bbe1* and of other inducible genes along the benzophenanthridine alkaloid biosynthetic pathway should help to elucidate the complex defense response signal transduction chain.

**BISBENZYLISOQUINOLINE ALKALOIDS**

The bisbenzylisoquinoline class of alkaloids contains over 270 members, all of which are dimers of tetrahydrobenzylisoquinolines connected by one to three ether linkages formed by phenol coupling. The structure of a prototype of these natural products, (+)-tubocurarine, which is the muscle relaxant isolated from tube-curare (a preparation of *Chondrodendron tomentosum* in wooden tubes), contains two ether linkages with the tetrahydrobenzylisoquinoline moieties combined in a head-to-tail orientation (Figure 1). Tube-curare has been traditionally used by South American Indians as an arrow poison. In modern medicine, tubocurarine chloride is used as a neuromuscular blocking agent to secure muscular relaxation in surgical operations. It is of interest that only one plant, *C. tomentosum*, is known to contain (+)-tubocurarine.
The bisbenzylisoquinoline family of alkaloids is rich in pharmacologically active constituents that range in activity from cytotoxins to antihypertensives to antimalarials. The structural variations in these alkaloids include substitutions on the phenyl rings, the regiospecificity of the ether linkages, and the stereospecificity of the isoquinoline moieties. The best understood biosynthesis is that of the dimeric alkaloids berbamunine and guattegaumerine, which are produced in cell suspension cultures of *Berberis stolonifera* (barberry). The single most important step in the biosynthesis of this category of alkaloids is ether linkage formation through phenol coupling. Any biotechnological production of the pharmacologically important bisbenzylisoquinolines requires correct stereo- and regioselectivity of ether bond formation. It has only recently been shown that the enzymes that catalyze either carbon–carbon or carbon–oxygen phenol coupling in plants are not nonspecific peroxidases or laccases but rather highly regio- and stereo-selective substrate-specific cytochrome P-450–dependent oxidases (Zenk et al., 1989; Stadler and Zenk, 1993). The reaction catalyzed by these enzymes is novel for cytochromes P-450 in that it proceeds without incorporation of oxygen into the product. In other words, this cytochrome P-450 functions as an oxidase rather than as a monoxygenase. It would be mechanistically interesting to discern how these enzymes act on molecular oxygen differently from the more common hydroxylases. Studies of the enzymatic mechanism require the ability to isolate large quantities of functional enzyme and to alter the enzyme's structure and observe the types of changes that occur in the enzymatic reaction. These approaches are, of course, dependent on molecular genetic manipulations. To this end, the first cDNA known to encode a plant cytochrome P-450 unequivocally involved in alkaloid biosynthesis has recently been cloned. This cDNA encodes berbamunine synthase (Figure 3) (Kraus and Kutchan, 1995).

**Berbamunine Synthase**

Berbamunine synthase (N-methylcoclaurine, NADPH:oxygen oxidoreductase [carbon–oxygen phenol coupling]; EC 1.1.3.34; CYP80) catalyzes formation of the ether linkage between one molecule of (R)-N-methylcoclaurine and one molecule of (S)-N-methylcoclaurine to form the bisbenzylisoquinoline alkaloid berbamunine. This cytochrome P-450 enzyme also couples two molecules of (R)-N-methylcoclaurine to form guattegaumerine. The cDNA encoding berbamunine synthase was isolated from a *B. stolonifera* cell suspension culture cDNA library (Kraus and Kutchan, 1995). An oligonucleotide primer based on the N-terminal amino acid sequence of the purified native oxidase was used as a screening probe. Translation of the nucleotide sequence of the clone revealed at least one notable amino acid residue difference in berbamunine synthase as compared with the sequences of cloned cytochrome P-450 monoxygenases. Structure–function studies on bacterial P-450cam suggest that three amino acid residues of the distal helix (helix I), Gly-248, Gly-249, and Thr-252, are essential for formation of the molecular oxygen binding pocket (Poulos et al., 1985). Mutation of Thr-252 to Ala or Val abolishes insertion of oxygen into the substrate (Imai et al., 1989). Thr-252 is present in berbamunine synthase, although there is no monoxygenation reaction in the carbon–oxygen phenol coupling process. However, the essential Ala or Gly at the position corresponding to 248 in P-450cam is replaced by a Pro in berbamunine synthase. Because this amino acid residue is one of three highly conserved residues of the oxygen binding pocket that are thought to be required for insertion of oxygen into the substrate, this may be a first indication of how berbamunine synthase functions as an oxidase.

Berbamunine synthase has been expressed in functional form in insect cell culture using a baculovirus expression vector (Kraus and Kutchan, 1995). The oxidase accepts electrons from either insect cell, porcine, or *Berberis* cytochrome P-450 reductases. Heterologously expressed berbamunine synthase can be obtained in near-homogeneous form and in large quantities (5 mg/L) after insect cell microsome solubilization followed by a single column–chromatography fractionation step. To obtain this amount of enzyme from *B. stolonifera* cells, 18,000 L of suspension culture would have to be extracted. A single gene probably codes for berbamunine synthase in the *B. stolonifera* genome, although two more weakly hybridizing DNA fragments are also present that may represent genes encoding other phenol coupling cytochromes.

**ECOCHEMICAL FUNCTION OF ALKALOIDS**

Their medicinal and toxicological properties clearly make alkaloids important to people, but their role in plants has been a longstanding question. Why should a plant invest so much nitrogen in synthesizing such a large number of alkaloids of such diverse structure? *C. roseus* alone contains over 100 different monoterpenoid indole alkaloids. That many alkaloids are cytotoxic provides insight into their potential function in the chemical defense arsenal of the plant. It needs to be systematically addressed whether alkaloids function as phytoalexins, as nitrogen storage forms, as UV protectants, or in any combination of these and other potential functions. The question of alkaloid function in plants and the relevance of that function have been reviewed (Hartmann, 1991; Caporale, 1995).

One alkaloid whose ecochemical functions have been thoroughly analyzed in recent years is nicotine, a highly toxic alkaloid that has been identified in the leaves, stems, and roots of a number of *Nicotiana* species. Nicotine sulfate, a byproduct of the tobacco industry, serves commercially as a very effective insecticide and fumigant. To date, no insect has evolved a resistance mechanism against nicotine. In mammals, ingestion of nicotine results in nausea, vomiting, diarrhea, mental confusion, convulsions, and respiratory paralysis. With such strong and varied physiological responses, nicotine would seem to be an effective deterrent against insect attack and herbivore grazing. This is indeed the case. A series of studies in the laboratory of I.T. Baldwin has demonstrated that nicotine, which is known to be synthesized in roots and transported
to leaves, is synthesized de novo from (15N)-nitrate in response to wounding of leaves of hydroponically grown N. sylvestris (Baldwin et al., 1994a). The amount of alkaloid that can be accumulated in the leaf is sufficient to reduce larval growth of the tobacco hornworm Manduca sexta (Baldwin, 1988). Most importantly, field tests with native populations of the summer annual N. attenuata demonstrated a systemic alkaloid induction in response to simulated leaf herbivory and browsing (Baldwin and Ohnmeiss, 1993).

Nicotine is clearly synthesized in response to wounding, but does the alkaloid have additional functions, for example, as a nitrogen storage form or as a UV protector? Although exogenously administered nicotine is catabolized to nornicotine and myosmine, most likely as a result of a detoxification mechanism, de novo–synthesized nicotine is not turned over. The plant does not recover nicotine nitrogen and reinvest it in other metabolic processes, even under conditions of nitrogen-limited growth, implying that nicotine is not used as a nitrogen storage form (Baldwin and Ohnmeiss, 1994). Nicotine supplied exogenously to D. stramonium, a species that accumulates tropine alkaloids but not nicotine, does not result in increased UV protection, although nicotine has a high molar extinction coefficient (2695 M⁻¹ cm⁻¹) at 262 nm (Baldwin and Huh, 1994). Taken together, these results suggest that nicotine functions as a phytoalexin in tobacco and has no additional role as a storage form of nitrogen or as a filter for UV radiation.

These results raise an important question: How does leaf wounding induce nicotine biosynthesis in the root? Jasmonates are known to induce accumulation of secondary metabolites in plant cell culture, and endogenous jasmonate pools increase rapidly in response to treatment of cells with a yeast elicitor (Gundlach et al., 1992). Leaf damage to N. sylvestris produces a similar increase in endogenous jasmonic acid pools in shoots within 30 min, and root pools of this signaling molecule increase within 2 hr (Baldwin et al., 1994b). In addition, application of methyl jasmonate as a lanolin paste to leaves results in increases in both endogenous jasmonic acid in roots and de novo nicotine biosynthesis. Jasmonate and 12-oxophytodienoic acid function as inducers of alkaloid biosynthesis both in culture and in plants. The next question that needs to be addressed is whether jasmonate is the systemic signaling molecule that is transported from the wounded leaf to roots, where it then induces transcription of the alkaloid biosynthetic genes.

PERSPECTIVES

Although the alkaloid field is a very old one, it is still in its infancy with regard to being fully understood, and our exploitation of the biotechnological potential of alkaloid biosynthesis has only just begun. We are still a long way from understanding how most alkaloids are synthesized in plants and how this biosynthesis is regulated. We also have much to learn about the chemical ecology of alkaloids so that we can better understand why these sophisticated and diverse structures evolved. What is clear is that alkaloids as integral components of medicinal plants have enjoyed a long and important history in traditional medicine. Our first drugs originated in the form of plant extracts, and some of our most important contemporary pharmaceuticals are either still isolated from plants or structurally derived from natural products. New antineoplastic, antiviral, and antimarial alkaloids are still being discovered in plants. As we accumulate the tools (biochemical knowledge, clones, and transformation and regeneration techniques) necessary for future developments in this field, we can look forward to genetically engineered microorganisms and eukaryotic cell cultures that produce medicinal alkaloids, to medicinal plants with optimized alkaloid spectra, and even to the production of important pharmaceuticals in transgenic plant cell cultures.

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