The expanding universe of alkaloid biosynthesis

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Characterization of many of the major gene families responsible for the generation of central intermediates and for their decoration, together with the development of large genomics and proteomics databases, has revolutionized our capability to identify exotic and interesting natural-product pathways. Over the next few years, these tools will facilitate dramatic advances in our knowledge of the biosynthesis of alkaloids, which will far surpass that which we have learned in the past 50 years. These tools will also be exploited for the rapid characterization of regulatory genes, which control the development of specialized cell factories for alkaloid biosynthesis.

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Abbreviations

ACS	acridone synthase
BPF-1	Box P Binding Factor-1
CHS	chalcone synthase
DAT	deacetylvindoline-4-O-acetyltransferase
D4H	deacetylvindoline-4-hydroxylase
DHS	deoxyhypusine synthase
HSS	homospermidine synthase
JA	jasmonic acid
MAT	minovincinine-19-O-acetyltransferase
MIA	monoterpenoid indole alkaloid
OMT	O-methyltransferase
ORCA	octadecanoid-derivative-responsive *Catharanthus roseus* APETALA2-domain
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PNAE	polyneuridine aldehyde esterase
RG	raucaffricine-O-β-glucosidase
SGD	strictosidine β-D-glucosidase
Str1	*Strictosidine synthase1*
T16H	tabersonine 16-hydroxylase
TDC	tryptophan decarboxylase

Introduction

The toxicity of plants, which contributes to their ability to protect themselves against predation, is partially related to the diversity of small molecules that they synthesize. Alkaloids, which display a large variety of pharmaceutical activities, compose one of the major classes of small molecules and accumulate in about 20% of all plant species. Although the importance of alkaloids in medicine has been highly publicized, the regulation of alkaloid biosynthesis, and the sites of alkaloid production and accumulation, is only now being elucidated. This review highlights recent developments in our understanding of selected alkaloid-biosynthesis pathways and provides a basic framework for rapid progress in the study of the biological roles played by these small molecules in plants. More generally, the stage is clearly set for the rapid characterization of whole biosynthetic pathways for the diversity of plant-derived small molecules that give specific plants their characteristic aroma, flavor, toxicity and pharmacological properties. This rapid expansion of knowledge should then lead to unlimited biotechnological applications for the production of improved crops that have enhanced nutritional and/or health-promoting properties.

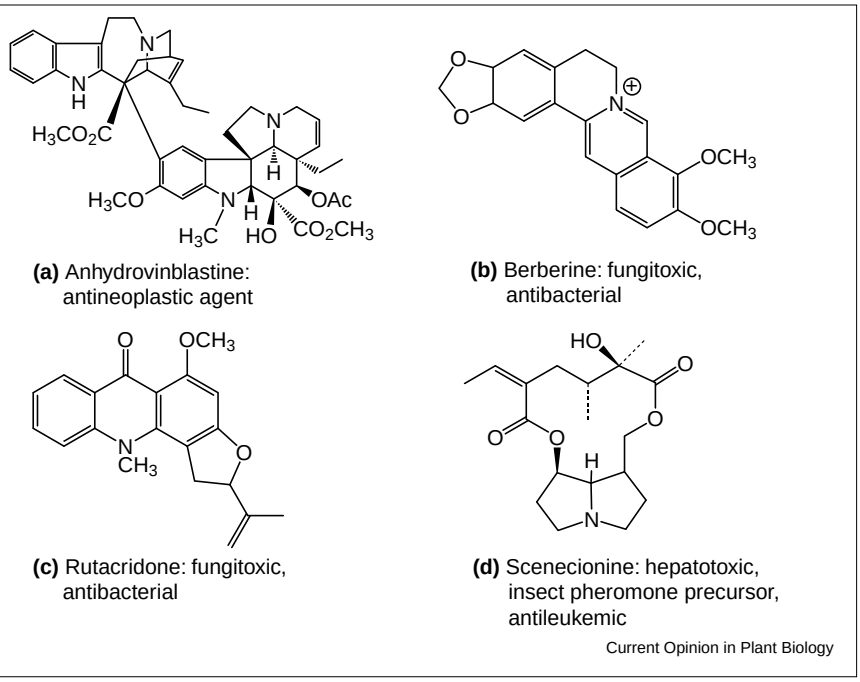
Biosynthesis of central intermediates

Most alkaloids are derived through the decarboxylation of amino-acid precursors (i.e. ornithine, lysine, tyrosine, tryptophan, and histidine) to yield their respective amines, or from anthranilic acid or nicotinic acid. The ability of plants to couple amines to different chemical partners produces a restricted number of versatile chemical backbones (i.e. central intermediates) from which the diversity of alkaloids is produced. For example, molecules such as strictosidine, norcoclaurine, 1,3-dihydroxy-*N*-methylacridone and homospermidine are central intermediates in the synthesis of monoterpenoid indole (Figure 1a), isoquinoline (Figure 1b), acridine (Figure 1c) and pyrrolizidine (Figure 1d) alkaloids, respectively. In fact, strictosidine and norcoclaurine appear to be particularly versatile sources of diversity: over 50% of the more than 12,000 characterized alkaloids are derived from these molecules.

The genes for some of these key reactions have been cloned. *Strictosidine synthase1* (*Str1*), which was cloned from *R. serpentina* and *C. roseus*, catalyses the formation of strictosidine from tryptamine and the monoterpenoid secologanin. Strictosidine-synthase-like genes have recently been identified in animals (including humans), and there are at least three *Str1*-like genes in soybean and 13 such genes in *Arabidopsis thaliana* (reviewed in [1]). In *Drosophila*, the cell-surface protein hemomucin is composed of a mucin-type repeat domain and an *Str1*-type domain. Hemomucin, which binds *Helix pomatia* lectin, may activate the immune response and may also be involved in haemolymph coagulation. The presence of *Str1*-like genes in *A. thaliana* and soybean, which are not known to produce indole alkaloids, suggests that *Str1*-like genes have other basic biological roles that precluded the evolution of Str1 activity in indole-alkaloid biosynthesis.

The key reaction in the biosynthesis of all acridone alkaloids, which are restricted to some genera of the Rutaceae, is catalyzed by acridone synthase (ACS). This enzyme, which converts *N*-methylanthraniloyl-CoA and 3 malonyl-CoAs into 1,3-dihydroxy-N-methylacridone, has a function

Figure 1



Examples of monoterpenoid alkaloids: (**a**) an indole, (**b**) an isoquinoline, (**c**) an acridine and (**d**) a pyrrolizidine.

that is similar to that of chalcone synthase (CHS; EC 2.3.1.74). Unlike CHS, however, ACS will not accept 4-coumaroyl-CoA as a substrate. The initial isolation of an *ACSI* clone (reviewed in [2•]), and the subsequent characterization of the tandemly arranged and 94% homologous *ACSII* gene [3], revealed how the ACS genes are evolutionarily related to the CHS gene family and classified them as plant polyketide synthases.

Most of the 360 known pyrrolizidine alkaloids [4•] are found within a scattered and restricted set of species within the Asteraceae, Boraginaceae, Fabaceae and Orchidaceae. Their scattered distribution within the angiosperms suggests that the biosynthesis pathway of the pyrrolizidine alkaloids may have arisen independently several times. Pyrrolizidine alkaloids are strong feeding deterrents for most herbivores and are highly toxic to vertebrates causing damage to the liver.

Pyrrolizidine alkaloids contain a characteristic necine base that is derived from putrescine and the aminobutyl moiety of spermidine, both of which are derived from arginine. Homospermidine synthase (HSS; EC 2.5.1.44), which catalyzes the formation of homospermidine from putrescine and spermidine, has recently been cloned from *Scenecio vernalis* [5]. The remarkable amino-acid identity between HSS from *S. vernalis* and deoxyhypusine synthase (DHS; EC 1.1.1.249) from *S. vernalis* (79%), tobacco (80%) and humans (61%), strongly suggests that HSS evolved from DHS. Both HSS and DHS transfer an aminobutyl moiety from spermidine to their individual substrate in a NAD^+-specific fashion. In contrast to HSS, however, DHS also participates in the activation of the protein factor 5A, which is required for the activation of cell proliferation in animal cells. Substrate specificity studies involving recombinant tobacco DHS, *S. vernalis* DHS and *S. vernalis* HSS confirmed that DHS catalyses both the aminobutylation of putrescine and the activation of factor 5A, whereas HSS only uses putrescine as an aminobutyl acceptor [5]. The existence of DHS in *S. vernalis* [5] and in tobacco [6] suggests its involvement in processes similar to those found in animals, and that the HSS of alkaloid-producing species was most likely recruited from this primary biological process. Selection pressure through herbivory may have been responsible for the evolution of an enzyme that does not activate protein factor 5A but retains the ability to transfer an aminobutyl moiety from spermidine to putrescine. The presence of the DHS gene, and hence the availability of homospermidine precursors for pyrrolizidine alkaloid synthesis, throughout the plant kingdom may explain why this pathway has successfully evolved several times [7•].

Monoterpenoid indole alkaloid biosynthesis

Assembly of secologanin

Secologanin provides the C9–C10 moiety in the biosynthesis of monoterpenoid indole alkaloids (MIAs) in *C. roseus*. In spite of extensive studies of the enzymes that convert geraniol into secologanin in the *C. roseus* model system, little is known about the genes involved. Recent 13C-glucose-feeding experiments with *C. roseus* cell cultures, showed that secologanin may be derived via the triose phosphate/pyruvate pathway [8]. Secologanin is formed via an initial rate-limiting hydroxylation of geraniol

by geraniol-10-hydroxylase, which leads to the formation of 10-hydroxygeraniol. Although geraniol-10 hydroxylase, a cytochrome P450 monooxygenase, was first purified to homogeneity from *C. roseus* (reviewed in [9•]), the geraniol-10 hydroxylase gene was first cloned and identified by functional expression in *A. thaliana*, which does not make MIAs [P1][10]. It is interesting that the *A. thaliana* gene could be used to probe corresponding sequences in maize [P1], which suggests that plants may generally produce 10-hydroxygeraniol for other uses. Recently, this gene was finally cloned and functionally characterized in *C. roseus* [9•,11].

Although the conversion of loganin to secologanin involves a unique oxidative cleavage of the methylcyclopropane ring, the enzyme that catalyses this reaction was characterized only recently [12•]. The pathway for secologanin biosynthesis is distributed within members of the Apocynaceae, Rubiaceae, Loganiaceae and Nyssaceae that have specialized capabilities for the biosynthesis of secoiridoids and/or MIAs. Initial studies showed that cell cultures from *Lonicera japonica* were capable of converting loganin into secologanin [13] and provided a useful potential source of secologanin synthase. The enzyme, which required NADPH, molecular oxygen and microsomal membranes for its activity, showed high specificity for loganin as substrate and was shown to be a member of the versatile cytochrome p450 family of genes.

This discovery provided key information for the identification of secologanin synthase from several uncharacterized *C. roseus* cytochrome p450 genes that had been cloned by a polymerase chain reaction (PCR) strategy [14]. Cytochrome p450s are known to be anchored to the cytoplasmic face of the endoplasmic reticulum and require NADPH cytochrome C reductase for activity. A protocol for fusing cytochrome p450 to NADPH cytochrome C reductase, which had previously been developed for animal systems [15], was adapted to investigate the biochemical function of cloned *C. roseus* cytochrome p450s. Using this approach, Schröder and colleagues ([14] and references therein) have been successful in functionally characterizing a number of *C. roseus* cytochrome p450 genes by expressing translational fusions in *Escherichia coli*. The enzyme assay to identify secologanin synthase used a ^{14}C-labelled loganin substrate, which allowed significantly better detection of the secologanin reaction product than that achieved in the assay originally used to characterize this enzyme in *L. japonica*.

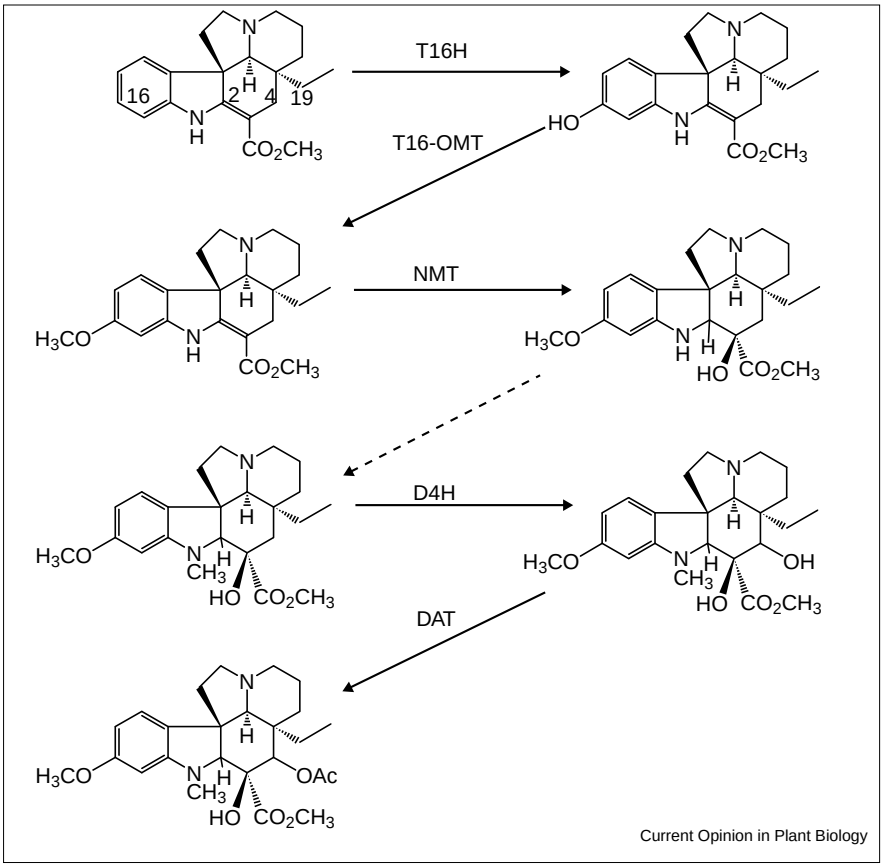
Assembly of MIAs

Strictosidine, which is deglycosylated by strictosidine β-D-glucosidase (SGD; EC 3.2.1.105) to yield a highly reactive dialdehyde, is converted by molecular rearrangement into the corynanthe, iboga and aspidosperma skeletons. These skeletons are elaborated to form the more than 200 alkaloids that are found in *C. roseus*. Although we do not know how this reactive dialdehyde is channeled into each of the major alkaloid types, the timing and site of SGD expression, together with those of downstream enzymes, may define which alkaloids are produced. The results of earlier studies showing that SGD exists as two isoforms with a high specificity for strictosidine were confirmed when SGD from *C. roseus* cultured cells was purified to homogeneity and characterized [16]. Denaturing SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) indicated that the protein has a molecular weight of 63 kDa, but native PAGE resolved the protein into three high molecular weight bands of *circa* 250, 500 and 630 kDa, which may comprise a 4-, 8- or 12-monomer aggregate. SGD was cloned from a cDNA library by a PCR-screening approach [16] and found to be a single-copy gene with homology to other plant β-glucosidases, such as prunasin hydrolase and amygdalin hydrolase from *Prunus serotina* and cyanogenic β-glucosidase from *Trifolium repens*. The clone was expressed in yeast and shown to possess SGD activity. The sgd mRNA and SGD activity were most abundant in leaf and root tissue.

R. serpentina is the source of ajmaline, a product that has been used in the treatment of heart disorders for the past four decades [17]. *R. serpentina* cell cultures have also been used as a model system for indole-alkaloid biosynthesis and to elucidate the complex biosynthetic pathway leading to ajmaline formation from strictosidine. In fact strictosidine synthase (EC 4.3.3.2), which catalyses the first step in indole-alkaloid biosynthesis, was cloned from *R. serpentina*. Substantial efforts to produce ajmaline in *R. serpentina* cell cultures have failed because they tend to accumulate a side-product known as raucaffricine. The re-utilization of raucaffricine for ajmaline biosynthesis appears to involve raucaffricine-O-β-glucosidase (RG; EC 3.2.1.125). The cDNA for RG was recently isolated [17] and shown to encode a 61-kDa protein belonging to the same -β-glucosidase family as SGD. Extensive biochemical studies with purified recombinant RG showed its high specificity for raucaffricine, but also that it accepts strictosidine as a substrate. Kinetic analysis revealed, however, that raucaffricine is the preferred substrate for RG and that there may be a separate strictosidine-specific SGD in *R. serpentina*.

The biosynthesis of ajmaline involves at least ten enzyme steps. Polyneuridine aldehyde esterase (PNAE) catalyzes the conversion of polyneuridine aldehyde into epi-vellosimine. This is the immediate precursor for the synthesis of the ajmalane skeleton leading to ajmaline biosynthesis. PNAE was purified to homogeneity and cloned by screening a corresponding cDNA library with oligonucleotide probes derived from PNAE peptide sequences [18]. The expression of PNAE as a histidine-tagged protein in *E. coli* showed that, unlike other esterases, this enzyme displays high substrate specificity and that it belongs to the α/β hydrolase superfamily.

Figure 2



Pathway for the conversion of tabersonine into vindoline. The numbering system is as for aspidospermine alkaloids in *Chemical Abstracts* [49]. The reaction catalyzing the hydration of the 2,3 double bond is represented by the dotted line and remains to be characterized. DAT, deacetylvindoline-4-O-acetyltransferase (EC 2.3.1.107); NMT, 16-methoxy 2,3-dihydro-3-hydroxytabersonine-N-methyltransferase; T16-OMT, 16-hydroxytabersonione 16-O-methyltransferase (EC 2.1.1.94).

Late stages of vindoline biosynthesis

C. roseus is the commercial source of the important anticancer agents vinblastine and vincristine, which are derived from the dimerization of catharanthine and vindoline. The late stages of vindoline biosynthesis (Figure 2) involve the hydroxylation of tabersonine to yield 16-hydroxytabersonine; the O-methylation of 16-hydroxytabersonine to form 16-methoxytabersonine; the hydration of the 2,3 double bond of 16-methoxytabersonine and its N-methylation and hydroxylation to yield deacetylvindoline; and finally, O-acetylation of the product to form vindoline. The conversion of tabersonine into vindoline requires three oxidative reactions involving a cytochrome p450 monooxygenase that converts tabersonine into 16-hydroxytabersonine, an unidentified hydration of the 2,3 double bond of 16-hydroxytabersonine and a 2-oxoglutarate dioxygenase that is involved in the formation of deacetylvindoline [8,19,20•].

Tabersonine 16-hydroxylase (T16H) is a cytochrome p450 monoxygenase that was recently cloned and assigned to the CYP71D subfamily of monooxygenases [21]. This T16H clone was fused to NADPH cyto-chrome C reductase and expressed in *E. coli*. A coupled radiolabelled-enzyme assay containing [methyl-14C]-S-adenosyl-L-methionine and tabersonine16-O-methyltransferase (EC 2.1.1.94) was used to detect the small amounts of 16 hydroxytabersonine generated from tabersonine by T16H. After termination of the reaction, [methyl-14C]-16 methoxy tabersonine was separated from unreacted AdoMet by extraction with ethyl acetate. The organic phase was counted directly by liquid scintillation counting or concentrated by evaporation for performance of analytical thin layer chromatography with reference standards. This assay considerably enhanced the sensitivity of product detection and was helpful in cloning this gene.

The alkaloids of *C. roseus* roots include oxidized O-acetylated forms of tabersonine [22] that are not intermediates of vindoline biosynthesis and may represent a potential competitive pathway for tabersonine in this underground organ. The isolation of O-acetyltransferases from *C. roseus* resulted in the description of two separate genes with 63% nucleic-acid identity whose deduced amino-acid sequences were 78% identical [23]. The active enzymes encoded by these genes, which were expressed as recombinant histidine-tagged proteins in *E. coli*, were named minovincinine-19-O-acetyltransferase (MAT) and deacetylvindoline-4-O-acetyltransferase (DAT) because they catalyzed the 19-O-acetylation of oxidized tabersonine derivatives (such as minovincinine and hörhammericine) and the 4-O-acetylation of deacetylvindoline, respectively.

Kinetic studies showed that the catalytic efficiency of recombinant MAT was poor compared to that of recombinant DAT. In fact, recombinant DAT's turnover rates for Acetyl-CoA and deacetylvindoline were *circa* 240 and 10,000 fold greater than those of recombinant MAT, respectively. Northern-blot analyses showed that MAT is expressed in cortical cells at the root tip, whereas DAT is expressed only in tissues such as leaves and stems.

Isoquinoline alkaloid biosynthesis

Several excellent recent reviews [19,20•,23] describe the biochemistry, cell biology and molecular biology of benzylisoquinoline alkaloid biosynthesis in detail. The most recent studies have focused on cloning and characterizing the O-methyltransferases (OMTs), which decorate the 6 and 4′ hydroxyl groups of S-norcoclaurine and 3′ hydroxy-N-methylcoclaurine, respectively. These enzymes compose part of the 13-step berberine biosynthesis pathway in *Thalictrum tuberosum* [24] and *Coptis japonica* [25].

Four *T. tuberosum* OMTs ([24]; see also Update) were cloned by a consensus-sequence-PCR-cloning and screening approach. All four clones were more than 93% identical at the amino-acid level and all four expressed enzymes had overlapping, but non-identical, substrate specificities. This study suggests that these OMTs, which exist as homodimers, might also occur as heterodimers thereby acquiring novel substrate specificities. Although this remains to be demonstrated, this theory was advanced to explain the surprising observation that the cloned OMTs did not display the strict substrate specificities that are typical of enzymes involved in alkaloid biosynthesis. Instead, they O-methylated a range of phenylpropanoid and alkaloid substrates.

The *C. japonica* 6-OMT and 4′-OMT were cloned by screening a cDNA library with oligonucleotide probes that were based on internal protein sequences obtained from two proteins. These proteins, whose molecular weights were 40 and 41 kDa, contained both OMT activities and were co-purified from extracts of induced cell cultures [25]. The two clones, like those isolated from *T. tuberosum*, appear to belong to the OMT-II group of plant genes and share distinct homology to caffeic OMT. Expression of these clones in *E. coli* revealed that the 40-kDa clone was the 6-OMT, which accepted only norcoclaurine as substrate. The 41-kDa protein was the 4′ OMT, which accepted only 3′ hydroxy-N-methylcoclaurine as substrate. The 4′-OMT, 6-OMT and Scoulerine OMT, which catalyses the last methylation in berberine biosynthesis, all displayed high substrate specificity for their respective substrates. In contrast to the OMTs of *T. tuberosum*, none of these enzymes accepted phenylpropanoid substrates. This raises the possibility that plants may have evolved different approaches to catalyze different methylations. In the case of *T. tuberosum*, a single amino-acid substitution in a catechol OMT was sufficient to expand its substrate acceptability to include alkaloids, whereas in *C. japonica*, these alkaloid-specific enzymes clearly belong to a different branch of the phylogenetic tree.

The penultimate step in morphine biosynthesis involves the conversion of codeinone into codeine by codeinone reductase. This enzyme was purified to homogeneity and selected peptides were sequenced to generate PCR primers [26]. Because of the low abundance of codeinone reductase mRNA, a combination of PCR and nested PCR was used to generate a genuine codeinone reductase fragment. This fragment was used to isolate full-length clones of the codeinone reductase gene by 5′- and 3′-RACE-PCR (rapid amplification of mRNA ends by PCR) and RT-PCR (reverse transcriptase-PCR). Four functional codeinone reductase clones with 95–96% amino-acid-sequence identity were isolated and were shown to belong to the family of NADPH-dependent reductases. Although three NADPH-dependent reductive steps are involved in the conversion of reticuline to morphine, the four codeinone reductase isoforms appear to catalyze only the highly specific conversion of codeinone to codeine.

Compartmentation of alkaloid biosynthesis

This review and previous reviews [19,20•,23] attribute the ability of plants to manufacture a large diversity of alkaloids to the evolution of new substrate specificities from existing biochemical functions [27••,28•,29••,30,31]. Mutation and natural selection appear to be responsible for the adaptations giving rise to novel chemistries and the ability of plants to survive in changing biotic and abiotic environments. The existence of large gene families within plants that are responsible for decorating the central intermediates of alkaloid biosynthesis support this hypothesis [27••,28•,29••,30,31].

Detailed biochemical and molecular studies have revealed that the biosynthesis of alkaloids is highly regulated in development and time. The expression of biosynthetic pathways in particular cells and tissues may produce either the accumulation of end-products in those cells and tissues or intermediates that are transported to other organs for further elaboration into different end-products. For example, the biosynthesis of tropane alkaloids takes place in the roots but they are transported to the leaves for accumulation and storage (reviewed in [32••]). The biochemistry and cell biology of vindoline biosynthesis has shown that this pathway is divided among the cytoplasm, the endoplasmic reticulum, the vacuole and the chloroplast [20•,32••]. In addition, *in situ* hybridization studies were combined with immunolocalization to identify the sites of biosynthesis of the vindoline. The results showed that at least three types of cells are involved and that movement of indole-alkaloid intermediates must occur between cells to allow the biosynthesis of vindoline [32••,33••]. Genes that are involved in the early steps of vindoline biosynthesis, such as tryptophan decarboxylase and strictosidine synthase, are expressed in the epidermis of stems, leaves and flower buds, and in the apical meristem of the root tips. In

contrast, the genes encoding enzymes for the last two steps in vindoline biosynthesis are expressed within the laticifer and idioblast cells of leaves, stems and flower buds [33••].

Recent immunolocalization studies revealed the presence of deacetylvindoline 4-hydroxylase (D4H, which catalyses the second-to-last step in vindoline biosynthesis) in cotyledons from etiolated *C. roseus* seedlings [34]. Although D4H protein was present within this etiolated tissue, the concentration of D4H was not correlated with the level of enzyme activity. These results further corroborated earlier findings suggesting that an inactive D4H isoform exists in etiolated *C. roseus* seedlings. Moreover, they suggest that light is essential for triggering D4H enzyme activity. It has also been suggested that light may cause laticifer-specific factors to interact at the transcription, translation, and/or posttranslation level to ultimately induce D4H activity.

Recent *in situ* hybridization studies with root-localized MAT have also shown that MAT is expressed within the same cortical cells near the root tip as both tryptophan decarboxylase (TDC) and STR1 [35]. This suggests that the whole pathway leading to tabersonine and its O-acetylated derivatives may be expressed in these cells. It also raises the possibility that tabersonine or a modified derivative is transported from these cells to above-ground laticifers for elaboration into vindoline. It remains to be shown whether the epidermis of above-ground plant parts, which is the other primary site of TDC and STR1 expression, is also a site of tabersonine biosynthesis.

Regulation of alkaloid biosynthesis

Most of the recent progress in understanding the regulatory factors that control alkaloid biosynthesis has been made using the *C. roseus* model system. A number of early studies showed that alkaloid biosynthesis could be regulated by a number of biotic and abiotic stimuli and is activated at particular stages of plant development (reviewed in [20•]). These studies showed that specific pathway genes, such as TDC and STR1, could be activated by various hormones or elicitors. For example, TDC is regulated at the transcriptional, translational and posttranslational levels (reviewed in [36•]). Ultra-violet-B-light-responsive [37] and elicitor-responsive [38] regions have been identified in the promoter of the TDC gene from *C. roseus*. This discovery led to the identification of the nuclear factors GT-1 and 3AF1, which interact with multiple sequences within the promoter of the TDC gene [39]. These studies suggest that GT-1 is involved in ultra-violet-light-induced expression of the TDC gene. Similarly, deletion analysis of tyrosine/dopa decarboxylase and berberine-bridge enzyme 5′ flanking regions from opium poppy revealed the presence of putative regulatory domains [40].

Recent promoter analyses of the STR1 gene in *C. roseus* cell cultures revealed that a GCC-box-like domain was necessary and sufficient to activate STR1 expression in response to treatment with jasmonic acid (JA) or a yeast fungal elicitor [41]. Further characterization of this promoter region using a yeast one-hybrid screen revealed that two ORCA (octadecanoid-derivative-responsive *C. roseus* APETALA2-domain) proteins bind the JA- and elicitor-responsive element in a sequence-specific manner. Studies with ORCA2 showed it could trans-activate the str1 promoter in the presence of JA and the fungal elicitor to rapidly induce STR1 expression [41]. More recently, ORCA3 was cloned using T-DNA activation tagging and was shown to be related to ORCA2; there are only slight differences in the sequences of these two proteins [41,42••]. Further, yeast one-hybrid screening of a *C. roseus* cDNA library using the STR1 promoter as bait identified a MYB-like protein, CrBPF-1, that has high homology to parsley Box P Binding Factor-1 (BPF-1) [42••]. CrBPF-1 differed from the ORCA transcription factors as its expression was rapidly activated by elicitor treatment but not by JA. These findings clearly illustrate the presence of two distinct transcription factors that are capable of affecting STR1 expression.

Further investigation of STR1 in *C. roseus* cell cultures demonstrated that yeast elicitation induced JA biosynthesis as well as activating TDC and STR1 [43]. The protein-kinase inhibitor K-252a abolished both the elicitor-induced biosynthesis of JA and the JA-induced expression of STR1 and TDC. These results suggest that the JA-biosynthetic pathway may coordinate the expression of TDC and STR1 and that protein kinases are involved in this mechanism [43]. In an attempt to improve the yield of monoterpene indole alkaloids in cell cultures, the effects of overexpressing STR1 and TDC on alkaloid production in *C. roseus* cell cultures were investigated [44]. The overexpression of TDC was toxic to the cells and was not necessary to increase the production of MIAs. In contrast, STR1 overexpression appeared to be necessary yet insufficient for the maintenance of a high level of alkaloid biosynthesis and was well tolerated by the cells.

Other STR1 promoter studies in transgenic tobacco plants revealed the presence of a putative CACGTG *cis*-acting element, known as a G-box, that directed seed-specific expression of STR1 [45]. In addition, enhancer sequences within the STR1 promoter region were shown to bind the tobacco nuclear-protein factor GT-1 [46].

The future: functional genomic and proteomic analyses of alkaloid biosynthesis

Functional genomic approaches, combined with computational and expression-based analyses, are only beginning to be used to accelerate our comprehensive understanding of specialized cellular metabolism. For example, a recent study with a peppermint oil gland secretory cell cDNA library demonstrated that 25% of randomly selected cDNA clones were involved in the synthesis of essential oils ([47••]; see also Update). This study confirms that cellular specialization greatly enhances the expression of genes involved in that specialization. Although this has yet to be

tried, random sequencing of cell-specific cDNA libraries from alkaloid-producing plants could be used to isolate whole alkaloid pathways. For example, the findings presented for the cell-specific compartmentation of alkaloid biosynthesis in *C. roseus* suggest that epidermal, idioblast and laticifer cells are specialized sites of alkaloid biosynthesis and may be enriched for these pathways [32••,34].

A paper describing a proteomic approach to analyzing the proteins in opium poppy latex, which is thought to be the major site of morphine biosynthesis, has recently been published [48]. This study focused on the analysis of latex-specific proteins by two-dimensional SDS-PAGE after separating the latex into cytosolic serum and alkaloid-containing vesicles. Internal peptide sequences were obtained for 75 protein spots and a putative function could be assigned to 69 of them. This type of analysis, together with analysis of expressed sequence tags, promises to help identify the genes that are required for the creation of the specialized 'cell factories' that are responsible for the biosynthesis of alkaloids and, more generally, of all secondary metabolites.

Conclusions

Alkaloids are derived from a restricted number of amino-acid precursors that are converted to versatile central intermediates from which the diversity of alkaloid structures found in plants are derived. The chemical decoration of intermediates involves oxidative reactions coupled to a diversity of substitution steps that appear to be catalyzed by enzymes encoded by a defined set of gene families. The functionalities of these enzymes have evolved to provide the substrate specificity necessary for chemical diversification. The ability to produce alkaloids requires cellular specialization, which converts cells into chemical factories that are capable of supplying precursors and converting them into particular end products. The data clearly show that different cells are responsible for the biosynthesis of different alkaloids (i.e. strictosidine in the epidermis, vindoline in idioblasts and laticifers, and 19-O-acetylated dervatives of tabersonine in roots). However, the complements of pathways expressed and alkaloids accumulated in each of these cell types remain to be established. Alkaloid biosynthesis is regulated in time, by development and by environmental factors that control the activity of transcriptional factors, such as ORCA, which program the whole process.

Update

A recent report [50] has suggested that the dimeric OMTs involved in the biosynthesis of isoquinoline alkaloids in *T. tuberosum* may be composed of heterodimeric combinations of different subunits, which give rise to different substrate specificities. Although this new idea does not explain the differences between the *T. tuberosum* and *C. japonica* O-methyltransferases, it does require some attention because, to date, there has been no evidence that plant OMTs can form heterodimers.

Another interesting recent study helps to confirm the value of sequencing cell-specific biochemical factories for isolating the pathways that are responsible for the assembly of secondary metabolites [51]. The glands of sweet basil accumulate phenylpropene defense compounds. The random sequencing of a cDNA library produced from the peltate glands of basil confirmed that the phenylpropanoid pathway is expressed at high levels in these cells; 13 % of the sequences obtained were for phenypropanoid biosynthetic genes.

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References and recommended reading

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- • of special interest
- •• of outstanding interest

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p450 genes. The review focuses on the large and difficult task of identifying the biochemical and physiological functions of all of these genes. The use of molecular approaches to facilitate the isolation of cytochrome p450s from plants is highlighted. The authors note that the techniques for functional and biochemical characterization of cytochrome p450s lag behind those for isolation of cytochrome p450 genes.

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The oxo-acid-dependent dioxygenases are soluble oxidative enzymes that catalyse the diverse hydroxylation/desaturation reactions of a series of interesting natural products. Prescott's review focuses on how dioxygenases have assisted other oxidative reactions in the evolution of novel plant chemistries. It also provides an idea of the abundance of these dioxygenases by providing information from the *Arabidopsis* genome.

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