Annu. Rev. Plant Physiol. Plant Mol. Biol. 2001. 52:29–66
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ALKALOID BIOSYNTHESIS IN PLANTS: Biochemistry, Cell Biology, Molecular Regulation, and Metabolic Engineering Applications

Peter J Facchini
Department of Biological Sciences, University of Calgary, Calgary, Alberta T2N 1N4, Canada; e-mail: pfacchin@ucalgary.ca

Key Words metabolic engineering, gene regulation, secondary metabolism, signal transduction, subcellular compartmentation

■ **Abstract** Recent advances in the cell, developmental, and molecular biology of alkaloid biosynthesis have heightened our appreciation for the complexity and importance of plant secondary pathways. Several biosynthetic genes involved in the formation of tropane, benzylisoquinoline, and terpenoid indole alkaloids have now been isolated. The early events of signal perception, the pathways of signal transduction, and the function of gene promoters have been studied in relation to the regulation of alkaloid metabolism. Enzymes involved in alkaloid biosynthesis are associated with diverse subcellular compartments including the cytosol, vacuole, tonoplast membrane, endoplasmic reticulum, chloroplast stroma, thylakoid membranes, and perhaps unique "biosynthetic" or transport vesicles. Localization studies have shown that sequential alkaloid biosynthetic enzymes can also occur in distinct cell types, suggesting the intercellular transport of pathway intermediates. Isolated genes have also been used to genetically alter the accumulation of specific alkaloids and other plant secondary metabolites. Metabolic modifications include increased indole alkaloid levels, altered tropane alkaloid accumulation, elevated serotonin synthesis, reduced indole glucosinolate production, redirected shikimate metabolism, and increased cell wall–bound tyramine formation. This review discusses the biochemistry, cell biology, molecular regulation, and metabolic engineering of alkaloid biosynthesis in plants.

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1040-2519/01/0601-0029$14.00

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INTRODUCTION

Alkaloids are a diverse group of low-molecular-weight, nitrogen-containing compounds found in about 20% of plant species. Many of the ∼12,000 alkaloids for which structures have been described function in the defense of plants against herbivores and pathogens (19, 178). The potent biological activity of some alkaloids has also led to their exploitation as pharmaceuticals, stimulants, narcotics, and poisons. Plant-derived alkaloids currently in clinical use include the analgesics morphine and codeine, the anticancer agents vinblastine and taxol, the gout suppressant colchicine, the muscle relaxant (+)-tubocurarine, the antiarrythmic ajmaline, the antibiotic sanguinarine, and the sedative scopolamine. Other important alkaloids of plant origin include caffeine, nicotine, cocaine, and the synthetic *O*,*O*-acetylated morphine derivative heroin.

Research in the field of plant alkaloid biochemistry began with the isolation of morphine in 1806. Remarkably, the structure of morphine was not elucidated until 1952 owing to the stereochemical complexity of the molecule. Since then, three major technical advances have led to substantial progress in our understanding of plant alkaloid formation. The first was the introduction in the 1950s of radiolabeled precursors that allowed the chemical elucidation of alkaloid biosynthetic pathways. The second involved the increased use during the 1970s of plant cell cultures as an abundant source of biosynthetic enzymes that could be isolated, purified, and characterized. Finally, the widespread application in the 1990s of molecular techniques to the alkaloid field facilitated the isolation of several genes involved in indole, tropane, and benzylisoquinoline alkaloid biosynthesis (Table 1). The early events of signal perception, the pathways of signal transduction, and the function of gene promoters have since been investigated in relation to the regulation of alkaloid metabolism. Tissue-specific localization studies have shown that sequential biosynthetic enzymes can occur in distinct cell types. The predicted translocation of pathway intermediates between cells further demonstrates the intricate cell biology of alkaloid biosynthesis. Isolated genes have also been used to genetically engineer the accumulation of alkaloids and other secondary metabolites in plants. In this review, recent advances in the biochemistry,

cell biology, molecular regulation, and metabolic engineering of plant alkaloid pathways are discussed.

BIOCHEMISTRY AND CELL BIOLOGY OF ALKALOID PATHWAYS

Terpenoid Indole Alkaloids

Terpenoid indole alkaloids (TIAs) comprise a family of ∼3000 compounds that includes the antineoplastic agents vinblastine and camptothecin, the antimalarial drug quinine, and the rat poison strychnine. Some TIAs have been proposed to play a role in the defense of plants against pests and pathogens (93). TIAs consist of an indole moiety provided by tryptamine and a terpenoid component derived from the iridoid glucoside secologanin. Tryptophan is converted to tryptamine by tryptophan decarboxylase (TDC; Figure 1), which is encoded by a single gene in *Catharanthus roseus* (31, 58) and by two autonomously regulated genes in *Camptotheca acuminata* (91). The *C. roseus TDC* gene exhibits both developmental and inducible regulation. In contrast, *C. acuminata TDC1* is expressed in tissues containing high levels of camptothecin including the shoot apex and bark, but the gene is not induced in response to elicitor treatment. However, *TDC2* is induced in elicitor-treated *C. acuminata* cell cultures, but is not developmentally expressed. The differential regulation of *TDC* genes in *C. acuminata* suggests that one participates in a developmentally controlled defense pathway, while the other is involved in an inducible defense mechanism.

The first committed step in secologanin biosynthesis is the hydroxylation of geraniol to 10-hydroxygeraniol. The enzyme geraniol 10-hydroxylase (G10H) was characterized as a P450 monooxygenase because it is membrane bound, dependent on NADPH and O_2, and displays light-reversible CO inhibition (106). G10H is specific for the C-10 position and exhibits similar affinity for geraniol and nerol, the *cis*-isomer of geraniol. The conversion of loganin to secologanin represents the last step in the pathway and is also catalyzed by a P450-dependent enzyme (180). The production of terpenoid precursors might play a regulatory role in TIA biosynthesis since the addition of secologanin or loganin to *C. roseus* cell cultures increases alkaloid accumulation (111, 113). Moreover, the level of G10H activity positively correlates with the accumulation of alkaloids when *C. roseus* cell cultures are transferred to alkaloid production medium (148). The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), which is involved in the biosynthesis of mevalonate, was cloned and characterized from *C. roseus* (97) and *C. acuminata* (16, 99). The differential expression of *HMGR* genes in response to wounding and methyl jasmonate (MeJA) was suggested to contribute to the regulation of TIA biosynthesis. However, secologanin was recently shown to be derived from the triose phosphate/pyruvate pathway (22); thus, the correlation between *HMGR* expression and TIA accumulation is likely coincidental.

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TABLE 1 Alkaloid biosynthetic enzymes for which the corresponding genes have been cloned

Enzyme	Function	Species	Type	Reference
Monoterpenoid indole alkaloid biosynthesis				
TDC	Tryptophan decarboxylase	*Catharanthus roseus*	Pyridoxal-5′-phospahate-dependent decarboxylase	31
		Camptotheca acuminata		91
STR	Strictosidine synthase	*Catharanthus roseus*	Vacuolar glycoprotein	104
		Rauwolfia serpentina		84
SGD	Strictosidine β-D-glucosidase	*Catharanthus roseus*	Membrane-associated glucosidase	55
T16H	Tabersonine 16-hydroxylase	*Catharanthus roseus*	P450-dependent monooxygenase	151
D4H	Desacetoxyvindoline 4-hydroxylase	*Catharanthus roseus*	2-Oxoglutarate-dependent dioxygenase	176
DAT	Deacetylvindoline acetyltransferase	*Catharanthus roseus*	Acetyl CoA-dependent acetyltransferase	164
Benzylisoquinoline alkaloid biosynthesis				
TYDC	Tyrosine decarboxylase	*Papaver somniferum*	Pyridoxal-5′-phospahate-dependent decarboxylase	38
6OMT	Norcoclaurine 6-*O*-methyltransferase	*Coptis japonica*	*S*-Adenosyl-L-methionine-dependent *O*-methyltransferase	114
4′OMT	3′-Hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase	*Coptis japonica*	*S*-Adenosyl-L-methionine-dependent *O*-methyltransferase	114
OMT II;1-4	Norcoclaurine 6-*O*-methyltransferase	*Thalictrum tuberosum*	*S*-Adenosyl-L-methionine-dependent *O*-methyltransferase	49

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CYP80A1	Berbamunine synthase	*Berberis stolonifera*	P450-dependent monooxygenase	81
CYP80B1	*N*-Methylcoclaurine 3′-hydroxylase	*Eschscholzia californica*	P450-dependent monooxygenase	133
		Papaver somniferum		72, 182
BBE	Berberine bridge enzyme	*Eschscholzia californica*	Flavinylated oxidoreductase	34
		Papaver somniferum		41
		Berberis stolonifera		21
SOMT	Scoulerine 9-*O*-methyltransferase	*Coptis japonica*	*S*-Adenosyl-L-methionine-dependent *O*-methyltransferase	171
COR	Codeinone reductase	*Papaver somniferum*	Aldo/keto reductase	173
Tropane alkaloid and nicotine biosynthesis				
ODC	Ornithine decarboxylase	*Datura stamonium*	Pyridoxal-5′-phospahate-dependent decarboxylase	112
PMT	Putrescine *N*-methyltransferase	*Atropa belladonna*	*S*-Adenosyl-L-methionine-dependent *N*-methyltransferase	167
		Nicotina tabacum		71
TR-I	Tropinone reductase-I	*Datura stramonium*	Short-chain dehydrogense	119
TR-II	Tropinone reductase-II	*Datura stramonium*	Short-chain dehydrogenase	119
H6H	Hyoscyamine 6β-hydroxylase	*Hyoscyamus niger*	2-Oxoglutarate-dependent dioxygenase	101
		Atropa belladonna		168
Purine alkaloid biosynthesis				
CS	Caffeine synthase	*Camellia sinensis*	*S*-Adenosyl-L-methionine-dependent *N*-methyltransferase	–

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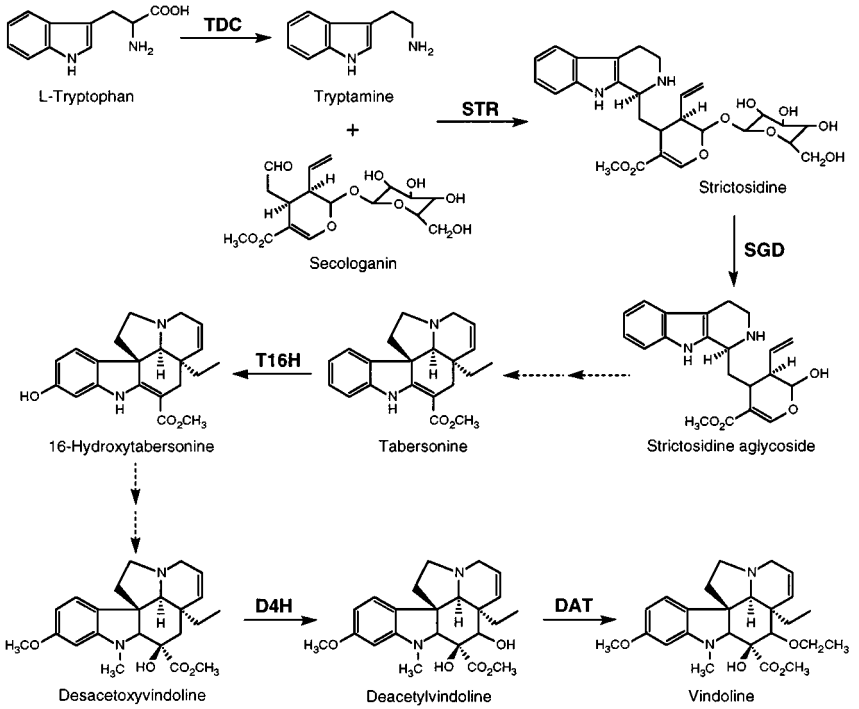

Figure 1 Reactions catalyzed by enzymes involved in monoterpenoid indole alkaloid biosynthesis for which the corresponding genes have been cloned. TDC, tryptophan decarboxylase; STR, strictosidine synthase; SGD, strictosidine β-D-glucosidase; T16H, tabersonine 16-hydroxylase; D4H, desacetoxyvindoline 4-hydroxylase; DAT, deacetylvindoline 4-*O*-acetyltransferase.

Tryptamine and secologanin are condensed by strictosidine synthase (STR) to form strictosidine, the common precursor to all TIAs (Figure 1). STR cDNAs have been isolated from *Rauvolfia serpentina* (84) and *C. roseus* (104, 132). The enzyme is encoded by a single gene in *C. roseus*, indicating that the multiple STR isoforms reported previously result from posttranslational modification of a single precursor (132). Strictosidine is deglucosylated by strictosidine β-D-glucosidase (SGD), which has been purified from *C. roseus* cell cultures (92). The native enzyme exhibits a high molecular mass, suggesting that it exists as an aggregate composed of multiple 63-kDa subunits. Digestion of SGD with trypsin causes the complex to disintegrate, solubilizing the enzyme without loss of activity. SGD is encoded by a single gene in *C. roseus* that shares ~60% homology with other plant glucosidases (55). Deglucosylated strictosidine is converted via several unstable intermediates to 4,21-dehydrogeissoschizine. Although several TIAs are produced from 4,21-dehydrogeissoschizine, few of the enzymes involved have been isolated (108). For example, none of the enzymes leading to catharanthine has been described.

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However, the biosynthesis of vindoline has been characterized in considerable detail. Vindoline is ultimately coupled to catharanthine by a nonspecific peroxidase to yield vinblastine (157).

The first of six steps involved in the conversion of tabersonine to vindoline consists of hydroxylation at the C-16 position by tabersonine 16-hydroxylase (T16H; Figure 1), which was detected in total protein extracts of young *C. roseus* leaves (163). Based on its requirement for NADPH and O_2, and its inhibition by CO, cytochrome *c*, and specific inhibitors, T16H was characterized as a P450-dependent monooxygenase. A T16H cDNA was isolated from *C. roseus* cell cultures using a cloning strategy based on the activation of the enzyme by light (151). Several P450 sequences were amplified by polymerase chain reaction (PCR), using degenerate primers specific to the conserved heme-binding domain. The PCR products were hybridized to RNA from induced and noninduced cells, and one showed induction kinetics consistent with T16H. The isolated cDNA, encoding a P450 homologue designated CYP71D12, was expressed in *Escherichia coli* as a translational fusion with cytochrome P450 reductase (CPR) from *C. roseus* (107). CYP71D12 was identified as T16H based on its ability to convert tabersonine to 16-methoxytabersonine. Genomic DNA hybridization analyses suggest the presence of at least two *T16H* genes (151), but only a single copy of the *CPR* gene (90), in *C. roseus*.

Subsequent to the 16-hydroxylation of tabersonine, the next three steps in vindoline biosynthesis are now accepted as 16-*O*-methylation, hydration of the 2,3-double bond, and *N*-methylation of the indole-ring nitrogen (8, 27, 28). An *S*-adenosyl-L-methionine (SAM)-dependent *O*-methyltransferase (OMT) has been reported that methylates 16-*O*-demethyl-4-*O*-deacetylvindoline (45). Initially, two consecutive hydroxylations at the C-3 and C-4 positions were proposed to follow the 16-hydroxylation of tabersonine (45). However, the isolation of a SAM-dependent *N*-methyltransferase (NMT) specific for the indole-ring nitrogen of 16-methoxy-2,3-dihydro-3-hydroxytabersonine indicated that the *O*-methylation step precedes *N*-methylation, and that 16-hydroxytabersonine is the natural substrate of the OMT (28, 33). The enzyme involved in hydrating the 2,3-double bond has not been isolated.

The second-to-last step in vindoline biosynthesis is catalyzed by a 2-oxoglutarate-dependent dioxygenase that hydroxylates the C-4 position of desacetoxyvindoline (D4H; Figure 1) (23). The enzyme requires ferrous ions and ascorbate, occurs as three unique charge isoforms, and exhibits an "ordered ter ter" mechanism with 2-oxoglutarate binding first, followed by O_2 and desacetoxyvindoline (24). Degenerate primers, designed from amino acid sequences derived from the purified protein, were used to isolate cDNA and genomic clones encoding D4H (174). Two different cDNAs were isolated, representing dimorphic alleles of a single-copy gene.

The final step in vindoline biosynthesis is catalyzed by acetylcoenzyme A: deacetylvindoline 4-*O*-acetyltransferase (DAT; Figure 1) (26, 44). The purified enzyme is strongly inhibited by tabersonine and coenzyme A (50% inhibition at

45 μM and 37 μM, respectively), and weakly inhibited by tryptamine, secologanin, and vindoline (28%, 25%, and 40% inhibition, respectively, at 500 μM), suggesting that DAT activity is modulated by pathway precursors and products (135). The original purification of DAT led to the incorrect conclusion that the enzyme consists of two subunits with molecular weights of 33 and 21 kDa. However, the isolated *DAT* gene encodes a 50-kDa polypeptide, suggesting that the protein was cleaved as an artifact of purification (164). Moreover, the protein that cross-reacts with anti-DAT antibody in seedlings and leaves also has a molecular weight of 50 kDa (164).

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Benzylisoquinoline Alkaloids

Benzylisoquinoline alkaloids (BIAs) are a large and diverse alkaloid group with ~2500 defined structures. The pharmacological activity of BIAs renders many of them useful as pharmaceuticals and is often a clue to their biological role in the plant (19). For example, the effectiveness of morphine as an analgesic, colchicine as a microtubule disrupter, and (+)-tubocurarine as a neuromuscular blocker suggests that these alkaloids function as herbivore deterrents. The antimicrobial properties of sanguinarine suggest that it confers protection against pathogens. The BIAs berberine, sanguinarine, and palmatine were specifically shown to confer protection against herbivores and pathogens (149).

BIA biosynthesis begins with a metabolic lattice of decarboxylations, *ortho*-hydroxylations, and deaminations that convert tyrosine to both dopamine and 4-hydroxyphenylacetaldehyde (142). The only enzyme involved in these early steps that has been purified (100), and for which the corresponding cDNA has been cloned (38, 98), is the aromatic L-amino acid decarboxylase (TYDC) that converts tyrosine and dopa to their corresponding amines (Figure 2). TYDC is encoded by a family of ~15 genes in *Papaver somniferum* (opium poppy) that can be divided into two subgroups based on sequence identity (38). Although the catalytic properties of the isoforms are similar, each *TYDC* subfamily exhibits a distinct developmental and inducible expression pattern (38, 40). TYDC cDNAs have also been reported from parsley (80) and *Arabidopsis thaliana* (172), which do not accumulate tyrosine-derived alkaloids. TYDC mRNAs were shown to be rapidly induced in response to elicitor treatment (40, 80, 172) and pathogen challenge (150) in various plants. Induction of TYDC mRNAs in parsley and *Arabidopsis* suggests that tyramine serves as the precursor to a ubiquitous class of defense-response metabolites, in addition to BIAs. Recent studies suggest that the synthesis and deposition in the cell wall of amides, composed of hydroxycinnamic acid-derivatives and tyramine, is central to the defense-response of many plants (105). Amides, together with other phenolics, are believed to reduce cell wall digestibility. The dual role of tyramine as a precursor for BIA and hydroxycinnamic acid amide biosynthesis suggests that the *TYDC* gene family in opium poppy encodes TYDC isoforms with diverse metabolic roles.

Dopamine and 4-hydroxyphenylacetaldehyde are condensed by norcoclaurine synthase (NCS) to yield the trihydroxybenzylisoquinoline alkaloid (*S*)-norcoclaurine, which is the central precursor to all BIAs in plants (Figure 2) (158, 159). Due to the inability of NCS to discriminate between 4-hydroxyphenylacetaldehyde and 3,4-dihydroxyphenylacetaldehyde, and the nonspecificity of the

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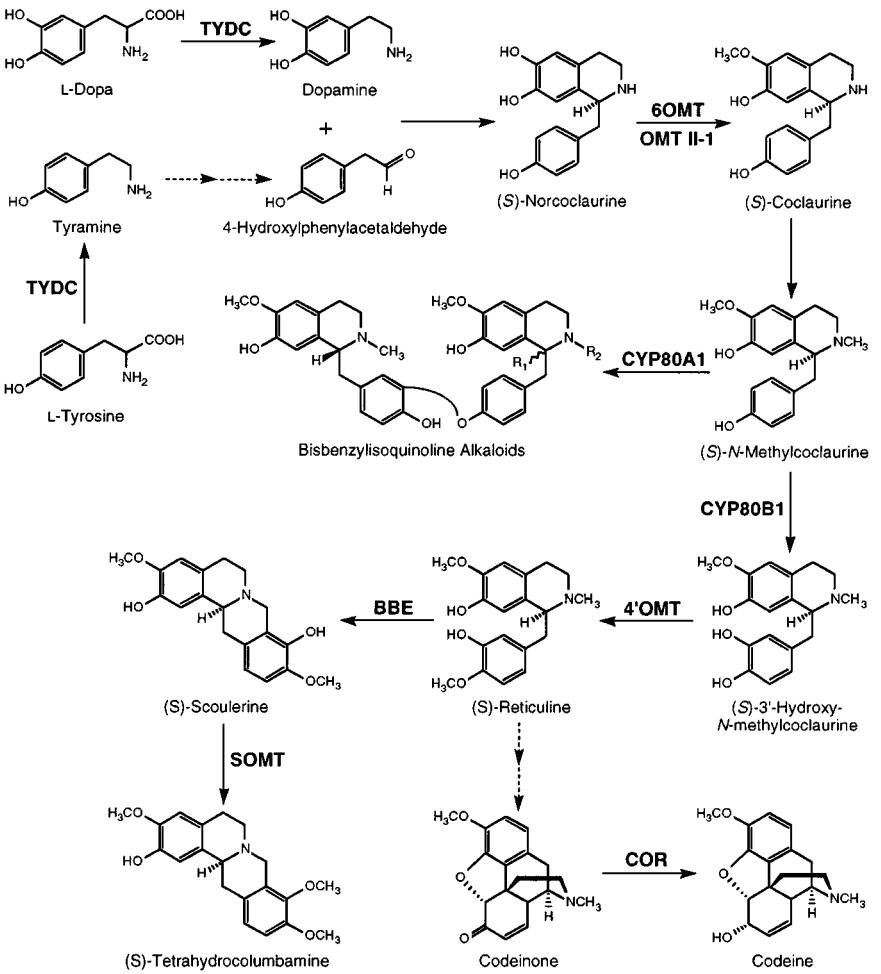

Figure 2 Reactions catalyzed by enzymes involved in benzylisoquinoline alkaloid biosynthesis for which the corresponding genes have been cloned. TYDC, tyrosine/dopa decarboxylase; 6OMT, norcoclaurine 6-*O*-methyltransferase; 4′OMT, 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase; OMT II-1, *O*-methyltransferase II-1; CYP80A1, berbamunine synthase, CYP80B1, (*S*)-*N*-methylcoclaurine 3′-hydroxylase; BBE, berberine bridge enzyme; SOMT, scoulerine *N*-methyltransferase; COR, codeinone reductase.

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subsequent methyltransferase reactions, it was originally thought that the tetrahydroxybenzylisoquinoline alkaloid (*S*)-norlaudanosoline was the precursor to BIAs (141). However, only norcoclaurine has been found to occur in plants.

(*S*)-Norcoclaurine is converted to (*S*)-reticuline by a 6-*O*-methyltransferase (49, 147), an *N*-methyltransferase (47), a P450 hydroxylase (133), and a 4′-*O*-methyltransferase (48, 147). The SAM-dependent 6-*O*- and 4′-*O*-methyltransferases (6OMT and 4′OMT, respectively) have been purified from cultured *Coptis japonica* cells (147), and the corresponding cDNAs isolated and characterized (114). Although the two enzymes display similar enzymological properties, they exhibit distinct substrate specificities. Moreover, the 6OMT follows a "ping-pong bi bi" mechanism, whereas the 4′OMT catalyzes an "ordered bi bi" reaction (114). Four homologous *O*-methyltransferase cDNAs (OMT II;1-4) have also been isolated from MeJA-treated *Thalictrum tuberosum* cell cultures (49). Heterologous expression of the OMT II;1-4 cDNAs showed that homodimers and various heterdimeric combinations of the four isoforms exhibit broad substrate specificity. The *O*-methylated substrates included simple catechols, phenylpropanoids, and various BIAs, suggesting that some of the isoforms are involved in both BIA and phenylpropanoid metabolism. For example, the homodimer of OMT II;1 efficiently *O*-methylates (*R*,*S*)-norcoclaurine (Figure 2) and various catechol and caffeic acid derivatives. Remarkably, OMT II;4 differs from OMT II;1 by only one amino acid, but its homodimer does not catalyze the alkaloid methylations. Both the 6OMT and 4′OMT from *C. japonica* show relatively low identity (24 and 35%, respectively) to the various catechol OMT II isoforms (114). The in vivo contribution, if any, of the OMT II enzymes to BIA biosynthesis remains to be established.

Originally, the aromatic-ring hydroxylation involved in the conversion of (*S*)-norcoclaurine to (*S*)-reticuline was thought to be catalyzed by a nonspecific phenol oxidase (89). However, a P450-dependent monooxygenase (CYP80B1; Figure 2) isolated from *Eschscholzia californica* (133) and opium poppy (72, 182) exhibits a K_m for (*S*)-*N*-methylcoclaurine 39-fold lower than that of the phenolase; thus, CYP80B1 is now known to convert (*S*)-*N*-methylcoclaurine to (*S*)-3′-hydroxy-*N*-methylcoclaurine. CYP80B1 is encoded by two to three genes in *E. californica* (133) and opium poppy (72, 182).

Intermediates of the (*S*)-reticuline pathway also serve as the precursors to ∼270 dimeric bisbenzylisoquinoline alkaloids such as berbamunine and (+)-tubocurarine. A phenol-coupling P450-dependent oxidase berbamunine synthase (CYP80A1) has been purified (160), and the corresponding cDNA isolated (81), from *Berberis stolonifera*. CYP80A1 couples two molecules of (*R*)-*N*-methylcoclaurine or one each of (*R*)- and (*S*)-*N*-methylcoclaurine by an ether linkage to form (*R*,*R*)-guattegaumerine or (*R*,*S*)-berbamunine, respectively (Figure 2). Additional variations in bisbenzylisoquinoline alkaloid structure include phenyl ring substitutions, the number of ether linkages, and regio- and stereoselection of monomers. CPR has also been purified from opium poppy, and the corresponding cDNAs isolated from opium poppy and *E. californica* (139). The CPR proteins

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share 69% amino acid identity and occur as two isoforms in each species. Coexpression of *E. californica* CPR and CYP80A1 in insect cell cultures resulted in an altered product profile compared to that obtained from the expression of CYP80A1 in the absence of the plant reductase.

(*S*)-Reticuline is a branch-point intermediate in the biosynthesis of many BIAs. Much research has focused on branch pathways that lead to benzophenanthridine alkaloids, such as sanguinarine and macarpine (85); protoberberine alkaloids, such as berberine and palmatine (66); and morphinan alkaloids, such as morphine and codeine (37). Most of the enzymes involved in these pathways have been isolated, many have been purified, and the corresponding cDNAs for three have been cloned. The first committed step in benzophenanthridine, protoberberine, and protopine alkaloid biosynthesis involves conversion of the *N*-methyl group of (*S*)-reticuline into the methylene bridge moiety of (*S*)-scoulerine by the berberine bridge enzyme (BBE; Figure 2). This conversion is unique in nature and cannot be achieved using synthetic chemical methods. The enzyme was purified from *Berberis beaniana* (161), the corresponding cDNAs were cloned from *E. californica* (34) and *B. stolonifera* (21), and *BBE1* genes have been isolated from opium poppy (41) and *E. californica* (68). The phenol coupling reaction catalyzed by heterologously expressed BBE (82) was recently identified as an ionic mechanism with a methylene iminium ion as the reaction intermediate (83).

(*S*)-Scoulerine can be converted to (*S*)-stylopine by two P450-dependent oxidases, (*S*)-chelanthifoline synthase (CFS) and (*S*)-stylopine synthase (SPS), which result in the formation of two methylenedioxy groups (9, 10). (*S*)-Stylopine is *N*-methylated by a substrate-specific methyltransferase, tetrahydroprotoberberine-*cis*-*N*-methyltransferase, that has been isolated from cultured *E. californica* and *Corydalis vaginans* cells (145), and purified from *Sanguinaria canadensis* cultures (122). The *N*-methylation step is followed by a third P450-dependent monooxygenase, (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase (MSH), which catalyzes the formation of protopine and has been isolated from *C. vaginans* cultures (143). Conversion of protopine to sanguinarine involves hydroxylation by another P450-dependent enzyme, protopine-6-hydroxylase (PPH), followed by a spontaneous intramolecular rearrangement to yield dihydrosanguinarine, the first alkaloid with the benzophenanthridine nucleus (171). Dihydrobenzophenanthridine oxidase (DBOX), a cytosolic enzyme originally isolated from *E. californica* cultures (154) and recently purified from *S. canadensis* cultures (4), catalyzes the oxidation of dihydrosanguinarine to sanguinarine. Root exudates from many Papaveraceae species, such as *S. canadensis* and *E. californica*, are intensely red owing to the accumulation of sanguinarine and other benzophenanthridine alkaloids. Two novel enzymes, a P450-dependent monooxygenase dihydrochelirubine-12-hydroxylase and a SAM-dependent 12-hydroxydihydrochelirubine-12-*O*-methyltransferase, have been discovered in yeast-elicited *Thalictrum bulgaricum* cultures (76). These enzymes catalyze the final two steps in the biosynthesis of macarpine, the most highly oxidized benzylisoquinoline alkaloid found in nature.

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In some plants, especially among the Berberidaceae and Ranunculaceae, (*S*)-scoulerine is methylated, rather than oxidized, to yield (*S*)-tetrahydrocolumbamine (Figure 2). The reaction is catalyzed by the SAM-dependent enzyme, scoulerine-9-*O*-methyltransferase (SOMT), which has been purified from *C. japonica* cells (146) and the corresponding cDNA isolated (170). Expression of the SOMT cDNA in *E. coli* produced a protein with a higher molecular weight than the native enzyme (51); thus, SOMT might be posttranslationally processed. Although the hydrophobic N-terminal region of SOMT is characteristic of a signal peptide, the enzyme has been reported to occur in the cytosol (116) and in the lumen of alkaloid-specific vesicles (52). The subsequent and second-to-last step in berberine biosynthesis involves the formation of a methylenedioxy bridge (52, 66). The enzyme activity originally thought to catalyze this reaction was actually a nonspecific peroxidase-mediated demethylation. The P450-dependent enzyme canadine synthase (CDS) was detected in members of the genera *Thalictrum* and *Coptis* and shown to catalyze methylenedioxy bridge formation in (*S*)-tetrahydrocolumbamine, but not in the quaternary alkaloid columbamine (144); thus, berberine biosynthesis cannot proceed via columbamine as once proposed. (*S*)-Canadine, also known as (*S*)-tetrahydroberberine, is oxidized to berberine either by (*S*)-canadine oxidase (CDO) or (*S*)-tetrahydroprotoberberine oxidase (STOX; 3). Although these enzymes catalyze the same reaction, their biochemical properties are quite distinct. STOX from *Berberis* is a flavinylated protein with a broad substrate range, whereas CDO from *Coptis* and *Thalictrum* contains iron and not flavin, proceeds via a different mechanism, and preferentially accepts (*S*)-canadine (66).

In some species of the genus *Papaver*, conversion of (*S*)-reticuline to its (*R*)-enantiomer represents the first committed step in morphinan alkaloid biosynthesis. An NADPH-dependent cytosolic enzyme 1,2-dehydroreticuline reductase, which catalyzes the stereospecific reduction of 1,2-dehydroreticuline to (*R*)-reticuline and is only found in plants that synthesize morphinan alkaloids, has been purified from opium poppy (25). Subsequent intramolecular carbon-carbon phenol coupling of (*R*)-reticuline by the P450-dependent enzyme salutaridine synthase (STS) results in the formation of salutaridine (56). The cytosolic enzyme, salutaridine:NADPH 7-oxidoreductase (SOR), found only in opium poppy and *P. bracteatum*, reduces salutaridine to (7*S*)-salutaridinol (57). Transformation of (7*S*)-salutaridinol into the morphinan alkaloid thebaine involves closure of an oxide bridge between C-4 and C-5 by the enzyme acetyl coenzyme A:salutaridinol-7-*O*-acetyltransferase (SAT), which has been purified from opium poppy cell cultures (87). SAT was also detected in *P. bracteatum*, but not in species that do not produce morphinan alkaloids.

In the remaining steps of the major pathway leading to morphine, thebaine is converted by enol-ether cleavage to codeinone, which is subsequently reduced to codeine. Ultimately, codeine is demethylated to yield morphine. The cytosolic enzyme codeinone reductase (COR), which catalyzes the NADPH-dependent reduction of (−)-codeinone to (−)-codeine, has recently been purified (88) and the corresponding cDNA isolated (173) from opium poppy (Figure 2). Four cDNAs

encoding different COR isoforms were cloned and expressed in *E. coli*. The four isoforms are members of a family of at least six alleles, and exhibit similar physical and catalytic properties. COR shares 53% amino acid identity with 6′-deoxychalcone synthase from soybean, further supporting an evolutionary link between the enzymes of phenylpropanoid and alkaloid biosynthesis (49, 173). Both COR and 6′-deoxychalcone synthase are members of the aldo/keto reductase family of NADPH-dependent oxidoreductases found in several primary metabolic pathways.

Tropane Alkaloids and Nicotine

Tropane alkaloids (TPAs) occur mainly in the Solanaceae and include the anticholinergic drugs atropine, hyoscyamine, and scopolamine, and the narcotic tropical anesthetic cocaine. Although nicotine is not a member of the tropane class, the *N*-methyl-Δ^1-pyrrolinium cation involved in TPA biosynthesis is also an intermediate in the nicotine pathway. *N*-Methyl-Δ^1-pyrrolinium cation formation begins with the decarboxylation of ornithine and/or arginine by ornithine decarboxylase (ODC; Figure 3) and arginine decarboxylase (ADC), respectively. These enzymes are involved in the formation of putrescine either directly by ODC, or via agmatine and *N*-carbamoylputrescine in the case of ADC; thus, the early steps of TPA and nicotine biosynthesis are also common to polyamine metabolism. ODC occurs in all living organisms, but ADC is not found in mammals and many lower

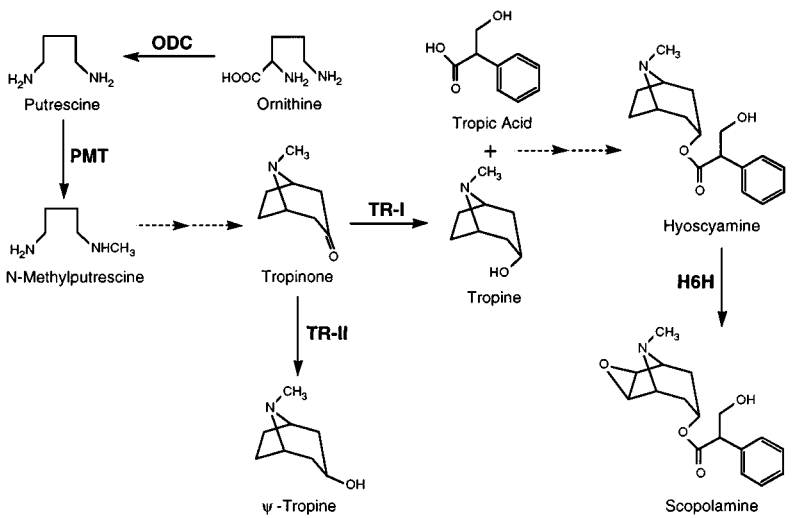

Figure 3 Reactions catalyzed by enzymes involved in tropane alkaloid biosynthesis for which the corresponding genes have been cloned. ODC, ornithine decarboxylase; PMT, putrescine *N*-methyltransferase; TR-I, tropinone reductase-I; TR-II, tropinone reductase-II; H6H, hyoscyamine 6β-hydroxylase.

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eukaryotes. An ODC cDNA isolated from *Datura stramonium* (112) was shown to be similar to other eukaryotic ODCs, and both prokaryotic and eukaryotic ADCs. In *D. stramonium*, ODC is encoded by a small gene family and ODC mRNAs are most abundant in roots compared to stems or leaves. ADC cDNAs have been isolated from oat (11) and tomato (136). Despite the existence of two routes to putrescine, arginine has been suggested to supply most of the putrescine for alkaloid biosynthesis (65).

The first committed step in TPA and nicotine biosynthesis is catalyzed by a SAM-dependent putrescine *N*-methyltransferase (PMT; Figure 3). The PMT cDNA isolated from tobacco (71) shows strong identity to spermidine synthase from human (73%), mouse (70%), and *E. coli* (58%). Such homology is consistent with the remarkably similar active site models determined for partially purified PMT from *Hyoscyamus albus* and spermidine synthase (70). Subsequently, *N*-methylputrescine is oxidatively deaminated by a diamine oxidase to 4-aminobutanol, which undergoes spontaneous cyclization to form the reactive *N*-methyl-Δ^1-pyrrolinium cation (66). The *N*-methyl-Δ^1-pyrrolinium cation is thought to condense with acetoacetic acid to yield hygrine as a precursor of the tropane ring, or with nicotinic acid to form nicotine, although the enzymology of these steps is not known.

Tropinone is located at a branch point in the TPA pathway and is the first intermediate with a tropane ring. Two related dehydrogenases, tropinone reductase I (TR-I) and tropinone reductase II (TR-II), reduce the 3-keto group of tropinone to the 3α- and 3β- groups of the stereospecific alkamines tropine and Ψ-tropine, respectively (Figure 3). cDNA clones for TR-I and TR-II were isolated from *D. stramonium* and expressed in *E. coli* (118). The proteins exhibit 64% amino acid identity and are similar to enzymes in the short-chain, nonmetal dehydrogenase family. TR-encoding genes were identified in other TPA-producing species, but not in tobacco, which accumulates nicotine rather than TPAs. The exchange of various domains of TR-I and TR-II was performed to create a series of chimeric enzymes that could be expressed in *E. coli* (119). A C-terminal domain of about 120 amino acids was shown to determine the stereo- and substrate-specificity of each enzyme. These results suggest that the stereospecificity of TR is determined by the orientation of tropinone in the substrate-binding site, composed mainly of the C-terminal domain. The N-terminal region constitutes the NADPH-binding site required for short-chain dehydrogenases. The basis for the reaction stereospecificities of TR-I and TR-II were determined from the crystal structures of the two enzymes at 2.4- and 2.3-Å resolution, respectively (121). These studies showed that the folding of the two enzymes is almost identical and that the NADPH-binding sites and positions of the active site residues are conserved. Structural models revealed the role of charged amino acids among the predominantly hydrophobic residues that comprise the substrate-binding site in determining the binding orientation of tropinone.

Hyoscyamine is produced by the condensation of tropine and the phenylalanine-derived intermediate tropic acid. Hyoscyamine can be converted to its epoxide

scopolamine by 6β-hydroxylation of the tropane ring followed by intramolecular epoxide formation via removal of the 7β-hydrogen (Figure 3). Cloning and heterologous expression of the H6H cDNA from *Hyoscyamus niger* showed that both reactions are catalyzed by a 2-oxoglutarate-dependent dioxygenase, hyoscyamine 6β-hydroxylase (H6H; 101).

Purine Alkaloids

Purine alkaloids such as caffeine, theobromine, and theacrine are widely distributed in the plant kingdom. Recent metabolic studies in tea and coffee have elucidated the biosynthesis of caffeine. The major route begins with xanthosine and proceeds through three *N*-methylations via 7-methylxanthosine, 7-methylxanthine, and theobromine (5, 6, 169), although a number of minor pathways have also been suggested (78, 152). The pathway contains three SAM-dependent *N*-methyltransferase activities found in young tea leaves, but absent in fully developed leaves (50). A partially purified enzyme preparation exhibited three activities, suggesting either that the *N*-methyltransferase steps in caffeine biosynthesis are catalyzed by a single enzyme, or by multiple enzymes with similar properties (78). However, an *N*-methyltransferase purified from the endosperm and leaves of coffee was active only toward 7-methylxanthine and theobromine (102). Moreover, an *N*-methyltransferase that catalyzes the methylation of methylxanthines and designated caffeine synthase (CS) was purified from young tea leaves (79). CS catalyzes two consecutive methylations involved in the conversion of 7-methylxanthine to caffeine, but is inactive toward xanthosine, indicating that the first methylation is catalyzed by a different enzyme. A CS cDNA has recently been isolated and expressed in *E. coli* (H Ashihara, personal communication). The predicted amino acid sequence shows that CS shares greater homology to salicylic acid *O*-methyltransferase than to other *N*-, *O*-, and *S*-methyltransferases, suggesting that CS belongs to a new methyltransferase class.

Subcellular Compartmentation of Alkaloid Biosynthetic Enzymes

Many alkaloid biosynthetic enzymes occur in subcellular compartments other than the cytosol. The compartmentation of these enzymes effectively sequesters toxic alkaloids and their biosynthetic intermediates away from the cytosol. The subcellular trafficking of pathway intermediates also creates an important level of metabolic regulation that could not occur if enzymes and substrates diffused freely in the cytosol. An understanding of the subcellular compartmentation of alkaloid pathways will reveal whether various enzyme characteristics observed in vitro, such as their inhibition by pathway intermediates, represent a true regulatory function in vivo.

Enzymes involved in vindoline biosynthesis have been localized to no fewer than five subcellular compartments. The conversion of tryptophan to tryptamine by TDC occurs in the cytosol (29, 162). Since STR is localized in the vacuole,

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tryptamine must be transported across the tonoplast before coupling to secologanin can occur (103). G10H, which catalyzes an early step in secologanin biosynthesis, is associated with provacuolar membranes (94). SGD, the enzyme catalyzing the deglucosylation of strictosidine, was suggested to be at least partly bound to the external face of the tonoplast (162). However, in vivo localization studies showed that SGD is associated with the endoplasmic reticulum (ER; 55). The P450-dependent monooxygenase T16H, which is involved in the C-16 hydroxylation of tabersonine, was also shown to be associated with the ER (163). NMT catalyzes the third-to-last step in vindoline biosynthesis and was found to be associated with thylakoid membranes (29, 33). However, chloroplast development is apparently not necessary since NMT activity was also detected in etiolated seedlings. The last two steps in vindoline biosynthesis, catalyzed by D4H and DAT, occur in the cytosol (23, 29). Vindoline must then be channeled back to the vacuole where nonspecific peroxidases necessary for coupling vindoline to catharanthine are localized (157). Overall, the complex compartmentation of the TIA pathway suggests the extensive subcellular trafficking of pathway intermediates. Although the vacuolar uptake of some TIAs, such as ajmalicine and serpentine, has been studied (15, 108), the mechanisms involved in channeling pathway intermediates to specific subcellular compartments are poorly understood.

Several BIA biosynthetic enzymes also occur in subcellular locations other than the cytosol. Of the five noncytosolic enzymes involved in the conversion of (*S*)-reticuline to dihydrosanguinarine, four (BBE, CFS, SPS, and MSH) are localized to a membrane fraction with a specific density of $\delta = 1.14$ g mL^{-1} (3, 9, 10, 143), while one (PPH) is associated with a membrane fraction with a density ($\delta = 1.11$ g mL^{-1}), consistent with that of the ER (171). The membrane-associated enzyme STS, which is involved in morphine biosynthesis, is also localized to a microsomal fraction with a density of $\delta = 1.14$ g mL^{-1} (56). With the exception of BBE, these noncytosolic enzymes are P450-dependent (14); thus, they must be membrane-bound proteins of the ER, or ER-derived compartments.

BBE appears to reside as a soluble protein within the lumen of a discrete subcellular compartment (3, 52). A putative N-terminal signal peptide was detected in the deduced amino acid sequence of BBE from *E. californica* (34). Other soluble enzymes involved in the biosynthesis of berberine are also associated with membranes of specific density $\delta = 1.14$ g mL^{-1}, including STOX (3), CDO (52), and columbamine *O*-methyltransferase (140). The association of these enzymes with a membrane fraction of greater density than the ER has led to speculation that distinct "alkaloid synthesizing vesicles" are found in certain cell types (3). Vesicles with a density of $\delta = 1.14$ g mL^{-1} and containing various alkaloids and biosynthetic enzymes have been visualized within vacuole-like compartments (3). Moreover, nascent BBE was found to contain a targeting domain comprised of an N-terminal signal peptide and an adjacent vacuolar sorting determinant (D Bird & P Facchini, unpublished results). Using the green fluorescent protein as a reporter, BBE was shown to be targeted to the ER lumen via the signal peptide. Subsequently, the protein is transported from the ER, possibly as a lumenal component of specific

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vesicles, to the vacuole. BBE is likely inactivated by the acidic conditions in the vacuole, suggesting that alkaloid synthesis occurs before the enzyme crosses the tonoplast membrane. The vacuolar accumulation of sanguinarine suggests that the entire contents of the transport vesicles, including BBE and various alkaloid intermediates, might be translocated directly from the ER to the vacuole.

Another example of novel subcellular compartmentation involves the biosynthesis of lysine-derived quinolizidine alkaloids, which occur in the mesophyll chloroplasts of legumes such as lupin (179). Recently, the biochemical localization of two acyltransferases that catalyze the last two acylations of (+)-*p*-coumaroylepilupinine and (−)-13α-tigloyloxymultifluorine showed that one enzyme occurs in the cytoplasm, whereas the other resides in the mitochondria of *Lupinus albus* (166). In animal cells, the acyl donor tigloyl-CoA is derived from isoleucine in the mitochondria, possibly explaining the mitochondrial localization of the terminal acyltransferase in plants. Although the quinolizidine nucleus appears to be synthesized in the chloroplast, subsequent modifications can occur only after alkaloid intermediates are transported to the cytosol and mitochondria. Ultimately, quinolizidine alkaloids are thought to accumulate in vacuoles of lupin epidermal cells, where their defensive properties are most effective (178).

REGULATION OF ALKALOID BIOSYNTHETIC GENES

Developmental Regulation and Tissue-Specific Localization

Alkaloid biosynthetic pathways are under strict developmental regulation in plants. This fact has severely restricted the exploitation of cultured plant cells for the production of valuable metabolites. For example, *C. roseus* cell cultures accumulate tabersonine and catharanthine, but not vindoline or vinblastine (108). Similarly, opium poppy cell cultures can be induced to accumulate sanguinarine, but do not synthesize morphine (37). The inability of dedifferentiated cell cultures to produce vindoline or morphine suggests that the biosynthesis of many alkaloids requires the differentiation of specific cell types.

The developmental regulation of TDC, STR, and four enzymes involved in vindoline biosynthesis (T16H, NMT, D4H, and DAT) has been studied in considerable detail. These enzymes are developmentally controlled in developing *C. roseus* seedlings, and some are further regulated by light (27, 30, 163). For example, *C. roseus* seedlings grown in the dark accumulate high levels of tabersonine and smaller amounts of four other vindoline pathway intermediates (8). The transfer of etiolated seedlings to light caused the turnover of tabersonine and other intermediates to vindoline, suggesting that some enzymes in the pathway are light regulated. The strict light activation of T16H in *C. roseus* seedlings (163) and cell cultures (151) was shown to be transcriptionally regulated. NMT activity increased ∼30% when etiolated *C. roseus* seedlings were exposed to light (30). D4H activity is also light induced (23), and the *D4H* gene has been shown to be under multilevel developmental and light-mediated regulation (175, 174). For

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example, D4H activity is low in etiolated seedlings despite an abundance of D4H transcripts. Exposure of etiolated seedlings to light caused a rapid increase in D4H activity without an increase in transcript levels (174). Despite the presence of only one *D4H* gene in *C. roseus*, several D4H isoforms were detected in etiolated and light-grown seedlings, suggesting that light modulates the developmental regulation of D4H at the posttranscriptional level (175). DAT activity was also shown to increase approximately tenfold in response to light, but the induction occurs primarily at the transcriptional level (30, 164). Phytochrome is clearly involved in the activation of vindoline biosynthesis by light (1) and has been shown to control expression of the *D4H* gene (175).

TDC and STR are most abundant in *C. roseus* roots, but also occur in photosynthetic organs (132). In contrast, T16H (163), D4H (174), and DAT (164) are restricted to young leaves and other shoot organs where vindoline biosynthesis occurs. In situ hybridization and immunocytochemical localization studies have shown that TDC and STR are localized to the epidermis of stems, leaves, and flower buds (32, 165). In roots, these enzymes occur in cells near the apical meristem. In contrast, D4H and DAT are associated with laticifers and idioblasts of shoot organs, but are absent from roots. Laticifers and idioblasts are distributed throughout the mesophyll in *C. roseus* leaves and are often several cell layers away from the epidermis; thus, vindoline biosynthesis involves at least two distinct cell types and requires the intercellular translocation of a pathway intermediate. The differential tissue specificity of strictosidine biosynthesis and the late steps of the vindoline pathway partially explain why vindoline is not produced in dedifferentiated *C. roseus* cell cultures.

Translocation of pathway intermediates also appears to occur in TPA biosynthesis (32). TPAs and nicotine are typically produced near the root apex, but accumulate within the vacuoles of leaves and roots (178). Histochemical localization of β-glucuronidase (GUS) activity in transgenic *Atropa belladonna* plants expressing a *PMT* promoter-GUS fusion showed that *PMT* expression occurs only in the root pericycle (167). Immunolocalization (64) and in situ hybridization studies (168) also demonstrated the pericycle-specific expression of H6H. In contrast, TR-I was immunolocalized to the endodermis and outer cortex, whereas TR-II was found in the pericycle, endodermis, and outer cortex (117). Similar, but not entirely identical, expression patterns of *TR-I* and *TR-II* promoter-*GUS* fusions were observed in transgenic *H. niger* (120). The localization of TR-I to a different cell type than PMT and H6H shows that an intermediate between PMT and TR-I moves from the pericycle to the endodermis and that an intermediate between TR-I and H6H moves back to the pericycle. Localization of PMT in the pericycle allows it efficient access to putrescine, ornithine, and arginine precursors unloaded from the phloem. Similarly, scopolamine produced in the pericycle can be readily translocated to the leaves via the adjacent xylem. Histochemical localization of GUS activity in transgenic roots of *H. niger*, *A. belladonna*, and tobacco expressing an *H6H* promoter-GUS fusion showed that the pericycle-specific expression of *H6H* is controlled by a mechanism that is specific to scopolamine-producing plants (77).

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The tissue-specific localization of TYDC has been demonstrated in opium poppy (38). TYDC mRNAs are most abundant in the metaphloem of opium poppy stems and roots, and are found only at low levels in developing seed capsules (39). Metaphloem is closely associated with laticifers in opium poppy (37); thus, detection of TYDC mRNAs in metaphloem supports the association of opium poppy BIA biosynthesis with cells ontogenically related to laticifers. NCS, which catalyzes the first committed step in BIA biosynthesis, is also most active in roots and stems of opium poppy (N Samanani & P Facchini, unpublished results). CYP80B1 transcripts are most abundant in stems, followed by roots, leaves, and floral tissues (72, 182). The activities of STS and SOR, which convert (*R*)-reticuline to salutaridinol, also occur abundantly in roots and shoots (56, 57). In contrast, COR, which catalyzes the penultimate step in morphine biosynthesis, is present throughout the plant, but is most abundant in shoot organs (72, 173). These results suggest that pathway intermediates are translocated between the roots and various shoot organs. Originally, morphine biosynthesis was thought to occur in laticifers (37). However, neither STS nor SOR could be detected in a fresh latex fraction from opium poppy capsules (56, 57), suggesting that laticifers are a site of alkaloid accumulation, but not alkaloid synthesis. Moreover, several enzymes (STS, SOR, SAT, and COR) have been detected in dedifferentiated opium poppy cell cultures despite the absence of laticifers (56, 57, 87, 88).

The root-specific accumulation of sanguinarine in opium poppy (39) suggests that the enzymes involved in its biosynthesis occur only in roots. However, the substantial levels of BBE mRNA (41) and enzyme activity (161) in shoot organs suggest that pathway intermediates involved in sanguinarine biosynthesis are translocated from shoots to roots. Similarly, although berberine accumulates in the primary roots of *Coptis japonica*, low levels of SOMT activity suggest that the primary root is not the main site of berberine biosynthesis (51). Indeed, the highest levels of SOMT are found in lateral roots and stems.

Signal Transduction and Inducible Expression

Protection from environmental stress is a major role for secondary metabolites in plants; thus, their biosynthesis is often induced by factors such as pathogen challenge and wounding (178). The accumulation of many alkaloids is induced by fungal elicitors, heavy metal ions, UV radiation, and osmotic shock (66, 85, 108). Initially, research was focused on the induction of alkaloid biosynthesis in elicitor-treated cell cultures. Recently, molecular tools have been used to investigate the regulation of inducible alkaloid pathways.

Treatment of *C. roseus* cell cultures with a fungal elicitor increases the accumulation of tryptamine and alkaloids such as catharanthine (35). Fungal elicitors rapidly induced *TDC*, *STR*, and *SGD* gene expression (55, 132), suggesting that the elicitor-mediated signal transduction pathway consists of relatively few steps that activate pre-existing transcription factors. Fungal elicitors also induced jasmonic acid (JA) biosynthesis in *C. roseus* cell cultures (110). JA functions as a messenger

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in the activation of secondary metabolism and other defense mechanisms by environmental signals (61, 115). Treatment of *C. roseus* seedlings with methyl jasmonate (MeJA) caused an increase in TDC, STR, D4H, and DAT activity levels and enhanced the accumulation of vindoline (2). TDC and STR transcript levels were also induced in *C. roseus* cell cultures by MeJA or the JA precursor α-linolenic acid (110). An inhibitor of JA biosynthesis, diethyldithiocarbamic acid, blocked the elicitor-induced formation of JA and the activation of TDC and STR. Moreover, elicitor-induced JA biosynthesis and MeJA-induced *TDC* and *STR* expression were blocked by the protein kinase inhibitor K-252a, suggesting the involvement of protein phosphorylation in the transduction pathway. The JA-mediated induction of TDC and D4H in *C. roseus* seedlings was shown to involve transcriptional and posttranslational controls (176). For example, MeJA treatment activated *TDC* gene expression and appeared to increase the stability of the TDC protein, but did not result in higher TDC activity in light- and dark-grown seedlings. Similarly, exogenous MeJA resulted in higher D4H activity and protein levels, but only in light-grown seedlings.

A functional JA-producing octadecanoid pathway is necessary for TIA biosynthesis by *C. roseus* cells cultured in an auxin-starved medium (53). Auxin inhibited TIA accumulation and reduced *TDC* transcription (58), but alkaloid biosynthesis could be induced by transferring the cells to an auxin-free medium. Exogenous MeJA restored the ability of cells grown in the presence of auxin to produce alkaloids (53). In cells cultured in auxin-free media, MeJA or JA treatment caused a further increase in alkaloid accumulation, whereas alkaloid production was reduced in auxin-starved cells treated with octadecanoid pathway inhibitors. These results suggest that JA is produced in response to auxin depletion and function in coordinating biochemical events that lead to alkaloid biosynthesis. Although JA is clearly involved in linking physiological and environmental signals to alkaloid biosynthesis, JA treatment of etiolated seedlings did not enhance TDC activity, nor could it replace the light requirement for *D4H* expression (176). JA appears to modulate events in TIA biosynthesis already under the control of other regulatory mechanisms.

Leaf damage caused by herbivores increases JA and nicotine levels in *Nicotiana sylvestris* roots (7). Exogenous MeJA also induced nicotine production in roots, suggesting that JA functions in transferring the damage signal from the shoot to the root. MeJA also induced ODC, PMT, and *S*-adenosylmethionine synthase (SAMS) transcript levels, and the accumulation of putrescine, *N*-methylputrescine, and nicotine in tobacco cell cultures (74). However, the induction of *SAMS* and *PMT* gene expression by MeJA was blocked by cycloheximide, whereas *ODC* expression was not, suggesting that multiple regulatory mechanisms are involved in the MeJA-mediated control of nicotine biosynthetic genes.

Cell cultures of many Papaveraceae species accumulate benzophenanthridine alkaloids, such as macarpine and/or sanguinarine, in response to treatment with fungal elicitors (36, 95, 153). In general, the membrane-associated biosynthetic enzymes are induced by elicitors, whereas most cytosolic enzymes are not (14).

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Several members of the *TYDC* gene family were rapidly and transiently expressed in opium poppy cell cultures in response to elicitor treatment (40, 42). CYP80B1 transcript levels were induced more than 20-fold in MeJA-treated *E. californica* cells (133) and fungal elicitor-treated opium poppy cells (72, 182). BBE was also transcriptionally activated in *E. californica* cells treated with a yeast elicitor (34) or MeJA (14, 85), and opium poppy cells treated with a fungal elicitor (41). The elicitor-mediated induction of other P450-dependent enzymes (CFS, SPS, MSH, and PPH) in the sanguinarine pathway has also been observed (10, 14, 171). Moreover, DBOX activity was reported to increase 4- and 14-fold in *S. canadensis* cells treated with MeJA or acetylsalicylic acid, respectively (73).

The induction of benzophenanthridine alkaloid biosynthesis in *E. californica* has been shown to occur at elicitor concentrations below the threshold required to stimulate events associated with the hypersensitive response, such as the production of phenolic compounds (138). Phenolic production could be selectively blocked by catalase at higher elicitor concentrations, suggesting that alkaloid biosynthesis is induced by a signal transduction pathway that is not mediated by reactive oxygen species involved in activating the hypersensitive response. Uncoupled induction mechanisms for phenylalanine ammonia lyase, TYDC, and downstream enzymes of sanguinarine biosynthesis have also been demonstrated in opium poppy (40). The elicitor-mediated induction of benzophenanthridine alkaloid biosynthesis requires a transient decrease in cytosolic pH caused by an efflux of protons from the vacuole (138). Artificial acidification of the cytosol was shown to induce alkaloid biosynthesis but not the hypersensitive response, whereas the depletion of vacuolar protons blocked the increase in alkaloid accumulation. Phospholipase A_2, a G-protein-controlled redox-dependent plasma membrane protein, might trigger the signal transduction pathway leading to the efflux of vacuolar protons (137). The role of G-proteins in the induction of benzophenanthridine alkaloid formation was also shown by treating *S. canadensis* cells with modulators of GTP-binding proteins and G-protein activators (96). The induction of alkaloid biosynthesis also appears to depend on an external source of Ca^{2+}, suggesting that Ca^{2+} and perhaps calmodulin participate in the signal transduction pathway (95).

Promoter Analysis

Recent studies have begun to reveal the location of *cis*-elements and the identity of transcription factors involved in the developmental and inducible regulation of *TDC*, *STR1*, and *CPR*. The activity of the *TDC* promoter linked to the *GUS* reporter gene was initially examined in transgenic tobacco plants and transfected protoplasts (59). Progressive 5′-truncations gradually reduced GUS activity levels until deletion to −112 essentially eliminated *TDC* promoter activity (59). Three functional regions involved in basal or elicitor-induced expression were identified in the *TDC* promoter from −160 to −37 by a loss-of-function assay (125). The −160 to −99 region was shown to act as the main transcriptional

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enhancer for basal expression, and two separate elicitor-responsive elements were found between −99 and −87, and between −87 and −37. In vitro binding of nuclear factors to the −572 to −37 region of the *TDC* promoter has also been described (126). Two binding activities that interact with multiple *TDC* promoter regions were identified as GT-1 and 3AF1 in tobacco and *C. roseus* nuclear protein extracts. Mutagenesis of the GT-1 binding sites did not affect basal or elicitor-induced expression, but did reduce *TDC* promoter activation by UV light. Only UV-B wavelengths were shown to induce TIA accumulation and TDC expression in *C. roseus*, suggesting the involvement of a specific receptor (123). Loss-of-function analysis revealed redundant UV-responsive elements in the *TDC* promoter between −99 and +198 (123).

The coordinate accumulation of transcripts suggests that *TDC*, *STR*, and *CPR* genes are regulated by common nuclear factors in response to elicitor treatment and UV light (108, 132). Deletion of the *CPR* promoter to −366 eliminated the elicitor-inducible expression observed with a 632-bp promoter (90). The −632 to −366 region of the *CPR* promoter also contains strong GT-1 binding sites. The main elicitor-responsive sequences of the *STR1* promoter were identified in the −339 to −145 region (131). Again, GT-1 was shown to bind to this and other regions of the *STR1* promoter. Despite the strong interaction of GT-1 with *TDC*, *STR1*, and *CPR* promoters, it remains to be determined whether GT-1 binding sites play a role in gene expression in vivo.

Other transcription factors are undoubtedly involved in the basal expression of the *TDC*, *STR1*, and *CPR* genes. A G-box motif at −105 was shown to bind G-box binding factors (GBFs) in vitro, but was not essential for the elicitor-induced expression of *STR1* in vivo (131). This G-box element also interacts with tobacco nuclear factors and the G-box-binding factor TAF-1 (124). Mutation of the G-box motif prevented binding of these factors and reduced the functional activity of constructs containing tetramers of the *STR1* G-box sequence. A G-box tetramer fused only to a TATA-box conferred seed-specific expression in transgenic tobacco, but required the enhancer region from the CaMV promoter for expression in leaves. These results suggest that sequences flanking the G-box motif determine *STR1* promoter activity in different tissues (124).

A GCC-box-like element in the −100 to −58 region of the *STR1* promoter is necessary and sufficient for JA- and elicitor-responsive expression (109). Two cDNAs encoding *C. roseus* AP2-domain (ORCA) proteins that bind the JA- and elicitor-responsive elements of *STR1* were isolated using a yeast one-hybrid screen. The ORCA2 protein *trans*-activates the *STR1* promoter, and expression of the *ORCA2* gene is induced by elicitor and JA treatment of *C. roseus* cell cultures. In contrast, the *ORCA1* gene is constitutively expressed. These results specifically identify a GCC-box-like element and ORCA2 in the JA- and elicitor-responsive expression of the *STR1* gene.

Deletion analysis has also revealed the location of regulatory domains necessary for *TYDC7* and *BBE1* promoter activities in a transient GUS assay system based on the microprojectile bombardment of cultured opium poppy cells (130). The

−393 to −287 region of the *TYDC7* promoter, and the −355 to −200 region of the *BBE1* promoter, were shown to be necessary for promoter function. Time-courses for the induction of TYDC7 and BBE1 mRNAs in wounded opium poppy cells were nearly identical to those for GUS activity in cells bombarded with promoter-*GUS* constructs when the −393 to −287 region of *TYDC7* and the −355 to −200 region of *BBE1* were present. These results suggest that the wound signal caused by the entry of DNA-coated microcarriers into opium poppy cells is sufficient to induce wound-responsive regulatory elements located from −393 to −287 in *TYDC7* and −355 to −200 in *BBE1*. Functional analysis of the *BBE1* promoter from *E. californica* showed that the −496 to −455 region is necessary for activity (68). Comparison of this region to the −355 to −200 region from opium poppy *BBE1* revealed a sequence with 55% nucleotide identity (130).

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METABOLIC ENGINEERING APPLICATIONS

Metabolic engineering is broadly defined as the improvement of cellular activities by the manipulation of enzymatic, transport, or regulatory functions using recombinant DNA technology. Several alkaloid biosynthetic genes have been used to genetically alter the production of various secondary metabolites in plants (Table 2). Transgenic plants with altered or novel enzyme activities have also become a powerful tool to study the metabolic control architecture of alkaloid pathways.

Terpenoid Indole Alkaloids

A *TDC* transgene was first introduced into *C. roseus* cells by infecting seedlings with an oncogenic strain of *Agrobacterium tumefaciens* (60). Tumorigenic calli expressing the *TDC* transgene showed increased TDC activity and tryptamine content, but alkaloid levels were not affected compared to wild-type controls. These results contradicted previous conclusions that tryptamine is a limiting substrate for TIA biosynthesis, at least in some tissues (108).

Nontumorigenic *C. roseus* cell cultures transformed with a *STR1* transgene showed tenfold higher STR activity and accumulated higher levels of strictosidine and other TIAs, compared to wild-type cultures, but TDC activity was not affected (18). In contrast, high TDC activity conferred by a *TDC* transgene introduced alone, or in combination with the *STR1* transgene, did not affect alkaloid accumulation. These results further suggest that STR catalyzes a rate-limiting step of alkaloid biosynthesis in *C. roseus* cell cultures (18). The influence of precursor availability on TIA accumulation was investigated by feeding various concentrations and combinations of tryptamine and loganin to a transgenic *C. roseus* cell line overexpressing *STR1* (177). High rates of tryptamine synthesis were found to occur even when TDC activity was low. Moreover, efficient STR activity was possible even when the tryptamine pool was small. However, the overall formation

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TABLE 2 Metabolic engineering of transgenic plants and tissue cultures using genes involved in alkaloid biosynthesis

Metabolic modification	Engineered enzyme(s)	Tranformed species	Source of gene(s)	References
Increased tryptamine	TDC	*Nicotiana tabacum*	*Catharanthus roseus*	155
Increased tyramine	TDC	*Nicotiana tabacum*	*Catharanthus roseus*	156
Increased serotonin	TDC	*Peganum harmala*	*Catharanthus roseus*	13
Reduced indole glucosinolates	TDC	*Brassica napus*	*Catharanthus roseus*	20
Redirection of shikimate metabolism; reduced phenylalanine; increased disease susceptibility	TDC	*Solanum tuberosum*	*Catharanthus roseus*	181
Increased tryptamine in crown gall tissue	TDC	*Catharanthus roseus*	*Catharanthus roseus*	60
Increased terpenoid indole alkaloids in cell cultures	TDC; STR	*Catharanthus roseus*	*Catharanthus roseus*	18
Increased quinoline alkaloids in root cultures	TDC; STR	*Cinchona officinalis*	*Catharanthus roseus*	54
Strictosidine production when supplied with exogenous secologanin	TDC; STR	*Nicotiana tabacum*	*Catharanthus roseus*	63
Increased cell wall–bound tyramine; decreased cell wall digestibility	TYDC	*Brassica napus*	*Papaver somniferum*	43
Increased cadaverine and anabasine	LDC	*Nicotiana tabacum*	*Hafnia alvei*	46, 69
Increased putrescine and nicotine	ODC	*Nicotiana tabacum*	*Saccharomyces cerevisiae*	62
Increased agmatine	ADC	*Nicotiana tabacum*	*Avena sativa*	17
Increased scopolamine	H6H	*Atropa belladonna*	*Hyoscyamus niger*	67, 183
Increased scopolamine	H6H	*Hyoscyamus muticus*	*Hyoscyamus niger*	75

of strictosidine was shown to require a sufficient supply of both secologanin and tryptamine, such that the efficient utilization of one depends on the availability of the other. Since precursor availability is a tissue-specific trait, different rate-limiting factors must occur in various cell lines, organs, and plants.

Transgenic tobacco expressing *C. roseus TDC* and *STR1* was used to establish a cell culture with high constitutive TDC and STR activity (62). This transgenic

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tobacco cell line accumulated tryptamine and produced strictosidine when secologanin was added to the culture medium. These results demonstrate that two consecutive steps in the TIA pathway can be cooperatively expressed in a foreign plant species that does not normally produce these metabolites. Examination of the integration frequencies and expression levels of *TDC* and *STR1* in tobacco showed that both transgenes were expressed in only 33% of the plants (86). Thus, the extensive phenotypic variation in alkaloid production in transgenic tissues (18) is partly caused by gene silencing phenomena affecting *TDC* and *STR1*.

Tropane Alkaloids, Nicotine, and Other Polyamine Derivatives

Some of the first attempts to metabolically engineer plant secondary products involved polyamine-derived alkaloids. Transgenic *Nicotiana rustica* root cultures expressing a yeast *ODC* gene produced higher levels of putrescine and nicotine (63). However, despite strong heterologous *ODC* expression, nicotine, putrescine, and *N*-methylputrescine levels increased only twofold, suggesting that ODC is not a rate-limiting step in nicotine biosynthesis. Overexpression of oat *ADC* in tobacco increased the accumulation of agmatine, the ADC reaction product (17). However, increased nicotine production was not detected despite suggestions that the putrescine required for nicotine biosynthesis is generated via ADC rather than ODC (66). It is possible that the additional agmatine was not accessible to the nicotine pathway.

Tobacco root cultures transformed with a bacterial lysine decarboxylase (*LDC*) gene produced higher levels of cadaverine, the product of the LDC reaction, and the alkaloid anabasine, produced by the coupling of cadaverine and *N*-methylputrescine (46). LDC activity, and cadaverine and anabasine accumulation, could be enhanced by fusing LDC to the RBCS transit peptide (69), demonstrating the importance of directing foreign enzymes to appropriate subcellular locations.

The *H6H* gene from *H. niger* was constitutively expressed in the hyoscyamine-rich plant *A. belladonna* (183). Transgenic plants exhibiting high H6H activity contained almost exclusively scopolamine. Similar results were obtained in transgenic root cultures of *A. belladonna* (67) and *Hyoscyamus muticus* (75). A large variation in TPA content was observed in root cultures of *H. muticus*, a species with a high capacity for tropane alkaloid accumulation, expressing *H6H*. One line produced over 100-fold more scopolamine than controls, but hyoscyamine conversion was still incomplete (75). Transgenic tobacco plants expressing an *H6H* were also capable of converting exogenous hyoscyamine and 6β-hydroxyhyoscyamine to scopolamine, which accumulated in leaves (184). Wild-type tobacco plants are unable to convert these intermediates to scopolamine.

Other Alkaloids

Cell cultures of *P. harmala* accumulate two simple and interrelated tryptamine-derived alkaloids: the harmane-type β-carboline alkaloids and serotonin. Serotonin

levels in transgenic *P. harmala* cell cultures with elevated heterologous TDC activity were ten- to 20-fold higher than in control cultures (13). In contrast, β-carboline alkaloid levels were not affected, demonstrating that tryptamine supply is limiting for serotonin, but not for β-carboline alkaloid, biosynthesis. The mechanism for the metabolic channeling of tryptophan into serotonin and β-carboline alkaloids in *P. harmala* is not known. It is possible that two TDC isoforms occur in separate subcellular locations, and the additional tryptamine produced in transgenic cultures is available to only one pathway.

The metabolic engineering of BIA pathways has not been reported owing to the previous lack of transformation protocols for BIA-producing plants. However, procedures have recently been developed for the transformation of opium poppy plants (128), root cultures (129), and cell cultures (12), and *E. californica* plants (127) and root cultures (129). These transformation systems provide the opportunity to alter the activity of individual enzymes of BIA biosynthesis, and to examine the consequences of such modifications on the accumulation of end-products and pathway intermediates. Efficient transformation protocols for these species will also facilitate progress in dissecting the molecular regulation of BIA biosynthetic genes.

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Other Uses of Alkaloid Biosynthetic Genes in Genetic Engineering

Enzymes that operate at the interface between amino acid and alkaloid metabolism have been used to alter substrate and product pools in a variety of plants. These studies provide insight into the control architecture of amino acid biosynthesis and relevant physiological processes. Tryptamine accumulation in tobacco plants expressing heterologous *TDC* was proportional to the level of TDC activity (60, 134, 155). Remarkably, plant growth was not affected despite the creation of a large sink for tryptophan and a large pool of soluble tryptamine. Moreover, no significant difference was detected in the activity of key aromatic amino acid biosynthetic enzymes (134), and IAA levels were identical in high-tryptamine and control plants (155). Transformed tobacco expressing *TDC* was also reported to accumulate more tyramine compared to controls (156). The most plausible explanation for this result is that the increased demand for tryptophan up-regulates the shikimate pathway resulting in increased tyrosine biosynthesis and, consequently, higher tyramine production via an endogenous TYDC activity.

Introduction of *TDC* into *Brassica napus* (canola) resulted in the redirection of tryptophan into tryptamine rather than indole glucosinolates (20). The indole glucosinolate content of seeds from transgenic plants was only 3% of that in wild-type seeds. In oilseeds, such as canola, the presence of indole glucosinolates decreases the value of the seed meal as an animal feed. This study is an elegant example of how a gene normally operating at an entry point in alkaloid biosynthesis can be used to divert metabolic flow and reduce undesirable product levels in crop species. In contrast, the introduction of *TDC* into potato altered the balance of

substrate and product pools involved in phenylpropanoid metabolism (181). The redirection of tryptophan to tryptamine caused a decrease in wild-type levels of tryptophan, phenylalanine, and phenylalanine-derived phenolic compounds such as chlorogenic acid and lignin in transgenic potato tubers; thus, artificial metabolic sinks can also alter substrate availability even if the foreign gene operates outside the pathway involved in substrate supply.

Canola was also transformed with *TYDC* transgenes encoding TYDC isoforms from opium poppy (43). Plants expressing high levels of *TYDC1* showed fourfold higher TYDC activity, a 30% decrease in cellular tyrosine pools, a twofold increase in cell wall-bound tyramine, and reduced cell wall digestibility compared to wild-type plants. This study supports the involvement of *TYDC* and tyramine in cell wall formation via the synthesis of hydroxycinnamic acid amides. The engineering of amide metabolism could also provide an effective strategy to reduce crop susceptibility to a broad spectrum of pathogens by decreasing cell wall digestibility.

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FUTURE PROSPECTS

Our understanding of the biological processes that permit the synthesis and accumulation of alkaloids in plants has advanced considerably over the past decade. This rapid progress has been facilitated by the availability of an impressive collection of alkaloid biosynthetic genes. These tools, combined with recent developments in plant genomics, will undoubtedly expedite the isolation of a multitude of genes involved in several alkaloid pathways. We have learned that plant alkaloid biosynthesis is more than a mere metabolic curiosity resulting in the formation of an immense array of biologically active products. Alkaloid pathways are highly regulated and involve novel cell-, tissue-, development-, and environment-specific controls. Many aspects of alkaloid biosynthesis, such as the elaborate subcellular compartmetation of enzymes and the intercellular translocation of pathway intermediates, reveal intriguing new variations in the complexity of plant metabolism. The expansion of our molecular toolbox will promote efforts to identify regulators associated with the development of cell types that can accommodate alkaloid pathways. Our emerging knowledge of the biochemistry, molecular biology, and cell biology of alkaloid biosynthesis will also lead to exciting opportunities to engineer alkaloid metabolism in transgenic plants. The inherent novelty of the pathways, and the socioeconomic importance of the products, are sure to encourage greater interest in alkaloid biosynthesis.

ACKNOWLEDGMENTS

I am grateful for support received from the Natural Sciences and Engineering Research Council of Canada, the Alberta Agricultural Research Institute, the University of Calgary Research Grants Committee, and Tasmanian Alkaloids Pty Ltd.

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Annual Review of Plant Physiology and Plant Molecular Biology
Volume 52, 2001

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