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# Biochemistry, Molecular Biology, and Metabolic Engineering of Benzylisoquinoline Alkaloid Biosynthesis

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**Abstract** Benzylisoquinoline alkaloids are a diverse group of natural products that include many pharmacologically active compounds produced in a limited number of plant families. Despite their complexity, intensive biochemical research has extended our knowledge of the chemistry and enzymology of many important benzylisoquinoline alkaloid pathways, such as those leading to the analgesic drugs morphine and codeine, and the antibiotics sanguinarine and berberine. The use of cultured plant cells as an experimental system has facilitated the identification and characterization of more than 30 benzylisoquinoline alkaloid biosynthetic enzymes, and the molecular cloning of the genes that encode at least 8 of these enzymes. The recent expansion of biochemical and molecular technologies has created unique opportunities to dissect the mechanisms involved in the regulation of benzylisoquinoline alkaloid biosynthesis in plants. Research has suggested that product accumulation is controlled by the developmental and inducible regulation of several benzylisoquinoline alkaloid biosynthetic genes, and by the subcellular compartmentation of biosynthetic enzymes and the intracellular localization and trafficking of pathway intermediates. In this paper, we review our current understanding of the biochemistry, cell biology, and molecular regulation of benzylisoquinoline alkaloid biosynthesis in plants. We also summarize our own research activities, especially those related to the establishment of protocols for the genetic transformation of benzylisoquinoline alkaloid-producing species, and the development of metabolic engineering strategies in these plants.

**Key words:** California poppy, *Eschscholzia californica*; genetic transformation, morphine; opium poppy, *Papaver somniferum*; sanguinarine, secondary metabolism, subcellular compartmentation.

## Introduction

Alkaloids are pharmacologically active nitrogenous compounds found predominantly, but not exclusively, in higher plants. Since the analgesic drug morphine was identified in 1806 as an active principle of *Papaver somniferum* (opium poppy) latex, the structures of more than 12,000 natural alkaloids have been elucidated. Benzylisoquinoline alkaloids are a large and diverse group of natural products with ~2,500 defined structures found primarily in five plant families: the Berberidaceae, Fumariaceae, Menispermaceae, Papaveraceae, and Ranunculaceae. In addition to morphine, several other benzylisoquinoline alkaloids are used as pharmaceuticals including codeine, papaverine, berberine, sanguinarine, colchicine, and (+)-tubocurarine. The structural complexity of these important alkaloids precludes chemical synthesis as an economical means of production; thus, plants remain the only commercial source.

The function of benzylisoquinoline alkaloids in plants includes protection against herbivory and infection by pathogens (Caporale 1995). Although the precise role of most plant secondary metabolites is not known, the potent pharmacological activity of benzylisoquinoline alkaloids that renders them useful as pharmaceuticals is often a clue to their biological function (Table 1). For example, the effectiveness of morphine as an analgesic, colchicine as a microtubule disrupter, and (+)-tubocurarine as a neuromuscular blocker suggests

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**Table 1.** Pharmacological activity and pharmaceutical applications of some benzylisoquinoline alkaloids.

| Alkaloid | Pharmacological Activity | Pharmaceutical Application | Source Plant |
|---|---|---|---|
| Berberine | Antibiotic | Intestinal infections | *Coptis japonica* |
| Codeine | Narcotic analgesic | Pain reliever | *Papaver somniferum* |
| | Antitussive | Cough suppressant | |
| Colchicine | Microtubule disrupter | Gout suppressant | *Colchicum autumnale* |
| Emetine | Parasiticide | Antiamoebic | *Uragoga ipecacuanha* |
| | | Oral emetic | |
| Morphine | Narcotic analgesic | Pain reliever | *Papaver somniferum* |
| Papaverine | Vasodilator | Muscle relaxant | *Papaver somniferum* |
| Sanguinarine | Antimicrobial | Oral hygiene | *Sanguinaria canadensis* |
| (+)-Tubocurarine | Neuromuscular blocker | Muscle relaxant | *Chondodendron tomentosum* |

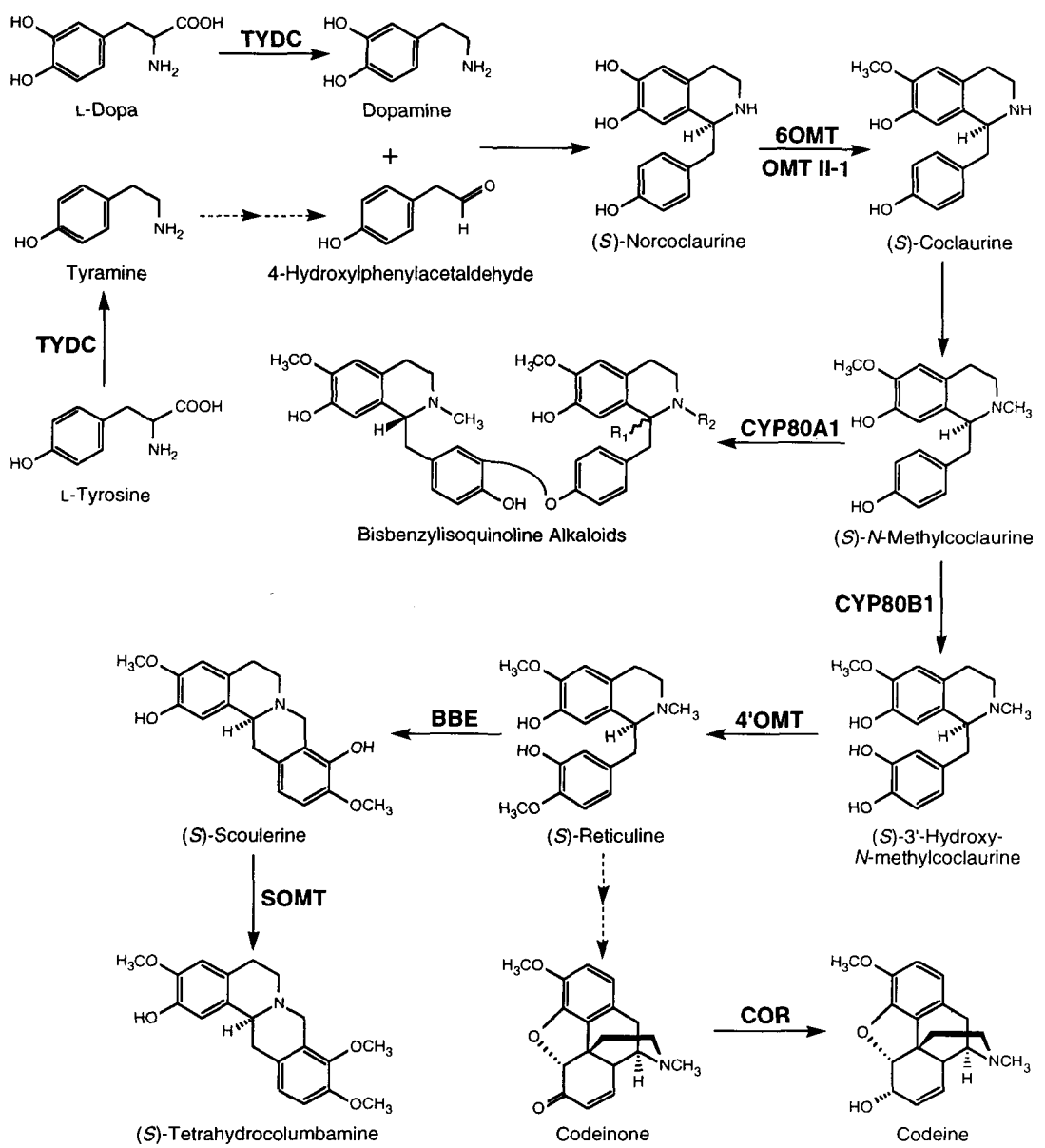


**Figure 1.** Reactions catalyzed by enzymes involved in benzylisoquinoline alkaloid biosynthesis for which the corresponding genes have been cloned. TYDC, tyrosine/dopa decarboxylase; 6OMT, norcoclaurine-6-*O*-methyltransferase; 4' OMT, 3' -hydroxy-N-methylcoclaurine-4' -*O*-methyltransferase; OMT II-1,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

that these alkaloids function as animal feeding deterrents. The antibiotic properties of sanguinarine suggests that it confers protection to the plant against pathogens. Recently, the benzylisoquinoline alkaloids berberine, sanguinarine, and palmatine were specifically shown to confer protection against herbivory and pathogen infection (Schmeller et al. 1997). Many plants, such as opium poppy, invest considerable metabolic resources into the biosynthesis of a diverse collection of benzylisoquinoline alkaloids suggesting that these compounds play essential ecochemical and/or physiological roles that remain to be discovered.

Despite our extensive appreciation for the chemistry and enzymology of several different benzylisoquinoline alkaloid biosynthetic pathways, the molecular mechanisms and biochemical control architecture that regulate metabolic flux through these pathways have only just begun to be understood. Recent applications of molecular techniques to study benzylisoquinoline alkaloid biosynthesis continue to expand the frontiers of our ability to understand and manipulate these pathways in plants. Ultimately, the prospect of engineering benzylisoquinoline alkaloid pathways for the custom biosynthesis of pharmaceuticals will require: (1) the availability of cloned alkaloid biosynthetic genes; (2) the ability to genetically transform alkaloid-producing plants; and (3) a thorough knowledge of the regulation of alkaloid biosynthetic pathways. In this review, we discuss the current state of knowledge regarding the chemistry, enzymology, and regulation of benzylisoquinoline alkaloid biosynthesis in plants. We also summarize recent developments in our own laboratory, especially those related to the metabolic engineering of benzylisoquinoline alkaloid pathways in *Eschscholzia californica* (California poppy).

## Biosynthesis of benzylisoquinoline alkaloids

Benzylisoquinoline alkaloid biosynthesis begins with a series of decarboxylations, *ortho*-hydroxylations, and deaminations that convert tyrosine to both dopamine and 4-hydroxyphenylacetaldehyde (Rueffer and Zenk 1987a). The aromatic L-amino acid decarboxylase (TYDC) which converts tyrosine and dopa to their corresponding amines (Figure 1) has been purified (Marques and Brodelius 1988), and the corresponding cDNAs have been cloned (Facchini and De Luca 1994; Maldonado-Mendoza et al. 1996). In opium poppy, TYDC is encoded by a family of ~15 genes that can be divided into two subgroups based on sequence identity (Facchini and De Luca 1994). Each *TYDC* subfamily shows a distinct developmental and inducible expression pattern, although the catalytic properties of the encoded isoforms are similar (Facchini and De Luca 1994; Facchini et al. 1996a). TYDC mRNAs were shown to be rapidly induced in response to elicitor treatment (Facchini et al. 1996a) in opium poppy cell cultures. The induction of TYDC mRNAs in parsley (Kawalleck et al. 1993) and *Arabidopsis* (Trezzini et al. 1993), which do not produce alkaloids, suggests that tyramine also serves as the precursor to a ubiquitous class of defense-response metabolites. The synthesis and deposition in the cell wall of amides, composed of hydroxycinnamic acid-derivatives and tyramine, was recently shown to be central to the defense-response in many plants (McLusky et al. 1999; Yu and Facchini 1999). Amides and other phenolics are believed to reduce cell wall digestibility and limit the ability of a pathogen to penetrate the cell (Facchini et al. 1999). The dual role of tyramine as a precursor for both benzylisoquinoline alkaloid and hydroxycinnamic acid amide biosynthesis suggests that the *TYDC* gene family in opium poppy encodes TYDC isoforms with diverse metabolic functions.

Dopamine and 4-hydroxyphenylacetaldehyde are condensed by norcoclaurine synthase (NCS) to yield (*S*)-norcoclaurine, which is the central precursor to all benzylisoquinoline alkaloids in plants (Figure 1; Stadler et al. 1987; Stadler et al. 1989). (*S*)-Norcoclaurine is converted to (*S*)-reticuline by a 6-*O*-methyltransferase (Sato et al. 1994; Frick and Kutchan 1999), an *N*-methyltransferase (Frenzel and Zenk 1990a), a P450 hydroxylase (Pauli and Kutchan 1998), and a 4' -*O*-methyltransferase (Frenzel and Zenk 1990b; Sato et al. 1994). The SAM-dependent 6-*O*- and 4' -*O*-methyltransferases (6OMT and 4' OMT, respectively; Figure 1) have recently been purified from cultured *Coptis japonica* cells (Sato et al. 1994), and the corresponding cDNAs isolated (Morishige et al. 2000). Although the two enzymes show similar catalytic properties, they possess distinct substrate specificities. Moreover, the 6OMT catalyzes a ping-pong bi bi reaction, whereas the 4' OMT follows an ordered bi bi mechanism (Morishige et al.

2000). Four homologous *O*-methyltransferase cDNAs (OMT II;1-4) have also been isolated from *Thalictrum tuberosum* cell cultures (Frick and Kutchan 1999). 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 include simple catechols, phenylpropanoids, and various benzylisoquinoline alkaloids suggesting that some of the isoforms are involved in both alkaloid and phenylpropanoid metabolism. For example, the homodimer of OMT II;1 efficiently *O*-methylates (*R,S*)-norcoclaurine (Figure 1), and various catechol and caffeic acid derivatives. In contrast, 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* exhibit only low identity (24 and 35%, respectively) to the various OMT II isoforms (Morishige et al. 2000). The *in vivo* contribution, if any, of the OMT II enzymes to benzylisoquinoline alkaloid biosynthesis remains to be determined.

Originally, the hydroxylation of the aromatic-ring involved in the conversion of (*S*)-norcoclaurine to (*S*)-reticuline was thought to be catalyzed by a nonspecific phenol oxidase (Loeffler and Zenk 1990). However, a P450-dependent monooxygenase (CYP80B1; Figure 1) isolated from California poppy (Pauli and Kutchan, 1998) and opium poppy (Yu and Facchini 2000; Huang and Kutchan 2000) exhibits a $K_m$ for (*S*)-*N*-methylcoclaurine 39-fold lower than that of the phenolase; thus, CYP80B1 is now accepted as the enzyme which converts (*S*)-*N*-methylcoclaurine to (*S*)-3' -hydroxy-N-methylcoclaurine. CYP80B1 appears to be encoded by two to three genes in California poppy (Pauli and Kutchan 1998) and opium poppy (Yu and Facchini 2000; Huang and Kutchan 2000).

Intermediates of the (*S*)-reticuline pathway also serve as the precursors to ~270 bisbenzylisoquinoline alkaloids such as berbamunine and (+)-tubocurarine. A phenol-coupling P450-dependent oxidase berbamunine synthase (CYP80A1) has been purified (Stadler and Zenk 1993), and the corresponding cDNA isolated (Kraus and Kutchan 1995), 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 1). A cytochrome-P450 reductase (CPR) has been purified from opium poppy, and corresponding cDNAs isolated from opium poppy and California poppy (Rosco et al. 1997). The CPR proteins share 69% amino acid identity and occur as two isoforms in each species. Coexpression of California poppy 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 benzylisoquinoline alkaloids. Much research has focused on branch pathways that lead to benzophenanthridine alkaloids, such as sanguinarine and macarpine (Kutchan and Zenk 1993), protoberberine alkaloids, such as berberine and palmatine (Hashimoto and Yamada 1994), and morphinan alkaloids, such as morphine and codeine (Facchini and Bird 1998). Most of the enzymes involved in these pathways have been isolated, many have been purified, and the corresponding cDNAs for three have been reported. 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 1). This conversion is unique in nature and cannot be achieved using synthetic organic chemistry. The enzyme was purified from *Berberis beaniana* (Steffens et al. 1985), the corresponding cDNAs were cloned from California poppy (Dittrich and Kutchan 1991) and *B. stolonifera* (Chou and Kutchan 1998), and *BBE1* genes have been isolated from opium poppy (Facchini et al. 1996b) and California poppy (Hauschild and Kutchan 1998). The phenol coupling reaction catalyzed by heterologous BBE (Kutchan et al. 1994) was identified as an ionic mechanism with a methylene iminium ion as the intermediate (Kutchan and Dittrich 1995).

(*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 (Bauer and Zenk 1989, 1991). (*S*)-Stylopine is *N*-methylated by a substrate-specific methyltransferase, tetrahydroprotoberberine-*cis*-*N*-methyltransferase, that has been isolated from cultured California poppy and *Corydalis vaginans* cells (Rueffer et al. 1990), and purified from *Sanguinaria canadensis* cultures (O' Keefe and

Beecher 1994). The *N*-methylation step is followed by another P450-dependent monooxygenase, (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase (MSH), which leads to the formation of protopine, and has been isolated from *C. vaginans* cultures (Reuffer and Zenk 1987b). Conversion of protopine to sanguinarine involves hydroxylation by a fourth P450-dependent enzyme, protopine-6-hydroxylase (PPH), followed by a spontaneous intramolecular rearrangement to yield dihydrosanguinarine. Dihydrobenzophenanthridine oxidase (DBOX), a cytosolic enzyme originally isolated from California poppy cultures (Schumacher and Zenk 1988) and recently purified from *S. canadensis* cultures (Arakawa et al. 1992) oxidizes dihydrosanguinarine to sanguinarine. Root exudates from many Papaveraceae species, such as *S. canadensis* and California poppy, are intensely red due to the accumulation of sanguinarine and other benzophenanthridine alkaloids. Two novel enzymes, the P450-dependent monooxygenase dihydrochelirubine-12-hydroxylase and the SAM-dependent 12-hydroxydihydrochelirubine-12-*O*-methyltransferase, have been discovered in *Thalictrum bulgaricum* cultures elicited with yeast extract (Kammerer et al. 1994). These enzymes catalyze the last two steps in the biosynthesis of macarpine, the most highly oxidized benzylisoquinoline alkaloid found in nature.

In some plants, especially among the Berberidaceae and Ranunculaceae, (*S*)-scoulerine is methylated, rather than oxidized, to yield (*S*)-tetrahydrocolumbamine (Figure 1). The reaction is catalyzed by the SAM-dependent enzyme, scoulerine-9-*O*-methyltransferase (SOMT), which has been purified from *C. japonica* cells (Sato et al. 1993) and the corresponding cDNA isolated (Takeshita et al. 1995). Heterologous expression of the SOMT cDNA resulted in a protein with a higher molecular weight than the native enzyme (Fujiwara et al. 1993); 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 (Muemmler et al. 1985) and in the lumen of alkaloid-specific vesicles (Galneder et al. 1988). The second to last step in berberine biosynthesis involves the formation of a methylenedioxy bridge (Galneder et al. 1988; Hashimoto and Yamada 1994). 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 (Rueffer and Zenk 1994); 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; Amann et al. 1986). Although these enzymes catalyze the same reaction, their biochemical properties are distinct. STOX from *Berberis* is a flavinylated protein with a broad substrate range, whereas CDO from *Coptis* and *Thalictrum* contains iron, proceeds via a different mechanism, and preferentially accepts (*S*)-canadine (Hashimoto and Yamada 1994).

In some species of the genus *Papaver*, conversion of (*S*)-reticuline to its (*R*)-epimer represents the first committed step in morphinan alkaloid biosynthesis. An NADPH-dependent cytosolic enzyme 1,2-dehydroreticuline reductase has been purified from opium poppy which catalyzes the stereospecific reduction of 1,2-dehydroreticuline to (*R*)-reticuline (De-Eknamkul and Zenk 1992). Subsequent intramolecular carbon-carbon phenol coupling of (*R*)-reticuline by the P450-dependent enzyme salutaridine synthase (STS) results in the formation of salutaridine (Gerardy and Zenk 1993a). The cytosolic enzyme, salutaridine: NADPH 7-oxidoreductase (SOR), found only in opium poppy and *P. bracteatum* reduces salutaridine to (7*S*)-salutaridinol (Gerardy and Zenk 1993b). Transformation of (7*S*)-salutaridinol into thebaine involves closure of an oxide bridge between C-4 and C-5 by acetyl coenzyme A:salutaridinol-7-*O*-acetyltransferase (SAT), which has been purified from opium poppy cell cultures (Lenz and Zenk 1995a). SAT was also detected in *P. bracteatum*.

In the remaining steps 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 (Lenz and Zenk 1995b) and the corresponding cDNA isolated (Unterlinner et al. 1999) from opium poppy (Figure 1). 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 (Frick and Kutchan 1999; Unterlinner et al. 1999). Both COR and 6' -deoxychalcone synthase are members of the aldo/keto reductase family of NADPH-dependent oxidoreductases.

## Subcellular compartmentation

Many alkaloid biosynthetic enzymes occur in subcellular compartments other than the cytosol in order to effectively sequester toxic alkaloids and their biosynthetic intermediates to appropriate locations in the cell. 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*.

Several benzylisoquinoline alkaloid biosynthetic enzymes occur in subcellular locations other than the cytosol. Of the five non-cytosolic 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 $mL^{-1}$ (Amann et al. 1986; Rueffer and Zenk 1987b; Bauer and Zenk 1989, 1991), while one (PPH) is associated with a membrane fraction with a density consistent with that of the ER ($\delta$ = 1.11 g $mL^{-1}$; Tanahashi and Zenk 1990). 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 $mL^{-1}$ (Gerardy and Zenk 1993a). With the exception of BBE, all of these non-cytosolic enzymes are P450-dependent (Blechert et al. 1995); 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 (Amann et al. 1986; Galneder 1988). A putative N-terminal signal peptide was detected in the deduced amino acid sequence of BBE from California poppy (Dittrich and Kutchan 1991). 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 (Amann et al. 1986), CDO (Galneder et al. 1988), and columbamine *O*-methyltransferase (Rueffer et al. 1986). The association of these enzymes with a membrane fraction of greater density than the ER has led to

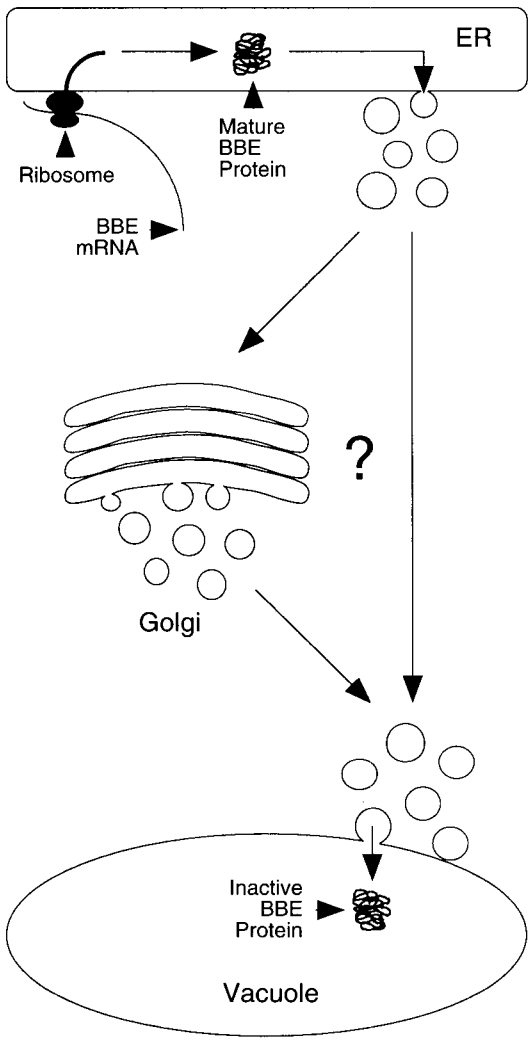


**Figure 2.** Schematic model showing possible trafficking pathways for BBE in sanguinarine-producing cells. The nascent BBE protein is targeted to the ER via the signal peptide, and co-translated into the ER lumen. The signal peptide is cleaved, and mature BBE is transported from the ER as a lumenal component of specific vesicles destined for the vacuole. BBE should be 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 sanguinarine, are translocated directly from the ER to the vacuole. Although a role for the Golgi in BBE sorting cannot be ruled out, a Golgi-dependent model would require an separate mechanism to sequester sanguinarine to the vacuole.

speculation that distinct 'alkaloid synthesizing vesicles' are found in certain cell types (Amann et al. 1986). 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 (Amman et al. 1986). 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 and P. Facchini, unpublished results). Using the green fluorescent protein as a reporter, the nascent BBE protein was shown to be targeted to the ER lumen via the signal peptide. Subsequently, mature BBE is transported from the ER, possibly as a lumenal component of specific 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 as shown in figure 2.

## Developmental and environmental regulation

The developmental biology of alkaloid biosynthesis in plants has proven to be remarkably complex. Research on the developmental aspects of monoterpenoid indole alkaloid and tropane alkaloid biosynthesis has revealed the involvement of several distinct cell types and the intercellular translocation of pathway intermediates (De Luca and St-Pierre 2000). The developmental biology of benzylisoquinoline alkaloid biosynthesis has not been investigated in as much detail, but recent studies also suggest distinct sites of biosynthesis and product accumulation. The relative levels of TYDC, CYP80B1, and BBE mRNAs in various opium poppy organs are shown in figure 3. 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 (Figure 3; Facchini and De Luca 1995). Metaphloem is closely associated with laticifers in opium poppy (Facchini and Bird 1998); thus, detection of TYDC mRNAs in metaphloem tissues supports the association of benzylisoquinoline alkaloid biosynthesis with cells related to laticifers. NCS, which catalyzes the first committed step in the pathway, is also most active in roots and stems of opium poppy (N. Samanani and P. Facchini, unpublished results). CYP80B1 transcripts are most abundant in stems followed by roots, leaves, and floral tissues (Figure 3; Huang and Kutchan 2000; Yu and Facchini 2000). The activities of STS and SOR, which convert (*R*)-reticuline to salutaridinol, also occur abundantly in roots and shoots (Gerardy and Zenk 1993a, 1993b). In contrast, COR, which catalyzes the penultimate step in morphine biosynthesis, is present throughout the plant, but is most abundant in shoot organs (Unterlinner et al. 1999; Huang and Kutchan 2000). These results suggest that pathway intermediates are translocated between the roots and various shoot organs. Originally, morphine biosynthesis was thought to occur in laticifers (reviewed by Facchini and Bird 1998). However, neither STS nor SOR were detected in a fresh latex fraction from opium poppy capsules

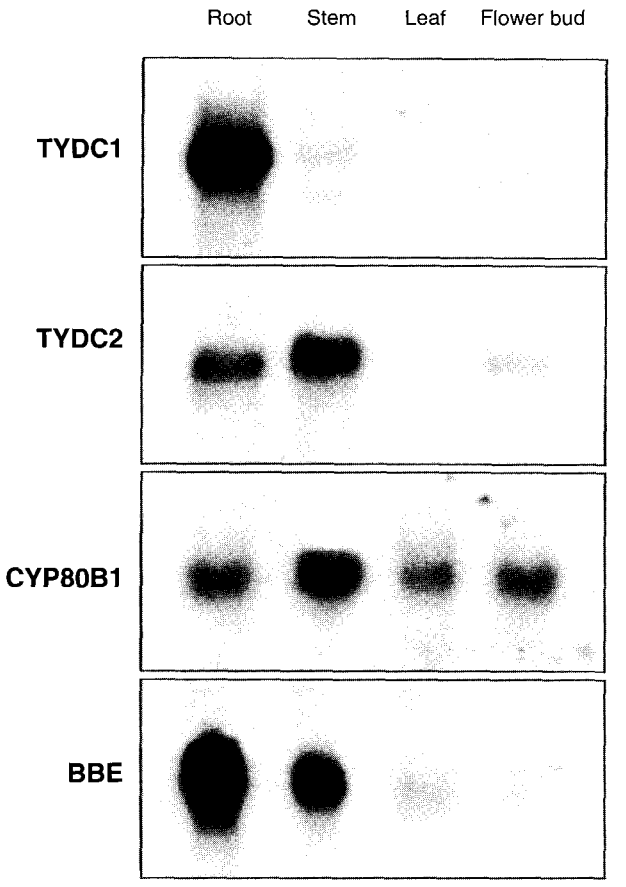


**Figure 3**. Northern blot hybridization analysis for TYDC1, TYDC2, CYP80B1, and BBE mRNAs in various organs of mature opium poppy plants. Fifteen µg of total RNA was fractionated on 1.0% formaldehyde agarose gels, transferred to nylon membranes, and hybridized at high stringency with $^{32}$P-labeled full-length probes prepared from cDNAs for each gene. Gels were stained with ethidium bromide prior to blotting to ensure equal loading.

(Gerardy and Zenk 1993a, 1993b) 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 (Gerardy and Zenk 1993a, 1993b; Lenz and Zenk 1995a, 1995b).

The root-specific accumulation of sanguinarine in opium poppy (Facchini and De Luca 1995) suggests that the enzymes involved in its biosynthesis occur only in roots. However, the substantial level of BBE mRNAs (Facchini et al. 1996b) and enzyme activity (Steffens et al. 1985) in shoot organs suggests that pathway intermediates involved in sanguinarine biosynthesis are translocated from shoots to roots (Figure 3). Similarly, although berberine accumulates in the primary roots of *Coptis japonica*, the low levels of SOMT activity and protein suggest that the primary root is not the main site of berberine biosynthesis (Fujiwara et al. 1993). Indeed, the highest levels of SOMT are found in lateral roots and stems.

Cell cultures of many Papaveraceae species also accumulate benzophenanthridine alkaloids, such as macarpine and/or sanguinarine, in response to treatment with fungal elicitors (Eilert et al. 1985; Schumacher et al. 1987; Mahady and Beecher 1994). In general, the membrane-associated biosynthetic enzymes are induced by elicitors, whereas most cytosolic enzymes are not (Blechert et al. 1995). The induction of TYDC, CYP80B1, and BBE mRNAs in opium poppy cell cultures treated with a fungal elicitor are shown in figure 4. Several members of the *TYDC* gene family are rapidly and transiently expressed in opium poppy cell cultures in response to elicitor treatment (Figure 4; Facchini et al. 1996a; Facchini et al. 1998). CYP80B1 transcript levels are induced more than 20-fold in MeJA-treated California poppy cells (Pauli and Kutchan 1998) and fungal elicitor-treated opium poppy cells (Figure 4; Huang and Kutchan 2000; Yu and Facchini 2000). BBE was also shown to be transcriptionally activated in California poppy cells treated with a yeast elicitor (Dittrich and Kutchan, 1991) or MeJA (Kutchan and Zenk 1993; Blechert et al. 1995), and opium poppy cells treated with a fungal elicitor (Figure 4; Facchini et al. 1996b). The elicitor-mediated induction of other P450-dependent enzymes (CFS, SPS, MSH and PPH) in the sanguinarine pathway has also been observed (Tanahashi and Zenk 1990; Bauer and Zenk 1991; Blechert et al. 1995). Moreover, DBOX activity was reported to increase 4- and 14-fold in *S. canadensis* cells treated with MeJA or acetylsalicylic acid, respectively (Ignatov et al. 1996).

The induction of benzophenanthridine alkaloid biosynthesis in California poppy 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 (Roos et al. 1998). The production of phenolic compounds can be selectively blocked by catalase at

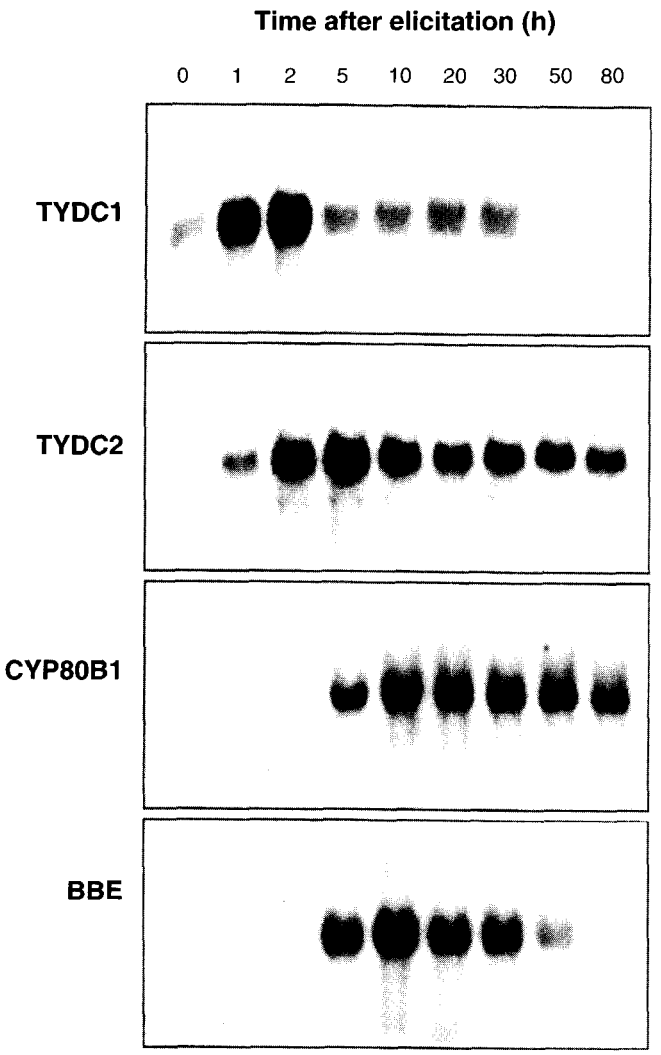


**Figure 4.** Northern blot hybridization analysis for TYDC1, TYDC2, CYP80B1, and BBE mRNAs in opium poppy cell suspension cultures treated with a *Botrytis* sp. elicitor preparation. Fifteen µg of total RNA was fractionated on 1.0% formaldehyde agarose gels, transferred to nylon membranes, and hybridized at high stringency with $^{32}$P-labeled full-length probes prepared from cDNAs for each gene. Gels were stained with ethidium bromide prior to blotting to ensure equal loading.

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 (Facchini et al. 1996a). The elicitor-mediated induction of benzophenanthridine alkaloid biosynthesis was shown to require a transient decrease in cytosolic pH caused by an efflux of protons from the vacuole (Roos et al. 1998). Artificial acidification of the cytosol has been found 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, has been identified as a possible trigger for the signal transduction pathway leading to the efflux of vacuolar protons (Roos et al. 1999). 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 (Mahady et al. 1998). The induction of alkaloid biosynthesis also appears to depend on the presence of external $Ca^{2+}$, suggesting that $Ca^{2+}$ participates in the signal transduction pathway (Mahady and Beecher 1994).

A loss-of-function assay revealed the location of regulatory domains involved in the activity of *TYDC7* and *BBE1* promoters in a transient ß-glucuronidase (GUS) expression system based on the microprojectile bombardment of cultured opium poppy cells (Park et al. 1999). The -393 to -287 region of the *TYDC7* promoter, and the -355 to -200 region of the *BBE1* promoter, were shown to be important for promoter function (Figure 5). Time-courses for the induction of TYDC7 and BBE1 mRNAs in wounded opium poppy cells were almost 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 suggests that the wound signal caused by the entry of DNA-coated microcarriers into opium poppy cells induced wound-responsive regulatory elements located from -393 to -287 in *TYDC7* and -355 to -200 in *BBE1*. Functional analysis of the *BBE1* promoter from California poppy showed that the region from -496 to -455 is necessary for activity (Hauschild et al. 1998). Comparison of this region to the -355 to -200 region from opium poppy *BBE1* revealed a sequence with 55% nucleotide identity (Park et al. 1999).

## Genetic transformation

Procedures have recently been developed in our laboratory for the stable genetic transformation of opium poppy plants (Park and Facchini 2000b) and root cultures (Park and Facchini 2000c), and California poppy plants (Park and Facchini 2000a) and root cultures

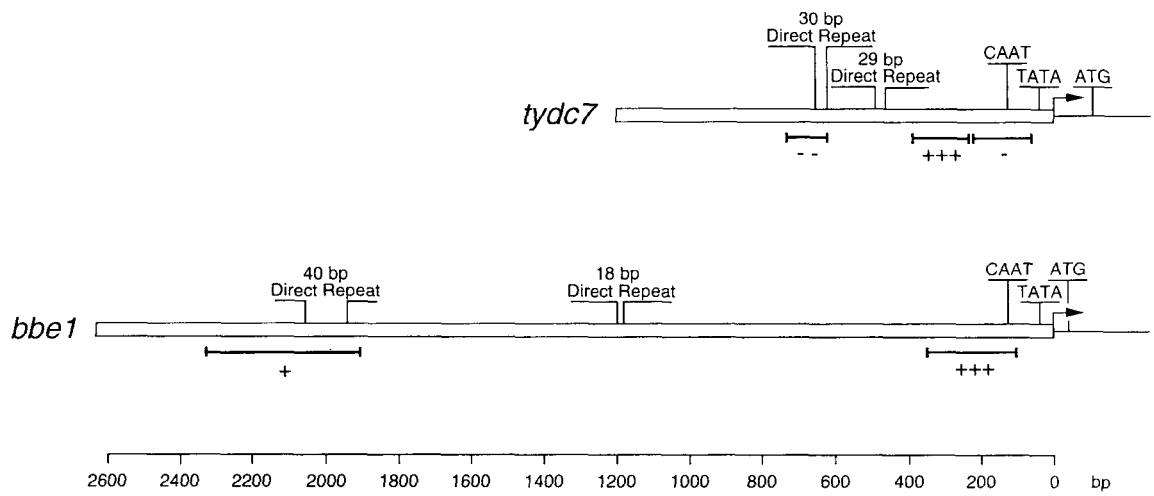


**Figure 5.** Regions in the opium poppy *tydc7* and *bbe1* promoters containing wound-responsive *cis*-elements. The open box represents the promoter region of each gene. Plus and minus signs represent the relative influence of the indicated regions on promoter activity in wound-induced opium poppy cell cultures. The locations of direct sequence repeats, the transcription start site (arrow), putative CAAT and TATA boxes, and the translation start codon (ATG) are shown in each gene.

(Park and Facchini 2000c). We have also established protocols for the transformation of *Chelidonium majus* and *Thalictrum rugosum* (S.-U. Park, N. Samanani, and P. Facchini, unpublished results). These protocols represent the first stable genetic transformation and regeneration methods to be reported for members of the Papaveraceae and Ranunculaceae. The *Agrobacterium*-mediated transformation of opium poppy cell cultures has also been reported (Belny et al. 1997).

An efficient *Agrobacterium*-mediated protocol for the stable genetic transformation and regeneration of California poppy plants via somatic embryogenesis was established using *A. tumefaciens* strain GV3101 (Park and Facchini 2000a). Excised cotyledons were co-cultivated with A. *tumefaciens* and cultured on selection media containing 50 mg $L^{-1}$ paromomycin. Four to five weeks after infection, paromomycin-resistant callus grew on the explants in the presence of 2.0 mg $L^{-1}$ 1-naphthaleneacetic acid (NAA) and 0.1 mg $L^{-1}$ 6-benzylaminopurine (BAP). Calli were cultured on somatic embryogenesis induction medium containing 1.0 mg $L^{-1}$ NAA and 0.5 mg $L^{-1}$ BAP, and somatic embryos were visible on the paromomycin-resistant calli within 3 to 4 weeks. Somatic embryos converted to paromomycin-resistant plants three to four weeks after they were transferred to a phytohormone-free plant regeneration medium. Stable transformation of the regenerated plants was confirmed by the detection of the neomycin phosphotransferase (NPTII) gene, the high levels of GUS mRNA and enzyme activity, and the cytohistochemical localization of GUS activity in all plant tissues. The normal alkaloid profile of California poppy was unaffected by the transformation process.

An efficient *Agrobacterium*-mediated protocol has also been developed for the stable genetic transformation of opium poppy plants via shoot organogenesis (Park and Facchini 2000b). Excised cotyledons were cocultivated with *A. tumefaciens* strain GV3101, and incubated on shoot induction medium consisting of B5 salts and vitamins, 30 g $L^{-1}$ sucrose, 2 mg $L^{-1}$ 6-benzylaminopurine, 5 mg $L^{-1}$ $AgNO_3$, and 3 g $L^{-1}$ Gelrite. Eight-week-old shoots growing in the presence of 30 mg $L^{-1}$ paromomycin were transferred to root induction medium consisting of B5 salts and vitamins, 0.5 mg $L^{-1}$ indole-3-acetic acid, 0.5 mg $L^{-1}$ indole-3-butyric acid, and either 5 mg $L^{-1}$ $AgNO_3$ or 40 mg $L^{-1}$ putrescine. As with California poppy, the genetic transformation of the regenerated plants was confirmed by the detection of the NPTII gene, the high levels of GUS mRNA and enzyme activity, and the cytohistochemical localization of GUS activity in all organs. Moreover, the transformation process did not alter the alkaloid content of opium poppy.

We have also developed a protocol for the establishment of transgenic root cultures using *Agrobacterium rhizogenes* (Park and Facchini 2000c). Five strains of *A. rhizogenes* were tested for their ability to produce hairy roots on wounded opium poppy seedlings and California poppy embryogenic calli. Three of the strains induced hairy root formation on both species. To further characterize the putative transgenic roots, explant tissues were co-cultivated with the most effective *A. rhizogenes* strain (R1000). Transgenic roots were selected using 50 mg $L^{-1}$ paromomycin. Four weeks after infection, paromomycin-resistant roots appeared on explants maintained on hormone-free medium. Isolated hairy roots were propagated in liquid medium containing 1.0 mg $L^{-1}$ indole-3-acetic acid to promote rapid growth. Again detection of the NPTII gene, high levels of GUS transcripts and enzyme activity, and GUS histochemical localization confirmed the stable transformation of root cultures. Transgenic roots grew faster than wild type roots, and California poppy roots grew more rapidly than those of opium poppy. Transformed roots of both species displayed anatomical features and benzylisoquinoline alkaloid profiles that were virtually identical to those of wild type roots, with the exception of a less compact arrangement of epidermal cells and more root hairs. These transgenic root cultures are a simple, rapid, and well-defined model system to investigate the molecular and metabolic regulation of benzylisoquinoline alkaloid biosynthesis, and to evaluate the genetic engineering potential of benzylisoquinoline alkaloid-producing plants.

## Metabolic engineering

Metabolic engineering is broadly defined as the improvement of cellular activities by the manipulation of enzymatic, transport, or regulatory functions using recombinant DNA technology. The genetic transformation systems we have developed provide an unprecedented opportunity to alter the activity of individual

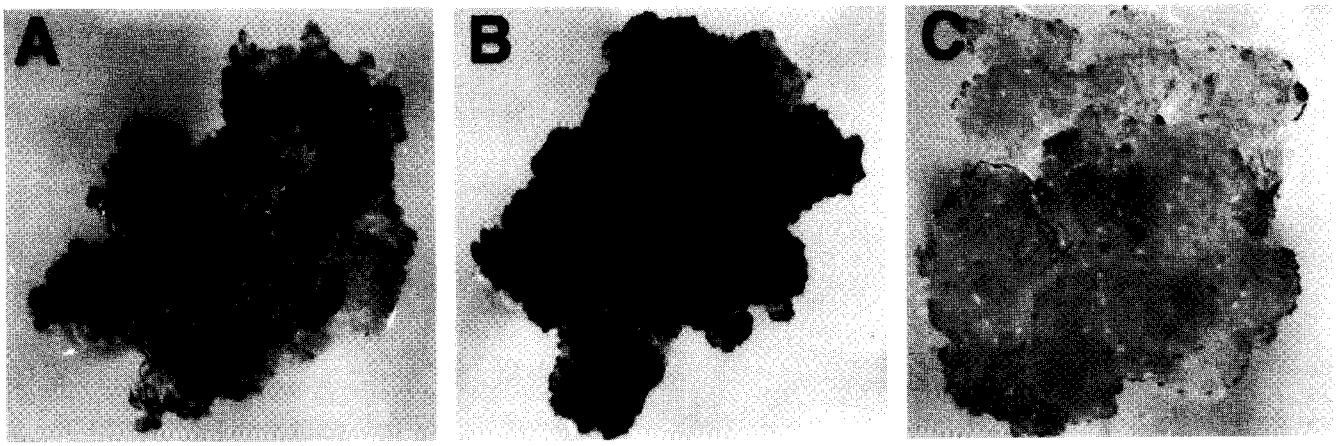


**Figure 6.** California poppy callus cultures transformed with (A) CaMV 35S::GUS; (B) CaMV 35S::BBE; and (C) CaMV 35S::antisense-BBE. Cultures were maintained on an agar-solidified medium consisting of B5 salts and vitamins, 30 g $L^{-1}$ sucrose, and 1 mg $L^{-1}$ 2,4-dichlorophenoxyacetic acid.

enzymes of benzylisoquinoline alkaloid biosynthesis, and to examine the consequences of such modifications on the accumulation of end-products and pathway intermediates. In figure 6, we present preliminary results from our initial attempts to metabolically engineer benzylisoquinoline alkaloid biosynthesis in transgenic plant tissues. We have transformed California poppy plants and cell cultures with *A. tumefaciens* strain GV3101 containing the opium poppy BBE cDNA in the sense orientation, or the California poppy BBE cDNA in the antisense orientation (S.U. Park and P. Facchini, unpublished results). In both cases, the transgene was driven by the constitutive cauliflower mosaic virus 35S promoter.

Our preliminary results in California poppy cell cultures show that cell lines transformed with constitutively expressed sense-BBE from opium poppy display an intense red-brown colour compared to control cultures transformed with a 35S::GUS contruct (Figure 6). In contrast, cell lines transformed with constitutively expressed antisense-BBE from California poppy show virtually a complete loss of red-brown colour (Figure 6). The benzophenanthridine alkaloids that accumulate in California poppy are typically orange to red in colour; thus, our observations suggest that cell lines transformed with the sense-BBE construct accumulate more of these alkaloids, whereas cell lines transformed with antisense-BBE accumulate little, if any, of the normal profile of benzophenanthridine alkaloids. Indeed, the $A_{280}$ of methanol extracts from these cell cultures (which can be used as a crude estimate of alkaloid content) was typically 40% higher for cell lines transformed with the sense-BBE construct, and 30% lower for cell lines transformed with the antisense-BBE construct, relative to control lines transformed with 35S::GUS (S.-U. Park and P. Facchini, unpublished results). Our continuing research is focused on the development and characterization of this, and other, genetically-mediated metabolic manipulations of benzylisoquinoline alkaloid pathways in a variety of plant species.

## Future directions

Our understanding of the biological processes that allow the synthesis and accumulation of benzylisoquinoline alkaloids in plants has improved considerably over the last decade. This rapid progress has been facilitated by the availability of an impressive collection of biosynthetic genes. We have learned that benzylisoquinoline alkaloid biosynthesis is more than a metabolic curiosity. Benzylisoquinoline alkaloid pathways are highly regulated and involve numerous cell-, tissue-, development-, and environment-specific controls. The expansion of our molecular toolbox will promote efforts to better comprehend the features associated with the development of cell types that can accommodate benzylisoquinoline alkaloid pathways. The expanding frontiers of biochemistry, molecular biology, and cell biology as they are applied to benzylisoquinoline alkaloid biosynthesis will also lead to exciting opportunities to metabolically engineer alkaloid pathways in transgenic plants.

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