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Opium poppy: blueprint for an alkaloid factory

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Abstract Opium poppy (*Papaver somniferum*) produces a large number of benzylisoquinoline alkaloids, including the narcotic analgesics morphine and codeine, and has emerged as one of the most versatile model systems to study alkaloid metabolism in plants. As summarized in this review, we have taken a holistic strategy—involving biochemical, cellular, molecular genetic, genomic, and metabolomic approaches—to draft a blueprint of the fundamental biological platforms required for an opium poppy cell to function as an alkaloid factory. The capacity to synthesize and store alkaloids requires the cooperation of three phloem cell types—companion cells, sieve elements, and laticifers—in the plant, but also occurs in dedifferentiated cell cultures. We have assembled an opium poppy expressed sequence tag (EST) database based on the attempted sequencing of more than 30,000 cDNAs from elicitor-treated cell culture, stem, and root libraries. Approximately 23,000 of the elicitor-induced cell culture and stem ESTs are represented on a DNA microarray, which has been used to examine changes in transcript profile in cultured cells in response to elicitor treatment, and in plants with different alkaloid profiles. Fourier transform-ion cyclotron resonance mass spectrometry and proton nuclear magnetic resonance mass spectroscopy are being used to detect corresponding differences in metabolite profiles. Several new genes involved in the biosynthesis and regulation of alkaloid pathways in opium poppy have been identified using genomic tools. A biological blueprint for alkaloid production coupled with the emergence of reliable transformation protocols has created an unprecedented opportunity to alter the chemical profile of the world's most valuable medicinal plant.

Keywords Benzylisoquinoline alkaloid biosynthesis · Cell and tissue culture · Cellular and subcellular localization · Expressed sequence tags · Functional genomics · Gene regulation · Genetic transformation · Metabolomics · DNA microarray

Abbreviations

4′OMT	(*R,S*)-3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase
6OMT	(*R,S*)-norcoclaurine 6-*O*-methyltransferase
7OMT	(*R,S*)-reticuline 7-*O*-methyltransferase
BBE	Berberine bridge enzyme

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BIA	Benzylisoquinoline alkaloid
CDO	(*S*)-canadine oxidase
CFS	(*S*)-cheilanthifoline synthase
CNMT	(*R,S*)-coclaurine *N*-methyltransferase
COR	Codeinone reductase
CYP719A1	(*S*)-canadine synthase
CYP80B1	(*S*)-*N*-methylcoclaurine 3′-hydroxylase
DBOX	Dihydrobenzophenanthridine oxidase
DRS	1,2-Dehydroreticuline synthase
DRR	1,2-Dehydroreticuline reductase
dsRNA	Double-stranded RNA
EMS	Ethylmethane sulfonate
ER	Endoplasmic reticulum
EST	Expressed sequence tag
FT-ICR MS	Fourier transform-ion cyclotron resonance mass spectrometry
GST	Glutathione *S*-transferase
H^1-NMR	Proton nuclear magnetic resonance mass spectroscopy
HSS	Homospermidine synthase
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MSH	(*S*)-*N*-methylstylopine 14-hydroxylase
NCS	(*S*)-norcoclaurine synthase
PCR	Polymerase chain reaction
P6H	Protopine 6-hydroxylase
PNMT	Putrescine *N*-methyltransferase
PTGS	Post-transcriptional gene silencing
RNAi	RNA interference
SAM	(*S*)-adenosylmethionine
SAT	Salutaridinol-7-*O*-acetyltransferase
SER	Sieve element reticulum
siRNA	RNA
SOMT	(*S*)-scoulerine 9-*O*-methyltransferase
SOR	Salutaridine:NADPH 7-oxidoreductase
SPS	(*S*)-stylopine synthase
STOX	(*S*)-tetrahydroprotoberberine oxidase
STR	Strictosidine synthase
STS	Salutaridine synthase
TDC	Tryptophan decarboxylase
TILLING	Targeting induced local lesions in genomes
TYDC	Tyrosine decarboxylase
TNMT	(*S*)-tetrahydroprotoberberine-*cis*-*N*-methyltransferase
TRV	Tobacco rattle virus
VIGS	Viral-induced gene silencing

Introduction

Alkaloids are a diverse group of nitrogen-containing secondary metabolites found in about 20% of plant species. Unlike other natural product categories, the different structural types of plant alkaloids emerged independently as the products of several unique biosynthetic pathways. Most biochemical research has focused on six groups—the benzylisoquinoline, monoterpenoid indole, tropane, purine, pyrrolizidine, and quinolizidine alkaloids (Facchini 2001). Due to their potent biological activity, many of the ~12,000 known plant alkaloids have long been exploited as pharmaceuticals, stimulants, narcotics, and poisons. In particular, the pharmacological attributes and socio-economic importance of several benzylisoquinoline alkaloids (BIAs) from opium poppy (*Papaver somniferum*) have been appreciated since before the dawn of civilization. The plant remains the only commercial source for the narcotic analgesics morphine, codeine, and semi-synthetic derivatives of thebaine, such as oxycodone.

The BIA class is composed of more than 2,500 compounds including morphine, and the antimicrobial agents sanguinarine and berberine. Morphine has been implicated in the stress-induced cross-linking of galacturonic-containing polysaccharides in cell walls of opium poppy (Morimoto et al. 2001), whereas sanguinarine and berberine are toxic to some herbivores and microbial plant pathogens and proposed to function as defense compound in certain members of a restricted number of plant families including the Papaveraceae, Ranunculaceae, and Berberidaceae (Schmeller et al. 1997). In opium poppy, morphine is the

major alkaloid in the latex, which is the multinucleate cytoplasm of the articulated laticifers in opium poppy, but sanguinarine is most abundant in roots (Facchini and De Luca 1995). However, only sanguinarine has been detected in dedifferentiated opium poppy cell cultures after treatment with a fungal elicitor (Facchini et al. 1996a), suggesting the absence of a morphine-specific biosynthetic enzyme or the requirement for a degree of cellular specialization to facilitate metabolic flux through the morphine branch pathway.

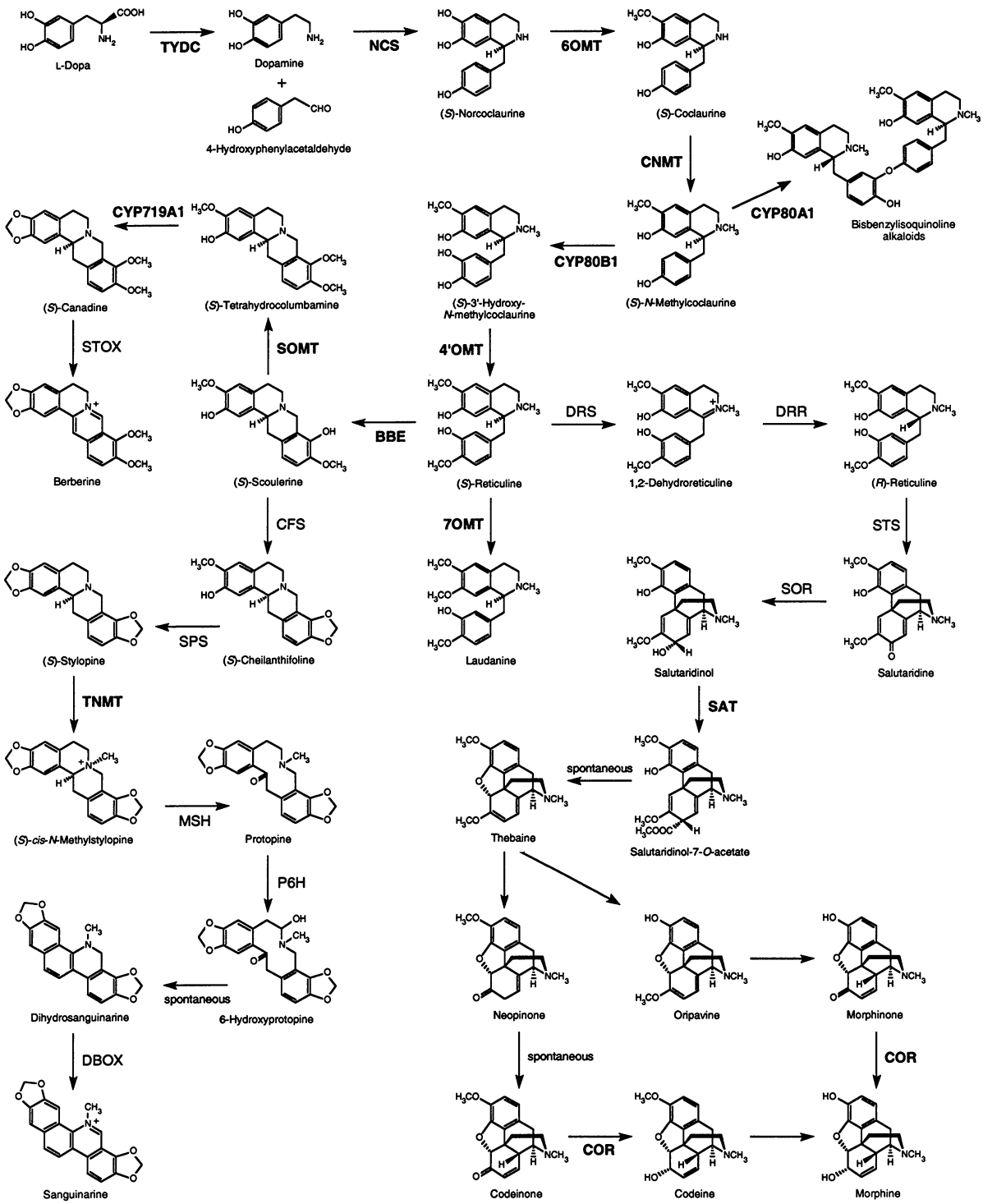
In this review, we summarize our holistic strategy—involving biochemical, cellular, molecular genetic, genomic, and metabolomic approaches—to draft a blueprint of the fundamental biological platforms required for an opium poppy cell to function as an alkaloid factory.

Fig. 1 Benzylisoquinoline alkaloid pathways leading to berberine, morphine, and sanguinarine. Enzymes for which corresponding cDNAs have been isolated are shown in bold. Abbreviations: 4′OMT, 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase; 6OMT, norcoclaurine 6-*O*-methyltransferase; 7OMT, reticuline 7-*O*-methyltransferase; BBE, berberine bridge enzyme; CFS, cheilanthifoline synthase; CNMT, coclaurine *N*-methyltransferase; COR, codeinone reductase; CYP719A1, canadine synthase; CYP80A1, berbamunine synthase; CYP80B1, *N*-methylcoclaurine 3′-hydroxylase; DBOX, dihydrobenzophenanthridine oxidase; DRR, 1,2-dehydroreticuline reductase; DRS, 1,2-dehydroreticuline synthase; MSH, *N*-methylstylopine 14-hydroxylase; NCS, norcoclaurine synthase; P6H, protopine 6-hydroxylase; SAT, salutaridinol 7-*O*-acetyltransferase; SOMT, scoulerine 9-O-methyltransferase; SOR, salutaridine:NADPH 7-oxidoreductase; SPS, stylopine synthase; STOX, (*S*)-tetrahydroxyprotoberberine oxidase; STS, salutaridine synthase; TNMT, tetrahydroprotoberberine *cis*-*N*-methyltransferase; TYDC, tyrosine decarboxylase

Benzylisoquinoline alkaloid biosynthesis

BIA biosynthesis begins with the conversion of tyrosine to both dopamine and 4-hydroxyphenylacetaldehyde by a lattice of decarboxylations, *ortho*-hydroxylations, and deaminations (Fig. 1) (Rueffer and Zenk 1987a). The aromatic amino acid decarboxylase (TYDC) that converts tyrosine and dihydroxyphenylalanine to their corresponding amines has been purified, and several cDNAs have been cloned (Facchini and De Luca 1994; Facchini et al. 2000). Dopamine and 4-hydroxyphenylacetaldehyde are condensed by norcoclaurine synthase (NCS) to yield (*S*)-norcoclaurine, the central precursor to all BIAs in plants (Stadler et al. 1987, 1989). NCS has been purified (Samanani and Facchini 2001, 2002) and corresponding cDNAs isolated from *Thalictrum flavum* (Samanani et al. 2004) and opium poppy (Liscombe et al. 2005). (*S*)-Norcoclaurine is converted to (*S*)-reticuline by a 6-*O*-methyltransferase (6OMT), a *N*-methyltransferase (CNMT), a P450 hydroxylase (CYP80B1), and a 4′-*O*-methyltransferase (4′OMT) (Fig. 1) (Frenzel and Zenk 1990; Sato et al. 1993; Pauli and Kutchan 1998). 6OMT and 4′OMT were purified (Sato et al. 1993), and corresponding cDNAs isolated and characterized from *Coptis japonica* (Morishige et al. 2000), *T. flavum* (Samanani et al. 2005), and opium poppy (Facchini and Park 2003; Ounaroon et al. 2003; Ziegler et al. 2005). CNMT has also been purified (Choi et al. 2001), and corresponding cDNAs isolated from *C. japonica* (Choi et al. 2002), *T. flavum* (Samanani et al. 2005), and opium poppy (Facchini and Park 2003). The aromatic ring hydroxylation involved in the conversion of (*S*)-norcoclaurine to (*S*)-reticuline is catalyzed by a P450-dependent monooxygenase (CYP80B1), which converts (*S*)-*N*-methylcoclaurine to (*S*)-3′-hydroxy-*N*-methylcoclaurine (Fig. 1) (Pauli and Kutchan 1998). cDNAs encoding CYP80B1 have been isolated from California poppy (*Eschscholzia californica*) (Pauli and Kutchan 1998), opium poppy (Huang and Kutchan 2000), and *T. flavum* (Samanani et al. 2005).

(*S*)-Reticuline pathway intermediates also serve as precursors to dimeric BIAs (Fig. 1). A phenol-coupling P450-dependent oxidase (CYP80A1) was purified, and the corresponding cDNA isolated from *Berberis stolonifera* (Stadler and Zenk 1993; Kraus and Kutchan 1995). CYP80A1 couples two molecules of (*R*)-*N*-methylcoclaurine or one each of (*R*)-and (*S*)-*N*-methylcoclaurine to form (*R,R*)-guattegaumerine or (*R,S*)-berbamunine, respectively. Phenyl ring substitutions, regiospecificity, the number of ether linkages, and monomer stereospecificity add



additional diversity to the bisbenzylisoquinoline alkaloids. A cytochrome P450 reductase (CPR) was purified from opium poppy, and corresponding cDNAs isolated from opium poppy and *E. californica* (Rosco et al. 1997). CPR is a co-enzyme of all P450-dependent enzymes.

(*S*)-Reticuline is a branch-point intermediate in the biosynthesis of most BIAs and many substituted derivatives are produced. For example, a cDNA encoding (*R,S*)-reticuline 7-*O*-methyltransferase (7OMT), which catalyzes the conversion of (*R,S*)-reticuline to laudanine, has been identified (Ounaroon et al. 2003). Most work has focused on branch pathways leading to the benzophenanthridine (e.g., sanguinarine), protoberberine (e.g., berberine), and morphinan (e.g., morphine and codeine) alkaloids (Facchini 2001). A multitude of relevant enzymes have been isolated, many have been purified, and several corresponding cDNAs have been cloned (Facchini 2001). The first committed step in benzophenanthridine and protoberberine alkaloid biosynthesis involves the conversion of (*S*)-reticuline to (*S*)-scoulerine by the berberine bridge enzyme (BBE) (Fig. 1). BBE was purified from *Berberis beaniana* (Steffens et al. 1985), corresponding cDNAs were cloned from *B. stolonifera*, *E. californica*, and *T. flavum* (Dittrich and Kutchan 1991; Chou and Kutchan 1998; Samanani et al. 2005), the recombinant enzyme was characterized (Kutchan et al. 1994; Kutchan and Dittrich 1995), and *BBE* genes were isolated from opium poppy and *E. californica* (Facchini et al. 1996b; Hauschild et al. 1998).

Benzophenanthridine alkaloid biosynthesis requires the conversion of (*S*)-scoulerine to (*S*)-stylopine by two P450-dependent oxidases, (*S*)-chelanthifoline synthase (CFS) and (*S*)-stylopine synthase (SPS), resulting in the formation of two methylenedioxy bridges (Bauer and Zenk 1989, 1991), (*S*)-Stylopine is *N*-methylated by tetrahydroprotoberberine-*cis*-*N*-methyltransfe rase (Rueffer and Zenk 1986), which has been isolated from *E. californica* and *Corydalis vaginans* (Rueffer et al. 1990), and purified from *Sanguinaria canadensis* cell cultures (O'Keefe and Beecher 1994). A P450-dependent monooxygenase, (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase (MSH), then catalyzes the formation of protopine (Rueffer and Zenk 1987b). Another P450-dependent enzyme, protopine-6-hydroxylase (P6H) followed by a spontaneous intramolecular rearrangement convert protopine to dihydrosanguinarine (Tanahashi and Zenk 1990). The oxidation of dihydrosanguinarine to sanguinarine occurs via dihydrobenzophenanthridine oxidase (DBOX) (Schumacher and Zenk 1988), a cytosolic enzyme purified from *S. canadensis* cultures (Arakawa et al. 1992). Two other enzymes, dihydrochelirubine-12-hydroxylase and 12-hydroxydihydrochelirubine-12-*O*-methyltransferase, catalyze the final two steps in the biosynthesis of macarpine, the most highly oxidized BIA found in nature (Kammerer et al. 1994).

In some plants, (*S*)-scoulerine is methylated, rather than oxidized, to yield (*S*)-tetrahydrocolumbamine (Fig. 1). The reaction is catalyzed by scoulerine 9-*O*-methyltransferase (SOMT) (Muemmler et al. 1985), which was purified and the corresponding cDNA isolated from *C. japonica* (Sato et al. 1994; Takeshita et al. 1995) and *T. flavum* (Samanani et al. 2005). The P450-dependent enzyme canadine synthase (CYP719A) was detected in members of the genera *Coptis* and *Thalictrum* and shown to catalyze methylenedioxy bridge formation in (*S*)-tetrahydrocolumbamine (Galneder et al. 1988). cDNA clones for CYP719A1 have been isolated from *C. japonica* (Ikezawa et al. 2003) and *T. flavum* (Samanani et al. 2005). (*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).

Conversion of (*S*)-reticuline to its (*R*)-epimer is the first committed step in morphinan alkaloid biosynthesis in certain species. The still poorly characterized enzymes 1,2-dehydroreticuline synthase and 1,2-dehydroreticuline reductase catalyze the stereospecific reduction of 1,2-dehydroreticuline to (*R*)-reticuline (De-Eknamkul and Zenk 1992; Hirata et al. 2004). 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 in opium poppy and *Papaver bracteatum* reduces salutaridine to (7*S*)-salutaridinol (Gerardy and Zenk 1993b). Conversion of (7*S*)-salutaridinol into thebaine is catalyzed by acetyl coenzyme A:salutaridinol-7-*O*-acetyltransferase (SAT). The enzyme was purified from opium poppy cell cultures and the corresponding

cDNA was isolated (Fig. 1) (Lenz and Zenk 1995a; Grothe et al. 2001). In the last steps of morphine biosynthesis, codeinone is produced from thebaine and then reduced to codeine, which is finally demethylated to yield morphine. Codeinone reductase (COR), which reduces (–)-codeinone to (–)-codeine, has been purified and the corresponding cDNA isolated from opium poppy (Fig. 1) (Lenz and Zenk 1995b; Unterlinner et al. 1999).

Genomics-based gene discovery

Much of the success in the isolation and characterization of genes encoding BIA biosynthetic enzymes has relied on the identification of gene-specific DNA fragments, which are used to screen an appropriate cDNA library for the corresponding full-length clone. The traditional biochemical approach toward the isolation of gene-specific probes involves the purification of the corresponding biosynthetic enzyme and subsequent determination of the amino acid sequences of endoprotease-derived peptide fragments, which are used to design degenerate oligonucleotide primers. Corresponding DNA fragments are amplified by polymerase chain reaction (PCR). This approach was used to isolate cDNAs encoding NCS from *T. flavum* (Samanani et al. 2004). Alternatively, gene probes can be amplified using degenerate primers targeted to conserved functional motifs in the putative gene of interest. Ikezawa et al. (2003) generated cDNA-derived probes using primers targeted to the conserved regions of eukaryotic P450-dependent enzymes. This strategy led to the isolation of the first cDNA encoding a methylenedioxy-bridge forming enzyme (CYP719A1), which catalyzes the formation of (*S*)-canadine as the penultimate step in berberine biosynthesis (Fig. 1). Conveniently, a cDNA from a related plant species can often serve as a heterologous probe to isolate the corresponding gene from opium poppy because BIA biosynthetic genes are typically conserved across species (Liscombe et al. 2005). For example, cDNAs from *C. japonica* were used as probes to isolate corresponding opium poppy clones encoding 6OMT, CNMT, and 4′OMT (Facchini and Park 2003). However, these types of library-screening approaches can be unsuccessful or simply unsuitable: the search for a cDNA encoding NCS from opium poppy being a prime example. The unstable nature of opium poppy NCS throughout purification ruled out the possibility of peptide microsequencing (Samanani and Facchini 2001), and the unique reaction catalyzed by NCS made it difficult to predict the presence of specific conserved motifs. Moreover, a cDNA encoding NCS was soon isolated from meadow rue but failed to hybridize to a homolog when used to screen a cDNA library prepared from elicited opium poppy cell cultures (D. Liscombe and P. Facchini, unpublished results). The development of genomics tools—expressed sequence tags (ESTs), in particular—has created new approaches for the discovery of BIA biosynthetic genes and recently facilitated the rapid isolation of new genes. Performing sequence similarity searches, such as BLAST, on our opium poppy EST database (described later in this review) has led to the isolation of cDNAs encoding two NCS isoforms (Liscombe et al. 2005) and TNMT (Liscombe and Facchini 2006, submitted).

The *T. flavum* NCS (TfNCS) cDNA (Samanani et al. 2004) provided a query sequence to identify a putative, partial cDNA encoding *P. somniferum* NCS (PsNCS) in our EST database. Screening of the cDNA library with the partial NCS cDNA led to the isolation of full-length cDNAs representing two distinct putative NCS isoforms, PsNCS1 and PsNCS2, which share 89% amino acid identity. Surprisingly, the PsNCS isoforms show less than 40% amino acid identity with TfNCS. Heterologous expression of full-length PsNCS1 or PsNCS2 ORFs in *Escherichia coli* produced recombinant proteins with NCS activity. NCS transcripts are abundantly expressed in opium poppy root and stem tissues, and exhibit inducible accumulation in cell suspension culture after wounding or treatment with a fungal elicitor (D. Liscombe and P. Facchini, unpublished results), as expected for BIA biosynthetic genes (Facchini and Park 2003). Although NCS proteins share considerable sequence identity with the pathogenesis-related (PR)-10 and major-allergen (MAP) protein families, NCS from opium poppy and *T. flavum* are unique among known MAP and PR10 proteins

due to the presence of N- and C-terminal oligopeptide extensions in the predicted nascent translation products. A putative signal peptide was predicted in TfNCS (Samanani et al. 2004) suggesting that the N-terminal extensions might be responsible for the subcellular targeting of NCS. Moreover, fusion of the first 25 amino acids from TfNCS or PsNCS to the N-terminus of the green fluorescent protein (GFP) resulted in the localization of fluorescence to distinct subcellular compartments in cultured opium poppy cells (N. Loukanina, D. Liscombe and P. Facchini, unpublished results).

Mining of our opium poppy EST database using the amino acid sequence from opium poppy CNMT, which catalyzes the conversion of (*S*)-coclaurine to (*S*)-*N*-methylcoclaurine (Fig. 1), led to the isolation of a full-length cDNA encoding TNMT (Liscombe and Facchini 2006, submitted). The predicted translation product of the opium poppy TNMT cDNA shares 45% and 48% amino acid identity with CNMT from opium poppy and *C. japonica* (Choi et al. 2002), respectively. TNMT transcripts were detected in all opium poppy organs, although they were most abundant in roots, stems, and leaves. Treatment of opium poppy cell cultures with a fungal elicitor induced the accumulation of *TNMT* gene transcripts and a corresponding increase in TNMT activity. Recombinant TNMT produced in *E. coli* was able to transfer a methyl group from (*S*)-adenosylmethionine to the tertiary nitrogens of (*R,S*)-stylopine, (*R,S*)-canadine and (*S*)-tetrahydropalmatine. However, recombinant TNMT did not accept (*S*)-scoulerine, 2,3,9,10-tetrahydroxyberbine, or the simple isoquinoline 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline as substrates (Liscombe and Facchini 2006, submitted). Therefore, the substrate specificities of recombinant TNMT are consistent with results obtained by other groups using native enzyme preparations (Rueffer and Zenk 1986; Rueffer and Zenk 1990; O'Keefe and Beecher 1994). The expressed *Arabidopsis thaliana* gene At4g33120 encodes an uncharacterized protein that is 74% identical to both CNMT and TNMT, and is one of many BIA biosynthetic gene orthologs that have been identified in plants not known to produce alkaloids (Facchini et al. 2004; Liscombe et al. 2005). However, expression of the At4g33120 ORF in *E. coli* produced a recombinant protein that failed to accept any of the isoquinoline substrates tested (Liscombe et al. 2005). These results support the notion that although Arabidopsis exhibits a latent genetic fingerprint for BIA biosynthesis, the biosynthetic capacity to produce these alkaloids has been lost.

A major challenge encountered during the characterization of recombinant BIA biosynthetic enzymes is the availability of native substrates. Most BIA pathway intermediates are present only in small quantities in the plant; thus, their isolation as natural products is generally impractical. As few BIAs are available for purchase from chemical suppliers, the chemical or enzymatic conversion of commercially available precursors is typically required, which is often not a trivial task. However, reverse genetics approaches involving post-transcriptional gene silencing (PTGS), such as RNA interference (RNAi) and virus-induced gene silencing (VIGS), circumvent an initial requirement for native substrates and allow the functional characterization of novel genes in planta. RNAi and VIGS exploit the homology-based defense mechanisms active in higher eukaryotes that evolved as strategies to combat viral infections (Hileman et al. 2005). In essence, double-stranded RNA (dsRNA) molecules, such as viral dsRNAs, are detected and degraded generating small-interfering RNAs (siRNAs), generally 20–22 nucleotides long. When the resulting siRNAs encounter an mRNA transcript sharing significant identity, they anneal to form another dsRNA that is subsequently degraded (Wesley et al. 2004). RNAi involves cloning unique sense and corresponding antisense fragments of the gene of interest into a specialized expression vector, such as pKannibal (Helliwell and Waterhouse 2005). The PTGS vector is then introduced into plant tissue by biolistic-or *Agrobacterium tumefaciens*-mediated genetic transformation. A constitutive promoter directs transgene expression and the resulting transcripts self-anneal to form hairpin loops possessing a double-stranded RNA region, which triggers the endogenous PTGS cascade. Th transgene transcripts and endogenous transcripts are then degraded, effectively silencing the target gene in

the plant. Allen et al. (2004) used a PTGS approach to silence COR genes in opium poppy, demonstrating the utility of this approach to investigate BIA biosynthesis. Recently, Hileman et al. (2005) reported an effective VIGS system for opium poppy. Using this system, target gene fragments are cloned into an *A. tumefaciens* T-DNA vector containing a modified tobacco rattle virus (TRV) cassette, which is introduced into plants by infiltration. Viral replication machinery then generates dsRNA transcripts of the target gene that subsequently induce the endogenous gene silencing mechanism. Gene silencing strategies such as these should be useful to characterize novel BIA biosynthetic genes.

Phthalideisoquinoline alkaloid biosynthesis

Battersby et al. (1968) first reported the incorporation of double-labeled (*S*)-scoulerine into the phthalideisoquinoline noscapine, also known as narcotine, in opium poppy plants. While noscapine and the related phthalideisoquinoline narcotoline (Fig. 2) are major metabolites of opium poppy (Frick et al. 2005), similar phthalideisoquinolines can be found in several other members of the Papaveraceae and Menispermaceae (Liscombe et al. 2005). Noscapine and derivatives have long been used as antitussive agents, and have recently been purported to possess anticancer properties (Ye et al. 1998). However, the biosynthesis of phthalideisoquinoline alkaloids remains poorly understood. Moniot and Shamma (1976) demonstrated the in vitro chemical conversion of berberine into the phthalideisoquinoline hydrastine, providing further support for a series of enzymatic transformations in vivo. Many of the potential precursors to noscapine have since been isolated from *Papaver* spp. or related phthalideisoquinoline-producing plants (Gozler et al. 1983; Sariyar and Shamma 1986; Sariyar et al. 1990; Sariyar 2002).

In Fig. 2, we propose a metabolic scheme for the biosynthesis of phthalideisoquinoline alkaloids. Noscapine possesses the same 2,3-methylenedioxy-9,10-dimethoxy substitution pattern as (*S*)-canadine, also known as (*S*)-tetrahydroberberine. Therefore, the first two steps leading to the phthalideisoquinolines from (*S*)-scoulerine are likely identical to those characterized in berberine-accumulating plants (Samanani et al. 2005), where a scoulerine-9-*O*-methyltransferase (SOMT) yields (*S*)-tetrahydrocolumbamine, which is then converted to (*S*)-canadine by a methylenedioxy bridge-forming enzyme (CYP719A) (Ikezawa et al. 2003). (*S*)-Canadine would then be *N*-methylated by TNMT to produce *N*-methylcanadine. We have recently demonstrated the ability of recombinant opium poppy TNMT to catalyze this reaction (Liscombe and Facchini 2006, submitted). Subsequently, *N*-methylcanadine would be hydroxylated at C-1 and the hydroxyl group then methylated by an *O*-methyltransferase (*O*MT) to yield 1-methoxy-*N*-methylcanadine (Sariyar and Shamma 1986). Hydroxylation at C-13 would lead to *N*-methopapaverberbine, which has been isolated from *Papaver* spp. (Iwasa and Kamigauchi 1996; Sariyar et al. 1990). Oxidation at C-8 of 1-methoxy-*N*-methylcanadine leads to the seco-berbine macrantaldehyde (Sariyar and Shamma 1986). Side reactions of macrantaldehyde include reduction to the benzylic alcohol macrantaline (Sariyar et al. 1990), which would then be hydroxylated at C-13 to produce narcotinediol (Sariyar and Shamma 1986). Hydroxylation of macrantaldehyde at C-13 could lead to the cyclic hemiacetal, narcotinehemiacetal (Sariyar and Shamma 1986). Finally, oxidation of the hemiacetal yields noscapine, possessing the lactone functionality characteristic of phthalideisoquinolines (Gozler et al. 1983). Figure 2 also accounts for narcotoline biosynthesis, which would presumably proceed in a similar manner to that of noscapine, except that the 1-hydroxyl group would not be methylated. Narcotoline could also be produced by demethylation of the 1-methoxyl group of noscapine. Therefore, we propose that at least two *O*-methyltransferases, two hydroxylases, some type of oxidase capable of breaking the N-7, C-8 berberine bridge, and a dehydrogenase to catalyze lactone formation are involved in noscapine biosynthesis. These represent novel genes, enzymes and activities that can be pursued using a genomics-based approach to elucidate phthalideisoquinoline metabolism in opium poppy.

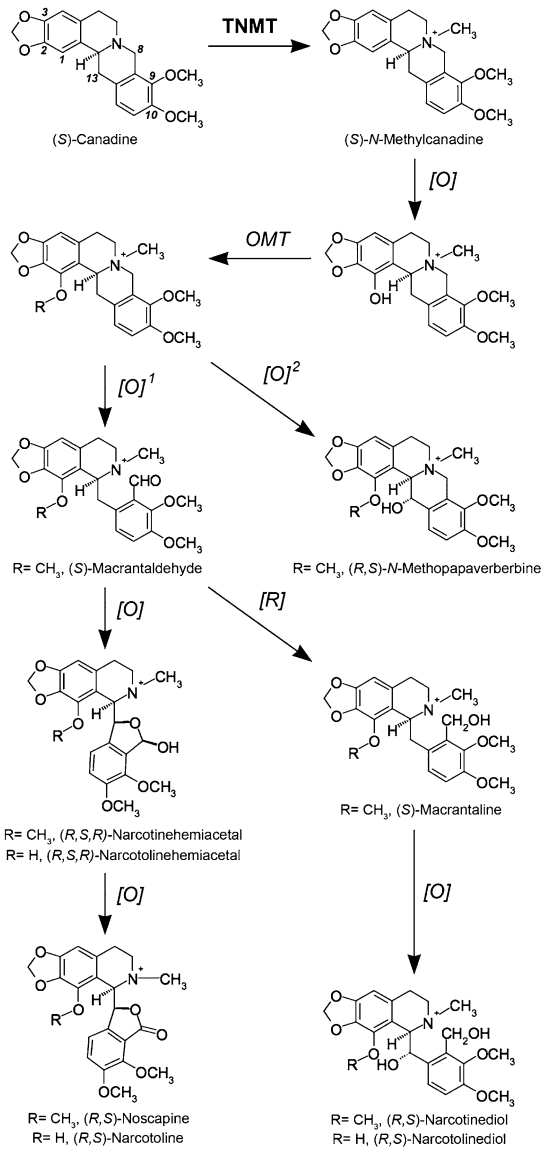


Fig. 2 Proposed noscopine biosynthetic pathway. Enzymatic steps for which corresponding cDNAs have been isolated and characterized are shown in bold. Putative enzymatic conversions, for which cDNAs have not been isolated or activities have not been detected are shown in *italics*. Abbreviations: *OMT*, *O*-methyltransferase; TNMT, tetrahydroprotoberberine-*cis*-*N*-methyltransferase; [*O*], oxidation; [*R*], reduction

Cellular and subcellular localization

Many secondary metabolites are sequestered to specialized cells or structures to protect the plant from their inherent cytotoxicity. Cellular localization patterns often reflect the purported ecophysiological functions of specific natural products. The cellular and subcellular compartmentalization of biosynthetic gene transcripts, enzymes, and pathway intermediates could play an important role in the separation of incompatible or competing reactions, and the concentration of enzymes and metabolites. Gene transcripts and enzymes involved in the biosynthesis of diverse secondary metabolites, including phenylpropanoids (Gang et al. 2001), flavonoids (Saslowsky and Winkel-Shirley 2001), terpenoids (Lange et al. 2000; Turner and Croteau 2004), glucosinolates (Andréasson et al. 2001), and alkaloids (Suzuki et al. 1999; St-Pierre et al. 1999; Irmler et al. 2000; Moll et al. 2002; Bird et al. 2003; Anke et al. 2004; Samanani et al. 2006), have been localized to a variety of specific cell types in plants.

A variety of complex spatial relationships couples the biosynthesis of phylogenetically unrelated—and related—alkaloids to the differentiation of diverse plant cell types. Putrescine *N*-methyltransferase (PNMT) and hyoscyamine 6β-hydroxylase catalyze the first and last steps in the biosynthesis of the tropane alkaloid scopolamine and are localized to the pericycle in the roots of *Atropa belladonna* and *Hyoscyamus muticus* (Hashimoto et al. 1991; Suzuki et al. 1999). PNMT also catalyzes the first step in nicotine biosynthesis and has been localized to the endodermis, outer cortex, and xylem in *Nicotiana sylvestris* (Shoji et al. 2000, 2002). In contrast, tropinone reductase I, an intermediate enzyme in the tropane alkaloid pathway, resides in the endodermis and nearby cortical cells (Nakajima and Hashimoto 1999); thus, intermediates of tropane alkaloid metabolism must be transported between cell types. Intercellular translocation of monoterpenoid indole alkaloid pathway intermediates was also postulated between internal phloem, epidermis, laticifers, and idioblasts in the leaves of *C. roseus* (St-Pierre et al. 1999; Irmler et al. 2000; Burlat et al. 2004; Murata and De Luca 2005). Pyrrolizidine alkaloids are synthesized in endodermal and cortical cells immediately adjacent to the phloem in roots of *Senecio vernalis* (Moll et al. 2002), or throughout the cortex in *Eupatorium cannabinum* roots (Anke et al. 2004). The biosynthesis and storage

of acridone alkaloids are also associated with endodermis in *Ruta graveolens* (Junghanns et al. 1998).

We have used immunofluorescence labeling and in situ transcript localization methods to define the cell type-specific biosynthesis of BIAs in opium poppy. Gene transcripts and enzymes for 6OMT, CNMT, CYP80B1, 4′OMT, BBE, SAT, and COR were localized to companion cells and sieve elements, respectively, of the phloem (Bird et al. 2003; Samanani et al. 2006). Pathway enzymes are assembled in companion cells and, subsequently, transported to sieve elements to participate in alkaloid biosynthesis (Fig. 3). However, alkaloids ultimately accumulate in laticifers, which are invariably adjacent or proximal to sieve element/companion cell pairs (Fig. 3). The localization of BIA biosynthetic enzymes to sieve elements was confirmed by: (1) the specific immunogold labeling of the peripheral cytoplasm characteristic of this cell type, (2) the co-localization of a sieve element-specific H^+-ATPase with all biosynthetic enzymes, and (3) the strict association of sieve plates with immunofluorescent cells (Samanani et al. 2006). The identification of laticifers was demonstrated using antibodies specific to major latex protein (MLP), which is characteristic of this cell type. In contrast, Weid et al. (2004) reported that enzymes involved in (*S*)-reticuline biosynthesis were localized to phloem parenchyma whereas COR, which catalyzes the penultimate step in the morphine pathway, was found in laticifers. The apparent absence of sieve plates in cells labeled with 4′OMT, SAT, and 7OMT antibodies was the sole indicator used to identify the cell type as phloem parenchyma rather than sieve elements (Weid et al. 2004). However, it cannot be ruled out that only immature sieve elements (i.e., lacking mature sieve plates) were labeled. Moreover, differences in the specificity and concentration of antibodies used in the two studies might also have produced differential labeling of cells with high or low levels of alkaloid biosynthetic enzymes. In addition, our experimental plants were grown in climate-controlled growth chambers (Bird et al. 2003; Samanani et al. 2006), whereas those used by Weid et al. (2004) were field grown. Different developmental programs, perhaps mediated by variations in environmental conditions, might affect the spatial limits of alkaloid biosynthetic gene expression. Interestingly, the in situ hybridization results presented by Weid et al. (2004) appear consistent with the companion cell-specific localization of alkaloid biosynthetic gene transcripts (Bird et al. 2003; Samanani et al. 2006).

The localization of BIA biosynthesis expands the role for sieve elements beyond the transport of solutes and information macromolecules

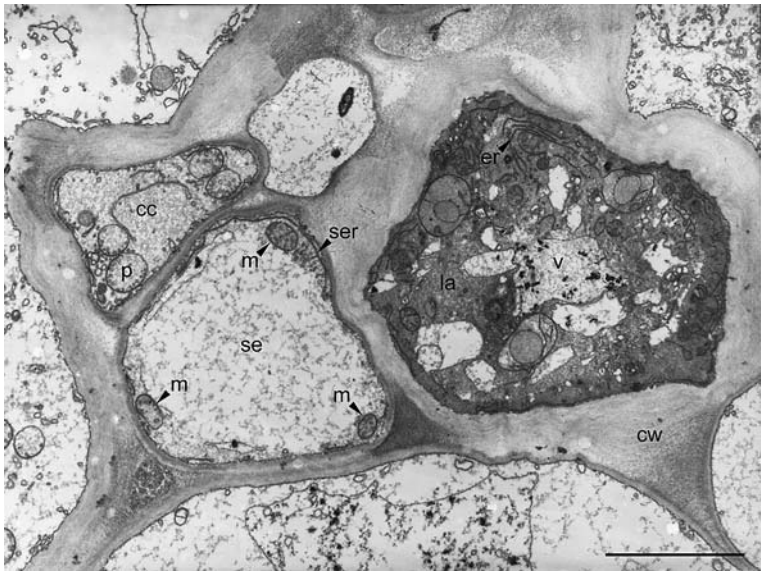


Fig. 3 Laticifers are typically adjacent to sieve elements and companion cells in the vascular bundles of opium poppy. Stem tissue was fixed using potassium permanganate and sectioned for viewing by transmission electron microscopy. Abbreviations: cc, companion cell; cw, cell wall; er, endoplasmic reticulum; la, laticifer; m, mitochondrion; p, plastid; se, sieve element; ser, sieve element reticulum; v, vacuole. Bar = 10 μm

(Oparka and Turgeon 1999). Other metabolic enzymes associated with sieve elements include monodehydroascorbate reductase (Walz et al. 2002), glutathione reductase (Szederkenyi et al. 1997), glutathione *S*-transferase, cinnamyl/sinapyl alcohol dehydrogenase, alcohol dehydrogenease, *S*-adenosyl methionine decarboxylase, pectate lyase (Vilaine et al. 2003), allene oxide cyclase (Hause et al. 2003; Vilaine et al. 2003), phosphoglycerate mutase, phosphoglycerate kinase, phosphopyruvate hydratase (Barnes et al. 2004), monodehydroascorbate reductase, lipoxygenase, aminocyclopropane carboxylate (ACC) oxidase, and ACC synthase (Walz et al. 2004). The detection of a multitude of proteins (Kehr et al. 1999) demonstrates the capacity of sieve elements to support diverse biochemical processes, such as the maintenance of an antioxidative environment (Walz et al. 2002), glutathione-dependent thiol-reduction (Alosi et al. 1988), and jasmonate (Stenzel et al. 2003) and L-ascorbic acid (Hancock et al. 2003) biosynthesis. Additional metabolic functions in sieve elements include glycolysis and other reactions involved in energy metabolism (Barnes et al. 2004) and the biogenesis of cell wall, lipids, polyamines, and vitamins (Vilaine et al. 2003).

The spatial separation of the sites of alkaloid biosynthesis and accumulation implicates the intercellular transport of pathway intermediates and/or products. Membrane bound P450-dependent enzymes, such as CYP80B1, are likely associated with the sieve element reticulum (SER), a form of endoplasmic reticulum (ER) also purported to function in the trafficking of proteins through plasmodesmata between sieve elements with companion cells (Oparka and Turgeon 1999; Van Bel and Knoblauch 2000; Lucas et al. 2001). The mechanisms involved in the translocation of alkaloids from sieve elements to laticifers in the opium poppy are not known. Possible mechanisms might include symplastic transport through plasmodesmata, or apoplastic transport across plasma membranes and cell walls via transporters. Immunolocalization of callose, which lines plasmodesmatal pores, using a monoclonal antibody specific for β1,3-linked glucans demonstrates the occurrence of symplastic connections between these cell types (Fig. 4).

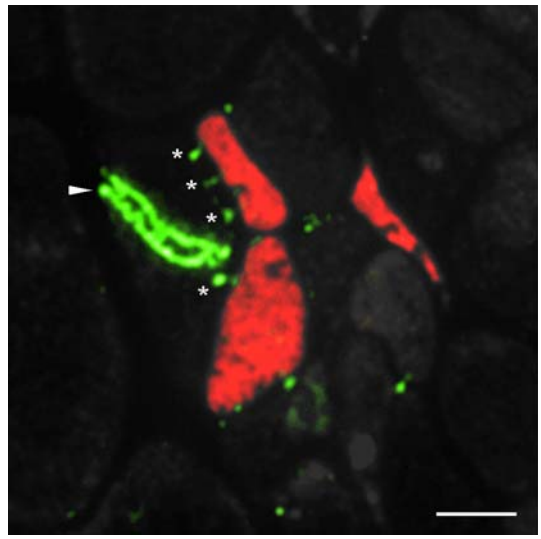


Fig. 4 Plasmodesmata create symplastic connections between sieve elements and laticifers. Stem cross sections were exposed to a mouse β-1,3-glucan monoclonal antibody (green; asterisks) to show plasmodesmata, and a rabbit major latex protein (MLP) polyclonal antibody (red) to identify laticifers. A sieve plate (green; white arrowhead) in an oblique orientation confirms the identity of a sieve element. Bar = 10 μm

However, an ATP-binding-cassette (ABC) multidrug-resistant transporter (CjMDR) of berberine was localized in the plasma membrane of *C. japonica* rhizome parenchyma (Shitan et al. 2003). ABC transporters have also been implicated in the transport of other secondary metabolites, such as terpenoids and phenolics (Yazaki 2006); and the export of cuticular wax in Arabidopsis (Pighin et al. 2004). The possible apoplastic translocation of alkaloids between sieve elements and laticifers is supported by the occurrence of several ESTs encoding ABC transporters in opium poppy (K. Zulak and P. Facchini, unpublished results).

In contrast to the three cell types required for alkaloid biosynthesis and accumulation in the plant (Bird et al. 2003; Samanani et al. 2006), the synthesis and storage of sanguinarine in opium poppy cell cultures appears to occur within a single dedifferentiated cell (Alcantara et al. 2005). Treatment of cultured opium poppy cells with a fungal elicitor resulted in the simultaneous induction of alkaloid biosynthetic gene expression and extensive dilation of the ER. The co-localization of CYP80B1, BBE, and sanguinarine to

the ER of cultured cells supports the association of the entire alkaloid biosynthetic pathway to the ER (Bird and Facchini 2001; Alcantara et al. 2005). Alkaloid-laden vesicles ultimately appeared to fuse with the central vacuole, where sanguinarine accumulates in cultured opium poppy cells. Similar vesicles were suggested to function in the intracellular transport of berberine in *Berberis stolonifera* cell cultures (Bock et al. 2002). The role of the ER in the biosynthesis and translocation of sanguinarine in cultured cells could reflect the mechanism of alkaloid transport through plasmodesmata via a possible continuum of SER in sieve elements and endomembranous vesicles in laticifers (Bird et al. 2003). The ER is contiguous through plasmodesmata desmotubules and has been shown to permit the movement of small molecules, such as dextrans (Cantrill et al. 1999). Moreover, alkaloid-containing vesicles in the laticifers of opium poppy likely result from dilations of the ER (Thureson-Klein 1970; Nessler and Mahlberg 1977).

A monophyletic origin for BIA biosynthesis is supported by the extensive sequence homology among biosynthetic enzymes from different plant families operating at corresponding points in the pathway (Samanani et al. 2005; Liscombe et al. 2005). Nevertheless, BIA metabolism involves endodermis, pericycle, protoderm, cortex, or pith in *Thalictrum flavum*, a member of the Ranunculaceae, rather than phloem tissues as in opium poppy (Samanani et al. 2005). The involvement of diverse cell types and the biosynthesis and accumulation of related BIAs in plants from different families suggests that alternative cellular compartmentalization and transport platforms have also evolved.

Induced alkaloid biosynthesis in cell cultures: genomics and metabolomics

Genomics technologies such as whole genome sequencing, EST databases, and functional genomics tools such as DNA microarrays have rapidly revolutionized the field of plant biology. However, only recently have these technologies been applied to studies on the biosynthesis of commercially valuable plant natural products such as essential oil production in mint (Lange et al. 2000) and sweet basil (Gang et al. 2001), and floral scent in rose (Guterman et al. 2002). Cell cultures of opium poppy are an ideal model system for the application of genomics due to the rapid transcriptional induction of BIA biosynthetic gene transcripts and sanguinarine accumulation in response to treatment with a fungal elicitor (Facchini et al. 1996a; Facchini and Park 2003).

We have developed a fully annotated EST database representing 7,225 unique sequences assembled from a total of 10,224 random clones sequenced from an elicited opium poppy cell culture cDNA library (Zulak et al. 2006). The most abundant ESTs found in the database include transcripts of defense-related proteins, enzymes involved in the biosynthesis of *S*-adenosylmethionine (SAM) and enzymes that use SAM as a methyl donor, as well as enzymes involved in alkaloid and phenylpropanoid biosynthesis. SAM synthetase is the most abundant EST in the database, representing 1.4% of all ESTs, which is not surprising since SAM-dependent methylations occur frequently in the biosynthesis of alkaloids and phenylpropanoids, among other compounds. Transcripts encoding enzymes involved in lipid metabolism were also common, the most abundant being lipid transfer proteins. From this collection we isolated cDNAs corresponding to the majority of enzymes involved in metabolic pathways from sucrose metabolism to the production of sanguinarine. Interestingly transcripts encoding two enzymes in the morphine branch pathway, salutaridinol-7-*O*-acetyltransferase (SAT) and codeinone reductase (COR) were also identified even though opium poppy cell cultures do not produce morphine.

Two generations of DNA microarrays have been constructed representing 6,000 and an additional 7,000 random cDNAs from the elicitor-induced cell culture EST database (Zulak et al. 2006). Hybridizations were performed to compare the gene transcript population from control cell cultures with those of cell cultures collected at various time points after the initiation of elicitor treatment (Fig. 5). Many gene transcripts were substantially induced, some within 1 h after the addition of the elicitor. Elicitor-induced, differential transcript abundance in the elicitor-treated

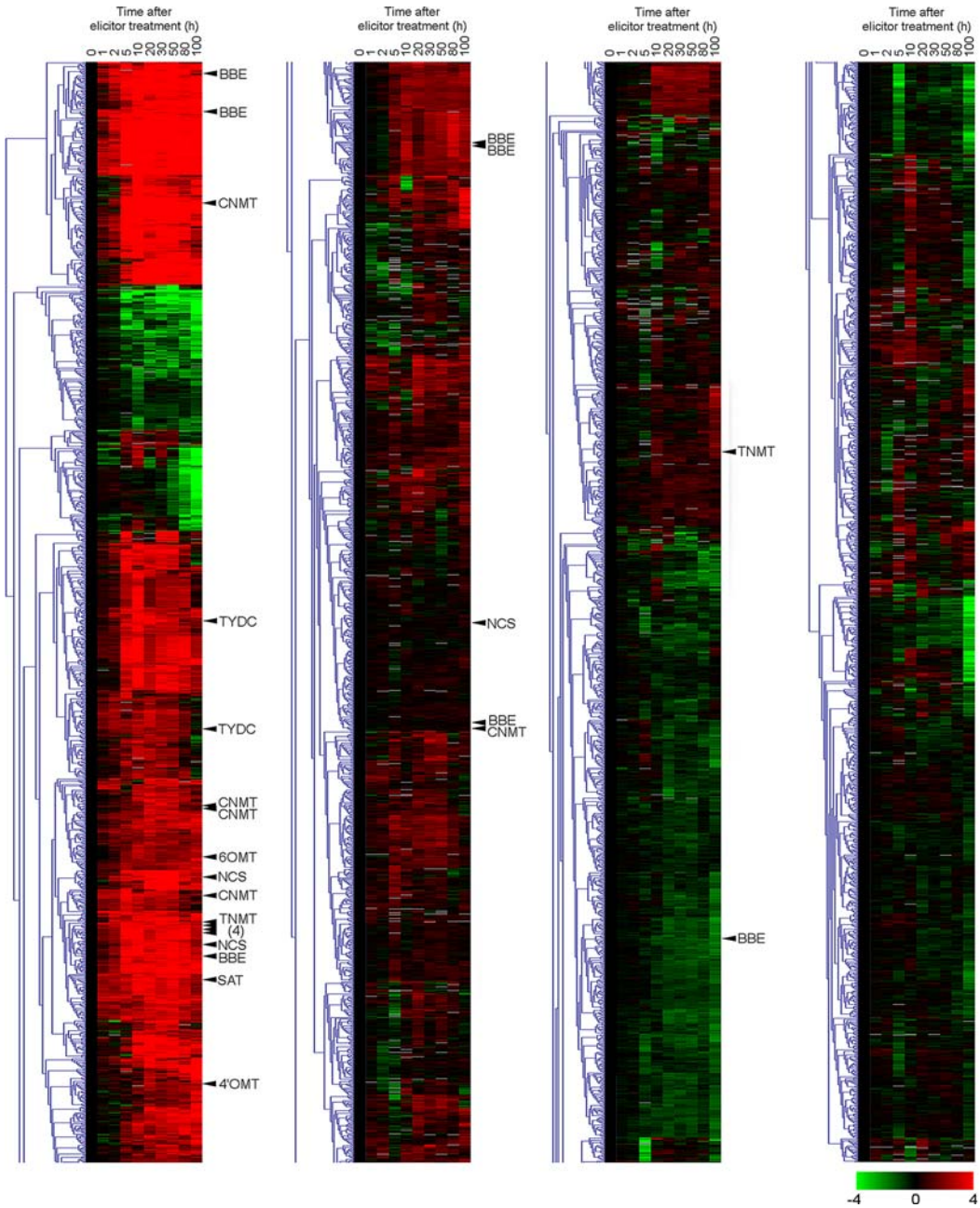


Fig. 5 The levels of diverse gene transcript populations are temporally and differentially modulated in opium poppy cell cultures treated with a fungal elicitor. Results of a typical DNA microarray experiment performed using RNA isolated at various time-points from elicitor-treated or control cell cultures were divided by corresponding average values obtained using RNA from cells collected prior to the addition of the elicitor (i.e., at $t = 0$) to generate the reported ratios, which were subsequently $\log_2$-normalized. The actual dynamic range of the ratios was –9.63 to 9.08, but was scaled from –4.00 (green) to 4.00 (red) to enhance visualization of the changes in relative abundance. Arrowheads show the location of data sets corresponding to alkaloid biosynthetic gene transcripts. Abbreviations: 4′OMT, 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase; 6OMT, norcoclaurine 6-*O*-methyltransferase; BBE, berberine bridge enzyme; CNMT, coclaurine *N*-methyltransferase; SAT, salutaridinol 7-*O*-acetyltransferase; TNMT, tetrahydroprotoberberine *cis*-*N*-methyltransferase; TYDC, tyrosine decarboxylase

cells reached a maximum after approximately 20–30 h. The levels of a smaller number of gene transcripts were suppressed after elicitor treatment (Fig. 5). Hierarchical clustering analysis revealed temporal coordination in the abundance of several groups of diverse gene transcripts (Fig. 5), with the largest group representing transcripts that did not show changes in abundance in response to elicitor treatment. The cluster showing the most rapid, substantial, and sustained induction was mostly composed of transcripts encoding proteins involved in plant defense-responses, such as the Bet v 1 family of pathogenesis-related proteins and xyloglucanase inhibitors. A small number of glutathione *S*-transferases (GSTs) and several plant hemoglobins were coordinately induced, but with a different profile than the Bet v 1 proteins. However, the majority of GSTs show only moderate or no induction. Although plant GSTs are induced upon pathogen infection (Wagner et al. 2002) not all GSTs respond to pathogen challenge (Dean et al. 2005). Similarly, perhaps only a small group of GSTs play a defensive role in opium poppy. Plant hemoglobins detoxify nitric oxide, a highly reactive signalling molecule produced during plant-microbe interactions as part of the defense response (Perazzolli et al. 2006).

Anti-microbial peptides were also induced, but did not cluster with other defense-response gene transcripts because of a more moderate and gradual induction profile, which reflects a degree of differential regulation among defense-related gene families. Lipid transfer proteins were the most highly represented among gene transcripts suppressed in response to elicitor treatment. Similarly, a reduction in the transcript levels of *DIR1*, which encodes a lipid transfer protein, was reported in *Arabidopsis* leaves inoculated with an avirulent strain of *Pseudomonas syringae* (Maldonado et al. 2002). Few of the ESTs in the opium poppy database corresponding to lipid-transfer proteins were induced, in contrast to the induction of antimicrobial lipid transfer proteins in grape (*Vitis* spp.) cell cultures treated with a fungal elicitor (Gomes et al. 2003). Such proteins were suggested to participate in the production or transmission of a mobile signal required for systemic acquired resistance in plants.

DNA microarray and RNA gel-blot analyses showed a coordinated and rapid induction of gene transcripts encoding enzymes involved in the biosynthesis of sanguinarine (Fig. 5). Transcripts for most members of alkaloid biosynthetic gene families or alleles were induced after the addition of the elicitor, although a small number encoding NCS, CNMT, BBE, and TNMT did not show induction. Gene transcripts encoding several enzymes involved in sucrose metabolism, the shikimate pathway, and aromatic amino acid biosynthesis were also induced upon elicitor treatment. The co-regulation of enzymes involved in secondary metabolic pathways involved in phenylpropanoid, flavonoid, and indole alkaloid biosynthesis, and primary pathways involved in precursor metabolism, such as shikimate, are tightly co-ordinated in Arabidopsis under stress conditions (Gachon et al. 2005). Flavonoid and primary metabolic supply pathways were also transcriptionally co-induced in parsley cells irradiated with UV light (Logemann et al. 2000).

We are pursuing a parallel proteomics initiative to analyze changes in the protein profile of the cell cultures to identify proteins changing in response to elicitor treatment. Two-dimensional, denaturing polyacrylamide gel electrophoresis performed on elicitor-treated cell culture protein extracts has shown that many polypeptides increase in abundance, temporally consistent with the induction of a multitude of gene transcripts (K. Zulak and P. Facchini, unpublished results). The opium poppy EST database will be used as a resource for the analysis of tryptic peptide fragment masses obtained by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS). Proteomics represents a crucial link between genotype and phenotype, and the importance of post-translational modifications, but does not yet provide widespread access to affordable technological advances that have increased the popularity of other functional genomics approaches, such as DNA microarrays (Rose et al. 2004). Proteomics initiatives have been undertaken in a small number of non-model plant species including opium poppy (Decker et al. 2000), and cell cultures of *Medicago truncatula*

(Lei et al. 2005), Korean ginseng (*Panax ginseng*) (Nam et al. 2005), and *Cannabis sativa* (Raharjo et al. 2004).

The coupling of transcriptomics, proteomics, and metabolomics has begun to facilitate the development of a *systems biology* approach to understand the integration of complex datasets (Urbanczyk-Wochniak et al. 2003). However, metabolic profiling is a relatively new methodology and many technological objectives, especially the separation, detection, and identification of compounds from complex samples, await refinement (Bino et al. 2004). Metabolic profiling has been used in several plant natural product applications. For example, proton-nuclear magnetic resonance (H^1-NMR) spectroscopy was used to compare the metabolic profiles of healthy and phytoplasma-infected *Catharanthus roseus* leaves (Choi et al. 2004a,b), and gas chromatography-mass spectrometry (GC-MS) was used to study the effects of biotic and abiotic elicitors on the metabolism of *Medicago truncatula* cell cultures (Broeckling et al. 2004).

We have used Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) to show temporal changes in the metabolite profile of elicitor-treated opium poppy cell cultures compared with controls (Zulak et al. 2006). A total of 992 independent analytes, which represented putative metabolites with a molecular mass between 106 and 1,128 Da, were detected in control and elicitor-treated cell cultures based on their mass to charge (m/z) ratio. Principle component analysis showed a separation of the metabolite profile in control and elicitor-treated cell cultures at most points in the time course. The separation was most noticeable in the metabolite profile of elicitor-treated cells from 20 h to 100 h post-elicitation, with the greatest separation observed at 80 h and 100 h. These data show that the metabolome of cultured opium poppy cell cultures was dynamic in response to elicitor treatment throughout the time course. The two modulations in metabolite profile primarily responsible for the difference between control and elicitor-treated datasets were the decrease in sucrose and the increase of sanguinarine levels. Of the 992 analytes detected, 144 were found only in elicitor-treated cells and another 232 showed a significant change in abundance in response to elicitor treatment. Several alkaloids were included among putative metabolites detected only in elicitor-treated cells. Sanguinarine and dihydrosanguinarine were the most abundant alkaloids, but molecular masses consistent with those of the alkaloid pathway intermediates *N*-methylcoclaurine, *N*-methylstylopine, and protopine, the methoxylated sanguinarine derivatives dihydrochelirubine and chelirubine, and the simple benzylisoquinoline alkaloid papaverine were also detected. Other molecular masses consistent with those of notable cellular metabolites were glucose, glucose 6-phosphate, citrate, adenosine, linolenic acid, and several phenylpropanoid pathway intermediates—ferulic acid, 5-hydroxyferulic acid, coumaroyl shikimate, and coniferaldehyde. In general, the cellular pools of many of these putative metabolites decreased more rapidly in elicitor-treated cell cultures compared with controls.

The use of ^{1}H-NMR spectroscopy for the characterization and quantification of plant metabolites has gained in popularity. Applications of ^{1}H-NMR spectroscopy include work on cereal root (Fan et al. 2001), Arabidopsis (Ward et al. 2003), and medicinal plant metabolism (Choi et al. 2004a, b; Kim et al. 2004a, b; Frederich et al. 2004). Unlike GC-MS, which detects only volatile compounds, ^{1}H-NMR simultaneously detects essentially all (proton-bearing) compounds. In FT-ICR MS, metabolite identification is limited by the ability to distinguish between isomers with identical molecular masses. Two-dimensional ^{1}H-NMR circumvents this problem and allows the detection and identification of metabolites without complex sample preparation (Defernez and Colquhoun 2003). We are currently using ^{1}H-NMR and multivariate statistical analyses (Antti et al. 2002) to characterize differences in the metabolite profiles of plant tissues and elicitor-treated opium poppy cell cultures.

Regulation of gene expression

In opium poppy, morphine, codeine and thebaine are most abundant in aerial organs, particularly in

mature capsules. In contrast, sanguinarine is the major alkaloid in roots, although substantial levels of morphine also accumulate (Williams and Ellis 1989; Facchini and De Luca 1995). Antimicrobial alkaloids, such as sanguinarine, occur in the roots (Ma et al. 2000) and shoots (Morteza-Semnani et al. 2003) of many members of the Papaveraceae. The induced accumulation of sanguinarine and other benzophenanthridine alkaloids via treatment of dedifferentiated cell cultures with hydrolyzed fungal cell wall extracts is also common among the Papaveraceae (Eilert et al. 1985; Schumacher et al. 1987; Mahady et al. 1998; Facchini et al. 1996a).

The induction of sanguinarine accumulation in opium poppy cell cultures displays features consistent with a phytoalexin response-coupling model, which has been well characterized for monoterpenoid indole alkaloid biosynthesis in *C. roseus*. The elicitation of cultured *C. roseus* cells using a fungal elicitor activates both jasmonate-dependent and jasmonate-independent signaling pathways, which result in the transcriptional activation of biosynthetic enzymes such as tryptophan decarboxylase (TDC) and strictosidine synthase (STR) (van der Fits et al. 2000). The activation of monoterpenoid indole alkaloid biosynthesis involves both Ca^{2+} and protein phosphorylation in *C. roseus*, as does the induction of BIA biosynthesis in bloodroot (*Sanguinaria canadensis*) cell cultures (Mahady- and Beecher 1994; Mahady et al. 1998).

The availability of five biosynthetic genes involved in the conversion of dopamine to the major branch point intermediate (*S*)-reticuline, and three biosynthetic genes from the morphine and sanguinarine branch pathways, has allowed preliminary studies on gene expression patterns in opium poppy (Huang and Kutchan 2000; Facchini and Park 2003). Gene transcript levels generally increase in developing seedlings and are consistently high in stems and flower buds, but are more variable in roots and leaves of mature plants (Facchini and Park 2003). With the exception of COR, the level of each transcript is substantially induced in response to elicitor treatment or wounding of cell cultures. Certain gene transcript levels generally correlate with the accumulation of morphine or sanguinarine, suggesting a degree of coordination in the developmental and inducible regulation of alkaloid biosynthetic genes in opium poppy. One notable exception involves the occurrence of SAT and COR gene transcripts in opium poppy cell cultures, which invariably fail to accumulate morphinan alkaloids (Facchini and Park 2003). The basis for the inability of cultured cells to produce morphine is not known, but could involve the absence of a key enzyme or inadequate cellular differentiation normally required to support the operation of the relevant branch pathway.

Beyond a basic characterization of differential gene transcript accumulation, little is known about the promoter *cis*-elements or transcription factors involved in alkaloid metabolism in opium poppy. The identification of *cis*-elements involved in the expression of BIA biosynthetic genes is currently limited to *TYDC7* and *BBE1*, for which promoter sequences have been reported (Park et al. 1999). Microprojectile bombardment of opium poppy cell cultures has been used to introduce reporter gene constructs for the loss-of-function analysis of *TYDC7* and *BBE1* promoters (Park et al. 1999). The –393 to –287 region of *TYDC7*, and the –355 to –200 region of *BBE1* were required to promote reporter gene function in bombarded opium poppy cells. Time-courses for the induction of TYDC7 and BBE1 mRNAs in wounded opium poppy cells are nearly identical to those for reporter gene activity in cells bombarded with constructs containing the –393 to –287 region of *TYDC7*, and the –355 to –200 region of *BBE1*, suggesting that the wound signal caused by the DNA-coated microcarriers induces wound-responsive regulatory elements located in these promoter regions. Functional analysis of the *BBE1* promoter from *E. californica* showed that the –496 to –455 region is required for basal activity (Hauschild et al. 1998). Comparison of this region to the –355 to –200 domain in opium poppy *BBE1* revealed 55% nucleotide identity within a 40-base pair sequence.

The successful application of advanced techniques to study the inducible and developmental regulation of monoterpenoid indole alkaloid metabolism in *C. roseus* foreshadows the direction of similar research in opium poppy. Several key *cis*-elements and transcription factors have

been identified (Facchini 2001). For example, a 396-base pair region of the strictosidine synthase (*STR)* promoter introduced into *C. roseus* cell cultures by microprojectile bombardment responded to treatment of the cells with a fungal elicitor or methyl jasmonate (Menke et al. 1999a). A 42-base pair jasmonate-and elicitor-responsive *cis*-element was identified within this promoter fragment and used as bait to screen a *C. roseus* cDNA library by yeast one-hybrid analysis (Menke et al. 1999b). An octadecanoid-responsive *C. roseus* AP2-domain transcription factor, ORCA2, was isolated. Using the same approach, a jasmonate-independent *cis*-element in the *STR*gene and a corresponding MYB-like transcriptional regulator with homology to a box-P binding factor were also identified (van der Fits et al. 2000); thus, at least two distinct transcriptional regulators are involved in the regulation of *STR* expression.

T-DNA activation tagging combined with the use of a toxic metabolic analog (i.e., 4-methyltryptophan) as an efficient selectable marker has provided an alternative approach to identify transcriptional regulators of primary and secondary metabolism in *C. roseus* (van der Fits and Memelink 2000; van der Fits et al. 2001). The *ORCA3* gene was cloned using T-DNA activation tagging and shown to enhance the expression of several monoterpenoid indole alkaloid biosynthetic genes when over-expressed in *C. roseus* cultures (van der Fits and Memelink 2000). The application of a similar strategy could provide an opportunity for the isolation of regulatory genes involved in BIA biosynthesis in opium poppy. Tyrosine derivatives (e.g., 3-fluorotyrosine) have been used as a selectable marker in opium poppy (Khanna et al. 2005). However, the availability of toxic analogs relevant to BIA metabolism is limited.

Our available genomics resources are providing an effective platform for the rapid identification of transcription factors involved in the regulation of alkaloid metabolism in in opium poppy. Among the large number of gene families encoding different regulatory proteins not involved in basal transcription in Arabidopsis, 28 groups are represented in our opium poppy EST database. Hierarchical clustering of microarray data from hybridizations designed to compare gene transcript populations in opium poppy cell cultures collected at various time points after elicitor treatment with those of control cultures (Fig. 5) revealed four major categories of temporal expression profiles among 142 putative transcription factors annotated within our EST collection (Fig. 6). Seventeen ESTs displayed transcript accumulation profiles similar to the temporal induction of alkaloid biosynthetic genes in elicitor-treated opium poppy cell cultures (Facchini and Park 2003). Several different categories—including three WRKY, two zinc-finger, and one each from the bZIP and AP2-domain families—are represented in this group, which is notable due the demonstrated role of related members in the regulation of biosynthetic genes involved in other secondary metabolic pathways (van der Fits and Memelink 2000; Vom Endt et al. 2002; Pauw et al. 2004). The isolation of a limited number of cDNAs encoding transcription factors with appropriate patterns of temporal and spatial regulation provides an excellent framework for gene silencing studies, using VIGS or RNAi, to assess their in vivo regulatory role in BIA metabolism.

As expected, comparison of the phylogeny of these transcription factors with their corresponding transcipt accumulation profiles showed that amino acid sequence homology is often, but not always, correlated with gene expression characteristics. Kalde et al. (2003) noted that the differential expression patterns of 13 Arabidopsis WRKY factors induced by SA and pathogen infection did not correlate with their phylogenetic relationships. However, a better correlation might be obtained by comparing the phylogenetic topologies derived from the DNA binding domains of transcription factors with their transcript accumulation profiles. The amino acid sequences of transcription factors show substantial variation outside the DNA binding domain; thus, changes in this region are more likely to reflect differences in the nucleotide sequence of the corresponding *cis*-element. For example, the change of a single amino acid in a MYB transcription factor from mouse resulted altered binding specificity to allow interaction with a *cis*-element in a petunia gene promoter (Solano et al. 1997). The ability to bind

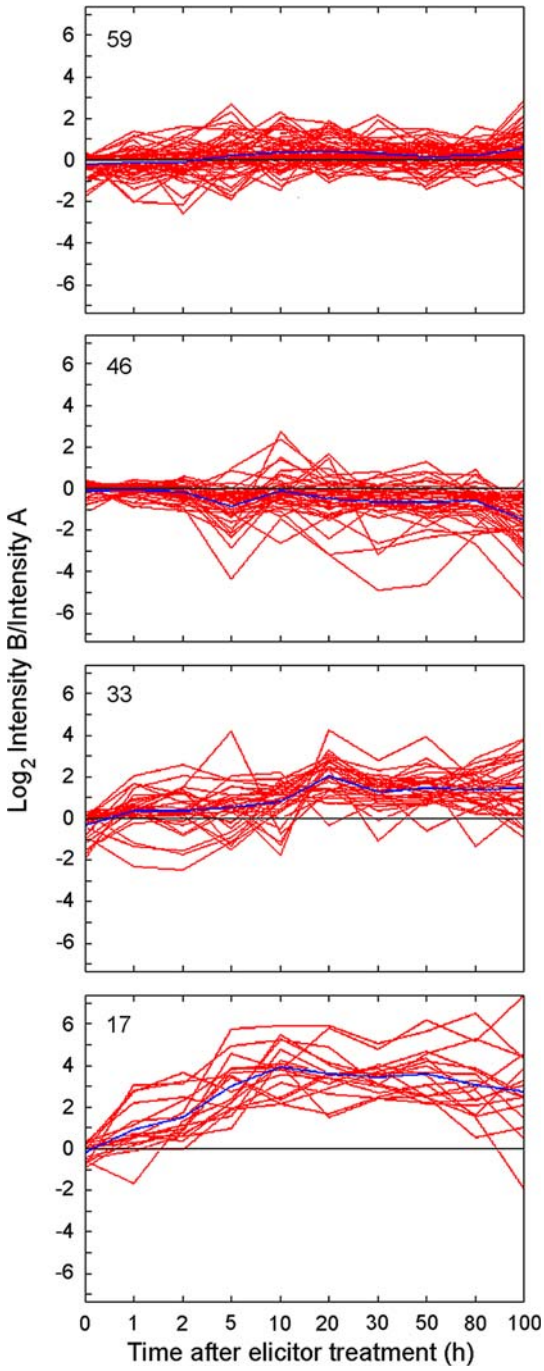


Fig. 6 The levels of diverse gene transcript populations encoding transcriptional regulators are differentially and temporally modulated in opium poppy cell cultures treated with a fungal elicitor. Cluster analysis of DNA microarray data was performed using a K-means algorithm with an arbitrary selection of four groups. The number of gene transcripts within each cluster is shown

the original *cis*-element in a mouse gene promoter was lost. Similarly, a mutant sensitive to low humidity in Arabidopsis was caused by an insertional mutation resulting in the addition of a single amino acid in the WRKYGQK motif of a WRKY transcription factor (Noutoshi et al. 2005).

Mutants and mutagenesis

Functional genomics is the study of gene function on a genome-wide scale and broadly includes (1) nucleotide and amino acid sequence homolog comparisons within and between organisms, (2) transcript profiling to determine large-scale gene expression patterns, and (3) protein interaction analyses to characterize metabolomes and other biochemical pathways. Both forward and reverse genetic approaches can be used (Østergaard and Yanofsky 2004). Forward genetics is best suited to model plants for which dense genetic and physical maps are available, and involves chromosome walking to find non-functional genes through the segregation analysis of mutant and wild type alleles (Bourgault et al. 2005). Reverse genetics often involves the targeted mutation of a specific gene and subsequent analysis of the resulting phenotype, and necessitates an extensive collection of T-DNA and/or transposon-tagged lines (Henikoff and Comai 2003). Alternatively, RNAi and VIGS can be used to investigate the in vivo function of a particular gene. Unfortunately, high efficiency transformation protocols are not available for many plants, including opium poppy, which precludes reverse genetic methods that rely on the insertion of mutational sequences or the expression of gene-silencing elements.

Methods to create non-transgenic mutants are mostly based on the mutagenic properties of certain chemicals or ionizing radiation. Fast neuron bombardment has been used to create deletion mutant libraries of *Arabidopsis* (Li et al. 2001), although chemical and/or radiation-based mutagenesis techniques are more popular because they are relatively simple and inexpensive to perform, and are effective in most, if not all, plant species (Waugh et al. 2006). Alkylating agents, such as ethylmethane sulfonate (EMS),

cause mostly point mutations (Koornneef et al. 1982) and generate a relatively high density of irreversible mutations (Henikoff and Comai 2003). EMS treatment produces GC → AT transitions and induces only low levels of chromosome breaks that result in aneuploidy, reduced fertility, and dominant lethality (Greene et al. 2003).

Opium poppy has been a popular target for EMS-and/or ionizing radiation-induced mutagenesis (Khanna and Singh 1971; Ilieva et al. 1975; Grover and Dhanju 1979; Ghiorghita et al. 1982; Sharma et al. 1999). Chemical mutagenesis of opium poppy created the *top1* mutant, which accumulates thebaine and oripavine, but not morphine or codeine (Millgate et al. 2004). Segregation analysis using F2 progeny suggested the alkaloid phenotype resulted from the mutation of a single genetic locus. Microarray analysis identified ten differentially suppressed transcripts in the *top1* mutant, but none of the corresponding genes appeared related to the *top1* phenotype, which was suggested to result from a mutation in the gene encoding a perceived 6-*O*-demethylase acting on thebaine and oripavine. An altered transport or structural component preventing the proper inter- or intra-cellular compartmentalization of thebaine and oripavine was also considered. Interestingly, the isolation of a spontaneous opium poppy mutant that accumulates thebaine rather than morphine has also been reported (Nyman 1980).

Until recently, the application of forward and reverse genetics to non-model plants (i.e., those lacking extensive genetic map or genome sequence resources) was limited since methods were not available for the localization of single-nucleotide polymorphisms. Recently, induced mutagenesis strategies coupled with the developing technology of TILLING (TargetedInducedLocalLesionsINGenomes) are making high-thoughput reverse genetics possible in virtually any plant (McCallum et al. 2000a, 2000b; Stemple 2004). In the context of novel gene discovery strategies, a combination of chemical mutagenesis and TILLING could be used to generate a collection of opium poppy lines with localized genetic mutations and associated alkaloid phenotypes.

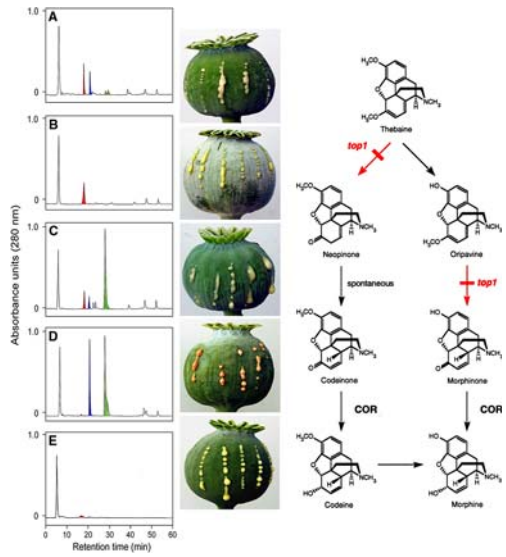


Fig. 7 Different commercial cultivars of opium poppy display diverse alkaloid phenotypes and provide a basis for the isolation of new genes using functional genomics. The alkaloid profiles and corresponding appearance of exuded latex for five cultivars are shown and include (**A**) high levels of morphine (red) and moderate levels of oripavine (blue) and thebaine (green); (**B**) high levels of morphine, but low levels of other alkaloids; (**C**) high levels of morphine, oripavine and thebaine; (**D**) high levels of oripavine and thebaine, but low levels of morphine, and (**E**) low levels of all alkaloids. The proposed metabolism of thebaine to morphine and putative reactions blocked in *top1* mutants are also shown

Several different alkaloid phenotypes were identified among a variety of commercial and ornamental opium poppy cultivars (Fig. 7). Variations in the levels of morphine, oripavine and thebaine (Fig. 7 A–D) are common. In particular, an EMS-mutagenized variety with a high-oripavine/thebaine, low morphine alkaloid profile, and orange–red latex similar to the *top1* mutant (Millegate et al. 2004) has been identified (Fig. 7D). Another variety contained only trace levels of alkaloids (Fig. 7E). The relative abundance of alkaloid biosynthetic gene transcripts was shown to correlate with the accumulation of specific alkaloids in some plant organs (J. Hagel and P. Facchini, unpublished results), suggesting that modulations in the levels of other mRNAs relevant to alkaloid synthesis and storage might also be detected. Experiments to determine the genetic nature of such alkaloid phenotypic

variants are underway using our 23,000-element opium poppy DNA microarray. However, alterations in alkaloid profile might result from either transcriptional or post-transcriptional variations of biosynthetic enzymes, transcription factors, transporters, or other cellular components; thus, a complementary approach such as TILLING might also be required.

Genetic transformation and metabolic engineering

Procedures have been developed for the transformation of opium poppy plants (Park and Facchini 2000a; Chitty et al. 2003), root cultures (Park and Facchini 2000b; Le Flem-Bonhomme et al. 2004), and cell cultures (Belny et al. 1997), *E. californica* plants (Park and Facchini 2000c), root cultures (Park and Facchini 2000b; Park et al. 2003), and cell cultures (Park et al. 2002), and *T. flavum* plants (Samanani et al. 2002). These transformation systems have provided the opportunity to alter the activity of individual enzymes of benzylisoquinoline alkaloid biosynthesis, and to examine the consequences of such modifications on the accumulation of pathway products and intermediates.

The efficiency of any transformation protocol is largely proportional to the successful development of effective tissue culture procedures. In this regard, the regeneration of intact opium poppy plants in vitro has proven rather difficult. Generally, the induction of opium poppy embryogenic callus is achieved after cultivation of explant material on a medium auxin, and sometimes cytokinin. Embryo conversion and development typically proceeds on hormone free medium. The regeneration of opium poppy plants through somatic embryogenesis has been reported with some variation in hormone concentration and explant source (Nessler 1982; Wakhlu and Bajwa 1986; Dieu and Dunwell 1988). However, converting opium poppy embryos invariably do not develop roots and require additional treatment to induce root formation (Ovecka et al. 1997; Kassemi and Jacquin 2001; Khanna et al. 2005). Moreover, the production of secondary embryos or shoot buds during the cultivation of opium poppy somatic embryos on hormone-free media is also common (Ovecka et al. 1997; Ovecka et al. 2000). A similar developmental pattern of regeneration was reported for other members of Papaveraceae, including *P. bracteatum* (Ilahi and Ghauri 1994), *P. orientale* (Schuchmann and Wellmann 1983), *Corydalis yanhusuo* (Sagare et al., 2000) and *E. californica* (Park and Facchini 1999, 2001). Variations in existing protocols for opium poppy regeneration most likely to involve genotype-specific characteristics of used cultivars. Shoot organogenesis is another option for the regeneration of opium poppy. We have reported a system for opium poppy regeneration via shoot organogenesis (Park and Facchini 2000a). Shoots were induced from isolated cotyledons on medium containing benzyladenine (BA). Recently, an optimized somatic embryogenesis procedure for the transformation of a commercial opium poppy cultivar was developed (Chitty et al. 2003). A major improvement in the protocol was achieved by buffering the culture medium used to maintain explants infected with *Agrobacterium tumefaciens*. However, transgenic plants were obtained for only two commercial cultivars among a wide range that were tested, supporting a correlation between genotype, and susceptibility to *A. tumefaciens* infection and/or regeneration capacity.

Antisense RNA-or RNAi-based post-transcriptional gene silencing (PTGS) were used to modify alkaloid biosynthesis in both California poppy and opium poppy, especially when single genes or a family of closely related genes were involved (Park et al. 2002, 2003; Allen et al. 2004; Frick et al. 2004). PTGS frequently results in plants or cell cultures with essentially knockout phenotypes when reliable transformation protocols are available. The antisense RNA-mediated suppression of BBE and CYP80B1 dramatically reduced the accumulation of benzylisoquinoline alkaloids in transgenic California poppy cell and root cultures (Park et al. 2002, 2003). Aromatic amino acid levels were also altered, suggesting that flux changes in benzylisoquinoline alkaloid metabolism resulted in the modulation of relevant primary metabolic pathways. Transgenic California poppy root cultures with increased levels of BBE displayed higher levels of sanguinarine and other

benzophenanthridine alkaloids compared with controls (Park et al. 2003). Transgenic opium poppy plants expressing an antisense-*BBE* gene also exhibited an altered ratio of latex alkaloids compared with wild type plants (Frick et al. 2004). Interestingly, root alkaloid ratios remained unchanged, suggesting the role of enzymes other than BBE in the control of sanguinarine biosynthesis. The RNAi-mediated replacement of morphine with non-narcotic (*S*)-reticuline highlights the potential commercial value of metabolic engineering in opium poppy (Allen et al. 2004). A chimeric hairpin RNA construct designed to silence all members of the *COR* gene family resulted in the accumulation of (*S*)-reticuline, which has pharmacological value as a potential hair-growth stimulant, and an antibacterial, antimalarial, and anticancer agent.

Biosynthetic enzymes present at pathway junctions have also been targets for the manipulation of benzylisoquinoline alkaloid metabolism. (*S*)-scoulerine 9-*O*-methyltransferase (SOMT) is thought to control the ratio of coptisine to berberine and columbamine in *C. japonica* cells (Sato et al. 2001). Overexpression of this gene caused a 20% increase in enzyme activity, and elevated levels of berberine and columbamine. Expression of *C. japonica SOMT* in cultured California poppy cells, which lacks this enzyme, resulted in the production of columbamine, which is normally not produced. The introduction of SOMT appeared to redirect flux toward columbamine at the expense of sanguinarine. The transient RNAi-mediated silencing of the *SOMT* gene in *C. japonica* protoplasts was shown to substantially reduce SOMT activity as a potentially useful system to systematically evaluate the function of alkaloid biosynthetic genes (Dubouzet et al. 2005).

Future prospects

Opium poppy has emerged as a versatile model system to investigate the biology of alkaloid metabolism. An impressive number of cDNAs encoding alkaloid biosynthetic enzymes have already been isolated and the pace of gene discovery will undoubtedly increase with the application of genomic technologies. Within the realm of genomics, EST databases and microarray chips have been established, but proteomic and metabolomic platforms require further refinement. Functional genomic strategies will benefit from the wealth of genetic diversity resulting from the intensive classical breeding of opium poppy and the development of new techniques, such as TILLING. However, the full potential of functional genomics in opium poppy is still restricted by the limited efficiency of available genetic transformation protocols, which also hinders the application of metabolic engineering for both experimental and commercial purposes.

Future research on the regulation of BIA biosynthesis in opium poppy should target the isolation of transcription factors involved in the coordinated regulation of pathway enzymes and other metabolic components. Similarly, little is known about the intra- and inter-cellular translocation of pathway intermediates, which might involve specific transporters or the symplastic movement of metabolites. In addition, a complex subcellular organization of the biosynthetic machinery involved in BIA metabolism has begun to emerge, but is poorly understood. A thorough understanding of metabolic regulation—at the transcriptional, cellular, and biochemical levels—is crucial to realize the biotechnological goal of rationally engineering BIA metabolism in opium poppy and, indeed, secondary metabolism in general.

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