Benzylisoquinoline Alkaloid Metabolism: A Century of Discovery and a Brave New World

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Benzylisoquinoline alkaloids (BIAs) are a structurally diverse group of plant specialized metabolites with a long history of investigation. Although the ecophysiological functions of most BIAs are unknown, the medicinal properties of many compounds have been exploited for centuries. These include the narcotic analgesics codeine and morphine, the antimicrobial agents sanguinarine and berberine, and the antitussive and anticancer drug noscapine. BIA biosynthesis involves a restricted number of enzyme types that catalyze landmark coupling reactions and subsequent functional group modifications. A pathogenesis-related (PR)10/Bet v1 'Pictet–Spenglerase', several O-methyl-, N-methyl- and O-acetyltransferases, cytochromes P450, FAD-dependent oxidases, non-heme dioxygenases and NADPH-dependent reductases have been implicated in the multistep pathways leading to structurally diverse alkaloids. A small number of plant species, including opium poppy (*Papaver somniferum*) and other members of the Ranunculales, have emerged as model systems to study BIA metabolism. The expansion of resources to include a wider range of plant species is creating an opportunity to investigate previously uncharacterized BIA pathways. Contemporary knowledge of BIA metabolism reflects over a century of research coupled with the development of key innovations such as radioactive tracing, enzyme isolation and molecular cloning, and functional genomics approaches such as virus-induced gene silencing. Recently, the emergence of transcriptomics, proteomics and metabolomics has expedited the discovery of new BIA biosynthetic genes. The growing repository of BIA biosynthetic genes is providing the parts required to apply emerging synthetic biology platforms to the development of production systems in microbes as an alternative to plants as a commecial source of valuable BIAs.

Abbreviations: BIA, benzylisoquinoline alkaloid; BBE, berberine bridge enzyme; CAS, canadine synthase; CheSyn, cheilanthifoline synthase; CNMT, coclaurine *N*-methyltransferase; CODM, codeine *O*-demethylase; COR, codeinone reductase; CYP, cytochrome P450; DBOX, dihydrobenzophenanthridine oxidase; DHBO, copper-dependent dihydrobenzophenanthridine oxidase; 3,4-DHPAA, 3,4-dihydroxyphenylacetaldehyde; DRR, 1,2-dehydroreticuline reductase; DRS, 1,2-dehydroreticuline synthase; 4-HPAA, 4-hydroxyphenylacetaldehyde; LC, liquid chromatography; NMCH, *N*-methylcoclaurine *N*-methyltransferase; NMR, nuclear magnetic resonance; MS, mass spectroscopy; MSH, *N*-methylstylopine 14-hydroxylase; NCS, norcoclaurine synthase; 6OMT, norcoclaurine 6-*O*-methyltransferase; 4′OMT, 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase; 7OMT, reticuline 7-*O*-methyltransferase P6H, protopine 6-hydroxylase; PR, PATHOGENESIS-RELATED; RNAi, RNA interference; SalAT, salutaridinol 7-*O*-acetyltransferase; SalR, salutaridine:NADPH 7-oxidoreductase; SalSyn, salutaridine synthase; SOMT, scoulerine *O*-methyltransferase; STOX, (S)-tetrahydroprotoberberine oxidase; StySyn, stylopine synthase; T6ODM, thebaine 6-*O*-demethylase; TNMT, tetrahydroprotoberberine *cis*-*N*-methyltransferase; VIGS, virus-induced gene silencing.

Introduction

Benzylisoquinoline alkaloids (BIAs) are a diverse group of specialized plant metabolites that includes approximately 2,500 known structures. Many BIAs possess potent pharmacological properties, including the narcotic analgesics morphine and codeine, the antimicrobials sanguinarine and berberine, the muscle relaxants (+)-tubocurarine and papaverine, and the cough suppressant and anticancer drug noscapine. Some of humankind's oldest medicines are BIAs derived from plants. Opium poppy (*Papaver somniferum*), for example, was one of the first domesticated crops and a traditional source of analgesia (Hagel et al. 2007). The healing properties of greater celandine (*Chelidonium majus*) were known throughout Europe and Asia during the Emperial Roman period (Pliny 1966), and New World aboriginal cultures exploited the antimicrobial effects of BIA-containing plants by using sap or root extracts to treat minor cuts and infections. Pomo women used

California poppy (*Eschscholtzia californica*) latex to dry up breastmilk during the weaning of infants (Loeb 1926, Barrett 1952), and Navajo peoples drank the tea of Fendler's meadow rue (*Thalictrum fendleri*) during ceremonial War Dance rites (Elmore 1944). Beyond medicine and ritual, human interactions with BIAs have also led to historical and modern problems including opiate addiction and political strife, such as the Anglo-Chinese Wars of the mid-19th century and recent incidents involving Afghanistan insurgents bolstered by drug trade profits (Piazza 2012). Even epidemics such as dropsy in India have resulted from the use of edible oils contaminated with the toxic alkaloid sanguinarine (Sharma 1999).
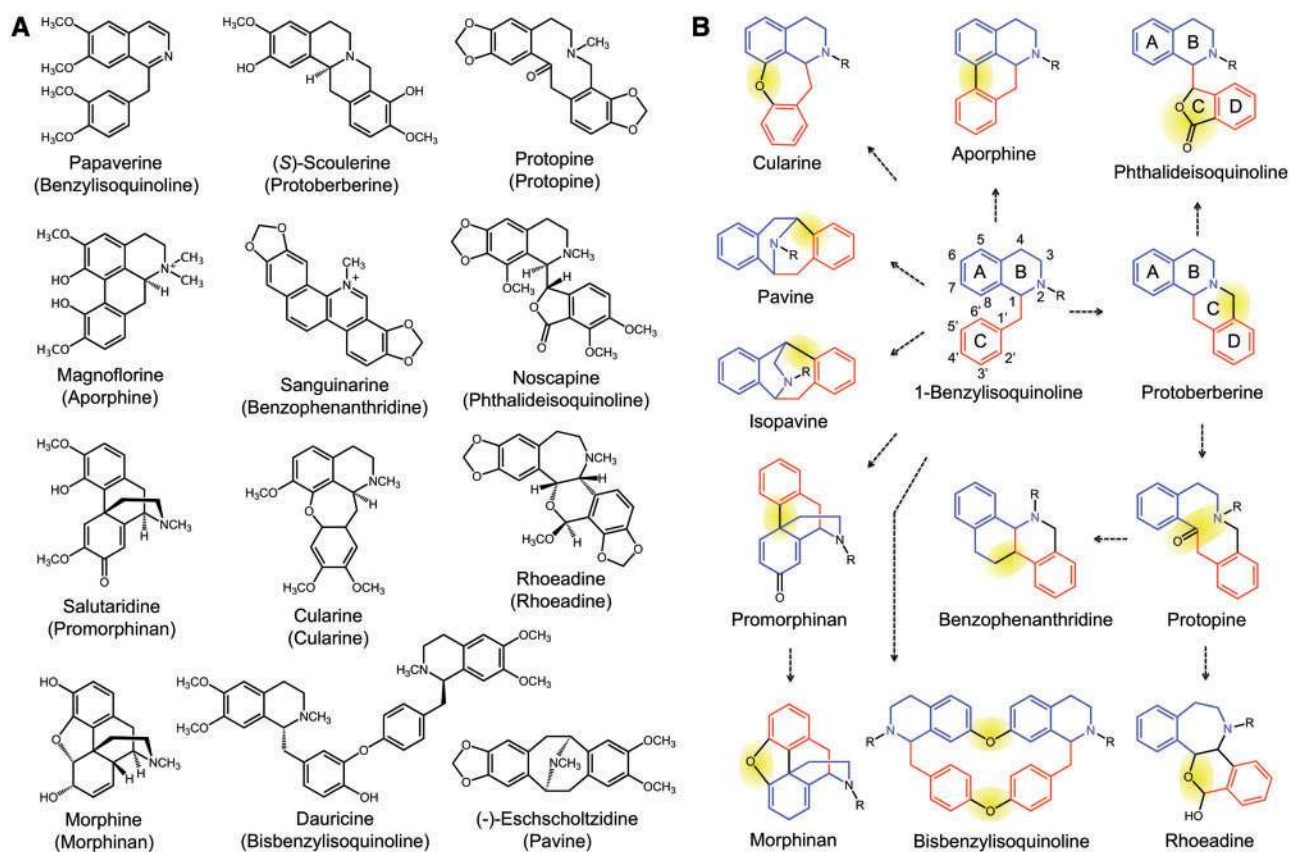
The economic, political and social importance of BIAs has prompted considerable research into how plants produce specific compounds. Research on BIAs began in earnest with the purification of morphine in 1806 from opium poppy latex, marking the first isolation of any alkaloid (Sertürner 1806) and initiating an explosion of scientific interest in plant-derived compounds with basic properties. As testament to the complex stereochemistry of BIAs, the structural characterization of morphine was difficult, and it was not until the 1950s that the first syntheses were published (Zenk and Juenger 2007). Early investigators began proposing reaction mechanisms (Winterstein and Trier 1910) and biosynthetic schemes to suggest how plants could produce such complex molecules. Empirial support for, or revision of, these schemes was initially achieved through radiolabel tracer studies (Spenser 1968), which laid the groundwork for biochemical investigations aimed at characterizing biosynthetic enzymes and their corresponding genes. Continued effort has allowed the complete, or near-complete, elucidation of entire metabolic pathways, such as those leading to berberine, sanguinarine and morphine (Ziegler and Facchini 2008, Hagel and Facchini 2010a). Recently, milestone discoveries based on novel in planta technologies such as virus-induced gene silencing (VIGS) have advanced our understanding of the biosyntheses of other alkaloids such as noscapine (Dang and Facchini 2012, Winzer et al. 2012) and papaverine (Desgagné-Penix and Facchini 2012).

Occurrence, Diversity, Ecology and Pharmacology

Interest in BIA biosynthesis raised fundamental questions concerning the biological function of these compounds. As secondary or specialized metabolites, BIAs do not appear essential for normal plant growth and development, but might play a key role in plant defense against herbivores and pathogens. Ironically, much more is known about the pharmacology of BIAs and relatively little study has been dedicated to determining their ecophysiological roles. Sporadic reports are available on the antifeedant properties of some BIAs toward pests (Krug and Proksch 1993, Park et al. 2000, Shields et al. 2008, Sellier et al. 2011) and the inhibitory effects on the propagation of microorganisms and viruses (Schmeller

et al. 1997). Mammals seem prone to the bitter taste of alkaloids. For example, the buffy-headed marmoset (*Callithrix flaviceps*), a primate native to South America, is known to avoid the BIA-rich fleshy fruit of the rainforest fevertree (*Siparuna guianensis*) when eating its nutritious seeds (Simas et al. 2001). Based on biochemical and molecular phylogenetic evidence, the occurrence of BIAs is thought to be of monophyletic origin (Liscombe et al. 2005). Although the accumulation of certain BIAs has been documented in diverse angiosperm taxa, these alkaloids are most common among the order Ranunculales, specifically the Papaveraceae, Ranunculaceae, Berberidaceae and Menispermaceae families (Ziegler and Facchini 2008). Prominent examples of BIAs that belong to different structural subgroups are shown in **Fig. 1A**. Protoberberine, benzophenanthridine and 1-benzylisoquinoline alkaloids show a broad taxonomic distribution and have even been isolated from gymnosperms (Xu and Lin 1999). Quaternary ammonium salts of protoberberine and benzophenanthridine alkaloids often possess antimicrobial activity, supporting their use in both traditional and modern medicine. The planar molecules sanguinarine and berberine have been identified as potential anticancer drugs owing to their roles as ligands that stabilize human telomeric G-quadruplex DNA (Bessi et al. 2012). Beyond its pharmaceutical applications, berberine exhibits potent antifeedant activity towards myriad insects including gypsy moth larvae (*Lymantria dispar*) (Shields et al. 2008), fourth instar larvae of fall webworms (*Hyphantria cunea*), adult Alder leaf beetles (*Agelastica coerulea*) (Park et al. 2000) and fruit flies (*Drosophila melongaster*) (Sellier et al. 2011). Antiherbivory activity, together with an ability to reduce larval growth and survival rates severely among certain generalist pests (Krug and Proksch 1993), has prompted consideration of berberine as a commercial insecticide (Shields et al. 2008).

The 1-benzylisoquinoline papaverine (**Fig. 1A**) is a major alkaloid in the latex of some opium poppy chemotypes and has been used as a non-specific vasodilator owing to its direct action on smooth muscle (McGuinness and Gandhi 2010). Although its use to treat cerebral vasospasm has largely been replaced by modern drugs, papaverine is still used topically and as an injectable to treat erectile dysfunction (Dinsmore 2005). Bisbenzylisoquinoline alkaloids are structural dimers of 1-benzylisoquinolines and several possess important pharmacological properties. For example, dauricine is an effective calcium channel blocker and antiarrhythmic agent found in Chinese herbal preparations of Moonseed (*Menispermum dauricum*) (Qian 2002). Another prominent bisbenzylisoquinoline alkaloid is (+)-tubocurarine, the principal neuromuscular blocking agent found in curare. South American aboriginals used the word 'curare' to describe poisons, particularly those derived from the vine *Chondrodendron tomentosum*, used to coat the tips of hunting arrows or blowpipe darts (Bowman 2006). Morphinan and promorphinan alkaloids are produced by certain genera of the Papaveraceae, Menispermaceae and Berberidaceae (Liscombe et al. 2005), and include valuable

Fig. 1 (A) Examples of BIAs found in plants. The structural subgroup to which each alkaloid belongs is indicated in parentheses. (B) BIA structural subgroups derived from the basic benzylisoquinoline subunit. Blue designates the part(s) of each molecule originating from the tetrahydroisoquinoline moiety derived from dopamine. Red designates the part(s) of each molecule originating with the benzylic moiety derived from 4-HPAA. Yellow highlights show landmark C–C or C–O bonds formed in the benzylisoquinoline subunit and defining each structural subgroup. Myriad substitutions, including hydroxylation, O- and N-methylations, glycosylation and so forth, build upon these scaffolds to generate naturally occurring diversity. For example, the -R group of nitrogen may be -H or -CH₃ (representing a secondary or tertiary amine, respectively), or a quaternary amine may be present via N,N-dimethylation or ring aromatization. Functional groups characteristic of certain structural categories (e.g. carbonyl groups of phthalideisoquinoline and protopine alkaloids) are shown. Stereochemistry is not indicated since both (R)- and (S)-configurations exist in many cases. Ring designations indicated for structures as discussed in text.

pharmaceuticals such as the narcotic analgesics morphine and codeine. Thebaine, a morphinan alkaloid extracted on a commercial scale from opium poppy straw, is the starting material for the manufacture of semi-synthetic opiates including oxycodone, oxymorphone, etorphine and buprenorphine (Hagel et al. 2007). Additionally, thebaine is the precursor in the syntheses of naloxone, naltrexone and other compounds used to treat opiate poisoning and addiction. Whether or not morphinan alkaloids play ecophysiological roles in plants is unclear. In opium poppy plants subjected to mechanical tissue damage, morphine was rapidly metabolized to bismorphine, which was incorporated into the cell wall and shown to increase resistance to hydrolysis by pectinase, suggesting a defensive function for morphine (Morimoto et al. 2001). Phthalideisoquinoline alkaloids are restricted to the genera *Eschscholtzia*, *Papaver*, *Dicentra* and *Glaucium* within the Papaveraceae, and *Cocculus* within the Menispermaceae (Shulgin and Perry 2002). The phthalideisoquinoline alkaloid noscapine, which also occurs in opium

poppy latex, has antitussive properties and was recently shown to possess anticancer activity (Barken et al. 2008). Pavine, isopavine, rhoeadine and cularine alkaloids represent small, relatively unrelated BIA subgroups found in most Papaveraceae genera (Liscombe et al. 2005) and some members of the Menispermaceae and Ranunculaceae, such as meadow rue (*Thalictrum flavum*). The reduced forms of several cularine and isocularine alkaloids are highly active at dopaminergic receptor sites (Protais et al. 1995), whereas oxidized forms show considerable cytotoxic activity (Suau et al. 2005).

Our long-standing interest in BIA metabolism is reflected by the large volume of literature on topics ranging from traditional medicine to structural biochemistry. Current schemes for BIA biosynthesis have emerged from decades of intensive research based on empirical observations and hypothetical deductions. For example, inferences have typically considered whether or not certain alkaloids occur naturally, the substrate acceptance profiles of isolated enzymes, comparisons with organic

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syntheses and analogies with other systems such as mammalian alkaloid metabolism. Over the years, BIA biosynthetic pathways have been modified and corrected with the availability of new data arising from advancing technologies. Precise knowledge of BIA biosynthetic networks is of paramount importance to the emerging field of synthetic biology, which aims to reconstruct plant pathways in microorganisms with the ultimate goal of creating alternative systems for the production of valuable bio-products (Facchini et al. 2012). Here we provide a critical review of the current status of plant BIA biosynthesis and discuss emerging prospects and applications.

Biosynthesis

Origins of structural diversity

Despite vast structural diversity, BIAs share a common biosynthetic origin beginning with the first committed intermediate (S)-norcoclaurine, which is formed through the condensation of two tyrosine derivatives, dopamine and 4-hydroxyphenylacetaldehyde (4-HPAA). Dopamine is incorporated into the tetrahydroisoquinoline component, whereas 4-HPAA comprises the C1-linked benzyl moiety. The resulting 1-benzylisoquinoline core of (S)-norcoclaurine and related BIAs such as the branch point intermediate (S)-reticuline is central to the biosynthesis of many other structural types, several of which are illustrated in **Fig. 1B**. Landmark C–C or C–O coupling reactions involving one or two 1-benzylisoquinoline units yield the core backbone structures of other alkaloid subgroups. The various BIA structural scaffolds undergo differential functionalization, such as aromatic ring hydroxylation, or N-methylation generating a tertiary or quaternary amine, to yield the diversity of known compounds. The availability or addition of hydroxyl groups provides opportunity for downstream reactions including O-methylation, O-acetylation and methylenedioxy bridge formation. Hydroxyl groups can also play active roles in catalysis during additional enzyme-mediated reactions. For example, the 3′-hydroxyl function of (S)-reticuline is critical for the formation of the protoberberine N7–C8 bridge (Winkler et al. 2008) and is probably required for the C–C phenol coupling reaction of (S)- or (R)-reticuline yielding aporphine (Ikezawa et al. 2008) and promorphinan (Gesell et al. 2009) alkaloids, respectively (**Fig. 1B**). In addition to a 3′-hydroxyl group, N7–C8 berberine bridge formation requires an N-methyl group in the 1-benzylisoquinoline structure, which undergoes nucleophilic attack as part of a concerted C–C coupling mechanism (Winkler et al. 2008). In some cases, the addition of a new group initiates the non-enzymatic restructuring of an alkaloid backbone. Hydroxylation of protopine at the C6 position leads to spontaneous ring opening, rearrangement and dehydration to yield the benzophenanthridine alkaloid, dihydrosanguinarine (Tanahashi and Zenk 1988). Formation of the oxide bridge between C4 and C5 of morphinan alkaloids requires O-acetylation of the promorphinan intermediate salutaridinol, which initiates spontaneous allylic elimination

of acetate at pH 8–9 (Lenz and Zenk 1995). Alternatively, under acidic conditions (pH 6–7), O-acetylation initiates the formation of an entirely different structure containing a nine-membered ring (i.e. dibenz[d,f]azinone alkaloids).

Enzymes of BIA metabolism

A restricted number of enzyme families have been implicated in BIA metabolism. Whereas some enzymes exhibit a relatively broad substrate range, others are highly substrate specific. Enzymes of the oxidoreductase, transferase and lyase (i.e. Pictet–Spenglerase) categories have been characterized from BIA-producing plants. **Table 1** provides a comprehensive list of these catalysts according to enzyme type (i.e. protein family or superfamily). The first committed enzyme of BIA metabolism, (S)-norcoclaurine synthase (NCS), catalyzes the condensation of dopamine and 4-HPAA through the formation of a C–C bond, yielding the basic 1-benzylisoquinoline core. As a member of the pathogenesis-related 10 (PR10) protein/Bet v1 allergen family, NCS is unique in BIA metabolism, with no known enzyme homologs. Functionally, NCS is one of only three isolated enzymes catalyzing a Pictet–Spengler condensation. Two other Pictet–Spenglerases are strictosidine synthase (Ziegler and Facchini 2008) and deacetylipecoside synthase (De-Eknamkul et al. 2000), which catalyze the first steps in the biosynthesis of monoterpenoid indole alkaloids and tetrahydroisoquinoline monoterpene glucosides, respectively. Although NCS is strictly stereoselective, forming only (S)-configured products, the enzyme was recently shown to cyclize dopamine with a wide variety of acetaldehydes to yield a diverse array of substituted tetrahydroisoquinoline alkaloids (Ruff et al. 2012). The apparent promiscuity of NCS could prove important for the development of general biocatalytic strategies for the synthesis of novel alkaloids. In addition to NCS, oxidoreductases such as cytochromes P450 (CYPs) and FAD-dependent enzymes (e.g. berberine bridge enzyme, BBE), catalyze the formation of C–C or C–O bonds to establish various BIA backbone structures (**Fig. 1B**). Three CYP families play key oxidative roles in BIA metabolism. Characterized members of the CYP80 family include berbamunine synthase (*Berberis stolonifera* CYP80A1), (S)-N-methylcoclaurine 3′-hydroxylase (California poppy CYP80B1 and opium poppy CYP80B3) and CYP80G2 from Japanese goldthread (Ikezawa et al. 2008, Ziegler and Facchini 2008). In addition to hydroxylation, members of this family catalyze intermolecular C–O and intramolecular C–C phenol coupling reactions. The formation of both bisbenzylisoquinoline and aporphine alkaloids is initiated by CYP80 enzymes. Salutaridine synthase, a member of the CYP719 family, also catalyzes a pivotal bridge-forming reaction through a C–C phenol coupling mechanism, yielding promorphinan alkaloids (Gesell et al. 2009).

Members of the CYP719A subfamily catalyze methylenedioxy bridge formation in the biosynthesis of alkaloids such as berberine and sanguinarine (**Fig. 1A**). Enzymes from Japanese goldthread and California poppy target methylenedioxy bridge formation exclusively at the A-rings of their

protoberberine substrates (**Fig. 1B**). For example, Japanese goldthread CYP719A1 yields (*S*)-canadine from (*S*)-tetrahydro-columbamine (Ikezawa et al. 2003), whereas California poppy CYP719A2 and CYP719A3 convert (*S*)-cheilanthifoline to (*S*)-stylopine (Ikezawa et al. 2007) (**Table 1**). Interestingly, CYP719A13 from Mexican prickly poppy (*Argemone mexicana*) performs both of these reactions although its close homolog CYP719A14 specifically forms methylenedioxy bridges on the opposite side of the protoberberine skeleton (i.e. the D-ring), yielding cheilanthifoline from (*S*)-scoulerine (Díaz Chávez et al. 2011). Recently, the involvement of a third family in BIA metabolism was reported, allowing the final two steps in sanguinarine biosynthesis to be elucidated at the molecular level. CYP82N2v2 from Japanese goldthread showed hydroxylase activity toward protopine and allocryptopine, producing the respective products dihydrosanguinarine and dihydrochelerythrine (Takemura et al. 2012). The second of the remaining steps is catalyzed by (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase (MSH). An opium poppy MSH clone isolated and expressed in *Saccharomyces cerevisiae* accepted the *N*-methylated protoberberine alkaloids *N*-methylstylopine and *N*-methylcanadine, producing protopine and allocryptopine, respectively (Beaudoin and Facchini 2013).

BBE is a FAD-dependent oxidoreductase unique in its ability to catalyze the formation of an intramolecular C–C bond between the phenyl moiety and the *N*-methyl group of various 1-benzylisoquinolines, yielding protoberberine alkaloids (**Fig. 1B**) (Ziegler and Facchini 2008). The cyclization reaction catalyzed by BBE has no known equivalent in organic chemistry and the underlying mechanism has been studied in detail (Winkler et al. 2008). FAD-dependent homologs of BBE include (*S*)-tetrahydroprotoberberine oxidase (STOX) (Gesell et al. 2011) and dihydrobenzophenanthridine oxidase (DBOX) (Hagel et al. 2012), which generally catalyze ring aromatization reactions (**Table 1**). Opium poppy DBOX oxidizes substrates belonging to benzophenanthridine, protoberberine and 1-benzylisoquinoline structural subgroups (**Fig. 1B**). DBOX preferentially catalyzes the oxidation of dihydrosanguinarine to sanguinarine, but also converts (*R*,*S*)-tetrahydropapaverine to papaverine and several protoberberine alkaloids to oxidized forms including (*R*,*S*)-canadine to berberine.

The formation of sanguinarine by DBOX has been suggested as a reversible cellular process via the action of the Rossmann fold-containing, NAD(P)H-dependent enzyme sanguinarine reductase (SanR) (Vogel et al. 2010). Model-guided structural analysis of SanR from California poppy supported a probable evolution from either NADPH-dependent epimerases or hydroxysteroid reductases. Sequence analysis of SanR revealed homology with members of the short-chain dehydrogenase/reductase (SDR) superfamily, specifically those of the atypical or 'extended' subgroup (Kavanagh et al. 2008). Another reductase involved in BIA metabolism, salutaridine reductase (SalR), is a 'classical' member of the SDR superfamily as defined by highly conserved cofactor and active site sequence motifs (Ziegler et al. 2009). SalR is responsible for the stereospecific,

NADPH-dependent reduction of salutaridine to 7(*S*)-salutaridinol in the morphinan alkaloid branch pathway. Mutational and crystallographic analyses of SalR from opium poppy have established a structural basis for substrate inhibition (Ziegler et al. 2009, Higashi et al. 2011). In contrast to SanR and SalR, a third NADPH-dependent enzyme in BIA metabolism, codeinone reductase (COR), belongs to the distinct aldo-keto reductase (AKR) superfamily. Members of this superfamily are functionally diverse, but share a common $(\alpha/\beta)_8$-barrel fold and reduce carbonyl groups to yield primary or secondary alcohols (Mindnich and Penning 2009). In the late steps of morphine biosynthesis, COR reduces the carbonyl groups of codeinone and morphinone to generate codeine and morphine, respectively (Unterlinner et al. 1999).

Recently, a new family of oxidoreductases in BIA metabolism with specific roles in the final steps of morphine biosynthesis have been identified. Thebaine 6-*O*-demethylase (T6ODM) and codeine *O*-demethylase (CODM) are members of the Fe(II) and 2-oxoglutarate (Fe^{2+}/2OG)-dependent dioxygenase family that occur uniquely in opium poppy and catalyze the *O*-demethylation of both morphinan and protoberberine alkaloids (Hagel and Facchini 2010a) (**Table 1**). Since CYPs perform functionally analogous reactions as part of endogenous morphine metabolism in mammals (Grobe et al. 2009), it was long presumed that morphinan alkaloid *O*-demethylation in opium poppy was also catalyzed by CYPs (Chou and Kutchan 1998). The discovery of T6ODM and CODM, which to date represent the only known Fe^{2+}/2OG-dependent dioxygenases catalyzing *O*-demethylation, provides a rational basis for the widespread occurrence of demethylases in BIA metabolism (Hagel and Facchini 2010b). Our recent awareness of dealkylation within the context of alkaloid biosynthesis is in contrast to the long-standing appreciation of the roles played by methyl- and acetyltransferases (Ziegler and Facchini 2008). Phylogenetic analysis shows that methyltransferases involved in BIA metabolism segregate into two disparate clades distinguished by the requirement for either *O*- or *N*-linked methyl moieties (Liscombe and Facchini 2008, Liscombe et al. 2009). In general, methyltransferases of BIA metabolism accept a wide variety of alkaloid substrates with diverse backbone structures, with some showing more flexibility than others with respect to substrate range. For example, enzymes such as meadow rue coclaurine *N*-methyltransferase (CNMT) accept diverse substrates including 1-benzylisoquinolines, protoberberines and pavine alkaloids (Liscombe et al. 2009) (**Table 1**). One acetylating enzyme belonging to the BAHD family (D'Auria 2006), salutaridinol 7-*O*-acetyltransferase (SalAT), also functions in BIA metabolism. SalAT adds an acetyl function to salutaridinol to initiate the spontaneous formation of the first pentacyclic alkaloid thebaine in the morphinan alkaloid pathway.

BIA metabolism in opium poppy and other plants

Opium poppy accumulates a large number of BIAs that belong to different structural subgroups, including morphinan, protoberberine, benzophenanthridine, phthalideisoquinoline,

Table 1 Enzymes of BIA metabolism, grouped according to protein family or superfamily, for which corresponding genes have been isolated

Enzyme name	Abbrev.	EC number	Major pathway(s)	Substrate(s)	Product(s)	Defining reaction	Initial reference
Pictet–Spengler condensation (PR10/Bet v 1 protein)							
Norcoclaurine synthase	NCS	4.2.1.78	Benzylisoquinoline	Dopamine and 4-Hydroxyphenyl-acetaldehyde or 3,4-Dihydroxyphenyl-acetaldehyde	(S)-Norcoclaurine or (S)-Norlaudanosoline		Samanani et al. (2004)
SAM-dependent O-methyltransferase							
Norcoclaurine 6-O-methyltransferase	6OMT	2.1.1.128	Benzylisoquinoline	(S)-Norcoclaurine	(S)-Coclaurine		Morishige et al. (2000)
3′-Hydroxy-N-methylcoclaurine 4′-O-methyltransferase	4′OMT	2.1.1.116	Benzylisoquinoline	(S)-3′-Hydroxy-N-methylcoclaurine	(S)-Reticuline		Morishige et al. (2000)
Reticuline 7-O-methyltransferase	7OMT	2.1.1.-	Benzylisoquinoline	(R,S)-Reticuline	(R,S)-Laudanine		Ounaroon et al. (2003)
Norreticuline 7-O-methyltransferase	N7OMT	2.1.1.-	Benzylisoquinoline	(R,S)-Norreticuline	(R,S)-Norlaudanine		Pienkny et al. (2009)
Scoulerine 9-O-methyltransferase	SOMT	2.1.1.117	Protoberberine	(S)-Scoulerine	(S)-Tetrahydro-columbamine		Takeshita et al. (1995)

(continued)

Table 1 Continued

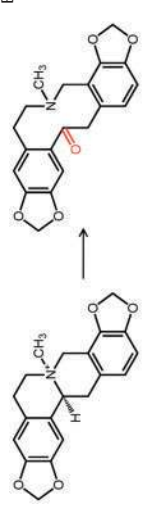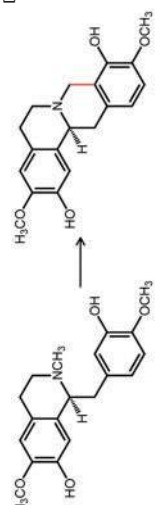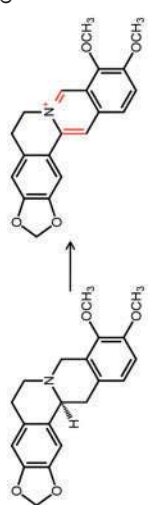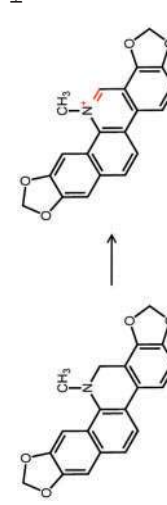| Enzyme name	Abbrev.	EC number	Major pathway(s)	Substrate(s)	Product(s)	Defining reaction	Initial reference
Columbamine O-methyltransferase	CoOMT	2.1.1.118	Protoberberine	Columbamine	Palmatine		Morishige et al. (2002)
SAM-dependent N-methyltransferase							
Coclaurine N-methyltransferase	CNMT	2.1.1.140	Benzylisoquinoline	(S)-Coclaurine	(S)-N-Methylcoclaurine		Choi et al. (2002)
Tetrahydroprotoberberine cis-N-methyltransferase	TNMT	2.1.1.122	Protoberberine, Benzophenanthridine, Phthalideisoquinoline	(S)-Stylopine or (S)-Canadine	(S)-N-Methylstylopine or (S)-N-Methylcanadine		Liscombe and Facchini (2007)
Pavine N-methyltransferase	PavNMT	2.1.1.-	Pavine	Pavine	N-Methylpavine		Liscombe et al. (2009)
P450-dependent monooxygenase							
N-Methylcoclaurine 3'-hydroxylase	NMCH CYP80B1	1.14.13.71	Benzylisoquinoline	(S)-N-Methylcoclaurine	(S)-3'-Hydroxy-N-methylcoclaurine		Pauli and Kutchan (1998)
Berbamunine synthase	BerSyn CYP80A1	1.14.21.3	Bisbenzylisoquinoline	(R,S)-Coclaurine	Berbamunine		Kraus and Kutchan (1995)

(continued)

Table 1 Continued

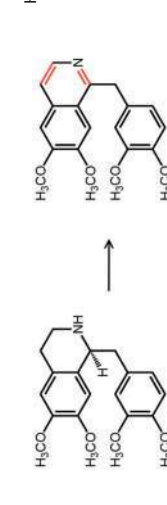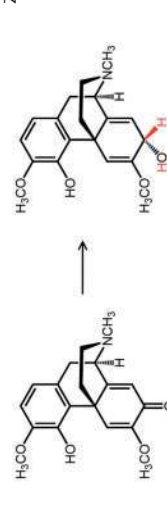| Enzyme name	Abbrev.	EC number	Major pathway(s)	Substrate(s)	Product(s)	Defining reaction	Initial reference
Corytuberine synthase	CorSyn CYP80G2	1.14.21.-	Aporphine	(S)-Reticuline	(S)-Corytuberine		Ikezawa et al. (2008)
Cheilanthifoline synthase	CheSyn CPY719 A14	1.14.21.2	Protoberberine, Benzophenanthridine	(S)-Scoulerine	(S)-Cheilanthifoline		Diaz Chavez et al. (2011)
Stylopine synthase	StySyn CYP719A2	1.14.21.1	Protoberberine, Benzophenanthridine	(S)-Cheilanthifoline	(S)-Stylopine		Ikezawa et al. (2007)
Canadine synthase	CanSyn CYP719A1	1.14.21.5	Protoberberine, Phthalideisoquinoline	(S)-Tetrahydro-columbamine	(S)-Canadine		Ikezawa et al. (2003)
Salutaridine synthase	SalSyn CYP719B1	1.14.21.4	Promorphinan, Morphinan	(R)-Reticuline	Salutaridine		Gesell et al. (2009)
Protopine 6-hydroxylase	P6H CYP82N2v2	1.14.13.55	Protopine, Benzophenanthridine	Protopine	6-Hydroxyprotopine (tautomerization to Dihydrosanguinarine)		Takemura et al. (2012)

(continued)

Table 1 Continued

Enzyme name	Abbrev.	EC number	Major pathway(s)	Substrate(s)	Product(s)	Defining reaction	Initial reference
N-Methylstylopine 14-hydroxylase	MSH CYP82N4	1.14.13.37	Protopine, Protoberberine, Benzophenanthridine	(S)-N-Methylstylopine	Protopine		Beaudoin and Facchini (2013)
FAD-linked oxidoreductase							
Berberine bridge enzyme	BBE	1.21.3.3	Protoberberine, Benzophenanthridine, Phthalideisoquinoline	(S)-Reticuline	(S)-Scoulerine		Dittrich and Kutchan (1991)
(S)-Tetrahydroproto-berberine oxidase	STOX	1.3.3.8	Protoberberine	(S)-Canadine	Berberine		Gesell et al. (2011)
Dihydrobenzophenan-thridine oxidase	DBOX	1.5.3.12	Benzophenanthridine, Benzylisoquinoline, Protoberberine	Dihydrosanguinarine or Tetrahydropapaverine or (S)-Scoulerine	Sanguinarine or Tetrahydropapaverine or Dehydroscoulerine		Hagel et al. (2012)
Tetrahydropapaverine oxidase	TPOX	1.5.3.12	Benzylisoquinoline, Benzophenanthridine, Protoberberine	Tetrahydropapaverine or Dihydrosanguinarine or (S)-Scoulerine	Tetrahydropapaverine or Sanguinarine or Dehydroscoulerine		Hagel et al. (2012)
NADPH-dependent short-chain dehydrogenase/reductase							
Salutaridine reductase	SalR	1.1.1.248	Promorphinan, Morphinan	Salutaridine	Salutaridinol		Ziegler et al. (2006)

(continued)

Table 1 Continued

Enzyme name	Abbrev.	EC number	Major pathway(s)	Substrate(s)	Product(s)	Defining reaction	Initial reference
Sanguinarine reductase	SanR	Not assigned	Benzophenanthridine	Sanguinarine	Dihydrosanguinarine		Vogel et al. (2010)
NADPH-dependent aldo-keto reductase							
Codeinone reductase	COR	1.1.1.247	Morphinan	Codeinone or Morphinone	Codeine or Morphine		Unterlinner et al. (1999)
Acetyl-CoA-dependent acetyltrasferase							
Salutaridine 7-O-acetyltransferase	SalAT	2.3.1.150	Promorphinan, Morphinan	Salutaridinol	Salutaridinol 7-O-acetate (spontaneous re-arrangement to Thebaine)		Grothe et al. (2001)
2-Oxoglutarate /Fe²⁺-dependent dioxygenase							
Thebaine 6-O-demethylase	T6ODM	1.14.11.31	Morphinan	Thebaine or Oripavine	Codeinone or Morphinone		Hagel and Facchini (2010)
Codeine O-demethylase	CODM	1.14.11.32	Morphinan, Protoberberine, Protopine	Codeine or Thebaine or Scoulerine	(S)- Morphine or Oripavine or (S)-3-O-Demethylscoulerine		Hagel and Facchini (2010)

Selected substrate(s), product(s) and defining reactions are shown, although many enzymes accept additional substrates and participate in more than one major pathway. Red highlights show structural modifications catalyzed by each enzyme. The initial reference reporting the molecular characterization of each enzyme is indicated. PR10, pathogenesis-related 10; SAM, S-adenosyl-ʟ-methionine.

aporphine, pavine, rhoeadine and protopine alkaloids (Hosztafi 1998). Depending on the chemotype, the most abundant alkaloids in the latex are the morphinans (i.e. morphine, codeine and thebaine), the phthalideisoquinoline noscapine and the 1-benzylisoquinoline papaverine (**Fig. 1A**). In contrast, the most abundant root alkaloids are the benzophenanthridines sanguinarine and dihydrosanguinarine (Frick et al. 2005). All known enzyme types involved in BIA metabolism are represented in opium poppy (**Fig. 2**; **Table 1**). Metabolic routes leading to the key alkaloids of opium poppy are illustrated in **Fig. 2** and show that the majority of relevant biosynthetic enzymes have been characterized. Opium poppy is one of a limited number of plant species that have been used as model organisms to investigate BIA metabolism (Facchini and De Luca 2008). Other model plants include California poppy and Mexican prickly poppy (Papaveraceae), Japanese goldthread (*Coptis japonica*) and meadow rue (Ranunculaceae), and barberry (*Berberis wilsonae*) (Berberidaceae) (**Fig. 3**).

BIA biosynthesis begins with a core pathway from (*S*)-norcoclaurine to the central intermediate (*S*)-reticuline that involves 6-*O*-methylation, *N*-methylation, 3′-hydroxylation and 4′-*O*-methylation (**Fig. 2A**). An NCS cDNA was first isolated from meadow rue (Samanani et al. 2004), and subsequent studies have provided valuable structural and mechanistic insights (Luk et al. 2007, Berkner et al. 2008, Ilari et al. 2009, Bonamore et al. 2010). Genes encoding norcoclaurine 6-*O*-methyltransferase (6OMT), coclaurine *N*-methyltransferase (CNMT) and 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT) have been isolated from opium poppy (Ounaroon et al. 2003, Ziegler et al. 2005), Japanese goldthread (Morishige et al. 2000) and meadow rue (Frick and Kutchan 1999). Although methyltransferase orthologs show extensive sequence similarity across species, the molecular basis for differences in regiospecificity is not known (Liscombe and Facchini 2008). Genes encoding the (*S*)-*N*-methylcoclaurine 3′-hydroxylase (NMCH) have also been isolated from several plants (Ziegler and Facchini 2008), and the recombinant enzyme is highly regio- and stereospecific (Pauli and Kutchan 1998).
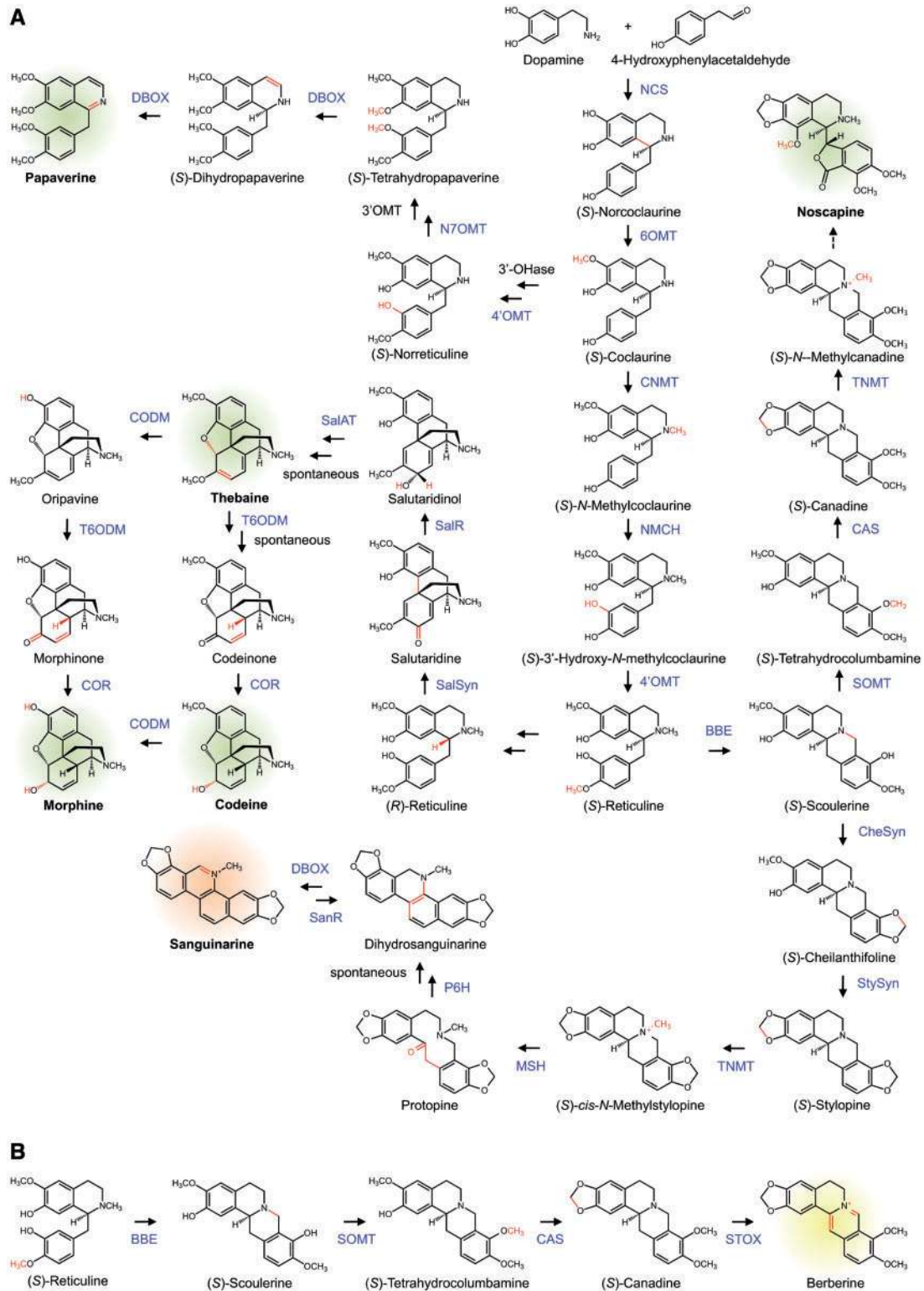
(*S*)-Reticuline is the branch point intermediate for most branch pathways including those leading to sanguinarine, noscapine, berberine and morphine (**Fig. 2**). Conversion of (*S*)-reticuline to the protoberberine (*S*)-scoulerine by BBE is a committed step in the biosynthesis of several alkaloids. The CYP-mediated addition of methylenedioxy bridges, *N*-methylation, and structural rearrangement resulting from CYP-catalyzed hydroxylation yields the benzophenanthridine, dihydrosanguinarine. Aromatization of this alkaloid is catalyzed by DBOX, the gene for which has recently been isolated from opium poppy (Hagel et al. 2012). SanR catalyzes the reverse reaction whereby sanguinarine is reduced to dihydrosanguinarine, which is thought to reduce the cytotoxic effects of sanguinarine within the cytoplasm of California poppy cells (Weiss et al. 2006). Alternatively, (*S*)-scoulerine can undergo 9-*O*-methylation by (*S*)-scoulerine *O*-methyltransferase (SOMT),

CYP-mediated methylenedioxy bridge formation, and *N*-methylation yielding *N*-methylcanadine, a purported intermediate in noscapine formation (Facchini et al. 2007, Winzer et al. 2012). (*S*)-Canadine can be oxidized to berberine by STOX (**Fig. 2B**). Although berberine is not a major alkaloid of opium poppy, it is abundant in Japanese goldthread, meadow rue and *Berberis* spp. (**Fig. 3**) (Grycová et al. 2007). The recent report of a clone encoding MSH from opium poppy (Beaudoin and Facchini 2013) marked the final step awaiting molecular elucidation, and now all genes encoding enzymes in sanguinarine and berberine biosynthesis have been isolated (Ziegler and Facchini 2008, Vogel et al. 2010, Gesell et al. 2011, Díaz Chávez et al. 2011, Takemura et al. 2012, Hagel et al. 2012, Beaudoin and Facchini 2013).

Morphine biosynthesis begins with the epimerization of (*S*)-reticuline to (*R*)-reticuline, which undergoes CYP-catalyzed C–C bond formation, reduction, *O*-acetylation and pH-dependent spontaneous rearrangement to yield thebaine (**Fig. 2A**). Two *O*-demethylases and a reductase complete the biosynthesis of morphine. Although the major route to morphine proceeds via codeinone, a minor route involves the intermediates oripavine and morphinone (Nielsen et al. 1983). Genes encoding all enzymes of the entire pathway leading to morphine have been cloned from opium poppy (Ziegler et al. 2008, Gesell et al. 2009, Hagel et al. 2010) with the exception of those responsible for reticuline epimerization. Two enzymes, an oxidase (1,2-dehydroreticuline synthase; DRS) (Hirata et al. 2004) and a reductase (1,2-dehydroreticuline reductase; DRR) (De-Eknamkul and Zenk 1992) have been isolated from opium poppy seedlings, and have been implicated in the epimerization process.
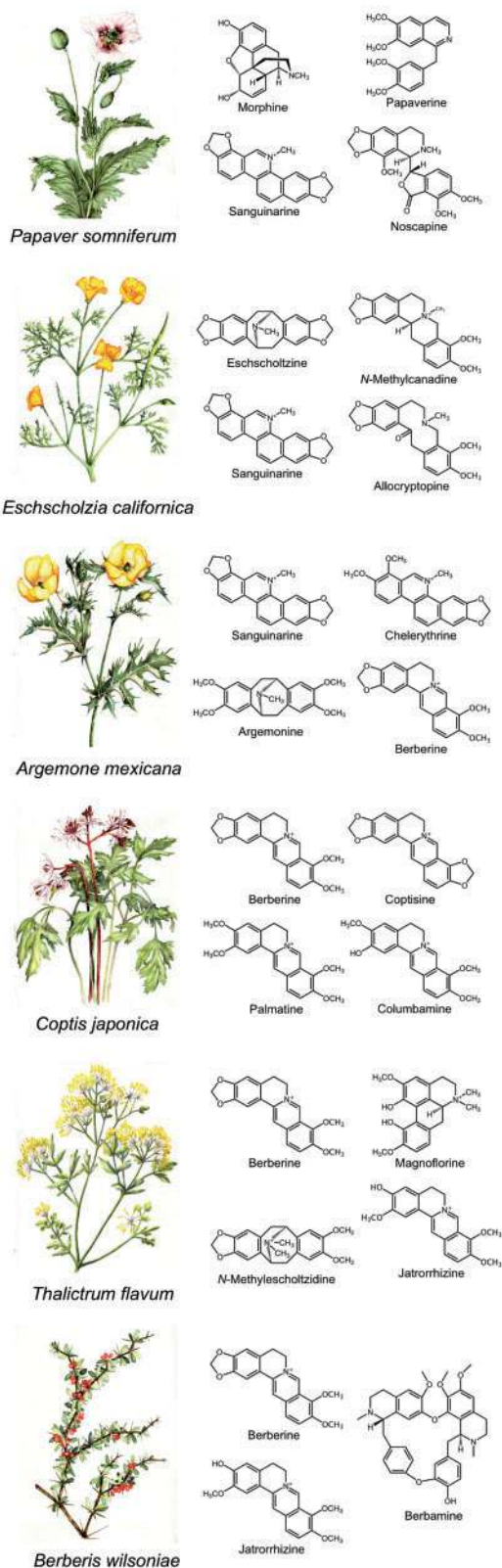
Tapping into the diversity of BIA metabolism

Decades of research have resulted in complete or near-complete isolation of biosynthetic genes involved in the biosynthesis of berberine, sanguinarine and morphine. Focused efforts using specific plants (**Fig. 3**) have allowed the establishment of platform resources, such as sequence (Liscombe et al. 2009, Desgagné-Penix et al. 2010, Desgagné-Penix et al. 2012, Farrow et al. 2012) and proteomic (Nawrot et al. 2007, Zulak et al. 2009, Angelova et al. 2010, Desgagné-Penix et al. 2010, Oldham et al. 2010) databases, cDNA micro- and macroarrays (Ziegler et al. 2005, Zulak et al. 2007, Dang et al. 2012), and nuclear magnetic resonance (NMR)- and mass spectroscopy (MS)-based metabolite profiling technologies (Zulak et al. 2007, Hagel et al. 2008, Zulak et al. 2008, Liscombe et al. 2009, Farrow et al. 2012). Such tools are invaluable for the identification and isolation of additional genes involved in the biosynthesis of the unique BIAs occurring in each species. Enzymes responsible for the biosyntheses of alkaloids such as noscapine, papaverine and derivatives of the pavine, bisbenzylisoquinoline, aporphine and protopine subgroups have not yet been identified. An unfortunate consequence of the focused effort on a small number of plants is the limited opportunity to discover enzymes involved in the production of alkaloids that are

Fig. 2 Representative BIA biosynthetic pathways. (A) Major alkaloid pathways of opium poppy. Products abundant in latex and roots are highlighted in green and orange, respectively. (B) Pathway leading from (S)-reticuline to berberine. Berberine (highlighted in yellow) occurs in various organs of many members of the Ranunculales. Corresponding genes have been isolated for enzymes shown in blue. Dotted arrows represent steps of BIA metabolism for which enzymes have not been characterized. Refer to **Table 1** for abbreviations.

Fig. 3 Plant species most extensively used to investigate BIA metabolism. Enzyme characterization and gene isolation studies have been largely restricted to a small number of plants accumulating a restricted suite of compounds. Representative alkaloids found in each species are shown.

produced exclusively or more abundantly in other species. Tapping into the immense structural diversity of BIAs requires the inclusion of plants from different families with unique alkaloid profiles. Transcriptomics and metabolite profiling projects targeting large numbers of plant species have been initiated to expand current resources (Facchini et al. 2012). Coupled with emerging technologies aimed at the high-throughput functional characterization of biosynthetic genes, such initiatives are central to future research on BIA metabolism.

Pathway Elucidation: Historical and Contemporary Approaches

Over a century of discovery

The biosynthesis of the 1-benzylisoquinoline backbone was hypothesized >100 years ago. Winterstein and Trier (1910) suggested that BIAs were formed with two L-DOPA derivatives, 3,4-dihydroxyphenylacetaldehyde (3,4-DHPAA) and dopamine, which combined to form a hypothetical alkaloid, norlaudanosoline. Almost simultaneously, Pictet and Spengler (1911) suggested that condensation between 2-arylethylamines and aldehydes resulted in ring closure, which provided a theoretical basis for the formation of norlaudanosoline and other alkaloids. At the turn of the 20th century, the mysterious ability of plants to synthesize highly complex molecules prompted speculation of 'reagents' able to catalyze transformations impossible under standard laboratory conditions. While expressing his opinion that such claims were 'exaggerated', Robinson (1917) nonetheless took the idea of enzymes into consideration when he proposed early schemes expanding on the biogenic origin of alkaloids. His work, which culminated in the 1947 Nobel Prize in Chemistry, coupled with other contributions relied heavily on chemical deduction (i.e. paper chemistry) followed by empirical studies focused on structural elucidation (Robinson 1955). Molecules were systematically degraded to reveal diagnostic fragments, or submitted to chemical transformation to identify reactive groups. The introduction of ^{14}C labeling in the late 1940s and 1950s initiated a paradigm shift toward experimental investigations of metabolic pathways (Battersby 1961, Ramstad and Agurell 1964). This breakthrough technology led to an explosion of data supporting the occurrence of complete BIA biosynthetic pathways in plants, from amino acid precursors to final products. Recognizing the structural similarities between 1-benzylisoquinolines and other alkaloid subgroups, researchers of the 1960s and 1970s linked together biosynthetic schemes connecting 1-benzylisoquinolines to more complex morphinan, protoberberine and aporphine structures (Battersby 1992). The groundwork established during this period provided biochemists with the metabolic blueprints required to search for the enzymes responsible for purported reactions, ushering in a new era of investigation providing insight into the order of multistep transformations, such as hydroxylation and O-methylation. Studying enzyme features,

such as substrate range and kinetic properties, provided the clues required to close gaps in the biosynthetic schemes. Such clues continue to enlighten our understanding of BIA metabolism. For example, the recent discovery of morphinan alkaloid *O*-demethylases that accept other BIA subgroups (Hagel and Facchini 2010a) has prompted a re-evaluation of protoberberine metabolism (Hagel and Facchini 2010b). Modern innovations in genomics have further revolutionized our ability to investigate BIA metabolism. The availability of biosynthetic genes and recombinant proteins, technical advances in metabolite profiling, and in vivo techniques such as VIGS are dramatically expanding our view of alkaloid metabolic networks. Not unexpectedly, the development of superior tools coupled with a more sophisticated understanding of secondary metabolism has prompted a re-examination of long-established paradigms.

L-Tyrosine to (S)-reticuline

Empirical support for the formation of BIAs from two units of L-tyrosine was provided by Battersby and Harper (1958), and by Leete (1958), via the isolation of [^{14}C]morphine after feeding labeled [2-^{14}C]tyrosine to opium poppy plants. A similar study using labeled tyrosine reported the isolation of radioactive papaverine (Battersby and Harper 1959) and narcotoline (Kleinschmidt and Mothes 1959). It was soon shown that dopamine, presumably formed by the decarboxylation of L-DOPA, was a direct precursor to the isoquinoline moiety (Spenser and Gear 1962, Battersby 1963). A role for dopamine discounted a competing contemporary theory suggesting that BIAs were derived from hydrated prephenate through a series of reactions, including decarboxylation, oxidation and Mannich condensation (Wenkert 1959). Based on the suggestion that norlaudanosoline represented the first BIA in the pathway (Winterstein and Trier 1910, Robinson 1917), it was realized that L-DOPA could also be a precursor to the benzyl moiety. However, a series of experiments conducted more than a decade later supported the involvement of 3,4-dihydroxyphenylpyruvic acid, which was proposed to condense with dopamine to form the amino acid norlaudanosoline-1-carboxylic acid (Wilson and Coscia 1975, Battersby et al. 1975a, Scott et al. 1978). Subsequent decarboxylation would yield norlaudanosoline. In a defining study, morphine was labeled following injection of radioactive norlaudanosoline-1-carboxylic acid to opium poppy capsules (Battersby et al. 1975a). However, the later isolation of the Pictet–Spenglerase enzyme (S)-norlaudanosoline synthase ruled out 3,4-dihydroxyphenylpyruvic acid as a BIA precursor (Rueffer et al. 1981). This enzyme, which would later be renamed (S)-norcoclaurine synthase (NCS), was shown to condense dopamine with 3,4-dihydroxy phenylacetaldehyde.

Initial support for the long-standing, yet erroneous inclusion of norlaudanosoline as the initial alkaloid in BIA metabolism was provided by studies whereby radioactive morphinan alkaloids were isolated after feeding labeled norlaudanosoline to opium poppy plants (Battersby and Binks 1960, Battersby

et al. 1964). Significantly, it was noted during these experiments that feeding reticuline in place of norlaudanosoline increased label incorporation, providing evidence of a core pathway involving conversion of norlaudanosoline to the central intermediate reticuline. Conversely, the fully *O*-methylated tetrahydropapaverine was ruled out as the central intermediate. Although the occurrence of a core pathway ending with reticuline proved correct, over two decades passed before the involvement of norlaudanosoline was questioned. Precursor feeding experiments involving *Annona reticulata* leaves showed that coclaurine and reticuline were derived from norcoclaurine, and that (S)-norcoclaurine was the specific precursor to protoberberine, benzophenanthridine and morphinan alkaloids (Stadler et al. 1987). Subsequent tracer studies using a variety of plant cell cultures and whole plants supported the involvement of (S)-norcoclaurine (Stadler et al. 1989, Stadler and Zenk 1990), prompting a revision of steps within the core pathway. Examination of labeled intermediates established the reaction order; specifically that (S)-norcoclaurine was stereoselectively metabolized to (S)-reticuline via 6-*O*-methylation, followed by *N*-methylation, 3'-hydroxylation and 4'-*O*-methylation (Stadler and Zenk 1990). All enzymes catalyzing these reactions, with the exception of a 3'-hydroxylase, had been isolated and characterized. Although a phenolase was initially thought to mediate the 3'-hydroxylation step (Loeffler and Zenk 1990), a CYP enzyme identified later was claimed to be the true catalyst of this reaction based on a lower K_m for (S)-*N*-methylcoclaurine and a rational argument based on the general roles of phenol oxidases compared with CYPs (Pauli and Kutchan 1998).

Morphinan branch

The major branch pathways utilizing (S)-reticuline as a common intermediate were also established based on tracer studies to test paper chemistry predictions and subsequently validated through the isolation of biosynthetic enzymes. Recent innovations in gene suppression and overexpression technologies have generally confirmed the established BIA biosynthetic schemes (Allen et al. 2004, Larkin et al. 2007, Allen et al. 2008; Wijikoon and Facchini 2012). It was recognized early that the C–C phenol coupling step essential for the formation of salutaridine (**Fig. 2**) would require reticuline in the (R)-configuration at C1, prompting the suggestion that (S)-reticuline is epimerized to (R)-reticuline through the ionic intermediate 1,2-dehydroreticuline. Labeling studies conducted with both (S)- and (R)-reticuline, and the predicted epimerization intermediate, supported this theory (Battersby et al. 1965). Additional support for the involvement of 1,2-dehydro reticuline was gained from a more conclusive tracer study wherein the labeled ion was efficiently incorporated into morphinan alkaloids (Borkowski et al. 1978). Moreover, 1,2-dehydroreticuline was detected in opium poppy with a native pool size about one-fifth that of reticuline. Of the two enzymes ostensibly responsible for reticuline epimerization, DRS has been purified 5-fold from opium poppy seedlings

(Hirata et al. 2004) and DRR has been purified and biochemically characterized (De-Eknamkul and Zenk 1992). Genes encoding these enzymes have not been isolated. All other biosynthetic genes in the morphinan alkaloid pathway have been identified.

Remarkably, the pathway leading from reticuline to morphine has undergone only minor revisions over several decades (Cordell 1981, Zeigler and Facchini 2008). One minor revision was prompted by the discovery of oripavine in opium poppy, which suggested a bifurcation in the final steps of morphine biosynthesis (Nielsen et al. 1983) (**Fig. 2A**). The substrate acceptance profiles of the recently identified enzymes T6ODM and CODM supported this bifurcation (Hagel and Facchini 2010a). Intramolecular condensation of (R)-reticuline to the morphinandienone salutaridine was initially detected using tracer feeding studies (Barton et al. 1965), the mediating CYP enzyme was subsequently isolated (Hodges and Rapoport 1982) and the corresponding gene recently identified (Gesell et al. 2009). Enzymes catalyzing each subsequent transformation, ending with the CODM-catalyzed demethylation of codeine to yield morphine, have been identified (**Fig. 2A**). The recent discovery of T6ODM and CODM marked the completion of the morphinan pathway in terms of biochemical characterization and the availability of corresponding genes. One remaining contentious point concerns the formation of thebaine. Whereas thebaine is thought to derive via spontaneous rearrangement of salutaridinol 7-O-acetate at basic pH (**Fig. 2A**), an enzyme mediating thebaine formation has also been proposed (Fisinger et al. 2007).

Protoberberine and benzophenanthridine alkaloids

Several alkaloid subgroups, including protoberberine, benzophenanthridine, protopine and phthalideisoquinoline alkaloids, are formed after the stereospecific conversion of (S)-reticuline to the protoberberine alkaloid (S)-scoulerine. In a cyclization reaction with no known equivalent in organic chemistry, the oxidation of (S)-reticuline results in the formation of a C–C bond between the N-methyl group and the phenolic ring. BBE catalyzes this hallmark reaction, and the underlying mechanism has been characterized (Winkler et al. 2008). Genes encoding BBE have been isolated from several plants including California poppy (Dittrich and Kutchan 1991) and opium poppy (Facchini et al. 1996, Hagel et al. 2012).

The initial steps toward the formation of sanguinarine and other benzophenanthridine alkaloids were clarified through early tracer-based experiments (Battersby et al. 1975b, Takao 1983), and subsequent advances at the biochemical and molecular levels have been reviewed (Zenk 1994, Zeigler and Facchini 2008, Beaudoin and Facchini 2013). The conversion of (S)-scoulerine to sanguinarine requires six enzymes, including three CYPs, an N-methyltransferase and a flavoprotein oxidase (**Fig. 2A**; **Table 1**). A corresponding gene has been isolated for each enzyme in the pathway (**Table 1**). Two different enzyme types have been implicated in the two-electron oxidation of

dihydrosanguinarine to sanguinarine. Recently, a flavin-dependent oxidase with homology to BBE was identified in opium poppy (Hagel et al. 2012). The enzyme was designated dihydrobenzophenanthridine oxidase (DBOX; **Table 1**) owing to shared characteristics with a partially purified enzyme from California poppy cell culture (Schumacher and Zenk 1988). A distinct dihydrobenzophenanthridine oxidase, designated DHBO to distinguish it from DBOX, was reported from bloodroot (Sanguinaria canadensis) cell culture (Arakawa et al. 1992). In contrast to opium poppy and California poppy DBOX, bloodroot DHBO is a copper-dependent enzyme with a relatively high molecular weight (77 kDa compared with 56–58 kDa for DBOX enzymes). A cDNA clone encoding DHBO is not yet available, although the enzyme was purified (Ignatov et al. 1997). Interestingly, in addition to dihydrosanguinarine, opium poppy DBOX accepted several protoberberines and the 1-benzylisoquinoline tetrahydropapaverine (Hagel et al. 2012). VIGS-mediated suppression of DBOX transcript levels in opium poppy reduced the abundance of sanguinarine, dihydrosanguinarine and papaverine in roots, supporting multiple biosynthetic roles for the enzyme.

Other enzymes involved in benzophenanthridine alkaloid metabolism include sanguinarine reductase (SanR; **Fig. 2A**; **Table 1**) (Vogel et al. 2010) and those responsible for the production of further oxidized and O-methylated compounds such as macarpine (Zenk 1994). Cell cultures of California poppy and Thalictrum bulgaricum were used to isolate four novel enzymes, including two CYPs and two O-methyltransferases, which catalyze the remaining steps leading to macarpine from dihydrosanguinarine (De-Eknamkul et al. 1992, Kammerer et al. 1994). These enzymes were highly substrate specific, accepting only dihydro derivatives in lieu of the fully aromatized benzophenanthridine alkaloids. Corresponding genes have not yet been isolated for these enzymes.

The origin of berberine from (S)-scoulerine was initially established through tracer experiments (Battersby et al. 1975b, Cordell 1981) although the specific order of biosynthetic events linking these two alkaloids was subsequently revised. O-Methylation, methylenedioxy bridge formation and aromatization are necessary steps, and it was originally thought that columbamine was the substrate for the methylenedioxy bridge-forming enzyme (Rueffer and Zenk 1985), implying that aromatization preceded bridge formation. However, the initially purported 'berberine synthase' was later identified as a peroxidase (Bauer et al. 1992) and the true bridge-forming enzyme was revealed as canadine synthase (CAS), a highly specific CYP. CAS accepted only (S)-tetrahydrocolumbamine as a substrate in both Berberidaceae and Ranunculaceae cell cultures (Rueffer and Zenk 1994), prompting a revision of the berberine biosynthetic pathway wherein the order was established as O-methylation by SOMT, methylenedioxy bridge formation by CAS and aromatization by STOX (**Fig. 3B**). This reaction sequence facilitated an in-depth study on the stereochemistry of berberine biosynthesis in terms of the formation of the berberine and methylenedioxy bridges (Bjorklund et al.

1995). All biosynthetic genes involved in the conversion of (S)-norcoclaurine to berberine have been identified, including the recent reports of genes encoding STOX (Gesell et al. 2011) from Mexican prickly poppy and barberry, or multifunctional enzymes with STOX activity (Hagel et al. 2012) from opium poppy.

Papaverine

Although papaverine was one of the first alkaloids for which a biosynthetic scheme was proposed, its formation has remained controversial. Two metabolic routes from (S)-norcoclaurine have been suggested. The first consists entirely of N-desmethylated intermediates (the NH pathway), whereas the second proceeds through several N-methylated intermediates including (S)-reticuline (the NCH$_3$ pathway). The NH pathway is supported by numerous tracer experiments whereby the feeding of labeled tyramine, dopamine or 4-hydroxyphenyl acetaldehyde resulted in the specific labeling of papaverine (Battersby and Binks 1960, Battersby and Harper 1962, Battersby 1963, Battersby et al. 1964, Battersby et al. 1965, Brochmann-Hanssen et al. 1971, Brochmann-Hanssen et al. 1975, Battersby et al. 1977, Brochmann-Hanssen et al. 1980). In one landmark study, label derived from norlaudanosoline, norreticuline, norlaudanine and tetrahydropapaverine, but not reticuline, was incorporated into papaverine (Uprety et al. 1975). The predicted biochemistry of the NH pathway includes 3′-hydroxylation of (S)-coclaurine, three O-methylations at C7, C3′ and C4′ yielding tetrahydropapaverine, and dehydrogenation (i.e. aromatization) producing papaverine (Pienkny et al. 2009, Desgagné-Penix and Facchini 2012). Whereas several permutations are possible, some studies have suggested a particular sequence of reactions. For example, 6-O-methylation is thought to occur before 7-O-methylation, and dehydrogenation probably takes place after completion of all four O-methylations (Brochmann-Hanssen et al. 1971, Brochmann-Hanssen et al. 1975). Some constraints are also obvious, such as the dependence of 3′-O-methylation on prior 3′-hydroxylation. The isolation of norreticuline 7-O-methyltransferase (N7OMT) (Pienkny et al. 2009) provided further support for the NH pathway. In a recent definitive study based on VIGS, transcript levels of six genes with putative roles in papaverine biosynthesis were systematically silenced in opium poppy (Desgagné-Penix and Facchini 2012). The differential accumulation of N-desmethylated compounds in response to the suppression of certain target genes highlighted the NH pathway as the primary route to papaverine.

Initial support for the occurrence of an NCH$_3$ pathway included an isotope dilution analysis interpreted to suggest that norreticuline was not a naturally occurring intermediate (Stadler and Zenk 1990). However, VIGS-based silencing of target genes coupled with liquid chromatography–tandem MS (LC-MS/MS) analysis confirmed the presence of norreticuline and other postulated N-desmethylated intermediates in opium poppy (Desgagné-Penix and Facchini 2012). The formation of papaverine via (S)-reticuline was proposed based on a study involving heavy isotope-labeled precursors (Han et al. 2010) whereby labeled (S)-coclaurine and (S)-reticuline were partially incorporated into papaverine and thebaine in opium poppy seedlings. The sequence of reactions thought to occur as part of the NCH$_3$ pathway includes 7-O-methylation of (S)-reticuline to form (S)-laudanine, 3′-O-methylation yielding (S)-laudanosine, N-demethylation forming tetrahydropapaverine, and finally dehydrogenation yielding papaverine. The first of these steps would be catalyzed by reticuline 7-O-methyltransferase (7OMT) (Ounaroon et al. 2003). Although a minor contribution of this route is possible, VIGS-based silencing of NMCH (**Fig. 2A**) and 7OMT did not affect papaverine levels (Desgagné-Penix and Facchini 2012).

Noscapine biosynthesis

Noscapine, also known as gnoscopine or narcotine, was among the first isolated alkaloids and is abundant in the latex of some opium poppy chemotypes. Its basic chemical structure was determined over a century ago (Roser 1889, Perkin and Robinson 1911) and it was proposed to derive from the 1-benzylisoquinoline structure combined with a one-carbon unit (e.g. a methyl group) (Robinson 1955). It was further postulated that the protoberberine skeleton could serve as a biosynthetic precursor of the phthalideisoquinoline backbone (Robinson 1955). An alternative theory suggested that phthalideisoquinoline alkaloid biosynthesis begins with hydrated prephenate, which rearranges to yield an O-carboxyphenylpyruvate compound (Wenkert 1959). Ensuing tracer feeding studies using labeled tyrosine quickly discounted this idea (Kleinschmidt and Mothes 1959, Gear and Spenser 1961, Battersby and McCaldin 1962). Like other BIAs, the A-, B- and D-rings of noscapine were derived from the condensation of two tyrosine derivatives (**Fig. 1**). However, the origin of noscapine's lactonic carbonyl carbon (forming ring C) was initially unclear. To test whether the berberine bridge carbon (C8 of the protoberberine skeleton; **Fig. 1B**) acted as the source of the 'one-carbon' unit proposed by Robinson (1955), both [^{14}C]formate and [methyl-^{14}C]methionine were tested as potential donors (Battersby et al. 1968). Labeled methionine was incorporated into noscapine at the carbonyl carbon, in addition to the O-methyl and methylenedioxy groups. Other tracer studies revealed that the N-methyl moiety of (S)-reticuline was specifically incorporated into the carbonyl carbon of ring C.

Over 40 years have passed since these early studies established the basic building blocks for noscapine biosynthesis. Until very recently, few studies have built upon these initial discoveries. The identification of several new phthalideisoquinoline alkaloids, including macrantaldehyde and papaveroxine, and hemiacetals such as egenine and narcotinehemiacetal (Sariyar and Phillipson 1977, Gözler et al. 1983, Sariyar and Shamma 1986), suggested a biosynthetic process whereby a tetrahydroprotoberberine alkaloid could be N-methylated and then oxidized in stages to a hemiacetal, which would undergo further oxidation to yield the carbonyl carbon of noscapine.

A scheme outlining this process in the context of opium poppy metabolism has been proposed (Facchini et al. 2007). Recently, a related scheme was assembled based on VIGS of purported gene candidates in opium poppy (Winzer et al. 2012). Suppressing the transcript levels of six out of 10 clustered genes revealed the accumulation of putative pathway intermediates. Although the corresponding enzymes were not characterized, the gene-silencing results supported their roles in noscapine metabolism.

Future Directions and Applications

Transcriptomics, proteomics and metabolomics

As with other areas of plant biology, the technological and methodological advances brought about as part of the post-genomics era have revolutionized the study of BIA metabolism. Whereas the identification of novel enzymes was previously dependent on laborious protein purification procedures coupled with functional assays, discoveries made over the past dozen years have relied largely on functional genomics tools including transcriptome libraries, proteomics analyses, and DNA micro- and macroarrays (Dang et al. 2012). Integration of these genomics resources has further enhanced our ability to identify novel genes. For example, comparative analyses of metabolite and transcript data from model systems have permitted the identification and isolation of numerous enzymes involved in BIA metabolism. The continued development of functional genomics resources will enable the bona fide application of systems biology (Schilmiller et al. 2012) approaches permitting the implementation of increasingly sophisticated gene discovery methods.

Whereas diminishing costs and advancing technological platforms have recently made large-scale sequencing projects attractive, modest sized cDNA libraries have been used repeatedly for the identification of novel genes (Ziegler and Facchini 2008). Such resources have mostly been established for BIA-accumulating plants traditionally used to investigate BIA metabolism (**Fig. 3**). Combining sequence information from such collections with metabolite profiling data has also been successful. For example, integration of MS-derived alkaloid profiles with modest (∼3,500) expressed sequence tag (EST) libraries from cell cultures of California poppy, oriental poppy (*Papaver bracteatum*) and meadow rue led to the identification of a novel *N*-methyltransferase that accepts pavine alkaloids (Liscombe et al. 2009). Similar resources have been described for cell cultures of 18 BIA-accumulating plant species (Farrow et al. 2012). The use of a cDNA macroarray derived from *Papaver* species exhibiting differential alkaloid accumulation profiles aided in the identification of 4′OMT (Ziegler et al. 2005), SanR (Ziegler et al. 2006), N7OMT (Pienkny et al. 2009) and SanSyn (Gesell et al. 2009). A cDNA fragment-based, 23,000-element microarray facilitated comparison of the transcriptomes of three opium poppy chemotypes with differential morphinan alkaloid profiles (Hagel et al. 2008). A gene
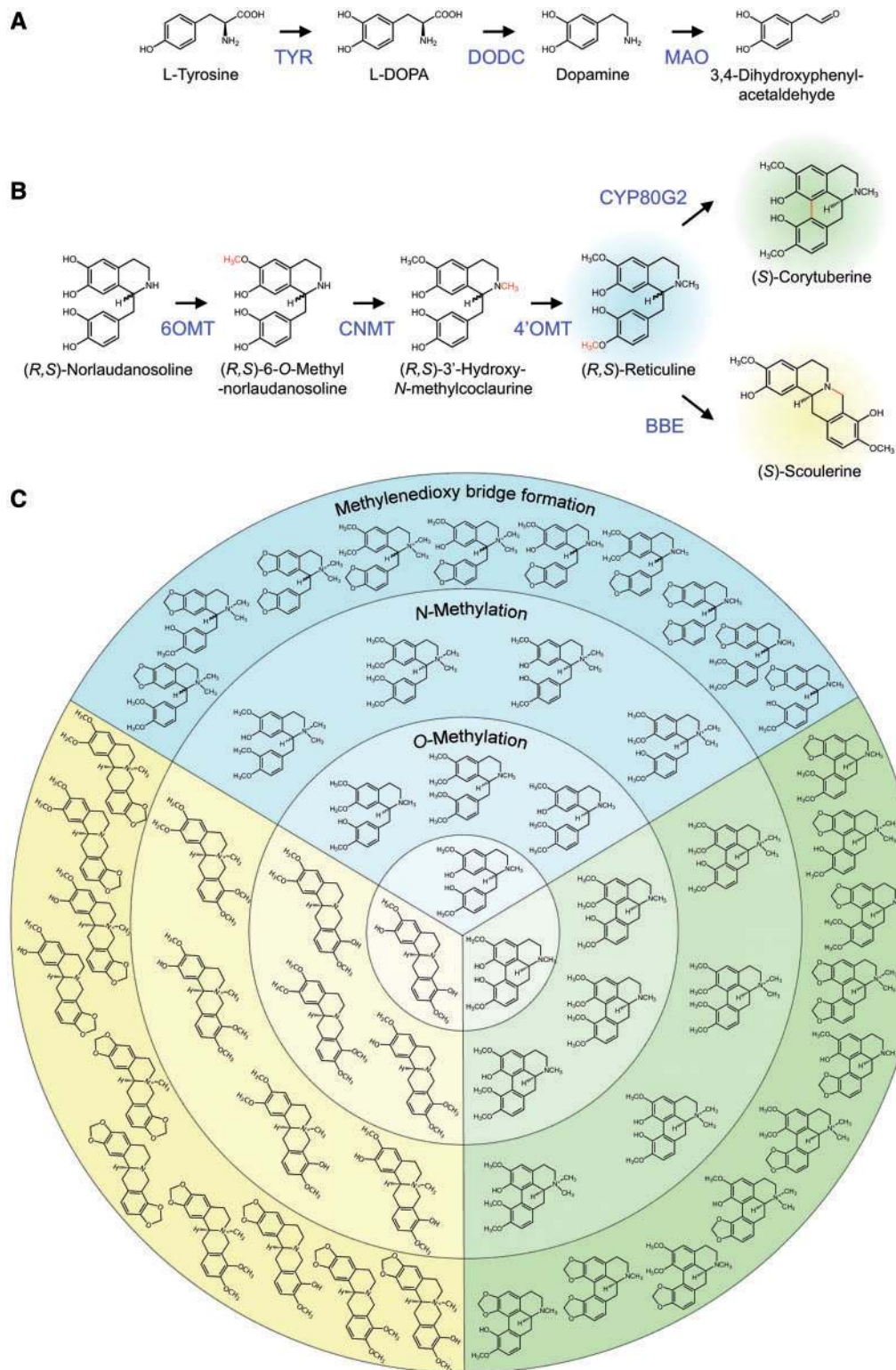
expressed in morphine-accumulating chemotypes, but not in a morphine-free cultivar, was identified as T6ODM, the first described dioxygenase of BIA metabolism (Hagel and Facchini 2010a). Array-based transcriptomics has also been used to study gene expression profiles in sanguinarine-accumulating opium poppy cell cultures (Zulak et al. 2007).

Continued resource development in our laboratory has included deep transcriptomes based on next-generation sequencing technologies for opium poppy and related species. Recently, transcriptome and proteome data acquired from elicitor-treated opium poppy cell cultures allowed cataloguing of known and putative genetic and biochemical components of secondary metabolism (Desgagné-Penix et al. 2010). Similar resources are available for eight different chemotypes of opium poppy exhibiting differential BIA accumulation profiles (Desgagné-Penix et al. 2012). Recently, a comparative transcriptomics approach using these resources led to the identification of three *O*-methyltransferases involved in noscapine biosynthesis (Dang and Facchini 2012). The establishment of deep transcriptomes derived from 20 non-model BIA-accumulating species is currently underway as part of a large-scale Canadian initiative (www.phytometsyn.com) aimed at tapping into the vast and diverse biosynthetic capacity of plants.

Expanding transcriptomics resources have been accompanied by advances in both proteomics and metabolomics methodologies. Whereas early proteomics studies in the context of BIA metabolism identified dozens of proteins (Decker et al. 2000, Ounaroon et al. 2003), technological advances including LC-MS/MS-based proteomics now permit the identification of hundreds (Zulak et al. 2009, Oldham et al. 2010) to thousands (Desgagné-Penix et al. 2010) of proteins. Two-dimensional gel electrophoresis coupled with microsequencing led to the discovery of opium poppy 6OMT and 7OMT (Ounaroon et al. 2003). Technological advances in metabolomics applied to the study of BIA metabolism have included quantitative ^{1}H NMR, Fourier transform-ion cyclotron resonance-mass spectrometry (FT-ICR-MS) and related tandem MS methods (Hagel and Facchini 2008). Opium poppy has been the subject of several metabolite-profiling studies (Frick et al. 2005, Schmidt et al. 2007, Hagel et al. 2008, Zulak et al. 2008, Desgagné-Penix et al. 2012). Integrated LC-UV-MS-NMR was applied to the identification of benzophenanthridine alkaloids from California poppy cell culture (Gathungu et al. 2012). Hyphenated techniques have been applied to the study of additional BIA-accumulating plants, including several members of the Berberidaceae, Fumariaceae and Papaveraceae (Iwasa et al. 2012). The popularity of MS-based approaches has prompted the development of relevant resources (Farrow et al. 2012) including a continuously updated BIA database (http://crdd.osdd.net/raghava/biadb/) (Singla et al. 2010).

In planta approaches

The role of candidate genes encoding enzymes putatively involved in BIA metabolism has been investigated in planta using a variety of technologies including overexpression

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Fig. 4 Synthetic biology and combinatorial biochemistry for BIA production. (A) De novo biosynthesis of BIAs in bacteria has been achieved through the expression of tyrosinase (TYR), DOPA decarboxylase (DODC) and monoamine oxidase (MAO), which convert endogenous tyrosine to the (S)-norlaudanosoline precursors, dopamine and 3,4-dihydroxyphenylacetaldehyde (Nakagawa et al. 2011). (B) Expression of genes encoding three enzymes [i.e. norcoclaurine 6-O-methyltransferase (6OMT), coclaurine N-methyltransferase (COMT) and 3′-hydroxy-N-methyl-coclaurine 4′-O-methyltransferase (4′OMT)] converts (R,S)-norlaudanosoline to (R,S)-reticuline, which can be converted to other BIA structural subgroups. (R,S)-Reticuline was produced in yeast as shown (Hawkins and Smolke 2008), whereas (S)-reticuline was produced in bacteria

(continued)

(Frick et al. 2007, Inui et al. 2007, Larkin et al. 2007, Allen et al. 2008, Inui et al. 2012), knock-out techniques (e.g. random mutagenesis) (Millgate et al. 2004) and knock-down approaches such as antisense RNA (Park et al. 2002, Park et al. 2003, Frick et al. 2004) and RNA interference (RNAi) methods (Fujii et al. 2007, Allen et al. 2008, Kempe et al. 2009). Gene overexpression and RNAi-based studies typically require the generation of stably transformed plants or cultured cells, although transient RNAi has been used with Japanese goldthread protoplasts (Dubouzet et al. 2005, Yamada et al. 2011). Recently, VIGS has emerged as an invaluable functional genomics tool to study BIA metabolism in intact plants. VIGS is a rapid and relatively straightforward technology that does not require stable transformation. Partial gene sequence information is sufficient to suppress target gene transcript levels, and VIGS may be used for both forward and reverse genetic applications. Another attractive feature of VIGS and related RNAi technologies is their ability to suppress gene families. However, several caveats unique to VIGS must be considered. For example, viral symptoms may arise that interfere with data interpretation, and off-target suppression can occur. Additionally, appropriate VIGS vectors and effective vector delivery protocols have yet to be established for most BIA-accumulating plants.

Since the description of VIGS in opium poppy (Hileman et al. 2005), the technique has been used to confirm the physiological roles of T6ODM and CODM in morphine biosynthesis (Hagel and Facchini 2010a), reveal the overall contribution of tyrosine aminotransferase to steady-state BIA levels (Lee and Facchini 2011) and investigate the function of three O-methyltransferases in the context of noscapine biosynthesis (Dang and Facchini 2012). VIGS was used systematically to knock down transcripts encoding all six morphine pathway enzymes (Wijikoon and Facchini 2012), which confirmed the physiological function of enzymes previously characterized only in vitro. Contentious models, such as papaverine biosynthesis, have also been substantially resolved using VIGS (Desgagné-Penix and Facchini 2012), and unprecedented insights into uncharacterized pathways, such as noscapine metabolism, have been generated (Winzer et al. 2012).

Synthetic biology and combinatorial biochemistry

As with other plant natural products, chemical synthesis of most BIAs is not a viable alternative for commercial production owing to the inefficiency of established protocols or the requirement for impractical separations. Of the pharmaceutical

BIAs used today, only papaverine is produced synthetically for commercial purposes. Other valuable drugs or drug precursors, such as thebaine, are derived from plants on an industrial scale. Recent interest in alternative production platforms has highlighted the use of microbes (Facchini et al. 2012, Xu et al. 2012) or genetically altered plant systems (Glenn et al. 2012). Microbes engineered to express genes encoding BIA-biosynthetic enzymes represent novel tools in the development of scalable manufacturing processes, and can be used for novel drug development. The metabolic engineering of microbial systems for the production of target biomolecules, often referred to as 'sythetic biology', is an emerging field aimed at the sustainable manufacture of chemicals currently derived from non-renewable or limited natural resources using inexpensive, readily available materials (Keasling 2010). The success of such engineering feats depends heavily on our ability to identify, isolate and characterize new genes—or variants of known genes—and delineate the diverse array of products potentially arising from the expression of recombinant plant genes in heterologous hosts (De Luca et al. 2012).

Recent synthetic biology initiatives involving BIAs have included the reconstitution of reticuline biosynthesis in yeast and bacteria. In 2008, two studies reported the production of (S)-reticuline and (R,S)-reticuline in Escherichia coli (Minami et al. 2008) and Saccharomyces cerevisiae (Hawkins and Smolke 2008), respectively. Production of (S)-reticuline in E. coli was initially achieved by supplementing the bacterial culture medium with dopamine, some of which was converted to 3,4-DHPAA by a heterologously expressed monoamine oxidase (MAO) from Micrococcus luteus (Minami et al. 2008). NCS from Japanese goldthread catalyzed the condensation of dopamine and 3,4-DHPAA to (S)-norlaudanosoline, which underwent successive methylations via 6OMT, CNMT and 4'OMT to yield (S)-reticuline (**Fig. 4A, B**). Recent modifications of this platform have included the de novo synthesis of dopamine through the expression of two additional enzymes, tyrosinase (TYR) and DOPA decarboxylase (DODC) (**Fig. 4A**) (Nakagawa et al. 2011). The use of bacterial enzymes facilitated the linking of BIA metabolism to the primary metabolism of E. coli, enabling a fermentation platform that creates plant products from simple carbon sources. In turn, E. coli-produced (S)-reticuline was converted to the aporphine alkaloid magnoflorine through co-culture with a strain of S. cerevisiae engineered to express the genes encoding CYP80G2 and an N-methyltransferase derived from

Fig. 4 Continued

(Minami et al. 2008, Nakagawa et al. 2011). The protoberberine and aporphine alkaloids (S)-scoulerine and (S)-corytuberine, respectively, are produced from (S)-reticuline via berberine bridge enzyme (BBE) and CYP80G2. (S)-Reticuline, (S)-scoulerine and (S)-corytuberine are central intermediates for possible additional functionalization. Note that the conversion of bacterially produced (S)-reticuline to (S)-scoulerine, (S)-corytuberine and (S)-magnoflorine (not shown) required co-culture with yeast expressing BBE, CYP80G2 and an N-methyltransferase (Minami et al. 2008), whereas yeast-produced reticuline was converted to (S)-scoulerine without co-culturing methods (Hawkins and Smolke 2008). (C) Core BIAs representing, for example, the 1-benzylisoquinoline (blue), aporphine (green) and protoberberine (yellow) subgroups can undergo differential functionalization through the expression of genes encoding known or putative enzymes, such as substrate- and regio-specific O- and N-methyltransferases, and methylenedioxy bridge-forming CYPs, to yield a potentially vast array of compounds.

Japanese goldthread. A similar approach was used to produce (S)-scoulerine using the gene encoding BBE (Minami et al. 2008) (**Fig. 4B**).

As an alternative platform, production of (R,S)-reticuline in *S. cerevisiae* from commercially available (R,S)-norlaudanosoline avoided the use of co-culturing methods. The biosynthesis of both (R)- and (S)-reticuline isomers, coupled with enzymes derived from three different plant sources (and humans), allowed the production of protoberberine alkaloids, and the morphine precursor salutaridine (Hawkins and Smolke 2008). To optimize heterologous expression and catalytic activities within the reconstituted pathways, enzyme variants from different plant species were evaluated prior to selecting the best candidates. This tuning strategy highlighted the importance of having a so-called 'toolbox' of biosynethic gene variants to avoid common problems such as failed or insufficient gene expression, enzyme inactivity or off-target/undesired effects. Furthermore, studies of BIA production in yeast and bacteria have underscored the importance of sourcing genes from various organisms including microbes and humans.

Progress leading to the commercial production of BIAs using synthetic biology appoaches will substantially hinge on the availability of an expanding toolbox of biosynethic gene variants, which in turn requires the availability of deep sequence resources from diverse plant species. Synthetic genes and protein engineering can complement the high-throughput characterization of natural enzyme variants. Access to natural, synthetic or engineered gene variants will create unprecedented opportunities to produce compounds not yet found in nature. For example, several natural and engineered enzymes sourced from plants and microbes have been shown to accept halogenated substrates (Runguphan et al. 2010, Glenn et al. 2011). Using combinatorial biochemistry, microbes (or plants) can serve as a 'chassis' on which rationally designed biosystems can be built. For example, the bioavailability of a 1-benzylisoquinoline such as reticuline could serve as a platform upon which to test the activities of previously uncharacterized enzyme variants, or 'parts'. The utility of the 'chassis-and-parts' paradigm is illustrated in **Fig. 4C**. Microbes accumulating reticuline, scoulerine and corytuberine via a reconstituted pathway (**Fig. 4B**) provide a chassis for the possible biosynthesis of many other alkaloids (**Fig. 4C**) using a catalog of characterized or novel enzyme variants. For example, platform microbial strains engineered to express various *O*-methyltransferases, *N*-methyltransferases and methylenedioxy bridge-forming enzymes can build upon a limited number of key BIA backbones (**Fig. 4C**). Clearly, enzyme variants that accept each of the compounds shown in **Fig. 4C** might not occur naturally, or might not be achievable through mutagenesis. However, the scheme serves to illustrate the potential of an expanding combinatorial biochemistry strategy. The complexity of possible structures increases exponentially with the addition of enzymes catalyzing other transformations, so the possibilities are almost endless.

Conclusion

The abilty of plants to produce a vast catalog of structually diverse BIAs has been studied for more than a century. Several well-supported, yet still evolving biosynthetic pathways include enzymes representing a number of families or superfamilies, including PR10/Bet v1 Pictet–Spenglerases, *O*- and *N*-methyltransferases, *O*-acetyltransferases, cytochromes P450, non-heme dioxygenases, short-chain dehydrogenase/reductases and aldo-keto reductases. The isolation of novel biosynthetic genes has relied on increasingly sophisticated methods. Technological innovations including next-generation sequencing can now be coupled with synthetic biology to standardize the process of engineering BIA metabolism in alternative production systems. However, the success of these engineering efforts will continue to rely on our expanding knowledge of BIA metabolism in plants. Clearly, the traditional depiction of linear reaction sequences catalyzed by single-function enzymes is highly oversimplified. Although it is often claimed that entire pathways are now characterized at the biochemical and genetic levels, important challenges remain, including an understanding of enzyme interactions, metabolic regulation and compartmentalization.

Funding

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