Biochemistry, genetics and genomics of opium poppy (*Papaver somniferum*) for crop improvement

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Abstract

Opium poppy is the only commercially viable source of narcotic raw materials used by the alkaloid pharmaceutical industry. Considerable advances in our knowledge of basic poppy biology and the alkaloid biosynthetic pathway have been driven by recent progress in transcriptomics, genomics and functional genetics. However, much work remains for this knowledge to be translated into improvements in crop performance. The genetic diversity of poppy is poorly characterized and the available information is highly fragmented. The recent release of a poppy genome sequence adds a new dimension to poppy genomic research, enabling characterization of diversity and identification of genes and molecular markers associated with valuable traits. This will create opportunities for functional genomics studies and the incorporation of more diverse germplasm into poppy improvement programs. This review discusses the current state of poppy genetic and genomic resources, highlights the advances made in elucidating the alkaloid biosynthetic pathway that led to the emergence of poppy as a model system to study secondary metabolism in plants, and presents perspectives for future research.

Keywords: alkaloid biosynthesis, benzylisoquinoline alkaloids, crop improvement, diversity, genetics and genomics, opium poppy, secondary metabolism, VIGS

1. Introduction

Opium poppy (*Papaver somniferum*) is one of the oldest recorded medicinal plants, described in Sumerian clay tablets dating to around 3000 BC. It is the source of several pharmaceutically important benzylisoquinoline alkaloids (BIAs), including the thebaine-derived narcotic analgesics morphine and codeine (Hagel and Facchini et al., 2010; Beaudoin and Facchini, 2014). Thebaine, the first opiate alkaloid in the biosynthesis pathway, is also used for the semi-synthesis of many derivatives including the prescription painkillers oxycodone and hydrocodone and the anti-opioid addiction drugs buprenorphine and naltrexone (Rinner and Hudlicky, 2011; Chen et al., 2018). Other important poppy BIAs include the antimicrobial agent sanguinarine, the muscle relaxant papaverine and the cough-suppressant and potential anticancer drug noscapine (Beaudoin and Facchini, 2014). Several recent attempts to use synthetic biology approaches, for example engineered microorganisms, as alternative sources of opiate production have yielded promising results (Galanie et al., 2015; Nakagawa et al., 2016; Li et al., 2018). However, poppy remains the only commercially viable source of BIAs. Despite poppy being a cheap source of pain relief, lack of or limited access to such medications in low and middle-income countries is a global health concern (Knaul et al., 2018). This is partly due to the strict regulations associated with poppy production, processing and trade, which result from its addictive nature. It is, however, believed that ensuring reliable and stable supplies of opium poppy can contribute significantly to meeting the current medically-necessary demand for opioids.

The global licit opium poppy industry is regulated under the United Nations Single Convention on Narcotic Drugs 1961 (https://treaties.un.org/pages/ViewDetails.aspx?src=TREATY&mtdsg_no=VI-15&chapter=6&clang=_en), which is administered by the International Narcotics Control Board (INCB; https://www.incb.org/). The INCB determines annual global production levels of narcotic plants based on estimated needs for each year. Australia, Spain, Turkey, Hungary, India and France are major producers and exporters of legal poppy material, whilst China and Korea

produce only for domestic use (INCB, 2017). Poppy production in Australia for therapeutic and research purposes started in the 1960's and 1970's and was restricted to Tasmania, due to the suitability of its soils and climate for poppy growing and its isolated location. Tasmania accounts for about half of global licit production. More recently, poppy production has expanded to other states including Victoria, New South Wales, the Northern Territory and South Australia.

Opium poppy is principally used as a commercial source of BIAs, which are extracted from fresh or dried capsules. Poppy seeds are used in the food chain, for example in baking and as a source of edible oil, rich in linoleic acid that is beneficial for cardiovascular health (Azcan et al., 2004; Kris-Etherton et al., 2004; Bozan and Temelli, 2008). Varieties grown for seed often contain no or negligible amount of alkaloids, although seeds can become contaminated with alkaloids from various sources (ESFA, 2018). The value of the global poppy seed market was approximately AUD 183.4 million (111,000 tonnes) in 2016 and is expected to increase at the rate of 1.2% per year over the next 10 years to about 123,000 tonnes in 2025 (https://www.foodmag.com.au/poppy-seed-global-consumption/). The global opioid market value was estimated around USD 25.4 billion in 2018 and is projected to grow at a compound annual growth rate (CAGR) of around 1.8% between 2019 and 2026 (https://www.grandviewresearch.com/industry-analysis/opioids-market). Commercial poppy varieties registered for cultivation in Europe are classified into three groups - pharmaceutical (industrial), culinary and dual - depending on the intended use (Labanca et al., 2018). Consequently, breeding programs should aim to develop not only varieties with high total or specific alkaloids but also high seed yielding varieties for the food industry.

Recent years have seen remarkable advances in functional genomic studies of major crops. These include development of next generation sequencing (NGS)-based tools for high throughput genotyping and gene identification, as well as analyses of gene and molecular networks regulating biological processes that underlie traits of interest (See Varshney et al., 2014; Bevan et al., 2017). Compared to the major agricultural crops, progress for opium poppy in these areas

has been limited and the available information is fragmented. In the following sections, we provide a comprehensive review of the current status of poppy genetic and genomic resources as well as recent progress in our understanding of the alkaloid biosynthesis pathway. We also discuss the significance of the recently released reference genome sequence for functional genomic studies in opium poppy and the implications such studies will have for genetic improvement of the crop.

2. Alkaloid biosynthesis in *P. somniferum*

BIAs are a diverse group of about 2,500 specialized plant metabolites, many of which have pharmacological properties (see Hagel and Facchini, 2013; Dastmalchi et al., 2018a). Opium poppy produces several BIAs, including the narcotic analgesics morphine and codeine. Prompted in part by their economic and social significance, considerable research effort has been devoted to investigating how BIAs are synthesized in plants. These efforts, supported by recent advances in technologies such as transcriptome and genome sequencing and virus-induced gene silencing (VIGS)-based functional studies of genes associated with BIA biosynthesis, have led to elucidation of many steps in the major pathways (see Beaudoin and Facchini, 2014; Schlager and Drager, 2016; Park et al., 2018). In this review, we focus on the commercially important pathways leading to the synthesis of thebaine, morphine, codeine, sanguinarine, noscapine and papaverine (**Figure 1**).

2.1. A central pathway leading to (*S*)-Reticuline

The biosynthesis of BIAs begins with the condensation of two L-tyrosine derivatives, 4-hydroxyphenylacetaldehyde (4-HPAA) and dopamine, via decarboxylation, meta-hydroxylation and transamination yielding (*S*)-norcoclaurine, the common precursor to all other BIAs produced in plants. Norcoclaurine synthase (NCS) catalyses the condensation of 4-HPAA and dopamine, producing (*S*)-norcoclaurine (Samanani and Facchini, 2002; Samanani et al., 2004; Liscombe et al., 2005) (**Figure 1**). NCS is a relatively inefficient enzyme that is also sensitive to substrate

concentration, suggesting it may act as 'gatekeeper' enzyme responsible for regulating entry into BIA metabolism (Lichman et al., 2015). Analysis of NCS transcripts from different species identified several genes encoding multi-domain fusion NCS proteins containing two or more tandem repeats of complete catalytic domains or partial domains in most members of the Papaveraceae (Li et al., 2016; Hagel and Facchini, 2017). Among the proteins that were confirmed to possess NCS activity, a proportional increase in catalytic efficiency appeared to correlate with the presence of multiple catalytic domains, as demonstrated by study of NCS homologs in bacteria and yeast, suggesting multiple domain fusions might be a possible evolutionary mechanism to enhance the efficiency of the enzyme (Li et al., 2016; Hagel and Facchini, 2017).

Conversion of (*S*)-norcoclaurine to (*S*)-coclaurine by norcoclaurine-6-*O*-methyltransferase (6OMT) is the first methylation step in the (*S*)-reticuline formation pathway of BIA metabolism (Morishige et al., 2000; Ounaroon et al., 2003). Coclaurine *N*-methyltransferase (CNMT) converts (*S*)-coclaurine to (*S*)-*N*-methylcoclaurine (Choi et al., 2001). Sequential methylation and hydroxylation of (*S*)-*N*-methylcoclaurine to (*S*)-3′-hydroxy-*N*-methylcoclaurine occurs *via* the activities of the (*S*)-*N*-methylcoclaurine 3′-hydroxylase (NMCH) or CYP80B3 enzyme (Pauli and Kutchan, 1998; Frick et al., 2007; Park et al., 2018). Finally, (*S*)-3′-hydroxy-*N*-methylcoclaurine is converted to (*S*)-reticuline by the enzyme 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase (4′OMT) (Morishige et al., 2000). (*S*)-reticuline is a key branch point intermediate for the thebaine/codeine/morphine, noscapine and sanguinarine pathways (**Figure 1**).

2.2. Papaverine synthesis

Papaverine is a vasodilator that relaxes smooth muscle, commonly used to treat ischemia and visceral spasm (Kang et al., 2018). Two routes have been proposed for papaverine biosynthesis. The first route is an N-methyl pathway commencing with (*S*)-reticuline and *N*-demethylation of an unspecified intermediate by a hypothetical enzyme (Han et al., 2010). The second route is an *N*-

desmethyl pathway commencing with (*S*)-coclaurine involving (*S*)-norreticuline and precluding the requirement for *N*-demethylation (Pienkny et al., 2009). Recent evidence from VIGS-mediated suppression of *CNMT* transcript and gene expression analysis of a high papavarine mutant, *pap1*, favors this second pathway as the major *in vivo* pathway (Desgagné-Penix and Facchini, 2012; Pathak et al., 2013). Once (*S*)-norreticuline is produced from (*S*)-coclaurine either via a 3′OHase or 3′OMT, it is converted to (*S*)-tetrahydropapaverine by 4'OMT or norreticuline 7-*O*-methyltransferase (N7OMT in the *N*-desmethyl pathway). The final step of papaverine synthesis is the conversion of (*S*)-tetrahydropapaverine to papaverine, catalysed by dihydrobenzophenanthridine oxidase (DBOX). DBOX also catalyses the final step of the sanguinarine synthesis pathway (Hagel and Facchini, 2012). (*S*)-Reticuline can be methylated by reticuline 7-*O*-methyltransferase (7OMT) to yield laudanine, which then has the capacity to get fully *O*-methylated by an unidentified 3′-*O*-methyltransferase (3′OMT) and yield laudanosine (Ounaroon et al., 2003). Subsequent N-demethylation of laudanosine yields the known precursor of papaverine, tetrahydropapaverine (Beaudoin and Facchini, 2014).

2.3. Noscapine and sanguinarine synthesis

Noscapine, the second most abundant alkaloid in poppy after morphine, is used as a non-narcotic antitussive drug and has also been shown to have anticancer properties (Ye et al., 1998; Barken et al., 2008). Sanguinarine is a potent anti-microbial agent. The biosynthesis of noscapine and sanguinarine separates at (*S*)-scoulerine, which is produced by the activity of the berberine bridge enzyme (BBE) that converts (*S*)-reticuline to (*S*)-scoulerine. This is the branch-point intermediate leading to protoberberine alkaloids (Dittrich and Kutchan 1991; Facchini et al., 1996; Kutchan, 1996; Winkler et al., 2006). (*S*)-scoulerine can then be converted to protoberberines such as (*S*)-canadine, (*S*)-stylopine and (*S*)-sinactine.

2.3.1. Noscapine synthesis

(*S*)-Canadine is a protoberberine that acts as the precursor for the protopine, allocryptopine and the phthalideisoquinoline, noscapine. (*S*)-Scoulerine is initially converted to (*S*)-tetrahydrocolumbamine *via* scoulerine 9-*O*-methyltransferase (SOMT1) (Takeshita et al. 1995; Dang and Facchini, 2014). (*S*)-Tetrahydrocolumbamine gets converted to (*S*)-canadine by the enzyme canadine synthase (CAS) or CYP719A21, a member of the CYP719A subfamily (Ikezawa et al., 2003; Chavez et al., 2011; Winzer et al., 2012). (*S*)-Canadine can be converted to a further oxidised protoberberine, berberine, *via* the FAD-linked enzyme (*S*)-tetrahydroprotoberberine oxidase (STOX) (Matsushima et al., 2012). Similar to sanguinarine, berberine acts as an anti-microbial agent.

The initial dedicated step of noscapine synthesis is the conversion of (*S*)-canadine to (*S*)-*N*-methylcanadine by the enzyme Tetrahydroprotoberberine ci(*S*)-*N*-MethylTransferase (TNMT). (*S*)-*N*-Methylcanadine is converted to (*S*)-1-hydroxy-*N*-methylcanadine by *N*-methylcanadine 1-hydroxylase (CYP82Y1) (Winzer et al., 2012; Dang and Facchini, 2014). (*S*)-1-Hydroxy-*N*-methylcanadine is converted to (13*S*,14*R*)-1-hydroxy-13-*O*-acetyl-*N*-methylcanadine by the activities of CYP82X2 and AT1. (13*S*,14*R*)-1-Hydroxy-13-*O*-acetyl-*N*-methylcanadine is converted to 4′-*O*-desmethyl-3-*O*-acetylpapaveroxine by CYP82X1 and a spontaneous reaction. It was previously suggested that 4′-*O*-desmethyl-3-*O*-acetylpapaveroxine is converted to narcotine hemiacetal by CXE1 and, ultimately, narcotine hemiacetal is converted to noscapine *via* noscapine synthase (NOS or SDR1), an $NADP^+$/NAD^+-dependent short-chain dehydrogenase/reductase (Winzer et al., 2012; Chen and Facchini, 2014; Chen et al., 2015). A more recent study identified an alternative route by which two *O*-methyltransferase heterodimers OMT2:OMT3 and OMT2:6OMT convert 4′-*O*-desmethyl-3-*O*-acetylpapaveroxine to 3-*O*-acetylpapaveroxine, which is then sequentially converted to narcotine hemiacetal and noscapine by CEX1 and NOS, respectively (Park et al., 2018). Contrary to a previous report, Park and colleagues further showed that narcotoline is not a substrate for OMT2:OMT3, completing the

elucidation of narcotoline and noscapine biosynthesis pathways (Li and Smolke, 2016; Park et al., 2018). Interestingly, many of the genes for noscapine biosynthesis are located within a 10-gene cluster spanning a 584-Kb region on chromosome 11 of *P. somniferum* and are co-expressed in stems (Winzer et al., 2012; Guo et al., 2018). Deletion of this whole cluster appears to be correlated with high levels of morphine and thebaine in some varieties (**See section 4.1 below**).

2.3.2. Sanguinarine synthesis

Sanguinarine biosynthesis involves seven enzymatic conversions starting from (*S*)-reticuline, which is converted to (*S*)-scoulerine by BBE (see **section 2.3** above). (*S*)-Scoulerine is converted to (*S*)-stylopine by two members of the CYP719 family proteins cheilanthifoline synthase (CFS) and stylopine synthase (SPS) (Ikezawa et al., 2007, 2009; Chávez et al., 2011). (*S*)-Stylopine is sequentially converted to (*S*)-*cis*-*N*-methylstylopine and protopine by enzymes tetrahydroprotoberberine *cis*-*N*-methyltransferase (TNMT) and (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase (MSH), respectively (Rüeffer and Zenk, 1987; Liscombe and Facchini, 2007). Protopine gets converted to 6-hydroxyprotopine *via* protopine 6-hydroxylase (P6H), which is another member of the CYP82 N subfamily (Tanahashi and Zenk, 1990; Takemura et al., 2013). 6-Hydroxyprotopine spontaneously converts to dihydrosanguinarine, which is a root-specific benzo[c]phenanthridine alkaloid and less toxic. Dihydrosanguinarine is then oxidised to sanguinarine (cytotoxic) by the FAD-linked enzyme dihydrobenzophenanthridine oxidase (DBOX) (Hagel and Facchini, 2012). Sanguinarine can be reduced to dihydrosanguinarine by sanguinarine reductase (SanR) (Weiss et al., 2006; Vogel et al., 2010).

2.4. Thebaine, Codeine and Morphine synthesis

The biosynthesis of morphine starts with the epimerization of (*S*)-reticuline to (*R*)-reticuline, which is a two-step process *via* a 1,2-dehydroreticuline intermediate. This is catalysed by a

recently described bifunctional fusion enzyme, reticuline epimerase (REPI; also called STORR for *(S)- to (R)* reticuline), which is composed of 1,2-dehydroreticuline synthase (DRS; CYP82Y2) and 1,2-dehydroreticuline reductase (DRR) (Farrow et al., 2015; Winzer et al., 2015) (**Figure 2**). (*R*)-reticuline is then converted to salutaridine by CYP salutaridine synthase (SalSyn; CYP719B1) (Gesell et al., 2009). Salutaridine then gets converted to salutaridinol by salutaridine reductase (SalR) (Ziegler et al., 2006). It was suggested that salutaridinol is *O*-acetylated by salutaridinol 7-O-acetyltransferase (SalAT) and forms salutaridinol 7-*O*-acetate (Lenz and Zenk, 1995; Grothe et al., 2001). Salutaridinol 7-*O*-acetate converts to thebaine (the first pentacyclic morphinan alkaloid) *via* spontaneous cyclisation at pH 8-9. At pH 6-7 spontaneous cyclisation results in its conversion to a dibenz[d,f]azonine alkaloid (Fisinger et al., 2007). However, a recent report demonstrated that SalR converts salutaridine to (*7S*)-salutaridinol, which is converted to (*7S*)-Salutaridinol 7-*O*-acetate by SalAT (Chen et al., 2018). Additionally, thebaine synthase (THS), a member of the pathogenesis-related 10 protein (PR10) superfamily, has been identified as a novel enzyme catalysing the final step of thebaine biosynthesis in plants, converting (*7S*)-salutaridinol 7-*O*-acetate to thebaine (Chen et al., 2018). Notably, THS is a member of a thebaine gene cluster in the poppy genome that includes the other four genes involved in thebaine biosynthesis upstream of THS (Chen et al., 2018).

The morphine pathway divides into two routes at thebaine; the major and the minor routes of morphine biosynthesis (**Table 2**). In the major route, thebaine is converted to neopinone by thebaine 6-*O*-demethylase (T6ODM) (Hagel and Facchini, 2010). Neopinone was thought to undergo spontaneous rearrangement to form codeinone. However, more recent data demonstrated that the isomerization of neopinone and codeinone is in fact catalysed by a novel enzyme neopinone isomerase (NISO), a third member of the pathogenesis-related 10 (PR10) protein family involved in alkaloid biosynthesis after NCS and the recently characterized THS (Ilari et al., 2009; Chen et al., 2018; Dastmalchi et al., 2019). Codeinone reductase (COR), which is an aldo-keto reductase, reduces codeinone to codeine, the narcotic analgesic (Unterlinner et al.,

1999). It has been shown recently that COR also irreversibly converts neopinone and neomorphinone to the metabolically 'trapped' alkaloids and carbon sinks neopine and neomorphine, respectively, and this explains why suppression of NISO results in neopine and neomorphine accumulation in plants (Dastmalchi et al., 2018b, 2019). Codeine is converted to morphine by the activity of codeine *O*-demethylase (CODM). In the minor route, CODM converts thebaine to oripavine, which is then converted to neomorphinone *via* T6ODM. Neomorphinone is isomerised by NISO to morphinone, which is ultimately reduced to morphine by COR (Beaudoin and Facchini, 2014; Dastmalchi et al., 2019).

3. Regulation of BIA metabolism

3.1. Cell types and localisation of morphine biosynthesis

The biosynthesis and storage of specialized metabolites in plants often involves multiple sub-cellular compartments and cell types (Hagel et al., 2008a; Pan et al., 2016). The cell type-specific accumulation of alkaloids is a result of their cytotoxicity and possibly due to specific biological functions, such as involvement in plant defence responses (Ziegler and Facchini, 2008). Advances in techniques such as *in situ* hybridization, immunofluorescence labelling and shotgun proteomics have enabled the cell-specific localisation of these metabolites and their intermediates to be identified in several species, including poppy (Onoyovwe et al., 2013) and Madagascar periwinkle (Yamamoto et al., 2016). Biosynthesis and storage of BIAs in the capsules of opium poppy involves three distinct cell types; companion cells, sieve elements and laticifers (Bird et al., 2003; Samanani et al., 2006) (**Figure 3**). It has been proposed that the majority of the BIA biosynthetic genes are transcribed and translated in companion cells, but the corresponding functional enzymes are translocated to sieve elements of the phloem where the alkaloids are made. Some alkaloids, in particular thebaine, are synthesized in the sieve elements then transported to adjacent laticifers for storage in large cytoplasmic vesicles by an unknown mechanism.

The majority of enzymes involved in morphine synthesis in opium poppy are expressed in companion cells, whilst genes encoding the enzymes that catalyse the last three steps of the morphine biosynthetic pathway (*T6ODM*, *CODM* and *COR*) are expressed mainly in laticifers (Beaudoin and Facchini, 2014) (**Figure 3**). The enzymes NCS, 6OMT, CNMT, NMCH, 4′OMT and SalSyn are expressed only in companion cells, and SalR and SalAT are expressed abundantly in companion cells and, to a lesser extent, in laticifers. *T6ODM*, *CODM,* and *COR* are also expressed at a much lower levels in companion cells (Onoyovwe et al., 2013). The enzymes NCS, 6OMT, NMCH, 4′OMT and SalSyn, SalR, SalAT are made in the companion cells, and are translocated from these companion cells to the sieve elements, most likely through the plasmodesmatal connections between these cells. The enzymes T6ODM, COR and CODM remain in laticifers, though a small proportion is found in sieve elements.

The alkaloids, (*S*)-norcoclaurine, (*S*)-coclaurine, (*S*)-*N*-methylcoclaurine, (*S*)-3′-hydroxy-*N*-methylcoclaurine, (*S*)-reticuline and (*R*)-reticuline are produced in the sieve elements. Since SalR and SalAT are present in laticifers, salutaridine and salutaridinol are synthesised in laticifers. The majority of thebaine, mainly produced in sieve element cells, is translocated to the laticifers and converted to codeinone, codeine and morphine by the action of T6ODM, COR and CODM, respectively. The small proportion of thebaine that remains in the sieve elements is converted to codeinone, codeine and morphine by the action of T6ODM, COR, CODM, which are present in small concentrations in the sieve elements themselves. NOS and 7OMT are two other BIA biosynthetic enzymes that are also abundant in the laticifers (Onoyovwe et al., 2013; Chen and Facchini, 20014). This indicates that the final steps of noscapine synthesis and 7-*O*-methylated derivatives of reticuline also occur in the latex and are separated from upstream enzymes. In addition to thebaine, another top candidate for transport from sieve element to laticifers is narcotine hemiacetal belonging to the noscapine pathway. Even though the exact translocation mechanisms of these products has not been described, it has been suggested that this may occur *via* symplastic and apoplastic transport routes through the plasmodesmata between sieve

elements and laticifers (Facchini and De Luca, 2008). Increased efficiency of this transport is a potential target for selection or modification to increase alkaloid content.

3.2. Subcellular trafficking in sanguinarine biosynthesis

Subcellular compartmentalisation and trafficking of enzymes involved in the sanguinarine biosynthesis pathway has been studied in cultured opium poppy cells (Hagel and Facchini, 2012). BBE and CYPs are associated with the endoplasmic reticulum (ER) (**Figure 4**; Bird and Facchini, 2001). Association of NCS with the ER lumen has also been reported, which necessitates that its substrates, 4HPPA and dopamine, are also translocated from the cytosol to the ER lumen (Beaudoin and Facchini, 2014). Norcoclaurine is exported after biosynthesis from the ER lumen to the cytosol, because 6OMT and other *O*- and *N*-methyltransferases are present in the cytosol. CNMT is also a cytosolic enzyme. Consequently, (*S*)-coclaurine gets converted to (*S*)-*N*-methylcoclaurine in the cytosol. (*S*)-*N*-Methylcoclaurine gets converted to (*S*)-reticuline by NMCH that is anchored to the cytosolic face of the ER.

(*S*)-Reticuline must be translocated to the ER lumen from the cytosol in order to be converted to (*S*)-scoulerine, because BBE is present in the ER lumen. As soon as (*S*)-scoulerine is produced it is exported to the cytosol, as four out of the five subsequent enzymes are cytochrome P450 (CYPs - CFS, SPS, MSH and P6H) that are attached to the cytosolic face of the ER. The fifth is the cytosolic enzyme TNMT. The final enzyme associated with sanguinarine synthesis is DBOX, which is associated with the ER like the STOX enzymes (Amann et al., 1988). Therefore, dihydrosanguinarine should enter the ER lumen to be converted to sanguinarine. Sanguinarine produced in the ER lumen cultured opium poppy cells by oxidation of dihydrosanguinarine subsequently enters the central vacuole *via* vesicle-mediated transport (Alcantara et al., 2005).

3.3. Regulation of gene expression in BIA Biosynthesis

Transcriptional regulation of alkaloid biosynthesis in opium poppy has only been studied comparatively recently (Kawano et al., 2012; Mishra et al., 2013). Ectopic expression of an *Arabidopsis thaliana* WRKY transcription factor in California poppy (*Eschscholzia californica*) causes an induction of BBE and NMCH transcripts and also an increase in the accumulation of benzo[c]phenanthridine alkaloids, including sanguinarine (Apuya et al., 2008). In opium poppy, several MYB and WRKY-binding elements occur within or near the promotor regions of biosynthetic genes in the reported noscapine gene cluster, suggesting noscapine biosynthesis may also be regulated by MYB and WRKY transcription factors (Winzer et al., 2012; Kakeshpour, 2015). However, there has been relatively little investigation of the regulation of individual genes and of regulation of cell type-specific expression (e.g companion cells vs. laticifers). More recently, an SSR motif repeat variation in the promoter of CYP82Y1, a key noscapine biosynthetic enzyme, was found to affect transcript abundance of the gene and the amount of noscapine produced (Abedini et al., 2018). Variant isoform usage may also be involved in regulation of BIA biosynthesis, since different codeine reductase (COR) isoforms affect enzyme activity and composition of the alkaloid profile (Dastmalchi et al., 2018b). A greater understanding of regulatory processes may allow for the increased expression of particular enzymes in different cells to be engineered and would also have implications for commercial production of opiate alkaloids in engineered microorganisms.

4. Opium poppy genetic and genomic resources

Considerable poppy genetic resources are maintained globally in seedbanks. For instance, a collection of 1,100 accessions, including 404 accessions originating from the poppy world collection at the N.I. Vavilov Institute of Plant Genetic Resources in Petersburg, are maintained at the Institute of Protection of Biodiversity and Biological Safety in the Slovak University of Agriculture (Brezinova et al., 2009). Over 1,000 accessions of poppy collected worldwide are

maintained at Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) genebank in Germany (https://www.ipk-gatersleben.de/en/genebank/). Reports from various genetic diversity studies conducted on poppy indicate collections of varying sizes exist in many other countries including India (Bajpai et al., 1999, 2001; Prajapati et al., 2002; Lahiri et al., 2018; Gupta, 2018) and Turkey (Celik et al., 2016; Valizadeh et al., 2017).

4.1. Natural and induced variations in metabolic pathways in *P. somniferum*

Several varieties are used for commercial poppy production. However, relatively little description of the molecular basis of variation in BIA biosynthesis in these varieties has been reported. This is due to the highly commercial nature of the crop and the reluctance of producers to share details of the origin and means of development with competitors. However, some varieties or mutants with blocks at various steps of the biosynthetic pathway have been characterised. The high papaverine *pap1* mutant has morphine, codeine and thebaine levels similar to wild type plants (Park et al., 2013). Gene expression analysis using RNA-seq determined that expression of many OMTs in the NH-pathway leading from (*S*)-norcoclaurine to papaverine is increased in *pap1*. The authors speculated that this may be due to the overexpression or mutation of a WRKY transcription factor that normally regulates the expression of multiple genes in the pathway, suggesting that a single mutation might lead to up-regulation of an entire biosynthetic pathway (Park et al., 2013). The Sujata cultivar, which was developed by mutation breeding following gamma-ray mutagenesis of an alkaloid accumulating variety, is latex free and contains very low amounts of alkaloids. Although the mutation responsible for the observed low alkaloid profile of Sujata is yet to be determined, this cultivar is characterized by reduced expression of *SalSyn* and *CODM*, suggesting a block in the biosynthetic pathway possibly affecting all alkaloids downstream of the candidate mutation (Sharma et al., 1999; Chaturvedi et al., 2014).

The noscapine-accumulating variety of *P. somniferum* (High Noscapine 1, HN1) is distinguished from the High Morphine 1 (HM1) and High Thebaine 1 (HT1) varieties developed in

Tasmania by the presence of a large gene cluster encoding ten enzymes (PMST1, PMST2, PSMT3, CYP82X1, CPY82X2, CYP82Y1, CYP719A21, PSAT1, PSSDR1 and PSCXE1) of the noscapine biosynthesis pathway (Winzer et al., 2012; Guo et al., 2018). These genes are missing from the genomes of HM1 and HT1. The HM1 and HT1 varieties of poppy are therefore mutants that have a large (approximately 500-kb) deletion of the chromosomal segment spanning this gene cluster. Other HM and HT cultivars may have lost some or all of these noscapine pathway genes as well (**Figure 5A**). The accumulation of morphine and thebaine in the HM1 and HT1 varieties is therefore most likely a consequence of the increased availability of metabolites diverted from the noscapine pathway, with high thebaine varieties also having reduced expression of T6ODM in the stem (Winzer et al., 2012).

The best-characterized variation in opium poppy is the *top1* (*thebaine oripavine poppy 1*, also known as Norman), which accumulates thebaine and oripavine due to a metabolic block in the conversion of thebaine to neopinine and oripavine to morphinone (Millgate et al., 2004) (**Figure 5B**). A similar, but independently isolated variant called T (high Thebaine) has a similar alkaloid profile to *top1* (Hagel et al., 2008b). T6ODM gene expression is reduced in T and demethylation of thebaine catalysed by T6ODM is blocked (Hagel and Facchini, 2010). The transgenic PsM1-2 mutant accumulates thebaine due to a T-DNA induced mutation of the CODM gene in a variety which also lacks COR and/or T6ODM (Kawano et al., 2012).

A series of three reticuline accumulating mutants [(*S*)-to (*R*)-reticuline, *storr*] has been described (Winzer et al., 2015). All three *storr* mutants accumulate (*S*)-reticuline, and the (*S*)-reticuline-derived laudanine and laudanosine. These mutants have defective STORR enzymes and cannot isomerise (*S*)-reticuline to (*R*)-reticuline in the usual two-step reaction with a 1,2-dehydroreticuline intermediate catalyzed by the bifunctional cytochrome P450/oxidoreductase STORR enzyme (Winzer et al., 2015) (**Figure 5C**). High thebaine/codeine varieties derived by mutagenesis have been patented. No molecular description of these varieties has been reported but they presumably lack functional CODM (**Figure 5D;** Fist et al., 2013).

4.2. The influence of natural diversity and heterosis on morphology and yield

An Indian landrace that exhibits dramatically altered capsule morphology increases alkaloid yield without directly affecting BIA biosynthesis (**Figure 6**) (Prajapati et al., 2001). This is caused by a recessive floral organ mutation called androcarpel organ (*aco*), which causes development of additional androcarpels (carpels derived from the inner two whorls of stamens). Mutant plants have higher morphine and codeine content (33% increase and 115% increase respectively in weight/weight of dry capsules) than their parental lines, but normal levels of other intermediates including codeinone, oripavine, reticuline, papaverine, and narcotine (Prajapati et al., 2001). This is most likely due to an increase in the amount of carpel wall tissue, and presumably the number of laticifer cells per capsule, present on a single capsule. The spontaneous OM mutant exhibits a similar, but less severe phenotype, resulting in increased morphine and codeine content (Singh et al., 2017).

Alkaloid content varies substantially between poppy genebank accessions of different reported geographic origin. Dittbrenner and colleagues found substantial variation in alkaloid content across 300 accessions, ranging from 0.6 to 2.5% total alkaloids as a percentage of dry capsule weight (Dittbrenner et al., 2007). However, they did not observe any significant correlation between alkaloid levels and 35 morphological characteristics, including flowering date, plant height, number and size of capsules, shape and hairiness of leaves. This suggests that none of these characters are reliable tools for selection during breeding. A recent study describes the analysis of *P. somniferum* F1 hybrids derived from crossing a wide range of elite parental lines (Valizadeh et al., 2017). Similar F1 hybrids are widely used in many crops including maize, rice, canola, sorghum and sunflower (Fu et al., 2014) as they exhibit heterosis (also known as hybrid vigour) that often leads to increased yield in the first generation of progeny derived by crossing. Valizadeh et al. reported increases in capsule yield (kg per hectare), seed yield (kg per hectare) and total alkaloid yields (kg alkaloid per hectare) of up to 100%, 80% and 160% above the best parent, respectively (Valizadeh et al., 2017). These figures are considerably higher than the

increases in crop yields due to heterosis reported in multiple agricultural crops, which are in the range of 15-50% (see Fu et al., 2014). Consequently, further validation is necessary to determine the true potential of hybrid vigour for increasing opium poppy yields.

4.3. Genomic resources for poppy

Several large projects have aimed to sequence the genomes of medicinal plants, including the Medical Plants Genomics Resource (http://medicinalplantgenomics.msu.edu/). However, many of the study species have large or complex genomes, which are correspondingly difficult and expensive to sequence, so most projects have focused initially on characterizing transcriptomes instead. Several consortia are sequencing a variety of medicinal and non-medicinal plants (PhytoMetaSyn, https://bioinformatics.tugraz.at/phytometasyn; 1,000 Plants, https://sites.google.com/a/ualberta.ca/onekp/). In addition, some studies have specifically targeted *P. somniferum* using a variety of approaches. The genes involved in morphine biosynthesis in poppy have been characterized in this manner. A complete summary of the available sequence resources for opium poppy is included in **Table 1**. Early approaches used Sanger and 454 sequencing of Expressed Sequence Tags (ESTs) of cloned mRNA, which is relatively low throughput. The 1,000 Plants initiative uses Illumina technology, which generates substantially more data at relatively low cost and in a shotgun-manner (i.e. not constrained to cloned mRNA, enabling comprehensive characterization of whole transcriptomes) (Unamba et al., 2015).

The recent report of a draft *P. somniferum* reference genome sequence is a major breakthrough (Guo et al., 2018). The genome assembly of a 2.72 Gb of sequence covered 94.8% of the estimated 2.87 Gb genome size and contained 51,213 protein coding genes that were supported by transcriptome data. Of the sequence assembled, 81.6% was assigned to the 11 chromosomes, providing a high quality reference genome that facilitates genetic analyses to map

and isolate candidate genes. As is frequently the case in species with large genomes, repetitive elements make up about 70% of the poppy genome (Guo et al., 2018).

4.4. Poppy transformation

There are several reports of successful transformation of *P. somniferum*. Here we distinguish two main types; transient transformation and stable transformation. Transient transformation is used to rapidly validate gene construct expression or function before proceeding to generating stable transformants. It does not result in heritable events i.e. any induced modification to gene expression or gene sequence is not inherited by progeny plants. Transient experiments typically use isolated cells or discrete tissues, including *in vitro* cultured cells, “hairy” roots or leaves as a target tissue to rapidly validate enzyme activity, specificity or sub-cellular localization (**Table 2**) (Park and Facchini, 2000a; Sharafi et al., 2013; Alagoz et al., 2016). Two methods of delivery have been described in poppy. Microprojectile bombardment uses DNA-coated metal beads into cultured cells whereas *Agrobacterium*-mediated transformation of cells is typically achieved by infiltration of bacteria carrying a DNA construct into young leaves (Park et al., 1999; Bird and Facchini, 2001; Park and Facchini, 2000a).

A variant of transient transformation is the use of virus-derived vectors to produce a long-lasting infection that can be propagated through multiple cells. The viruses are engineered to carry sequences homologous to endogenous plant loci, which results in reduced gene expression through RNA silencing, termed VIGS (Baulcombe, 1999; Hileman et al., 2005). Several groups have applied VIGS to reduce expression of genes in *P. somniferum* and *P. bracteatum*. The VIGS persists for several weeks and, usefully, spreads into the developing stem and capsule (Hileman et al., 2005; Wege et al., 2007). To conduct VIGS young leaves and shoot apices of 18-21 day old, two-leaf stage *P. somniferum* plants are infiltrated with an *Agrobacterium tumefaciens* bacterial strain carrying a Tobacco Rattle Virus (TRV)-derived DNA construct that expresses a virus designed to silence the gene of interest (see **Figure 7** for a proof-of-concept demonstration).

464 The plant is allowed to grow and stems below the first flower are collected 1-2 days prior to
465 anthesis for chemical analysis. This approach has been used successfully by several groups to
466 reduce gene expression and to validate the role of enzymes at different steps of the alkaloid
467 biosynthesis pathway in opium poppies (**Table 2** and **Table 3**).

468 Stable transformation aims to produce plants that carry introduced DNA or induced
469 mutations that are stable and inherited by progeny plants. This approach requires the production
470 of transformed cells cultured *in vitro* under sterile conditions on specific combinations of
471 phytohormones to produce plantlets, which give rise to shoots that will produce flowers and
472 eventually seed. Several methods have been developed for *P. somniferum* by different research
473 groups, variously using cotyledons (embryonic leaves), root explants or hypocotyl (embryonic
474 stem) explants as the target for *Agrobacterium*-mediated transformation (Park and Facchini,
475 2000a, b; Chitty et al., 2003) (**Table 3**). Most recently, the induction of somatic embryos (seedlings
476 developed from root explants) has been reported to take 8-12 months to produce transgenic T1
477 seed (Pathak et al., 2012) (**Table 3**).

478

479 **4.5. Poppy gene modification**

480 Gene editing enables generation of very specific, targeted changes to the DNA of an organism
481 (Ma et al., 2016). This can be applied to modify an organism's genotype to achieve a specific
482 outcome, such as a desirable alteration of a trait, in a controlled and relatively rapid manner. The
483 changes to the DNA may be indistinguishable from naturally occurring mutations and are
484 consequently regulated as such by many nations. For example, two recent cases in the United
485 States have seen a gene-edited corn variety and a gene-edited mushroom classified by the US
486 Department of Agriculture (USDA) as not requiring regulation for cultivation or sale, but these new
487 varieties may still be subject to the Food and Drug Administration (FDA) and the Environmental
488 Protection Agency (EPA) regulations (Waltz, 2016a, b).

Gene editing technologies are relatively new and have only recently been applied to opium poppy (Alagoz et al., 2016). It was demonstrated that the CRISPR/Cas gene editing technology could be used in *P. somniferum* leaves in a transient assay to mutate the 4′OMT2 gene and reduce morphine biosynthesis (Alagoz et al., 2016). More recently, a CRISPR/Cas9 edited variant of NCS with increased enzyme activity was used to achieve 8-fold increase in noscapine production in yeast (Li et al., 2018). These proof-of concept papers indicate that creating opium poppy with targeted mutations in genes of interest is now feasible.

5. Modification of alkaloid levels by metabolic engineering

5.1. Gene over-expression

Only a few reports describe the effects of heritable increases in gene expression on alkaloid levels in opium poppy, with most studies using transient assays that do not produce heritable effects. However, the few studies describing stable lines do report promising increases in alkaloids, suggesting that the approach is viable. For example, over-expression of COR causes an increase in morphine and codeine accumulation (22 and 50%, respectively) (Larkin et al., 2007). Over-expression of the SalAT gene increases the total alkaloid content of opium poppy by approximately 40% (Allen et al., 2008) and overexpression of the NMCH gene resulted in up to a 450% increase (Frick et al., 2007). The overexpression of the 6OMT and 4′OMT in California poppy cells increased the total alkaloid content, leading to the proposal that 6OMT plays a rate limiting role.

Not all alkaloid biosynthetic genes increased total alkaloid content when over-expressed and the corollary effects on gene expression in the alkaloid biosynthetic pathway were often complex. Stable over-expression of 4′OMT had limited effects on total alkaloid content in cultured California poppy (*E. californica*) cells (Inui et al., 2007). Transient over-expression of 4′OMT had different effects in opium poppy. It increased expression of CNMT, SalAT and 7OMT and decreased expression of the COR gene in the stem (Gurkok et al., 2016). Morphine and

noscapine levels in the stem increased by two-fold but alkaloid content of the capsule was reduced significantly (75%), with the greatest reductions being from lost morphine and noscapine content (Gurkok et al., 2016). Over-expression of the 7OMT gene increased COR, SAT, 6OMT and 4′OMT transcript abundance in the stem but reduced CNMT, 6OMT and COR transcript abundance in the capsule (Gurkok et al., 2016). It also increased 6OMT and decreased CNMT and TYDC transcript abundance in the leaf while reducing CNMT transcript abundance in leaf, stem and capsules (Gurkok et al., 2016). These effects caused significant increase of alkaloid accumulation in stem and leaf tissues of *P. somniferum* cv Ofis 95, particularly morphine, but a significant decrease of the noscapine concentration in the capsule (Gurkok et al., 2016).

5.2. Gene suppression

VIGS-based gene silencing has been instrumental in linking gene functions to BIA biosynthetic pathways and elucidating the major pathways in opium poppy. VIGS has been used to silence the genes responsible for the last 6 steps of morphine synthesis, based upon a tobacco rattle virus (TRV) system. Morphine content decreased when SalSyn, SalR, T6ODM and CODM genes were silenced (Wijekoon and Facchini, 2012). In another study, six genes (*6OMT, CNMT, NMCH, 4'OMT2, N7OMT* and *7OMT*) potentially involved in papaverine biosynthesis were individually suppressed in a papaverine-rich cultivar to determine their influence on papaverine synthesis, revealing a major route to papaverine in poppy (Desgagne-Penix and Facchini, 2012). When *4'OMT* expression was suppressed in a morphine rich variety, it caused a reduction in the total alkaloid content in stem tissues, but significantly increased total alkaloid content in the capsule (Gurkok et al., 2016). Silencing of another gene, reticuline 7-*O*-methyltransferace (*7OMT*) that converts (S)-reticuline to (S)-laudanine, also reduced total alkaloid content by 66% in the stem, as well as reducing expression levels of selected BIA biosynthesis genes in different tissues (Gurkok et al., 2016). Further VIGS assays have been conducted to determine functions of some of the genes involved in the noscapine biosynthetic pathway (*PSMT1, PSMT2, CYP719A21,*

CYP82X2, PSSDR1 and *PSCXE1)* (Winzer et al., 2012). Suppression of these genes lead to accumulation of intermediates in the pathway, allowing elucidation of a novel noscapine biosynthetic pathway in poppy. The recent discovery and VIGS based functional analyses of the genes THS and NISO provided further details of the metabolic machinery used for alkaloid production in poppy (Chen et al., 2018; Dastmalchi et al., 2019). Such information is crucial for the on-going efforts to engineer opiate alkaloid pathways in microbial systems.

RNA interference (RNAi), a double stranded RNA (dsRNA) molecule-based mechanism for post-transcriptional gene silencing, is a powerful tool to study or validate gene function in plants. This technique has been used to elucidate the BIA biosynthetic pathway in poppy. RNAi-based suppression of COR resulted in the accumulation of (*S*)-reticuline at the expense of morphine, codeine, thebaine and oripavine (Allen et al., 2004). The accumulation of an intermediate several enzymatic steps upstream of the target gene suggested the presence of a feedback mechanism regulating gene expression or metabolic channels (Allen et al., 2004). Similarly, RNAi-suppression of BBE (berberine bridge enzyme) changed the ratio of alkaloids in latex but not in roots, which led to the suggestion that enzymes other than BBE are important in the control of sanguinarine biosynthesis in poppy (Frick et al., 2004). Expression of antisense *CYP80B3* (*NMCH*) cDNA in poppy resulted in up to 84% reduction in total alkaloids in the latex, demonstrating that *CYP80B3* (*NMCH*) is a key regulation step in morphine biosynthesis (Frick et al., 2007). DNA-encoded hairpin RNA-mediated suppression of *SalAT* led to the accumulation of the intermediate alkaloids salutaridine and salutaridinol, which also suggested a physical interaction between the enzymes SalR and SalAT as demonstrated by results of yeast two-hybrid assays (Allen et al., 2008; Kempe et al., 2009).

MicroRNAs (miRNAs) are non-coding small RNAs that regulate gene expression post-transcriptionally in eukaryotes (He and Hannon, 2004). Although the role of miRNAs in regulating several biological processes related to biotic and abiotic responses in plants is well documented, their involvement in regulating secondary metabolism in plants has only begun to be determined

567 recently (see Khraiwesh et al., 2012; Gupta et al., 2017). Boke and colleagues recently identified
568 novel miRNAs including pso-miR13, pso-miR2161, and pso-miR408 that are potentially involved
569 in the alkaloid biosynthetic pathway in poppy (Boke et al., 2015). The BIA biosynthetic genes of
570 7OMT, 4'OMT, and BBE have been identified as potential targets of miR13, pso-miR2161, and
571 pso-miR408, respectively, and are highly co-expressed with their target genes in different tissues
572 (Boke et al., 2015).
573
574 ## 6. Prospects for improving traits of interest in poppy
575 The achievements made in defining the alkaloid biosynthetic pathway are remarkable, even more
576 so when considered in the context that the poppy reference genome sequence has been released
577 only recently (Guo et al., 2018). Consequently, poppy serves as a model system to study BIA
578 biosynthesis in plants (Beaudion and Facchini et al., 2014). As a key trait of commercial interest,
579 several attempts have been made to generate cultivars with higher total alkaloids or modified
580 alkaloid composition. The approaches followed so far mainly involve the identification and
581 characterization of spontaneous and induced mutants altered in alkaloid biosynthesis (Nyman,
582 1978 and 1980; Sharma et al., 1999; Millgate et al., 2004).
583 Germplasm collections provide the genetic and phenotypic diversity that have underpinned
584 improvement of many other crops, but this resource appears under-exploited for poppy.
585 Considerable diversity, including in alkaloid content, has been reported in opium poppy
586 germplasm from different regions (Bernáth and Tétényi, 1979, 1981; Yadav et al., 2006;
587 Dittbrenner et al., 2007, 2012; Verma et al., 2016). Nevertheless, much of the information is highly
588 fragmented, making direct comparison between data from various reports difficult. Access to the
589 various poppy global and regional collections also remains limited. Comprehensive
590 characterization and evaluation of the available germplasm is a prerequisite to unravel the extent
591 of poppy genetic diversity. Such knowledge would inform germplasm management, including
592 establishment of a poppy core collection. A core collection, which typically consists of ~10% of all

593 available accessions while capturing the majority of the allelic diversity, facilitates access to and
594 utilization of the available germplasm for crop improvement (van Hintum et al., 2000).

595 Wild relatives of poppy such as *Papaver setigerum* are also under-exploited resources that
596 could be used for improving traits of interest in commercial poppy cultivars. To this end, these
597 resources need to be characterized in detail. *P. setigerum* is the species most closely related to
598 *P. somniferum* both genetically and morphologically (Hosokawa et al., 2004; Choe et al. 2012;
599 Lane et al., 2018). These two are also the only species known to produce the morphinan alkaloids
600 morphine and codeine, while *P. bracteatum* produces only thebaine (Ziegeler et al., 2005, 2006).
601 Resolving the relationships amongst these species using genomics and transcriptomics can
602 therefore contribute significantly to advancing our understanding of the evolution of BIA
603 biosynthesis. Considering that successful interspecific hybridisation has been demonstrated
604 between some of these species, including between *P. somniferum* and *P. setigerum*, there is
605 potential for improving the existing commercial cultivars through introgression of useful traits from
606 wild relatives (Shukala et al., 1995).

607 Both transient and stable transformation techniques are available for targeted modifications
608 of genes of interest in poppy. These techniques have been restricted to research applications due
609 to both the inherent limitations of the technologies and the manner in which genetically modified
610 crops are regulated in many countries (Frick et al., 2007). However, since poppy breeding *via*
611 traditional approaches has been slow, genetic modification merits revisiting now that it would be
612 facilitated by the poppy reference genome (Shukla et al., 2013; Valizadeh et al., 2017; Lahiri et
613 al., 2018).

614 The significance of the recently released poppy reference genome sequence for genetic
615 improvement of the crop is many fold. It allows better application in poppy of the various NGS-
616 based techniques, including bulked segregant analysis, to rapidly identify major genes and
617 quantitative trait loci controlling traits of interest. These techniques have significantly accelerated
618 crop improvement in many other species (see Varshney et al., 2014; Schneeberger, 2014; Onda

and Mochida, 2016). Combining desirable traits from different varieties can be used to produce poppy varieties with improved alkaloid content. To this end, NGS-based techniques provide powerful tools to study the diversity present in poppy germplasm and accelerate the identification of lines/accessions with desirable traits to be used as parents for breeding via marker-assisted selection. The same techniques are also used to rapidly identify gene(s) controlling desirable traits using progeny segregating for the traits under consideration.

Cell-specific resolution RNA-seq has proved powerful to unravel the transcriptome dynamics associated with regulation of many important biological processes (see Martin et al., 2013; Libault et al., 2017). Alkaloid biosynthesis in opium poppy involves three distinct cell types; companion cells, sieve elements and laticifers (Bird et al., 2003; Samanani et al., 2006). A greater understanding of cell-specific gene expression in the developing capsule might allow increased expression of particular enzymes in different cells in order to manipulate alkaloid levels and composition. Moreover, analyses of the regulatory mechanisms, such as transcription factors and chromatin conformation, coordinating expression of alkaloid biosynthetic pathway genes within their specific cells would increase our fundamental knowledge of poppy biology.

Opium poppy is easily amenable for genetic and genomic studies. Despite its large genome size, it is diploid (2n=2×=22) and now has a reference genome sequence. It is a self-fertile species and cross-fertilization is easily achieved *via* cross-pollination, with successful crosses leading to the production of hundreds of seeds per plant. The plant completes its life cycle (from germination to harvest) in about three months. These attributes make it easy to study the inheritance of traits of interest and generate mapping populations segregating for these traits. Genetic stocks are also easily generated and maintained. In sum, it is a highly appealing model for the study of plant secondary metabolism and tissue-specific genome regulation.

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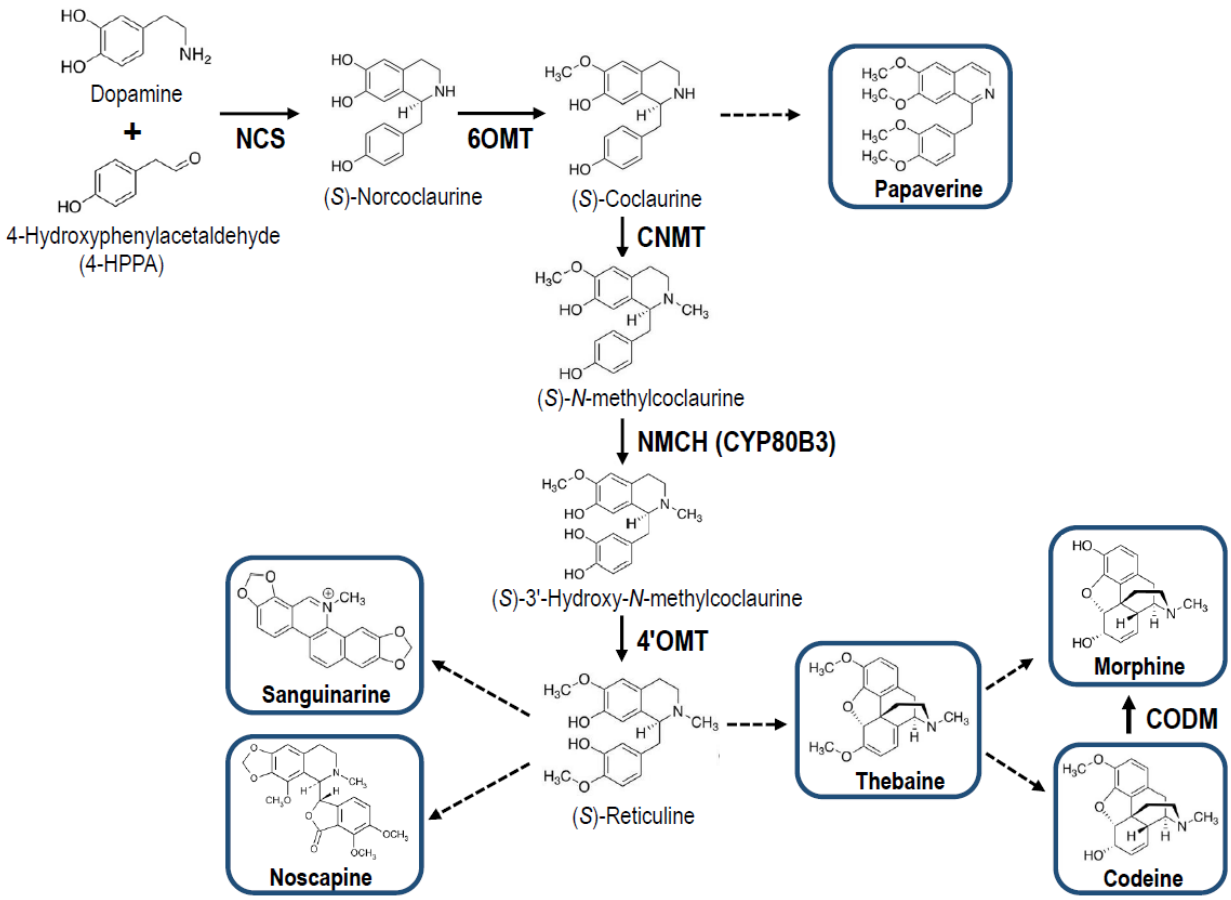

1047

1048 **Figure 1.** Overview of BIA biosynthesis in opium poppy. The major pathways are shown, with
1049 branches leading to papaverine, noscapine, sanguinarine, thebaine, codeine and morphine. NCS,
1050 Norcoclaurine synthase; 6OMT; Norcoclaurine 6-*O*-methyltransferase; CNMT, Coclaurine *N*-
1051 methyltransferase, NMCH, (*S*)-*N*-Methylcoclaurine 3′-hydroxylase; CYP, Cytochrome P450;
1052 4′OMT, 3′-Hydroxy-*N*-methylcoclaurine 4′-hydroxylase; CODM, Codeine *O*-demethylase
1053 (Adapted by permisssion of Springer Nature from Dastmalchi et al., 2018).

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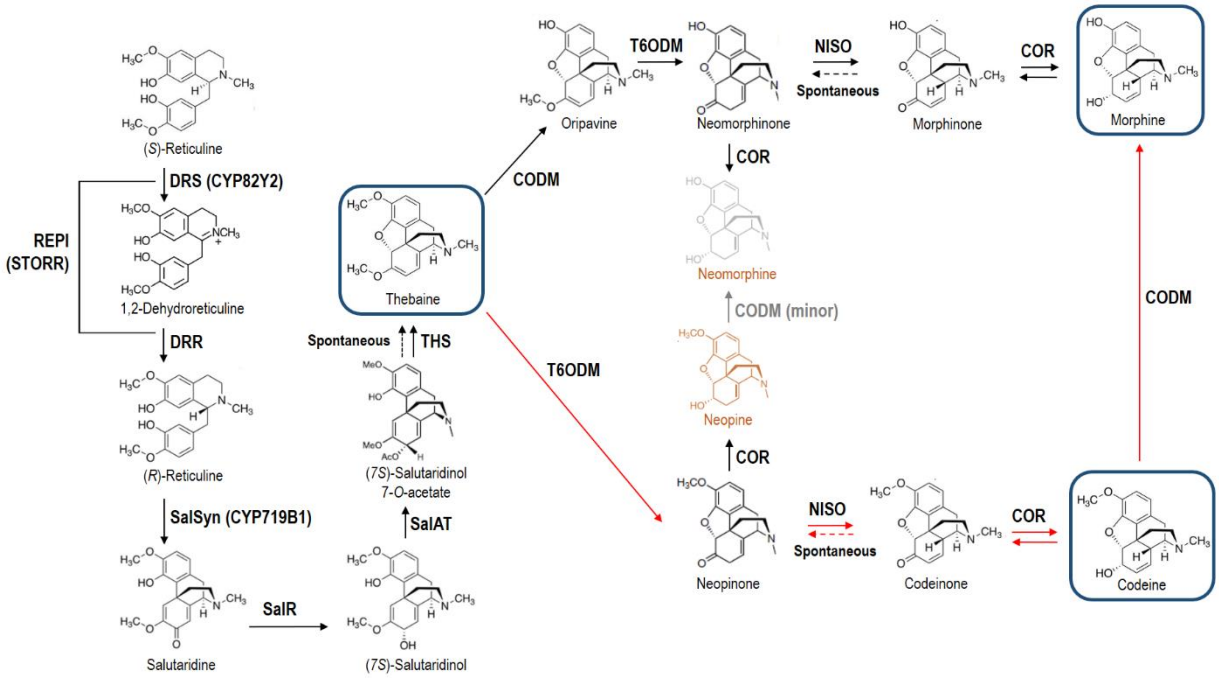

1055

1056 **Figure 2.** Overview of thebaine, codeine and morphine biosynthesis in opium poppy. All steps
1057 from the (*S*)-reticuline precursor are shown, and key enzymes are indicated in bold. The major
1058 route from thebaine to morphine is shown in red, and spontaneous conversion steps are shown
1059 in dashed lines. The reduction by COR of neopinone and neomorphinone to the metabolically
1060 'trapped' alkaloids neopine and neomorphine, respectively is shown in orange. REPI, Reticuline
1061 epimerase; DRS, 1,2-Dehydroreticuline synthase; CYP, Cytochrome P450; DRR, 1,2-
1062 Dehydroreticuline reductase; STORR, (*S*)-to-(*R*)-reticuline; SalSyn, Salutaridine synthase; SalR,
1063 Salutaridine reductase; SalAT, Salutaridinol 7-*O*-acetyltransferase, THS, Thebaine synthase;
1064 CODM, Codeine *O*-demethylase; T6ODM, Thebaine 6-*O*-demethylase; NISO, Neopinone
1065 isomerase; COR, Codeinone reductase (Adapted by permisssion of Springer Nature from
1066 Dastmalchi et al., 2018, 2019; Chen et al., 2018).

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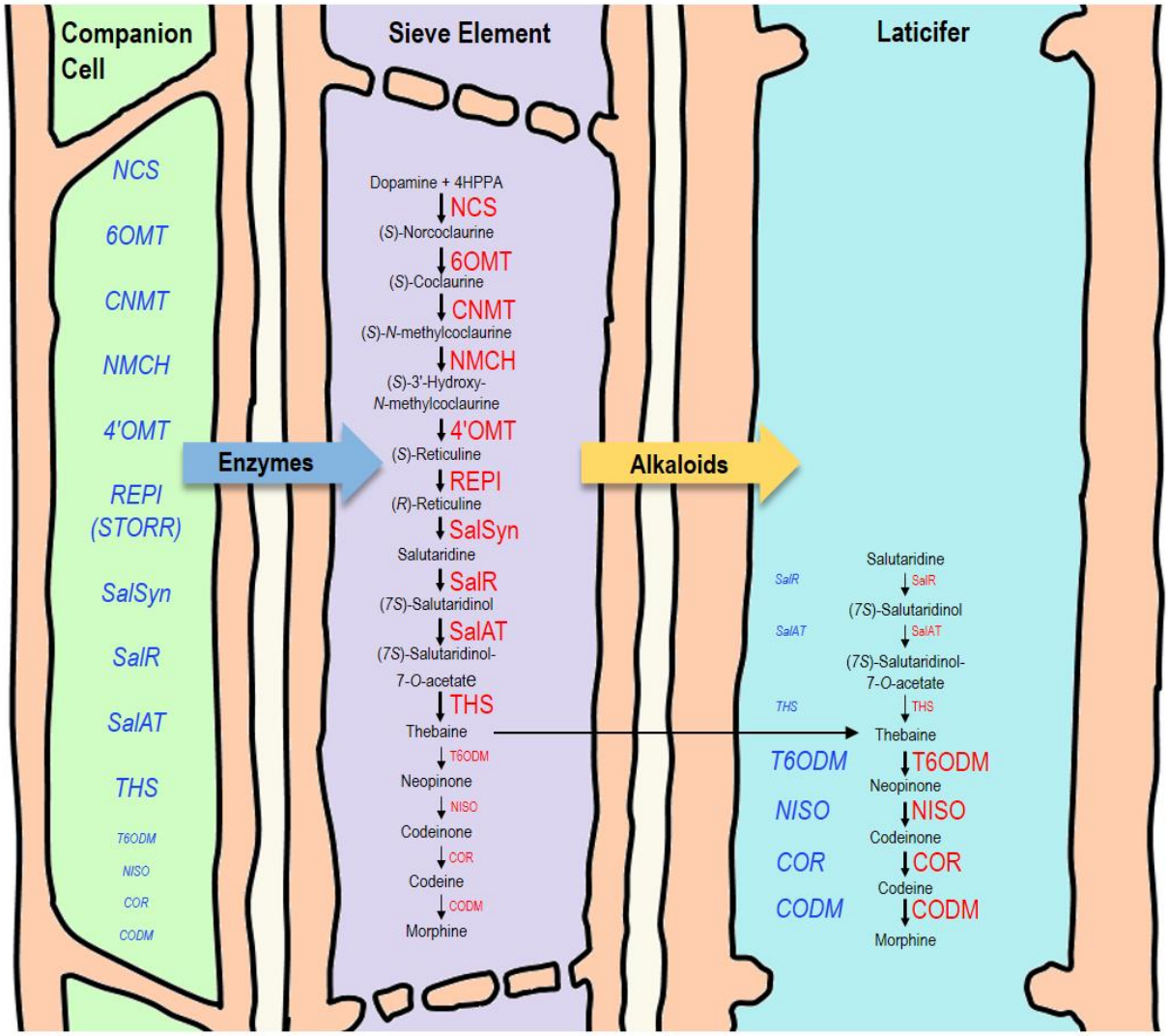


Figure 3. Coordination of BIA biosynthesis between three cell types in opium poppy. Gene expression (transcripts, in blue italics), enzymes (proteins, in red) and products (black) are shown. Possible points of translocation of enzymes and alkaloids between the cellular compartments of companion cells, sieve elements and laticifers are shown with blue and yellow arrows, respectively. Thebaine is the major intemediate alkaloid translocated between sieve elements and laticifers as indicated by horizontal black arrow. Abbreviations are given in the legends of Figure 1 and Figure 2 (Adapted by permisssion of Springer Nature from Beaudoin and Facchini, 2014).

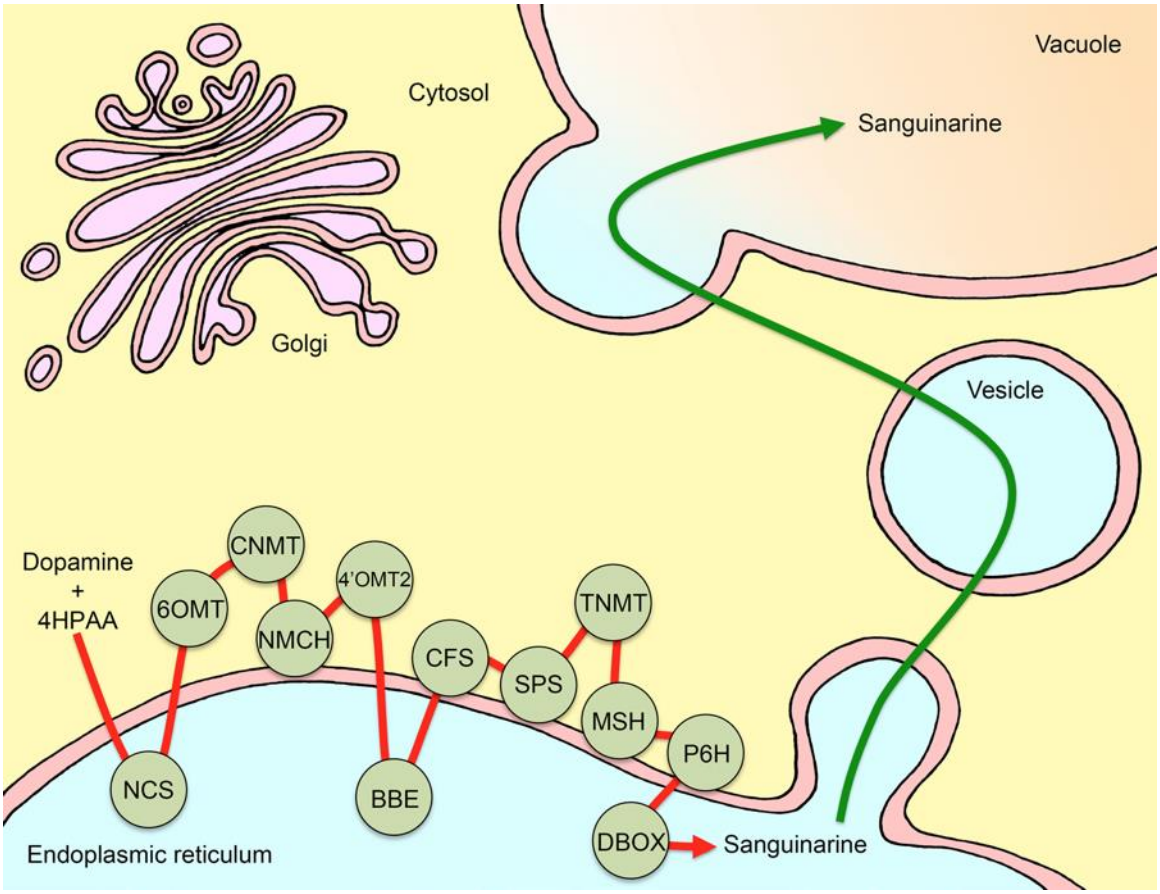
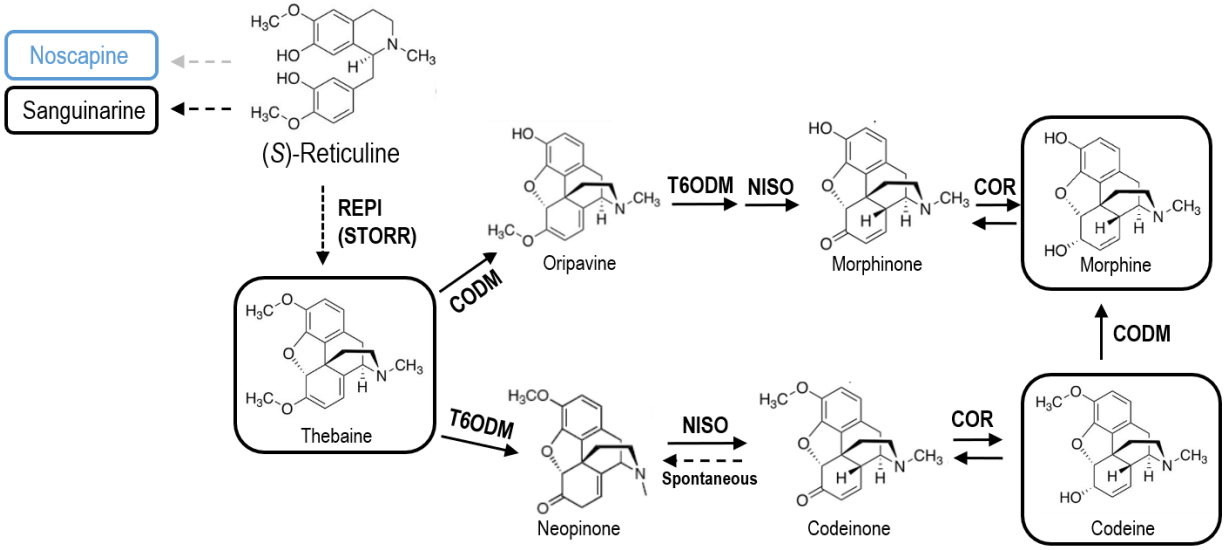


Figure 4. The proposed compartmentalisation of enzymes involved in sanguinarine synthesis in cultured opium poppy cells. The red arrow indicates the flow of the reactions and the green arrow indicates the traffiking of sanguinarine from the ER lumen to the central vacuole. BBE, Berbrine bridge enzyme; CFS, Cheilanthifoline synthase; SPS, Stylopine synthase; TNMT, Tetrahydroprotoberberine *N*-methyltransferase; MSH, *N*-Methylstylopine 14-hydroxylase; P6H, Protopine 6-hydroxylase; DBOX, Dihydrobenzophenanthridine oxidase (Reproduced by permission of Springer Nature from Beaudoin and Facchini, 2014).

(a)



(b)

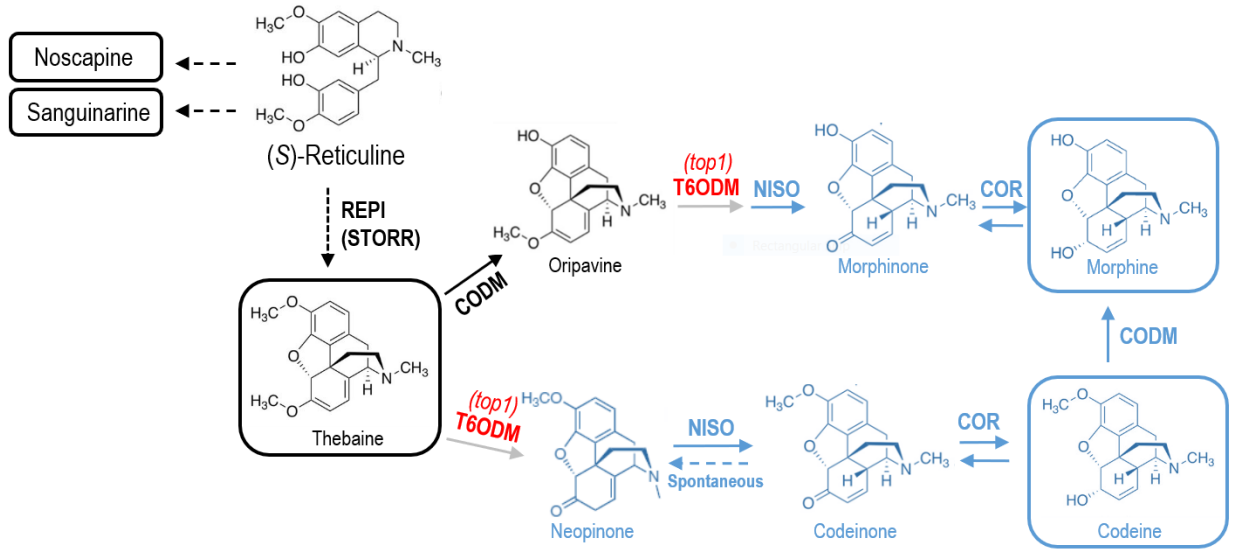
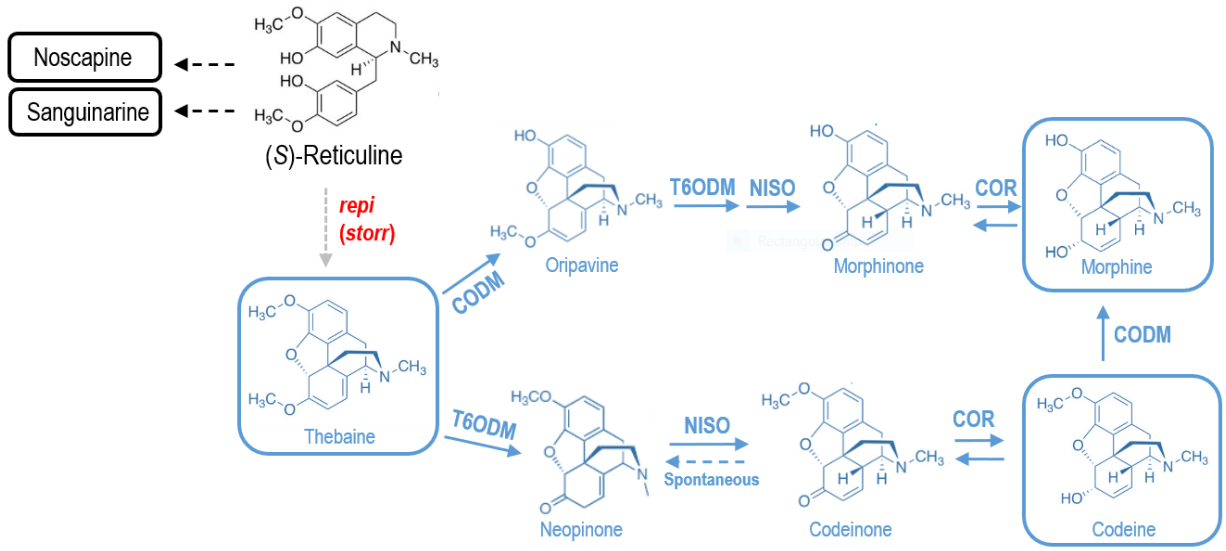


Figure 5 (Continued…)

(c)



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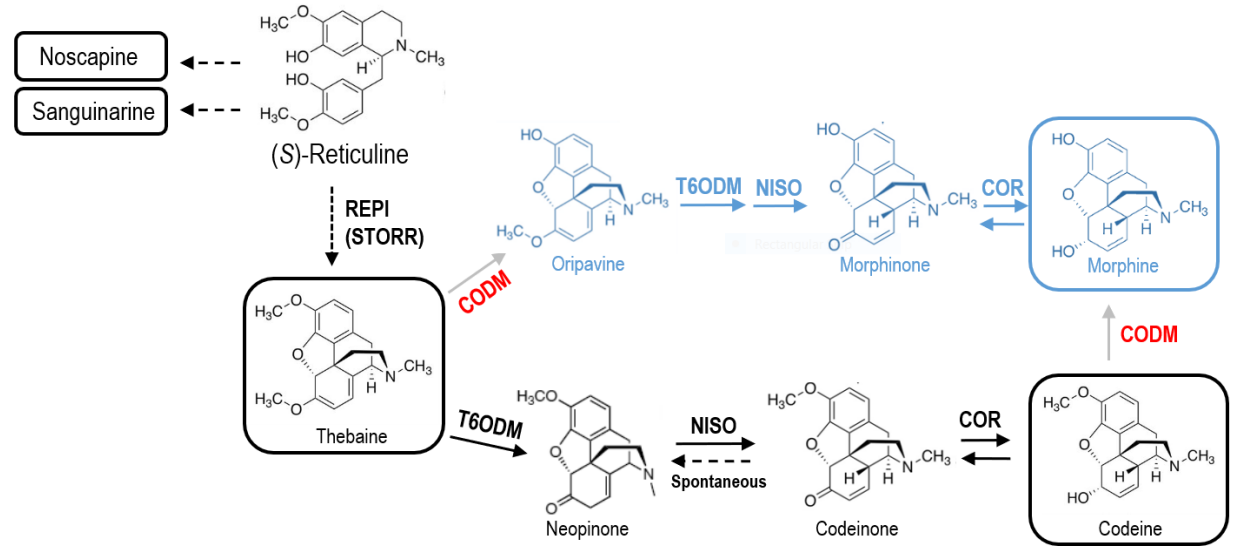

Figure 5. Characterised variation in morphine biosynthesis. **A**: Noscapine mutants lack many of the enzymes in the noscapine branch, and accumulate high levels thebaine and morphine. **B:** The *top1* (and T) mutants lack a functional T6ODM, and accumulate thebaine and oripavine. **C:** *storr* mutants accumulate (*R*)-reticuline. **D:** The thebaine/codeine varieties are likely to lack a functional CODM. Mutations are indicated in red, and downstream steps are shown in blue. Abbreviations are given in the legend of Figure 2.

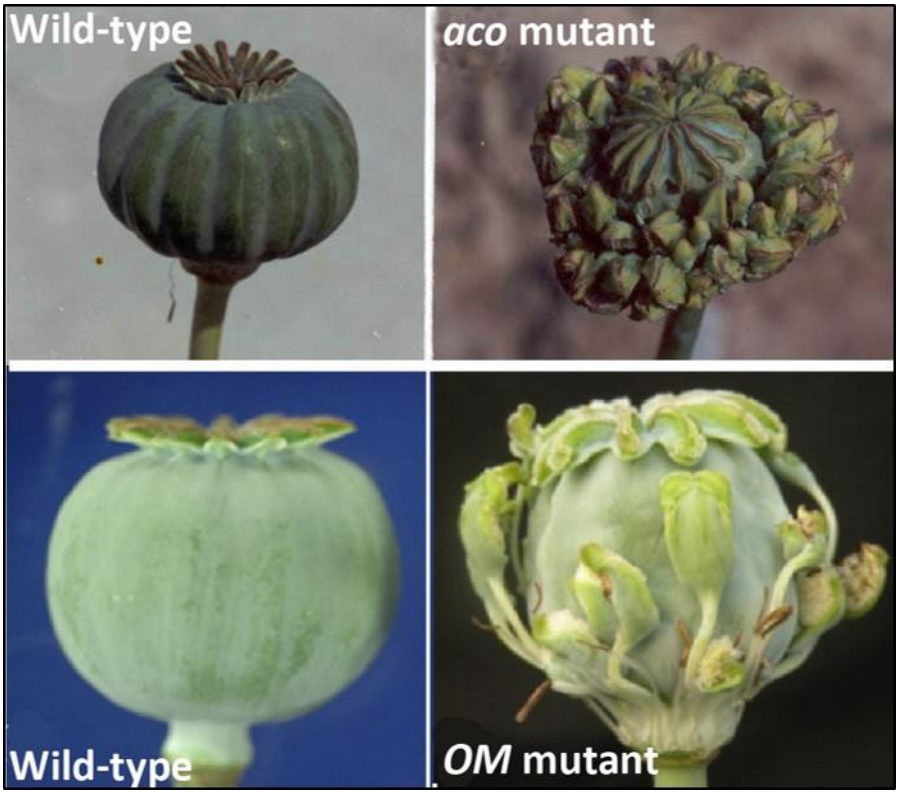

Figure 6. The *androcarpel organ* (*aco*) and *OM* poppy mutants. The *aco* mutant has an increased number of carpels due to the transformation of some of the stamens of the inner whorls of the flower into small carpels, and the OM mutant has a similar but less severe phenotype (Adapted by permission of Current Science and Elsevier from Prajapati et al., 2001 and Singh et al., 2017, respectively).

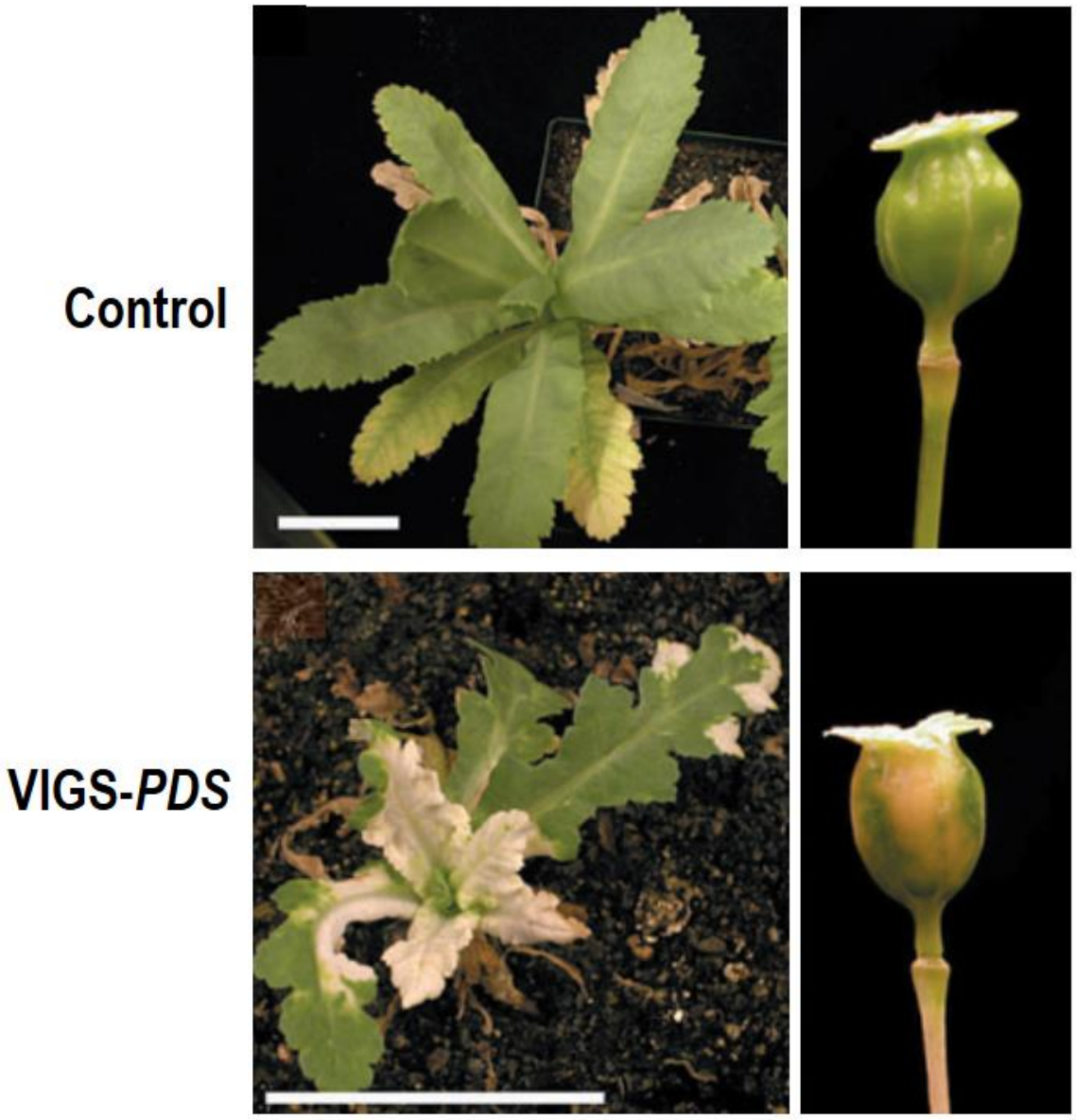

Figure 7. Virus induced silencing (VIGS) of the *PHYTOENE DESATURASE* (*PDS*) gene in *P. somniferum*. PDS is involved in carotenoid production, and silencing of the *PDS* gene using a PDS-specific VIGS construct leads to the loss of green pigmentation. Similar approaches have been used to reduce gene expression of alkaloid biosynthesis genes in stems and capsules. Scale bars: 3.5 cm (Adapted by permission of John Wiley and Sons from Hileman et al., 2005).

1117 **Table 1.** Sequence resources for Opium Poppy (*Papaver somniferum*)

Database	No. of Samples	Cultivar/Variety	Tissues/ Samples	Amount of sequence	Technology	Reference
Not publically available	n.r.	n.r.	Seedlings	1,709 clones	Sanger - ESTs	Ziegler et al., 2005
NCBI, Expressed Sequence Tag (EST)		Marianne (low alkaloid, seed variety)	Suspension cell culture	10,224 clones	Sanger - ESTs	Zulak et al., 2007; Hagel and Facchini, 2010
	1	Marianne	Cell cultures treated with a fungal elicitor	Total of ~182 Mb (427,369 ESTs with an average length of 426 bp)	Roche 454 GS-FLX	Desgagne-Penix et al., 2010
NCBI, Short Read Archive (SRA)	8	40 (high codeine) Marianne Przemko (low alkaloid, seed variety) Natasha (high noscapine) Deborah (high morphine) T (high thebaine, high oripavine) Roxanne (high papaverine) Veronica (high papaverine)	Stem	Total of ~1.35 Gb (3,155,265 ESTs with an average length of 426 bp)	Roche 454 GS-FLX	Desgagne-Penix et al., 2012
NCBI	12	High morphine 1 (HM1), High thebaine 1 (HT1), High noscapine 1 (HN1)	Upper stem (two stages of growth) Capsule (two stages of growth)		Roche 454 GS-FLX	Winzer et al., 2012
NCBI		High noscapine 1 (HN1)	Young seedlings	6 BAC sequences	Illumina HiSeq	Winzer et al., 2012
The 1,000 Plants Project	5	Munich	Stem Leaf Root Developing fruit (capsule) Flower-bud	50.3 million reads (10.4 Gb) 5.3M spots, 2.3 Gb 14.5M spots, 2.1 Gb 5.2M spots, 2.2 Gb 12.8M spots, 1.9 Gb 12.5M spots, 1.9 Gb	Illumina	https://www.bioinfodata.org/Blast4OneKP/search

Database	No. of Samples	Cultivar/Variety	Tissues/ Samples	Amount of sequence	Technology	Reference
NCBI GenBank	n.a.	Various	Various	859 sequences from 139 genes	Various	https://www.ncbi.nlm.nih.gov/gquery/?term=Papaver+somniferum
NCBI, WGS		High Noscapine 1 (HN1)	Young leaf	~685 Gb sequence data (about 239× coverage the estimated 2.87 Gb poppy genome)	Illumina, PacBio, Nanopore, 10× Genomics	Guo et al., 2018
NCBI, RNA-seq	7	High Noscapine 1 (HN1)	Leaf, Petal, Stamen, Capsule, Stem, Fine root, Tap root	~26 Gb paired-end (PE) reads for each tissue	HiSeq 3000	Guo et al., 2018
NCBI, BAC seq		High Noscapine 1 (HN1)		15 BAC sequences	Illumina HiSeq and MinION	Guo et al., 2018

NCBI, National Centre for Biotechnology Information (USA); n.r., not reported; n.a., not applicable

Table 2. Transient genetic modification approaches used to modify gene expression in Opium Poppy (*Papaver somniferum*)

Approach	Summary	Target Genes	Transformation method	References
Virus induced gene silencing (VIGS)	Reduction of gene expression in stem	*PDS*	*Agrobacterium* infiltration	Hileman et al., 2005
		T6ODM, CODM, DIOX2	*Agrobacterium* infiltration of young leaves	Hagel and Facchini, 2010
		6OMT, CNMT, NMCH, 4'OMT2, N7OMT, 7OMT	*Agrobacterium* infiltration of young leaves	Desgagne-Penix and Facchini, 2012
		SalSyn, SalR, T6ODM, CODM, SalAT, COR	*Agrobacterium* infiltration of young leaves	Wijekoon and Facchini, 2012
		PSMT1, PSMT2, CYP719A21, CYP82X2, PSXCE1, PSSDR1	*Agrobacterium* infiltration of young leaves	Winzer et al., 2012
		CODM, T6ODM	*Agrobacterium* infiltration of young leaves	Farrow and Facchini, 2013
		4'OMT, 7OMT	*Agrobacterium* infiltration of young leaves	Gurkok et al., 2016
		OMT2, OMT3	*Agrobacterium* infiltration of young leaves	Park et al., 2018
		STORR (REPI)	*Agrobacterium* infiltration	Winzer et al., 2015; Farrow et al., 2015
		T6ODM, CODM, BBE1	*Agrobacterium* infiltration of young leaves	Sohrabi et al., 2018
		CODM	*Agrobacterium* infiltration of young leaves	Dastmalchi et al., 2018
		THS	*Agrobacterium* infiltration of young leaves	Chen et al., 2018
		NISO	*Agrobacterium* infiltration of young leaves	Dastmalchi et al., 2019
CRISPR/Cas gene editing	Targeted gene mutagenesis in leaves	*4'OMT2*	*Agrobacterium* infiltration of leaves	Alagoz et al., 2016
Subcellular localisation	Cultured poppy cell line	*NCS, 6OMT, CNMT, NMCH, 4'OMT, BBE, CheSyn, StySyn, TNMT*	Microprojectile bombardment of DNA	Hagel and Facchini, 2012
		BBE	Microprojectile bombardment of DNA	Bird and Facchini, 2001

PDS, Phytoene Desaturase; T6ODM, Thebaine 6-O-demethylase; CODM, Codeine O-demethylase; 6OMT; Norcoclaurine 6-O-methyltransferase; CNMT, Coclaurine N-methyltransferase; NMCH, (S)-N-Methylcoclaurine 3'-hydroxylase; 4'OMT, 3'-Hydroxy-N-methylcoclaurine 4'-hydroxylase; N7OMT, Norreticuline 7-O-methyltransferase; 7OMT, Reticuline 7-O-methyltransferase; SalSyn, Salutaridine synthase; SalR, Salutaridine reductase; SalAT, Salutaridinol 7-O-acetyltransferase, COR; Codeinone reductase CYP, Cytochrome P450; BBE1, Berbrine Bridge Enzyme; STORR, (S)-to-(R)-reticuline; REPI, Reticuline epimerase; THS, Thebaine synthase; NISO, Neopinone isomerase.

Table 3. Stable genetic modification approaches used to modify gene expression in Opium Poppy (Papaver somniferum)

Technique	Approach	Summary	Target genes	Transformation method	References
Stable Transformation	Overexpression (OX)	Increase in gene expression	*SalAT*	*Agrobacterium* transformation of hypocotyl explants	Allen et al., 2008
			COR	*Agrobacterium* transformation of hypocotyl explants	Larkin et al., 2007
			CYP80B3 (NMCH)	*Agrobacterium* transformation of hypocotyl explants	Frick et al., 2007
			PAT	*Agrobacterium* transformation of root explants	Facchini et al., 2008
			4′OMT2	*Agrobacterium* transformation of hypocotyl explants	Shorabi et al., 2018
	RNA interference (hpRNAi or antisense RNA)	Reduction of gene expression	*SalAT* (hpRNAi and antisense RNA)	*Agrobacterium* transformation of hypocotyl explants	Allen et al., 2008 Kempe et al., 2009
			COR (hpRNAi)	*Agrobacterium* transformation of hypocotyl explants	Allen et al., 2004
			BBE (antisense RNA)	*Agrobacterium* transformation of hypocotyl explants	Frick et al., 2004
			CYP80B3 (NMCH) (antisense RNA)	*Agrobacterium* transformation of hypocotyl explants	Frick et al., 2007

SalAT, Salutaridinol 7-O-acetyltransferase; COR; Codeinone reductase; CYP, Cytochrome P450; NMCH, (S)-N-Methylcoclaurine 3′-hydroxylase; 4'OMT, 3′-Hydroxy-N-methylcoclaurine 4′-hydroxylase; BBE1, Berbrine Bridge Enzyme.