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Isolation and characterization of a cDNA encoding (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase from opium poppy, a key enzyme in sanguinarine biosynthesis

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ABSTRACT

Sanguinarine is a benzo[*c*]phenenthridine alkaloid with potent antimicrobial properties found commonly in plants of the Papaveraceae, including the roots of opium poppy (*Papaver somniferum*). Sanguinarine is formed from the central 1-benzylisoquinoline intermediate (*S*)-reticuline via the protoberberine alkaloid (*S*)-scoulerine, which undergoes five enzymatic oxidations and an *N*-methylation. The first four oxidations from (*S*)-scoulerine are catalyzed by cytochromes P450, whereas the final conversion involves a flavoprotein oxidase. All but one gene in the biosynthetic pathway from (*S*)-reticuline to sanguinarine has been identified. In this communication, we report the isolation and characterization of (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase (MSH) from opium poppy based on the transcriptional induction in elicitor-treated cell suspension cultures and root-specific expression of the corresponding gene. Along with protopine 6-hydroxylase, which catalyzes the subsequent and penultimate step in sanguinarine biosynthesis, MSH is a member of the CYP82N subfamily of cytochromes P450. The full-length MSH cDNA was expressed in *Saccharomyces cerevisiae* and the recombinant microsomal protein was tested for enzymatic activity using 25 benzylisoquinoline alkaloids representing a wide range of structural subgroups. The only enzymatic substrates were the *N*-methylated protoberberine alkaloids *N*-methylstylopine and *N*-methylcanadine, which were converted to protopine and allocryptopine, respectively.

1. Introduction

Plants produce a multitude of specialized metabolites that are not necessary for normal plant growth and development. Most of these molecules are assembled through complex enzymatic networks and facilitate the interaction between plants and their environment through, for example, the attraction of pollinators or the deterrence of herbivores [1–3]. Benzylisoquinoline alkaloids (BIAs) are a large and structurally diverse group of approximately 2500 nitrogenous compounds mostly distributed in four related plant families, including the Papaveraceae [4]. Many BIAs have potent pharmacological activities and include the narcotic analgesics morphine and codeine, the cough suppressant and potential anticancer drug noscapine [5], the antimicrobial agents sanguinarine and berberine, and the vasodilator papaverine.

BIA biosynthesis begins with the condensation of two tyrosine derivatives, dopamine and 4-hydroxyphenylacetaldehyde to yield (*S*)-norcoclaurine, which is then modified via 3′-hydroxylation, one *N*-methylation, and two *O*-methylations to (*S*)-reticuline (Supplementary Fig. S1). The 3′-hydroxylation of (*S*)-*N*-methylcoclaurine is reportedly catalyzed by a cytochrome P450 monooxygenase (*S*)-*N*-methylcoclaurine 3′-hydroxylase (NMCH) belonging to the CYP80B subfamily [6,7]. The central 1-benzylisoquinoline intermediate (*S*)-reticuline is converted to the protoberberine alkaloid (*S*)-scoulerine by the flavoprotein oxidase, berberine bridge enzyme (BBE), the corresponding gene for which has been isolated and characterized from California poppy (*Eschscholzia californica*) [8] and opium poppy (*Papaver somniferum*) [9] (Fig. 1). (*S*)-Scoulerine is an intermediate in the formation of several BIA structural subgroups including protoberberine (e.g. berberine), protopine (e.g. allocryptopine), benzo[*c*]phrenanthridine (e.g. sanguinarine) and phthalideisoquinoline (e.g. noscapine) alkaloids, whereas other structural subgroups such as morphinan (e.g. morphine) and aporphine (e.g. magnoflorine) are derived from different oxidative C–C couplings of (*S*)-reticuline [4]. In general, P450s catalyze C–C and C–O couplings, aromatic and aliphatic hydroxylations and ring rearrangements in plant specialized metabolism [10]. In humans and some plants, P450s are also involved in the *N*- and *O*-dealkylation of specialized metabolites [11].

Abbreviations: BIA, benzylisoquinoline alkaloid; BBE, berberine bridge enzyme; CFS, cheilanthifoline synthase; CID, collision-induced dissociation; DBOX, dihydrobenzophenanthridine oxidase; ESI, electrospray ionization; MSH, (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase; NMCH, (*S*)-3′-hydroxy-*N*-methylcoclaurine hydroxylase; P450, cytochrome P450 monooxygenase; P6H, protopine 6-hydroxylase; SPS, stylopine synthase; TNMT, tetrahydroprotoberberine *cis*-*N*-methyltransferase.

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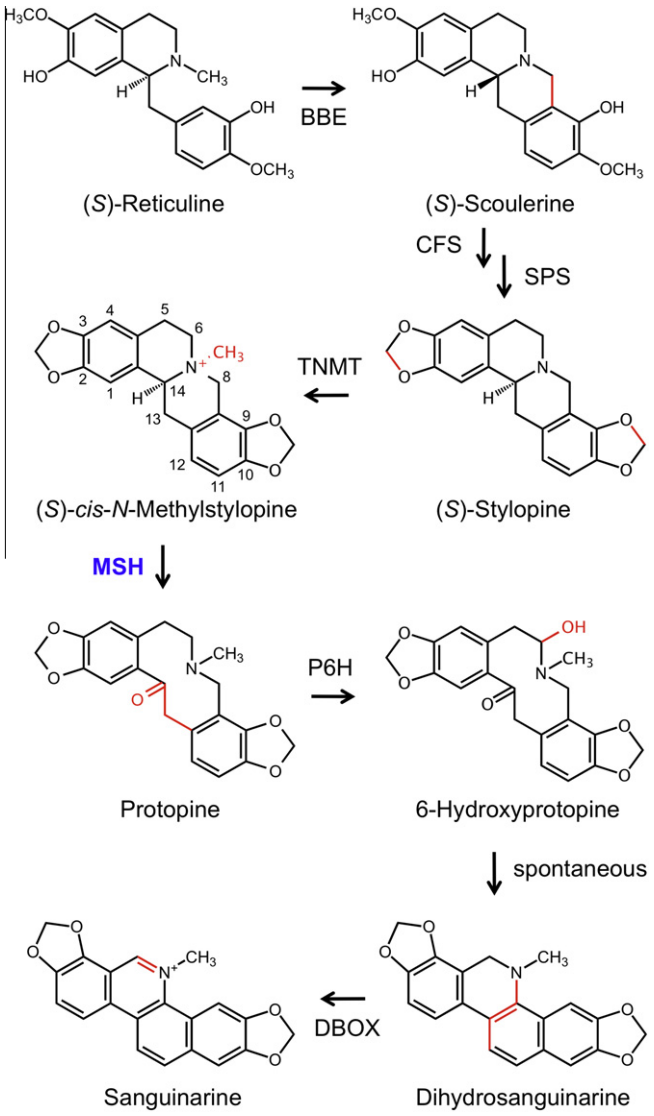

Fig. 1. Sanguinarine biosynthesis involves seven enzymatic conversions from (*S*)-reticuline beginning with oxidation by the berberine bridge enzyme (BBE) yielding (*S*)-scoulerine, which is further oxidized by two members of the CYP719A subfamily, cheilanthifoline synthase (CFS) and stylopine synthase (SPS), that introduce two methylenedioxy bridges. (*S*)-Stylopine is then *N*-methylated by tetrahydroprotoberberine *cis*-*N*-methyltransferase (TNMT), which is oxidized to protopine by (*S*)-*cis*-*N*-methyl-stylopine 14-hydroxylase (MSH). Hydroxylation of protopine by protopine 6-hydroxylase (P6H) produces an unstable intermediate that spontaneously rearranges to form dihydrosanguinarine. MSH and P6H belong to the CYP82 family. Oxidation of dihydrosanguinarine by dihydrobenzophenanthridine oxidase (DBOX) yields sanguinarine.

The conversion of (*S*)-scoulerine to sanguinarine begins with the formation of two methylenedioxy bridges by cheilanthifoline synthase (CFS) and stylopine synthase (SPS), both of which are members of the CYP719 family, forming (*S*)-stylopine (Fig. 1). CFS and SPS have been isolated and characterized from California poppy [12,13] and Mexican prickly poppy (*Argemone mexicana*) [14]. Subsequent *N*-methylation of (*S*)-stylopine by tetrahydroprotoberberine *cis*-*N*-methyltransferase (TNMT) [15] yields (*S*)-*cis*-*N*-methylstylopine, which is converted by (*S*)-*cis*-*N*-methylstylopine 14-hydroxylase (MSH) to protopine. MSH activity was isolated in microsomal fractions from various plant cell cultures and the enzyme was characterized as a P450 [16]. A second hydroxylation by protopine 6-hydroxylase (P6H) causes spontaneous ring rearrangement to yield the benzo[*c*]phenanthridine alkaloid dihydrosanguinarine, which is oxidized by the flavoprotein oxidase dihydrobenzophenanthridine oxidase (DBOX) to sanguinarine. Recently, genes encoding P6H from California poppy [17] and DBOX from opium poppy [18] have been isolated. Variations in the occurrence of methylenedioxy bridges or substituted dimethoxy moieties on protopine and benzo[*c*]phenanthridine alkaloids are common, but not all derivatives have been detected in opium poppy (Supplementary Fig. S1).

MSH remains the only enzyme involved in the conversion of (*S*)-norcoclaurine to sanguinarine for which the corresponding gene has not yet been identified. In this communication, we report the isolation and partial characterization from opium poppy of a cDNA encoding MSH, which belongs to the CYP82N subfamily. We show the coordinated induction of *MSH* mRNA levels with other sanguinarine biosynthetic gene transcripts in elicitor-treated opium poppy cell suspension cultures [19], and the root-specific expression of *MSH* in the plant. Heterologous expression of *MSH* in *Saccharomyces cerevisiae* and *in vitro* enzyme analysis showed that MSH accepted only the *N*-methylated protoberberines (*S*)-*cis*-*N*-methylstylopine and (*S*)-*cis*-*N*-methylcanadine yielding protopine and allocryptopine, respectively. Enzymatic activity was not detected using other BIAs, belonging to a wide variety of structural subgroups, as potential substrates.

2. Material and methods

2.1. Plant materials and chemicals

Opium poppy (*P. somniferum*) plants and cell suspension cultures were grown as described previously [9,20]. Plant materials for real-time quantitative PCR were harvested one day before anthesis (60–80 d after seed germination), frozen in liquid nitrogen and immediately extracted. The elicitor used to treat plant cell suspension cultures was prepared from the fungus *Botrytis cinerea* as described previously [9]. *N*-Methylstylopine and *N*-methylcanadine were produced from stylopine and canadine, respectively, using recombinant TNMT from *E. californica* [15] and partially purified by solid-phase extraction on Strata X-CW SPE columns (Phenomenex; Torrance, CA; http://www.phenomenex.com). Other chemicals were obtained as described previously [15,18,20–22].

2.2. Selection of MSH candidates

Cytochromes P450 candidate genes were selected based on: (1) transcriptional induction in response to elicitor treatment of opium poppy cell suspension cultures [19] and (2) root-specific expression in the plant. Full-length gene sequence data was obtained *in silico* from root and stem RNA-seq databases for the opium poppy chemotype Bea's Choice [18].

2.3. Real-time quantitative PCR analysis (RT-qPCR)

Total RNA from opium poppy root, stem, leaf and carpel was purified [23], and reverse transcription and quantitative PCR were performed [18] as described previously with primers specific for *PsMSH* (forward: 5′-TCATCCAAGCGATCATCAAAGA-3′; reverse: 5′-GGCTACTTCGCAGTCCTCCAT-3′) and ubiquitin as the endogenous control.

2.4. Phylogenic analysis

Phylogenic analysis and amino acid alignment were performed with ClustalW [24] and visualized using Geneious (Biomatters; Newark, NJ; http://www.geneious.com). Abbreviations and Genbank accession numbers: *P. somniferum* PsMSH (CYP82N4; *N*-methylstylopine 14-hydroxylase), KC154003; *P. somniferum* PsP6H

(CYP82N3; protopine 6-hydroxylase), KC154002; *P. somniferum* PsCFS (CYP719A25; cheilanthifoline synthase), GU325749; *P. somniferum* PsSPS (CYP719A20; stylopine synthase), GU325750; *P. somniferum* PsNMCH (CYP80B3; 3′-hydroxy-*N*-methylcoclaurine hydroxylase), AF191772; *E. californica* EcCYP719A5 (cheilanthifoline synthase), AB434654; *E. californica* EcCYP719A3 (stylopine synthase), AB126256; *E. californica* EcCYP719A2 (stylopine synthase), AB126257; *E. californica* EcCYP82N2v2 (protopine 6-hydroxylase), AB598834; *E. californica* EcCYP82B1, AF014802; *E. californica* EcCYP80B1 (3′-hydroxy-*N*-methylcoclaurine hydroxylase), AF014801; *P. somniferum* PsCYP719B1 (salutaridine synthase), EF451150; *Coptis japonica* CjCYP719A1 (canadine synthase), AB026122; *Mentha x piperita* MmCYP71A32 (menthofuran synthase), AF346833; *Ammi majus* AmCYP71AJ1 (psoralen synthase), AY532370; *Catharanthus roseus* CrCYP72A1 (secologanin synthase), L10081; *Helianthus tuberosus* HtCYP76B1 (7-ethoxycoumarin *O*-deethylase), Y09920; *Arabidopsis thaliana* AtCYP79B2 (camalexin biosynthesis), NM_120158; *Berberis stolonifera* BsCYP80A1 (berbamunine synthase), U09610; *Hyoscyamus niger* HnCYP80F1 (littorine mutase), DQ387048; *C. japonica CjCYP80G2* (corytuberine synthase), AB288053; *Sesamum indicum* SiCYP81Q1 (methylenedioxy bridge formation), AB194714; *A. thaliana* AtCYP82C2, O49394; *Nicotiana tabacum* NtCYP82E4v1 (nicotine *N*-demethylase), DQ131886; *A. thaliana* AtCYP82G1, NM113423; *Zea mays* ZmCYP92A1, AY072297; *Glycyrrhiza echinata* GeCYP93C2 (2-hydroxyisoflavanone synthase), AB023636; *Lithospermum erythrorhizon* LeCYP98A6 (4-coumaroyl-4′-hydroxyphenyllactic acid 3-hydroxylase), AB017418; *Cucurbita maxima* CmCYP701A1 (*ent*-kaurine oxidase), AF212990; *A. thaliana* AtCYP705A5 (flavonoid 3′-monooxygenase), NM_124173; *A. thaliana* AtCYP71A13 (camalexin biosynthesis), NM_128630; *C. roseus* CrCYP71BJ1 (tabersonine/lochnericine 19-hydroxylase), HQ901597; *P. somniferum* PsCYP82X1, JQ659002; *P. somniferum* PsCYP82X2, JQ659004; *P. somniferum* PsCYP82Y1, JQ659005.

2.5. S. cerevisiae expression vectors

A synthetic *PsCPR* gene [25] codon-optimized for expression in *S. cerevisiae* (DNA 2.0, Menlo Park, CA; www.dna20.com) was amplified (forward primer: 5′-GGATCCAACAATGGGGTCAAACAACC-3′; reverse primer: 5′-CTCGAGCCATACATCTCTCAAGTATC-3′), and the amplicon was inserted in-frame with the c-Myc tag in pESC-leu2d using *Bam*HI and *Sal*I restriction sites introduced by PCR, yielding pESC-leu2d::PsCPR. *PsMSH* was amplified (forward primer: 5′-AAAAATGCGAACCGAATCAATC-3′) and reverse primer: 5′-TCTAGAAATATCTCGAGTCGAGGTTTGA-3′) from cDNA synthesized using total RNA isolated from elicitor-induced opium poppy cell suspension cultures, and the amplicon inserted into pESC-leu2d::PsCPR in-frame with the FLAG tag using *Not*I and *Spe*I, yielding the dual expression plasmid pESC-leu2d::*PsMSH/CPR*.

2.6. Heterologous expression in S. cerevisiae

The pESC-leu2d::*PsMSH/CPR* vector was introduced into *S. cerevisiae* strain YPH499 (Agilent Technologies; Santa Clara, CA; http://www.agilent.com) and heterologous gene expression was performed as described previously [26] except that the induction culture also contained 1% (w/v) raffinose. The microsomal fraction was prepared by glass bead disruption [27].

2.7. Enzyme assays

In vitro enzyme assays were performed using native and boiled microsomal fractions in 50 mM HEPES, pH 7.5, 2.5 mM NADPH, 100 μM alkaloid substrate except for *N*-methylstylopine and *N*-methylcanadine, which were used at a concentration of 10 μM and 20 μg of microsomal protein in a total volume of 50 μL. Assays were incubated at 30 °C for 90 min and were stopped by adding 100 μL of MeOH. The precipitate was discarded and the supernatant evaporated to dryness in a Speed-Vac concentrator (Savant; Ramsey, MN; http://www.thermo.com) and resuspended in 50 μL of buffer A (95:5, 10 mM ammonium acetate, pH 5.5:acetonitrile). All enzyme assays were performed in triplicate.

2.8. Liquid chromatography-tandem mass spectrometry

Analysis of enzyme assays was performed using a 1200 liquid chromatograph and a 6410 triple quadropole mass spectrometer (Agilent Technologies). Ten microliters of the reaction mixtures were injected onto a Poroshell 120 SB C18 column (2.1 mm × 50 mm, 2.7 μm particle size, Agilent Technologies) and eluted at a flow rate of 0.7 mL/min. Liquid chromatography was initiated at 100% solvent A, ramped to 60% solvent B (acetonitrile) using a linear gradient over 6 min, further ramped to 99% solvent B using a linear gradient over 1 min, held constant at 99% solvent B for 1 min and returned to original conditions over 0.1 min for a 3.9 min equilibration period. Eluate was applied to the mass analyzer using an electrospray ionization probe operating in positive mode with the following conditions: capillary voltage, 4000 V; fragmentor voltage, 100 V; source temperature, 350 °C; nebulizer pressure, 50 PSI; gas flow, 10 L/min. For full-scan analysis, quadrupole 1 and 2 were set to RF only, whereas the third quadrupole scanned from 200–700 *m/z*. For collision-induced dissociation (CID) analysis, the precursor *m/z* was selected in quadrupole 1 and collision energy of 25 eV was applied in quadrupole 2. The resulting fragments were resolved by quadrupole 3 scanning from 40 *m/z* to 2 *m/z* greater than the precursor *m/z* and compared with previously published spectra [28].

3. Results

3.1. Identification of a cDNA candidate encoding MSH from opium poppy

The search for candidate cDNAs encoding MSH was performed using uncharacterized sequences annotated as P450s from a previous microarray analysis that demonstrated the coordinated induction of all known sanguinarine biosynthetic genes [19] and corresponding proteins [29] in elicitor-treated opium poppy cell suspension cultures. Genes showing induced transcript levels that correlated with the accumulation of sanguinarine included the *PsMSH* candidate (Genbank accession number KC154003), in addition to *PsNMCH, PsCFS*, *PsSPS* and *PsP6H (*KC154003). The predicted 526 amino acid sequence of opium poppy MSH contained conserved motifs found in many P450s (Supplementary Fig. S2). Phylogenic analysis comparing P450s involved in BIA metabolism and other plant specialized metabolic pathways shows that opium poppy MSH belongs to the CYP82 family (Fig. 2). Interestingly, opium poppy MSH shows strong similarity to recently reported P450s (CYP82X1, CYP82X2 and CYP82Y1) putatively involved in the biosynthesis of noscapine in opium poppy [30] and Californica poppy P6H (CYP82N2v2) [17] (Fig. 2). MSH shares 49%, 48%, 53% and 54% amino acid sequence identity to CYP82X1, CYP82X2, CYP82Y1 and P6H, respectively.

3.2. Expression of PsMSH and PsCPR in S. cerevisiae

Heterologous co-expression of *PsMSH* and *PsCPR* was confirmed by immunoblot analysis of microsomal fractions prepared from *S. cerevisiae* transformed with the pESC-leu2d::*PsMSH/CPR* vector using c-Myc and FLAG antibodies to detect tagged MSH and CPR

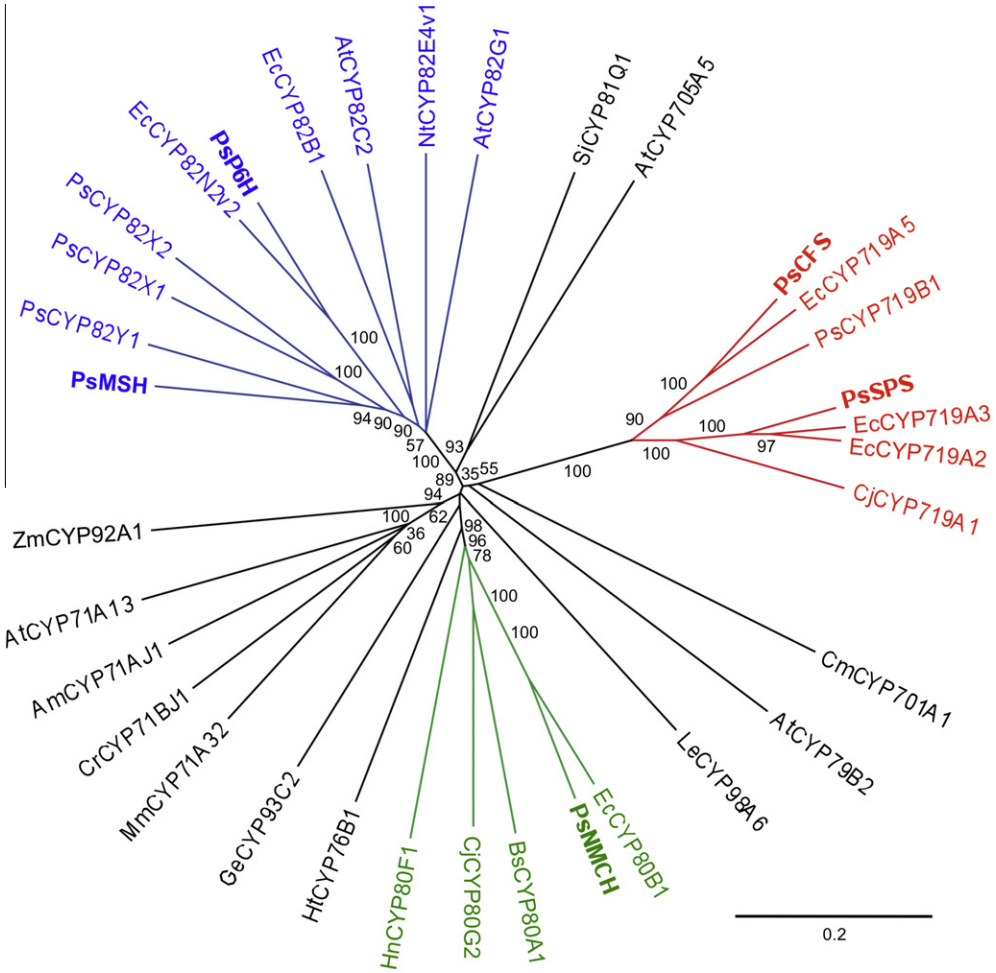

Fig. 2. Unrooted neighbor-jointing phylogenetic tree of selected cytochromes P450 involved in plant specialized metabolism. PsMSH forms a distinct clade with other enzymes of the CYP82 family (blue) and displays the most similarity with clade members involved in BIA metabolism. The CYP82 clade is distinct from the other P450 families, CYP80 (green) and CYP719 (red), involved in BIA metabolism. Genbank accession numbers are listed in the Materials and methods. The branch length is proportional to the estimated divergence distance of each protein. The scale bar (0.2) corresponds to a 20% change. The percentage of replicate trees in which associated taxa clustered together in the bootstrap test is shown next to each branch. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

proteins tagged with c-Myc and FLAG, respectively (Fig. 3A). MSH tagged with c-Myc was not detected in microsomal fractions prepared from *S. cerevisiae* transformed with the pESC-leu2d::*PsCPR*, which lacked the *PsMSH* expression cassette.

3.3. Determination of substrate specificity and range of PsMSH

Microsomal fractions containing opium poppy MSH accepted both *N*-methylstylopine and *N*-methylcanadine as substrates, forming protopine and allocryptopine, respectively (Fig. 3B and C). The identity of substrates and products was confirmed by collision-induced dissociation mass spectrometry (Supplementary Fig. S3). Turnover efficiencies using the described reaction and analysis parameters were 9.8% and 6.5% for the conversion of *N*-methylcanadine to allocryptopine and *N*-methylstylopine to protopine, respectively. Boiled enzymes yielded no detectable products. Twenty-three other compounds representing several BIA structural subgroups were also tested as potential substrates for opium poppy MSH. However, no reaction products were detected by full-scan mass spectrometry analysis. Moreover, no peaks were detected corresponding to compounds with a *m/z* of −2 or +16 compared with the *m/z* of the potential substrate, ruling out the occurrence of either C–O or C–C bond formation, or hydroxylation reactions.

3.4. Expression analysis of PsMSH in opium poppy

Since sanguinarine accumulates only in opium poppy roots, RT-qPCR was performed to determine the relative transcript abundance of P450 candidates in various plant organs. Sanguinarine biosynthetic gene transcript levels should be relatively high in roots, but were expected to be absent in other plant organs. *PsMSH* transcript levels were more than 100-fold greater in roots than in other organs (Fig 4A). The RT-qPCR results were in agreement with RNA-seq data for *PsMSH*, with FPKM values of 141 and 1, respectively, in opium poppy roots and stems [18]. *PsMSH* transcript abundance was induced in opium poppy cell suspension cultures within 5 h after addition of the elicitor, reached maximum levels in 10 h and gradually returned to near-baseline levels after approximately 100 h (Fig. 4B).

4. Discussion

The formation of 14-hydroxylated *N*-methylated protoberberine alkaloids is a crucial step in the formation of the protopine and subsequently benzo[*c*]phenanthridine backbone structures, which add to the impressive structural diversity of BIA derivatives in plants. In this study, we identified the enzyme in opium poppy

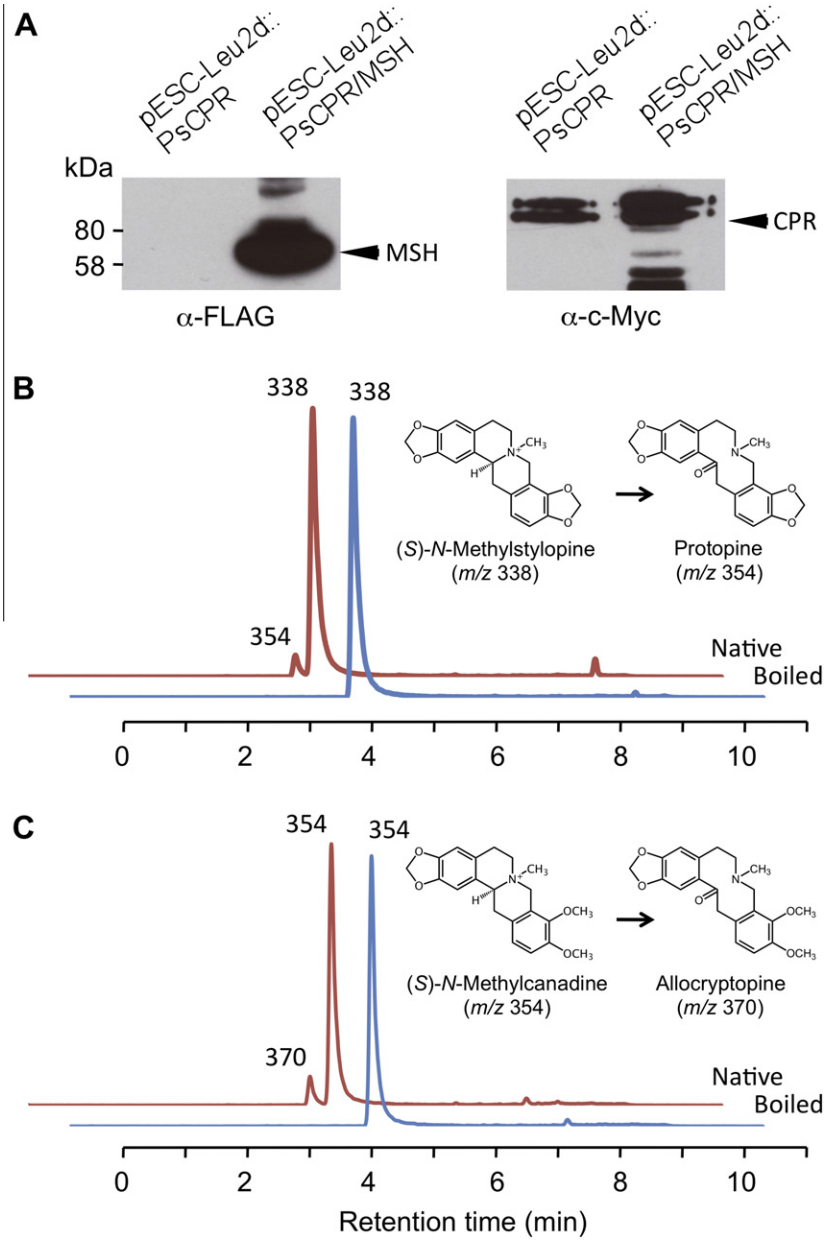

Fig. 3. Microsomes from *S. cerevisiae* expressing *PsMSH* hydroxylate *N*-methylated protoberberine alkaloids. (A) Immunoblot of microsomal protein (3 μg) from *S. cerevisiae* expressing *PsMSH* and *PsCPR*, or *PsCPR* alone, showing accumulation of the expected protein products. (B and C) Extracted ion chromatograms of microsomes expressing *PsMSH* and *PsCPR* (red) or expressing *PsCPR* alone (blue). (B) Assays incubated with *N*-methylstylopine (*m/z* 338) produced a product at *m/z* 354 corresponding to protopine. (C) Assays incubated with *N*-methylcanadine (*m/z* 354) showed a product peak at *m/z* 370 corresponding to allocryptopine. Collision-induced dissociation analysis to confirm the identity of substrates and products are provided in Supplementary Fig. S3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

responsible for the conversions of (*S*)-*N*-methylstylopine and (*S*)-*N*-methylcanadine to protopine and allocryptopine, respectively, as a member of the CYP82N subfamily. Other members of the CYP82 family include California poppy P6H (CYP82N2v2) [17], Arabidopsis (*A. thaliana*) methoxypsoralen hydroxylase (CYP82C2) [31] and homoterpene synthase (CYP82G1) [32] and tobacco (*N. tabacum*) nicotine *N*-demethylase (CYP82E4v1) [33]. As expected from the enzymatic characterization of MSH from *Corydalis vaginans* cell culture [16], opium poppy MSH displayed all essential P450 domains and amino acids (Supplementary Fig. S2) as described for California poppy P6H [17]. Although the broad substrate range of *C. vaginans* MSH for different BIAs was not tested, the relative activity of the enzyme for *N*-methylated protoberberines was similar to opium poppy MSH, with *N*-methylcanadine preferred over *N*-methylstylopine. The limited substrate range of MSH is a common feature of several P450s involved in BIA metabolism including California poppy P6H, in which only protopine alkaloids were accepted [17], and several CYP719A variants that functioned only on protoberberine alkaloids [12,14,34].

Phylogenetic analysis showed that opium poppy MSH forms a distinct clade together with other reported members of the CYP82 family involved in BIA metabolism (Fig. 2). Similarity to functionally uncharacterized P450s (CYP82X1, CYP82X2, CYP82Y1) purported to participate in noscapine biosynthesis [30] is intriguing since noscapine-producing opium poppy chemotypes also accumulate substantially higher levels of cryptopine and protopine in latex compared with noscapine-free varieties, despite the unlikelihood that protopine alkaloids are involved in phthalideisoquinoline

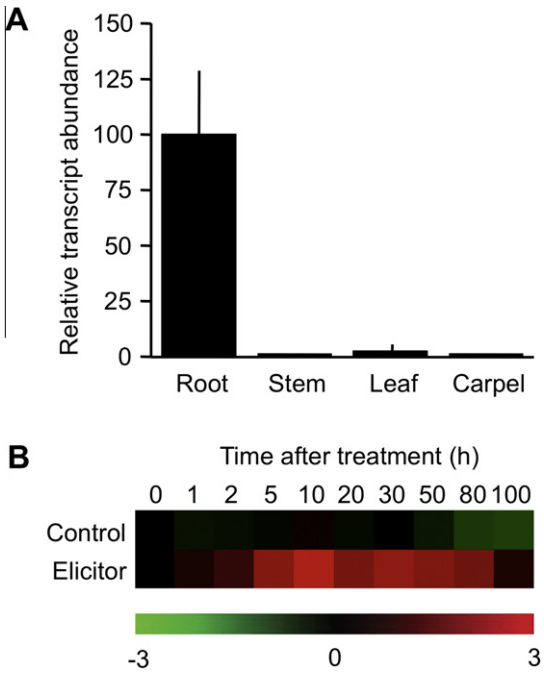

Fig. 4. Relative transcript abundance of *PsMSH* in opium poppy plant organs and elicitor-treated cell suspension cultures. (A) Real-time quantitative PCR analysis was performed on cDNA synthesized using 300 ng of RNA isolated from plant tissues and normalized to ubiquitin as the internal control. (B) Microarray analysis of cell suspension cultures showing the induction of *PsMSH* over a period of 100 h after elicitor treatment compared with control cultures treated with sterile water.

alkaloid biosynthesis [35]. Latex is the cytoplasm of laticifers, which are specialized cells found in all plant organs, that occurs most abundantly in aerial organs including stems, leaves and seed capsules. Interestingly, *PsMSH* is not expressed at appreciable levels in the stems of noscapine-producing chemotypes suggesting that one or more of CYP82X1, CYP82X2 or CYP82Y1 might be involved in protopine metabolism in aerial organs. In contrast, MSH appears solely responsible for protopine and benzo[*c*]phenanthridine alkaloid biosynthesis in roots and elicitor-treated cell suspension cultures.

Although (*S*)-*N*-methylcanadine was the preferred substrate for opium poppy MSH compared with (*S*)-*N*-methylstylopine, its reaction product allocryptopine is far less abundant than cryptopine or protopine in opium poppy latex [36] or roots [18]. Interestingly, relatively low levels of allocryptopine are found in both noscapine-free and noscapine-producing chemotypes despite the role of *N*-methylcanadine in phthalideisoquinoline alkaloid biosynthesis [30], suggesting spatial separation of protopine and phthalideisoquinoline alkaloid metabolism. The possibility of organ-specific biosynthetic pathways is reinforced by the occurrence of two CYP719A variants, SPS and canadine synthase (CAS), the latter of which is clustered in the opium poppy genome with other putative noscapine biosynthetic genes [30]. As reported for homologous enzymes from California poppy SPS [12,14,34] and Japanese goldthread (*C. japonica*) CAS [12,14,34], opium poppy SPS and CAS likely have a common substrate range.

The isolation of a cDNA encoding MSH from opium poppy completes the isolation of genes encoding all predicted biosynthetic enzymes involved in the conversion of dopamine and 4-hydroxyphenylacetaldehyde to sanguinarine. All 12 biosynthetic genes are coordinately induced in elicitor-treated opium poppy cell suspension cultures (Fig. 4B) [19]. In addition to providing an essential tool to further investigate the regulation of sanguinarine biosynthesis, the availability of genes encoding all biosynthetic enzymes from (*S*)-norcoclaurine synthase (NCS) through DBOX (Supplementary Fig. S1) creates the opportunity to complete the reconstitution of the pathway in heterologous systems [36] as a potential alternative commercial source for plant-derived complex metabolites.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.12.129.

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