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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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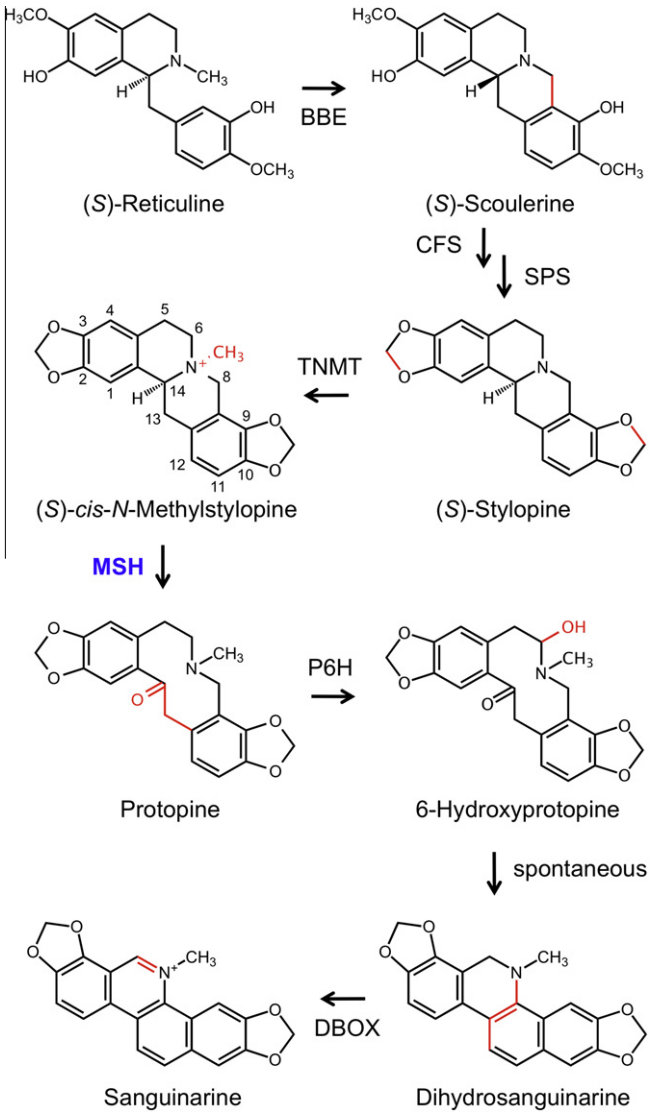

Fig. 1. Sanguinarine biosynthesis involves seven enzymatic conversions from (*S*)-reticuline beginning with oxidation by the berberine bridge enzyme (BBE) yielding (*S*)-scoulerine, which is further oxidized by two members of the CYP719A subfamily, cheilanthifoline synthase (CFS) and stylopine synthase (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