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Molecular cloning and characterization of a cytochrome P450 in sanguinarine biosynthesis from *Eschscholzia californica* cells ☆,☆☆

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ABSTRACT

Benzophenanthridine alkaloids, such as sanguinarine, are produced from reticuline, a common intermediate in benzylisoquinoline alkaloid biosynthesis, via protopine. Four cytochrome P450s are involved in the biosynthesis of sanguinarine from reticuline; i.e. cheilanthifoline synthase (CYP719A5; EC 1.14.21.2.), stylopine synthase (CYP719A2/A3; EC 1.14.21.1.), *N*-methylstylopine hydroxylase (MSH) and protopine 6-hydroxylase (P6H; EC 1.14.13.55.). In this study, a cDNA of P6H was isolated from cultured *Eschscholzia californica* cells, based on an integrated analysis of metabolites and transcript expression profiles of transgenic cells with *Coptis japonica* scoulerine-9-*O*-methyltransferase. Using the full-length candidate cDNA for P6H (CYP82N2v2), recombinant protein was produced in *Saccharomyces cerevisiae* for characterization. The microsomal fraction containing recombinant CYP82N2v2 showed typical reduced CO-difference spectra of P450, and production of dihydrosanguinarine and dihydrochelerythrine from protopine and allocryptopine, respectively. Further characterization of the substrate-specificity of CYP82N2v2 indicated that 6-hydroxylation played a role in the reaction.

1. Introduction

Isoquinoline alkaloids are a large group of alkaloids that include many pharmacologically useful compounds, e.g., the analgesic morphine, the antitussive codeine, and the antimicrobial agents berberine and sanguinarine. These various types of isoquinoline alkaloids (morphinans, protoberberines, and benzophenanthridines) are biosynthesized from a central precursor, (*S*)-reticuline (**1**) (Fig. 1) (Preininger, 1986; Kutchan, 1998; Sato et al., 2007; Ziegler and Facchini, 2008). Although the molecular process of this chemical diversity has not yet been clarified, recent studies have shown that addition of a branch pathway and relatively broad substrate-specificity of endogenous enzymes may be involved in the metabolic diversification (Takemura et al., 2010). In this, the most critical step would be oxidative steps catalyzed by cytochrome P450 (P450). P450s have been shown to play essential roles in plant secondary metabolism (Chapple, 1998; Werck-Reichhart et al., 2002; Mizutani and Sato, 2010).

California poppy (*Eschscholzia californica*), a Papaveraceae plant, is a traditional medicinal plant of Native Americans and has been intensively investigated because of the variety and pharmacological effects of its alkaloids. While *E. californica* is known to produce aporphine-, pavine-, protoberberine-, protopine-, and benzophenanthridine-type alkaloids (Kutchan, 1998; Fabre et al., 2000), the biosyntheses of benzophenanthridine-type alkaloids has been most intensively studied at the enzyme level (Zenk, 1994; Kutchan, 1998). Biosynthesis of the major metabolite, sanguinarine (**8**), requires seven reaction steps from (*S*)-reticuline (**1**), including the four P450 reaction steps of two methylenedioxy bridge-forming (cheilanthifoline synthase (CYP719A5) [EC 1.14.21.2.] and stylopine synthase (CYP719A2/A3) [EC 1.14.21.1.]), *N*-methylstylopine hydroxylase (MSH), and protopine 6-hydroxylase (P6H; EC 1.14.13.55.), respectively (Fig. 1).

In benzophenanthridine alkaloid biosynthesis, protopine 6-hydroxylase (P6H) converts protopine (**5**) to dihydrosanguinarine (**7**) (Tanahashi and Zenk, 1990). Tanahashi and Zenk (1990) characterized P6H using [6-^{3}H] protopine (**5**) and microsomal fractions of elicitor-treated cultured *E. californica* cells, and measured the re-

Abbreviations: *Cj*SMT, *Coptis japonica* (*S*)-scoulerine-9-*O*-methyltransferase; CYP719A2/A3, stylopine synthase; CYP719A5, cheilanthifoline synthase; EST, expressed sequence tag; LC–MS, liquid chromatography–mass spectrometry; P6H, protopine 6-hydroxylase; P450, cytochrome P450; (*S*)-THB, (*S*)-tetrahydroberberine; (*S*)-THC, (*S*)-tetrahydrocolumbamine.

☆ This manuscript is dedicated to the late Prof. Meinhart Zenk for his great contributions on isoquinoline alkaloid biosynthesis studies.

☆☆ Nucleotide sequence of full-length cDNA of *Ec*CYP82N2v2 (AB598834) was deposited in the DDBJ/Genbank/EMBL database.

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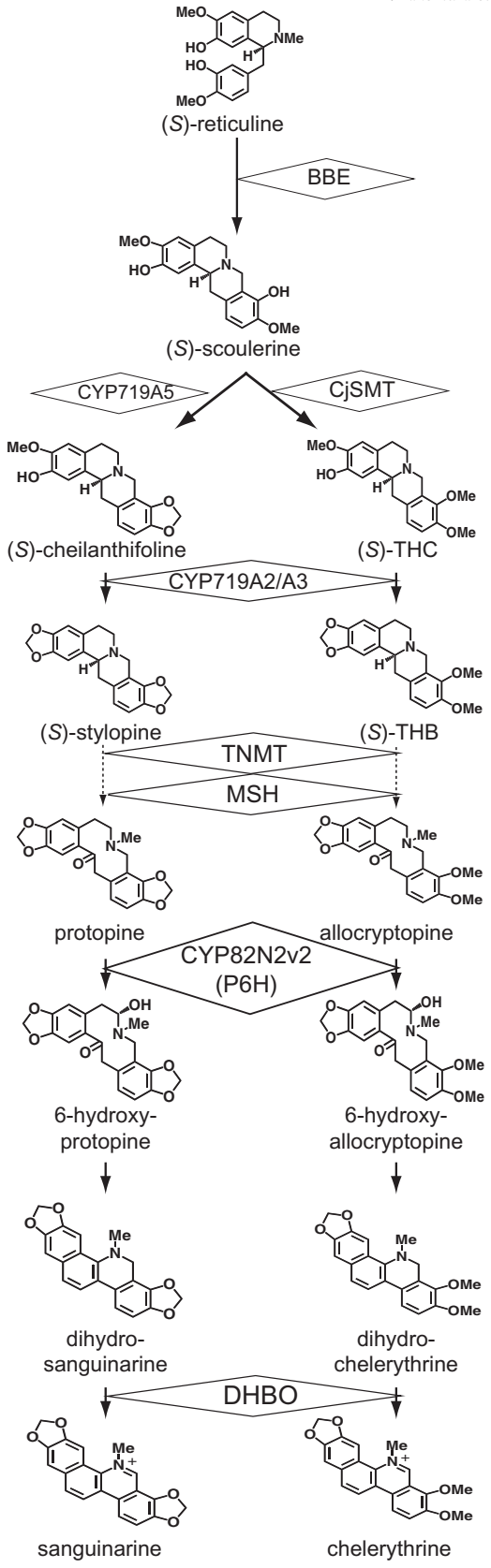

Fig. 1. Biosynthetic pathways for isoquinoline alkaloids. Both native and metabolically modified pathways in transgenic *E. californica* cells with *Cj*SMT are shown. Down-regulation of P6H (CYP-A/CYP82N2v2) induced the accumulation of allocryptopine in transgenic *E. californica* cells (Takemura et al., 2010). BBE: berberine bridge enzyme, CYP719A5: cheilanthifoline synthase, SMT: scoulerine-9-*O*-methyltransferase, CYP719A2 and CYP719A3: stylopine synthase, TNMT: tetrahydroprotoberberine-*N*-methyl transferase, MSH: methylstylopine hydroxylase, P6H: protopine-6-hydroxylase, DHBO; dihydro-benzophenanthriddine oxidase.

lease of ^{3}H from the substrate through formation of dihydrosanguinarine (**7**). It was estimated therein that the 6-hydroxylation of protopine (**5**) and further non-enzymatic intramolecular rearrangement produced dihydrosanguinarine (**7**) from 6-hydroxyprotopine (**6**), since no stable intermediate was detected.

In this report, the isolation of cDNA of a P450 is described, which converts protopine (**5**) to dihydrosanguinarine (**7**), from cultured *E. californica* cells based on an integrated analysis of metabolites and transcript expression profiles (Takemura et al., 2010). The candidate P450 (CYP82N2v2) was heterologously expressed in *Saccharomyces cerevisiae* and a recombinant microsomal protein was used for the P6H assay. Further characterization of the substrate-specificity supports a unique reaction characteristic of CYP82N2v2. The potential role of the broad substrate-specificity of CYP82N2v2 is discussed from the perspective of metabolic diversification.

2. Results

2.1. Isolation of P6H cDNA candidate

In a previous study (Takemura et al., 2010), the relationship between the accumulation of metabolite and biosynthetic gene expression in transgenic *E. californica* cells was reported with ectopic expression of scoulerine-9-*O*-methyltransferase of *Coptis japonica*. Enhanced variation of gene expression of biosynthetic enzymes provided a considerable variation of metabolites, and a good correlation was found between the gene expression of biosynthetic enzymes and metabolites (Takemura et al., 2010). An EST clone (*Ec*CYP-A in Takemura et al., 2010) identified from four independent putative P450 sequences from an EST library of California poppy (http://pgn.cornell.edu/index.pl) showed a good negative correlation with the accumulation of allocryptopine (**11**), which is a potential substrate for protopine 6-hydroxylase in transgenic *E. californica* cells with the overexpression of *Cj*SMT (Fig. 1). This means that *Ec*CYP-A might be protopine 6-hydroxylase, the inhibition of which induces the accumulation of protopine (**5**) in biosynthesis. Since *Ec*CYP-A only contained the N-terminal sequence of P450, a full-length cDNA was isolated from transgenic cultured California poppy cells using 3′ RACE.

Isolated cDNA clone of *Ec*CYP-A contained 1572 nucleotides with an open reading frame of 514 amino acids (DDBJ/GenBank™/EMBL Accession No. AB598834). The predicted amino acid sequence had conserved eukaryotic P450 domains: a helix K region, an aromatic region, and a heme-binding region. The P450 nomenclature committee (http://drnelson.uthsc.edu/CytochromeP450.html) named it CYP82N2v2, since CYP82N2v1 with only eight amino acid sequence difference had been registered in P450 nomenclature database. CYP82N2v2 also had a hydrophobic endoplasmic reticulum sorting signal at the N-terminal region.

CYP82N2v2 was compared with other P450s, especially those in isoquinoline alkaloid biosynthesis (*Bs*CYP80A1, *Ec*CYP80B1, *Cj*CYP80G2, *Ec*CYP719A2/A3, *Ec*CYP719A9, and *Ps*CYP719B1) (Kraus and Kutchan, 1995; Pauli and Kutchan, 1998; Ikezawa et al., 2007, 2008, 2009; Gesell et al., 2009). CYP82N2v2 was distant from the CYP719 and CYP80 families on the phylogenetic tree. CYP82N2v2 had the highest sequence similarity to *At*CYP82C2 (45% identity), followed by *At*CYP82G1 (42% identity) and *Nt*CYP82E4v1 (39% identity) among the functionally-characterized P450s (Fig. 2). Among P450s that are known to play a role in isoquinoline alkaloid biosynthesis, CYP82N2v2 showed the highest sequence similarity (43% identity) to CYP80B1 (*N*-methylcoclaurine-3′-hydroxylase). CYP82N2v2 also had highly conserved Gly/Ala residues in the helix I region, whereas the CYP719 family, unique to isoquinoline biosynthesis, has less-conserved residues in this region (Mizutani and Sato, 2010; Fig. 3).

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