Phytochemistry xxx (2012) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

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

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ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Eschscholzia californica
Papaveraceae
Benzylisoquinoline alkaloids
Cytochrome P450
Protopine 6-hydroxylase
Sanguinarine biosynthesis

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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doi:10.1016/j.phytochem.2012.02.013

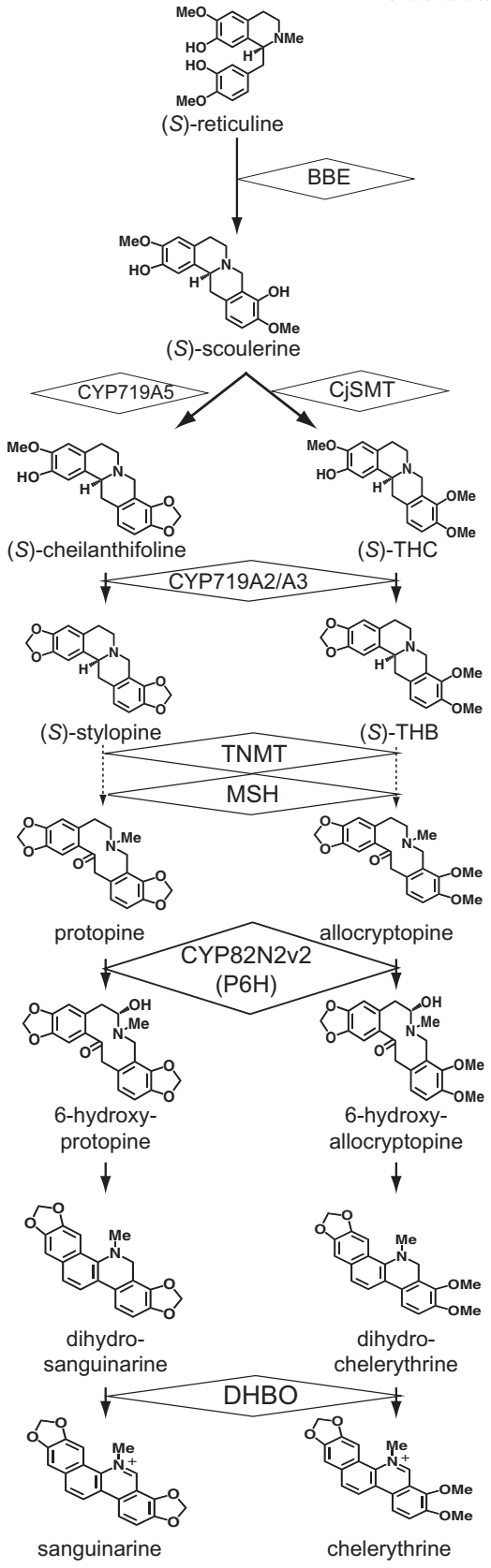

Fig. 1. Biosynthetic pathways for isoquinoline alkaloids. Both native and metabolically modified pathways in transgenic *E. californica* cells with *Cj*SMT are shown. Down-regulation of P6H (CYP-A/CYP82N2v2) induced the accumulation of allocryptopine in transgenic *E. californica* cells (Takemura et 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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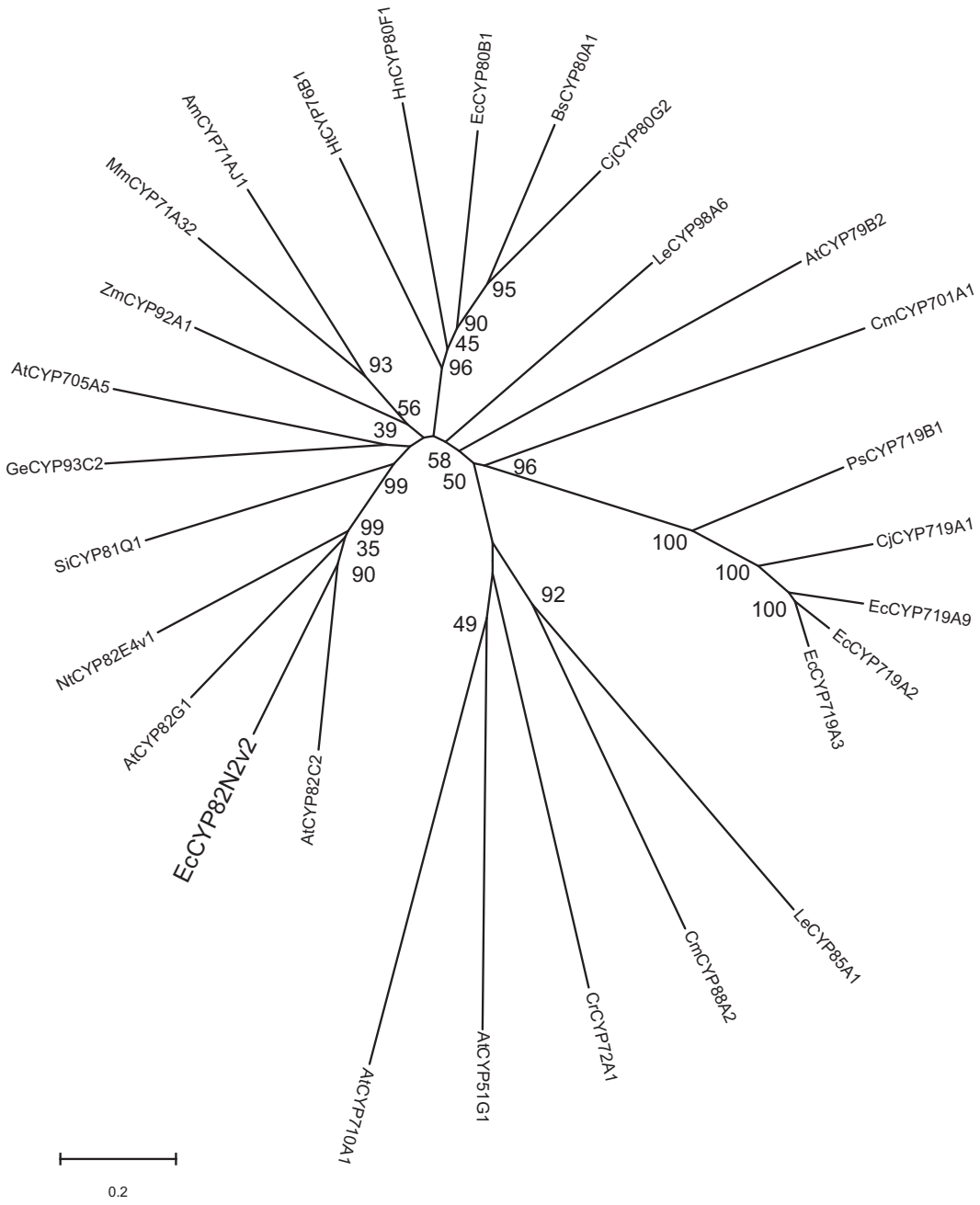

Fig. 2. Phylogenetic tree of plant cytochrome P450 families. Plant cytochrome P450 amino acid sequences obtained from GenBank or SwissProt were used for tree building. Accession Nos. are: NM101040, *Arabidopsis thaliana At*CYP51G1 (sterol 14-demethylase); AF346833, *Mentha x piperita Mm*CYP71A32 (menthofuran synthase); AY532370, *Ammi majus Am*CYP71AJ1 (psoralen synthase); L10081, *Catharanthus roseus Cr*CYP72A1 (secologanin synthase); Y09920, *Helianthus tuberosus Ht*CYP76B1 (7-ethoxycoumarin *O*-deethylase); AF069495, *Arabidopsis thaliana At*CYP79B2 (conversion of amino acid to aldoxime); U09610, *Berberis stolonifera Bs*CYP80A1 (berbamunine synthase); DQ387048, *Hyoscyamus niger Hn*CYP80F1 (littorine mutase/monooxygenase); AB288053, *Coptis japonica Cj*CYP80G2 (corytuberine synthase); AB194714, *Sesamum indicum Si*CYP81Q1 (two methylenedioxy bridge formation); O49394, *Arabidopsis thaliana At*CYP82C2 (monooxygenase); DQ131886, *Nicotiana tabacum Nt*CYP82E4v1 (nicotine *N*-demethylase); NM113423 *Arabidopsis thaliana At*CYP82G1 (DMNT/TMTT homoterpene synthase); DQ374443, *Lycopersicon esculentum Le*CYP85A1 (6-deoxocastasterone oxidase); AF212991, *Cucurbita maxima Cm*CYP88A2 (ent-kaurenoic acid oxidase); AY072297 *Zea mays Zm*CYP92A1 (monooxygenase); AB023636, *Glycyrrhiza echinata Ge*CYP93C2 (2-hydroxyisoflavanone synthase); AB017418, *Lithospermum erythrorhizon Le*CYP98A6 (4-coumaroyl-4′-hydroxyphenyllactic acid 3-hydroxylase); AF212990, *Cucurbita maxima Cm*CYP701A1 (ent-kaurene oxidase); BAB11063, *Arabidopsis thaliana At*CYP705A5 (flavonoid 3′-monooxygenase); NM129002, *Arabidopsis thaliana At*CYP710A1 (sterol C-22 desaturase); EF451150, *Papaver somniferum Ps*CYP719B1 (salutaridine synthase); AF014801, *Eschscholzia californica Ec*CYP80B1 ((*S*)-*N*-methylcoclaurine 3′-hydroxylase); AB026122 *Coptis japonica Cj*CYP719A1 (canadine synthase); AB126257 *Eschscholzia californica Ec*CYP719A2 (stylopine synthase); AB126256 *Eschscholzia californica Ec*CYP719A3 (stylopine synthase); AB434655 *Eschscholzia californica Ec*CYP719A9 (involved in alkaloid biosynthesis). 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 the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches.

2.2. P450 nature of CYP82N2v2

To determine the P450 nature of CYP82N2v2, its expression vector was constructed and introduced into *S. cerevisiae*. The microsomal fraction prepared from transgenic *S. cerevisiae* cells showed a reduced CO-difference that was characteristic of a P450, which had a peak at 450 nm (Fig. 4). The CYP82N2v2 content in the microsomal fraction was estimated to be 80 pmol P450 per mg microsomal protein.

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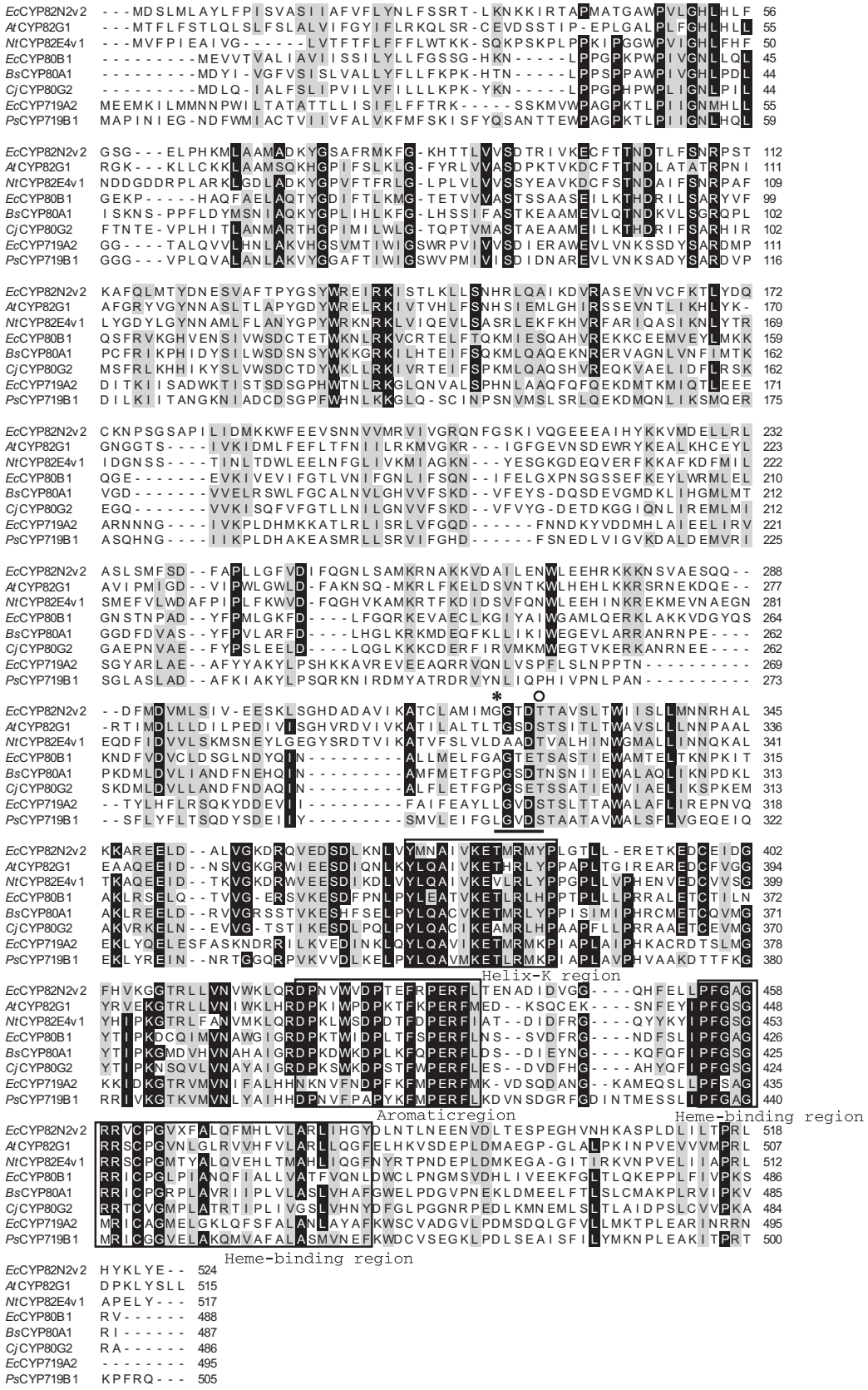

Fig. 3. Amino acid sequence alignment of CYP82N2v2 and related P450s. Boxes indicate the conserved regions of eukaryotic P450; i.e., the helix K region, aromatic region, and heme-binding region. Underline indicates consensus sequence in the Helix I region. Asterisk indicates conserved Gly/Ala, open circle indicates conserved Thr, which are conserved in monooxygenase-type P450. *Ec*CYP80B1, *Bs*CYP80A1, *Cj*CYP80G2, *At*CYP82G1, *Nt*CYP82E4v1, *Ec*CYP719A2, and *Ps*CYP719B1 are (*S*)-*N*-methylcoclaurine-3′-hydroxylase (Accession No. AF014801), berbamunine synthase (Accession No. P47195), corytuberine synthase (Accession No. BAF80448), homoterpene synthase (NM113423), nicotine *N*-demethylase (DQ131886), stylopine synthase (Accession No. AB126257), and salutaridine synthase (Accession No. ABR14720), respectively. Highly conserved amino acid residues are shown in white in black box-shading, while similar amino acid residues are shown in black in gray box-shading.

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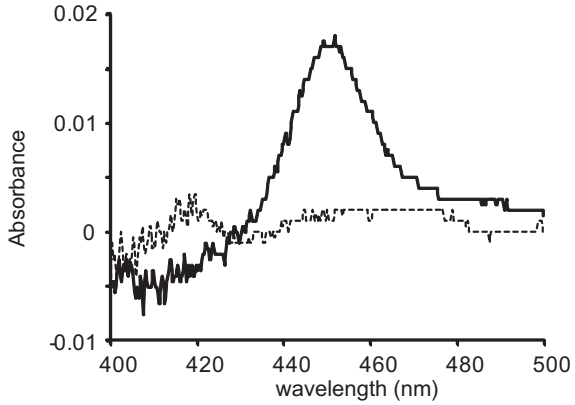

Fig. 4. Reduced CO-difference spectra of CYP82N2v2. A microsomal fraction containing CYP82N2v2 was prepared from transgenic *S. cerevisiae* and used for reduced CO-difference spectrum measurement at 1.65 mg protein/ml. Spectra of the microsomal fraction from *S. cerevisiae* transformed with empty vector (dotted line) and CYP82N2v2-expressing *S. cerevisiae* (solid line) are shown.

2.3. Enzymological activity of CYP82N2v2

To determine the enzymological activity of CYP82N2v2, the microsomal fraction was reacted with either protopine (**5**) or allocryptopine (**11**) as a substrate under P450 assay conditions. Recombinant CYP82N2v2 clearly showed protopine (**5**) or allocryptopine (**11**) conversion activity to produce dihydrosanguinarine (**7**) or dihydrochelerythrine (**13**), respectively (Fig. 5). The identities of the products were confirmed by direct comparison with authentic standards (Fig. 5 and Supplementary Fig. S1). During the experiments, an intermediate, such as 6-hydroxyprotopine (**6**) from protopine (**5**), was not detected even in the single ion mode in LC–MS.

To understand the reaction properties of CYP82N2v2, the dependence of the reaction on NADPH and oxygen was determined. As shown in Table 1, the absence of NADPH or removal of O_2 from the reaction mixture using a glucose/glucose oxidase/catalase system (Bauer and Zenk, 1991) clearly inhibited the activity of CYP82N2v2. Furthermore, ketoconazole, a known P450 inhibitor, also inhibited its activity. These results clearly indicated that CYP82N2v2 reacted as P450 to convert protopine (**5**) or allocryptopine (**11**) to dihydrosanguinarine (**7**) or dihydrochelerythrine (**13**), respectively.

2.4. Substrate specificity of CYP82N2v2

To obtain more insight about the reaction mechanism of CYP82N2v2, the substrate-specificity of CYP82N2v2 was examined using several types of alkaloids at 10 μM (Supplementary Fig. S2). Among protopine-type alkaloids, corycavine (**15**) and 13-oxoprotopine (**17**) (see Fig. 6 for structure) in addition to protopine (**5**) and allocryptopine (**11**) were converted by CYP82N2v2 to give new products.

The reaction product of corycavine (**15**) was determined to be corynoloxine (**16**) by direct comparison with a standard compound using LC–MS (Fig. 6A–C). On the other hand, the reaction product of 13-oxoprotopine (**17**) by CYP82N2v2 showed a reduction of 2 *m*/*z* by LC–MS (Fig. 6D and E, Supplementary Fig. S4). Among protopine-type substrates, protopine-*N*-oxide (18, Supplementary Fig. S2) did not show any new product when reacted with CYP82N2v2. Simple benzylisoquinolines such as reticuline (**1**), and protoberberines such as scoulerine (**2**) and *N*-methyl stylopine (19, Supplementary Fig. S2) also did not generate new product.

2.5. Enzyme kinetics of CYP82N2v2

To understand the physiological role of CYP82N2v2 in California poppy cells, the substrate affinities for protopine (**5**) and allocryptopine (**11**) were determined using recombinant microsomal fractions. CYP82N2v2 was reacted with different concentrations of substrates and product formation was quantified by HPLC. The CYP82N2v2 reaction followed Michaelis–Menten-type reaction kinetics and the apparent k_m values for protopine (**5**) and allocryptopine (**11**) were estimated to be 6.45 and 8.70 μM, with estimated V_{max} values of 24.5 and 23.0 pkat/μg P450, respectively (Supplementary Fig. S3). The P450 content was estimated from the CO-difference spectrum. These data suggest that CYP82N2v2 can convert both protopine (**5**) and allocryptopine (**11**) to the corresponding benzophenanthridine-type alkaloids at comparable rates, as shown in transgenic California poppy cells with *Cj*SMT, which efficiently metabolized the new product produced by *Cj*SMT (see Fig. 1). In comparison with other P450s in the biosynthetic pathway for isoquinoline alkaloids, CYP82N2v2 has a higher k_m value than that of CYP719A1 for (*S*)-tetrahydrocolumbamine ((*S*)-THC) (**9**) (0.269 μM), and a lower k_m value than that of CYP80B1 for (*S*)-*N*-methylcoclaurine (15 μM).

3. Discussion

3.1. Isolation of cDNA of a key P450, CYP82N2v2, in benzophenanthridine biosynthesis

The conversion of protopine (**5**) to dihydrosanguinarine (**7**) is the crucial step in benzophenanthridine alkaloid biosynthesis. In this study, it was determined that CYP82N2v2 catalyzed conversion of protopine (**5**) to dihydrosanguinarine (**7**) by the P450 reaction. CYP82N2v2 had conserved eukaryotic P450 regions i.e., helix K region, aromatic region and heme-binding region at the C-terminal end (Fig. 3), and depended on NADPH/O_2 (Table 1). Whereas some P450s in isoquinoline alkaloid biosynthesis such as CYP719 and CYP80G2 have unique substitutions at a conserved Gly/Ala residue in the helix I region, *Ec*CYP82N2v2 has conserved Gly, suggesting that *Ec*CYP82N2v2 would basically function as a monooxygenase-type enzyme. Conserved Gly is also found in *At*CYP82C2 (Accession No. NM119348), which functions as methoxypsoralen hydroxylase (Kruse et al., 2008), whereas *At*CYP82G1 (Accession No. NM113423), which functions as homoterpene synthase for oxidative degradation of geranyllinalool/nerolidol (Lee et al., 2010), has a Thr residue, and CYP82E4v1 (Accession No. DQ131886), which functions as nornicotine synthase for demethylation (Siminszky et al., 2005), has an Asp residue (Fig. 3). While several members of the CYP80 and CYP719 families have been reported in isoquinoline biosynthesis, CYP82N2v2 is the first gene in the CYP82 family that has been shown to be involved in isoquinoline alkaloid biosynthesis. It would be interesting to determine how CYP82N2v2 evolved, since the CYP719 family is unique to isoquinoline alkaloid biosynthesis. Since some P450 genes are clustered in the genome (Shimada et al., 2003), characterization of the genome structure of isoquinoline alkaloid-producing plants would be useful.

3.2. Reaction mechanism of CYP82N2v2

Recombinant CYP82N2v2 expressed in *S. cerevisiae* converted protopine (**5**) to dihydrosanguinarine (**7**) without detectable formation of any intermediate. Tanahashi and Zenk (1990) used 6-^{3}H protopine (**5**) and detected the removal of HO^{3}H from the substrate by the microsomal fraction of *E. californica* cells during formation of [11-^{3}H] dihydrosanguinarine (**7**). It was concluded

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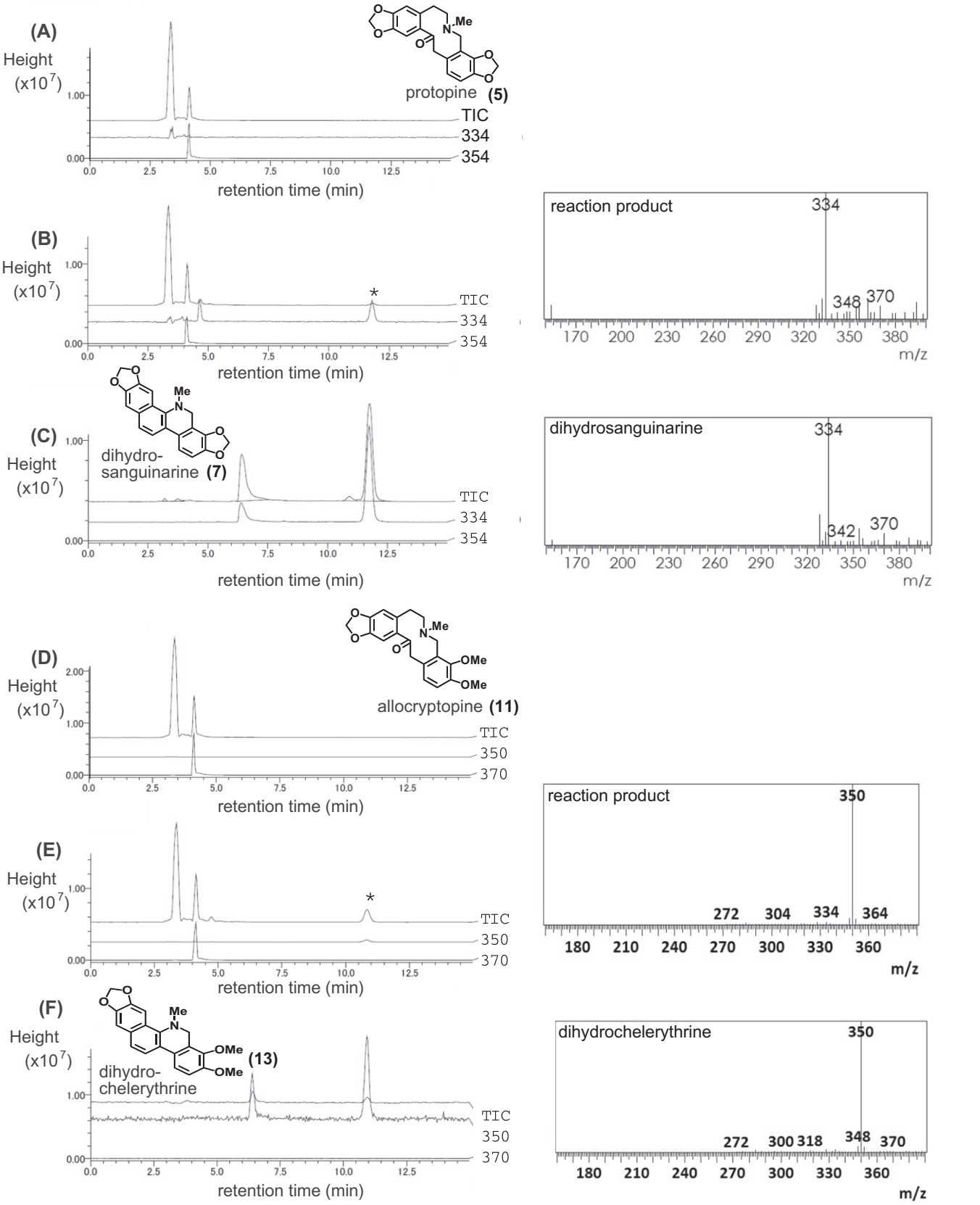

Fig. 5. CYP82N2v2 reaction with protopine (**5**) or allocryptopine (**11**) as a substrate. (A) Vector control reaction with protopine (**5**) (*m*/*z* 354, $[M+H]^+$), (B) CYP82N2v2 reaction with protopine (**5**), (C) authentic dihydrosanguinarine (**7**) (*m*/*z* 334, $[M+H]^+$), (D) vector control reaction with allocryptopine (**11**) (*m*/*z* 370, $[M+H]^+$), (E) CYP82N2v2 reaction with allocryptopine (**11**), and (F) authentic dihydrochelerythrine (**13**) (*m*/*z* 350, $[M+H]^+$). Reaction conditions were as shown in Section 5. Total ion chromatogram (TIC) and single ion chromatogram for each *m*/*z* were monitored. Asterisks show the reaction products. Mass fragment patterns of reaction products are shown in comparison with those of standard chemicals.

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Table 1
NADPH or oxygen dependency of CYP82N2v2 activity. The reaction was carried out under standard assay conditions with protopine as the substrate, after the removal of NADPH or oxygen with glucose/glucose oxidase, or the addition of ketoconazole. The CYP82N2v2 activity of control assay was 6300 pmol/30 min/8.5 ng P450. n.d. means that the activity was less than 2% of control assay.

Addition	Relative activity
	%
None	100
−NADPH	n.d.
40 mM glucose + 5 units of glucose oxidase + 10 units of catalase	15
40 mM glucose + boiled glucose oxidase + 10 units of catalase	92
25 μM ketoconazole	n.d.

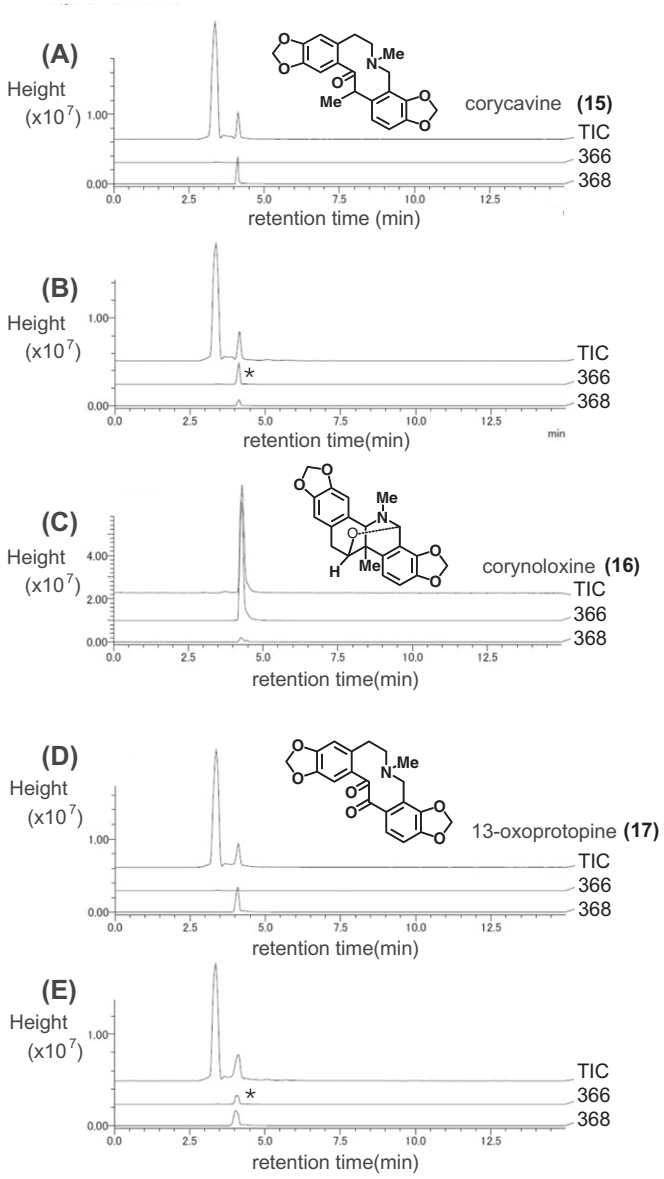

Fig. 6. CYP82N2v2 reaction with corycavine (**15**) or 13-oxoprotopine (**17**) as the substrate. Vector control reaction with corycavine (**15**) (A), CYP82N2v2 reaction with corycavine (**15**) (m/z 368, $[M+H]^+$) (B), authentic corynoloxine (**16**) (m/z 366, $[M+H]^+$) (C), vector control reaction with 13-oxoprotopine (**17**) as substrate (D), and CYP82N2v2 reaction with 13-oxoprotopine (**17**) (E). Asterisks show the reaction products.

that hydroxylation of protopine (**5**) at the 6-position and successive spontaneous conversion produces dihydrosanguinarine (**7**). The sequence information for CYP82N2v2 as a simple monooxygenase supports this speculation.

Characterization of the substrate-specificity of CYP82N2v2 further provided additional support for the 6-hydroxylation of protopine (**5**): when CYP82N2v2 was reacted with corycavine (**15**), the formation of corynoloxine (**16**) was detected (Fig. 6). Its formation was speculated to occur through an enamino aldehyde intermediate in equilibrium with 6-hydroxy protopine-type alkaloids (Supplementary Fig. S5, Iwasa et al., 1989). In fact, when CYP82N2v2 was reacted with 13-oxoprotopine (**17**) (Fig. 6), CYP82N2v2 produced a novel compound with an m/z of 366. This product had a unique MS fragment pattern, which resembled that of the amino alcohol-type intermediate and was distinct from the substrate 13-oxoprotopine (**17**) (Fig. S4). The limited reaction with 13-oxoprotopine (**17**) also indicates that the hydrogen at the 13-position plays a critical role in benzophenanthridine alkaloid biosynthesis. Although the first 6-hydroxylation step is not detectable, the identification of CYP82N2v2 provides a useful tool for understanding the molecular mechanism of this reaction.

3.3. Physiological role of CYP82N2v2

The identification of CYP82N2v2 also provides useful information on the regulation of benzophenanthridine alkaloid biosynthesis. In fact, CYP82N2v2 can react with several protopine-type alkaloids, such as protopine (**5**), allocryptopine (**11**), 13-oxoprotopine (**17**) and corycavine (**15**). Both protopine (**5**) and allocryptopine (**11**) are particularly good substrates for CYP82N2v2, but this broad reactivity is not common for P450s in alkaloid biosynthesis, as shown for *Ec*CYP719A2 or *Cj*CYP719A1 (Ikezawa et al., 2003, 2007). The results herein also suggest that a CYP82N2v2 homolog may be involved in the formation of corynoloxine (**16**) from corycavine (**15**) found in *Corydalis incisa* cells (Supplementary Fig. S5, Iwasa et al., 1989). CYP82N2v2 would be a good example of the importance of endogenous enzymes for metabolic diversification.

4. Conclusions

A cDNA of P450 (CYP82N2v2) in sanguinarine biosynthesis was isolated from *E. californica*, based on an integrated analysis of metabolites and the mRNA expression profile of transgenic cells with *C. japonica* scoulerine-9-*O*-methyltransferase (Takemura et al., 2010). Recombinant CYP82N2v2 produced in *S. cerevisiae* clearly showed protopine (**5**) or allocryptopine (**11**) conversion to produce dihydrosanguinarine (**7**) or dihydrochelerythrine (**13**), respectively. Further characterization of the substrate-specificity of CYP82N2v2 indicated that 6-hydroxylation played a role in the reaction. Although the first 6-hydroxylation step is not detectable, the identification of CYP82N2v2 provides a useful tool for understanding the molecular mechanism of this reaction.

5. Experimental

5.1. Plant material

E. californica seeds were obtained from Takii Seed Co., Ltd. (Kyoto, Japan). Suspension cultures of *E. californica* with ectopic expression of *C. japonica* scoulerine-9-*O*-methyltransferase (*Cj*SMT) cDNA were established and maintained in Linsmaier–Skoog medium (Linsmaier and Skoog, 1965) with 3% sucrose, 10 μM α-naphthaleneacetic acid, and 1 μM 6-benzylaminopurine on a gyratory

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shaker (90 rpm) at 26 °C in the dark in a 21-day growth cycle, as described previously (Takemura et al., 2010).

5.2. Chemicals

Protopine (**5**), allocryptopine (**11**) and sanguinarine (**8**) were purchased from SIGMA–ALDRICH, Co., (St. Louis, MO, USA). 13-Oxoprotopine (**17**), corycavine (**15**), protopine-*N*-oxide (18), corynoloxine (16), (*S*)-*N*-methylstylopine (19), dihydrosanguinarine (**7**) and dihydrochelerythrine (**13**) were from the collection of K. Iwasa. (*S*)-Reticuline (**1**) and (*S*)-scoulerine (**2**) were gifts from Mitsui Chemicals, Inc. (Tokyo, Japan).

5.3. Preparation of cDNAs for P6H candidate gene

cDNA was prepared from transgenic cells at day 14 of culture. Since cDNA candidate (*Ec*CYP-A) clone for P6H contained only 5′-termini, 3′-RACE (rapid amplification of cDNA ends) was performed using a GeneRacer kit (Invitrogen) with a total RNA sample (1 μg), gene-specific primer, 3′-GSP (5′-ATCATTTCATGGGTGGGAAA-3′) and the universal primer (5′-GCTACGTAACGGCATGACAGT-3′) following the manufacture's instructions. The resultant PCR product of ~1.5 kb was subcloned into pGEM T-easy vector and the nucleotide sequence was determined.

5.4. Alignment analysis

The nucleotide sequence was deposited in DDBJ/GenBank™ (AB598834) and also in the P450 nomenclature committee, who named it CYP82N2v2. CYP82N2v2 was aligned using Clustal W (Thompson et al., 1994; Higgins et al., 1996) and BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). GenBank™ accession numbers for the sequences used are *Bs*CYP80A1 (U09610), *Ec*CYP80B1 (AF014800), *Cj*CYP80G2 (AB288053), *Ec*CYP719A2 (AB126257), *Ps*CYP719B1 (EF451150), *Nt*CYP82E4v1 (DQ131886), *At*CYP82C2 (NM119348), and *At*CYP82G1 (NM113423).

5.5. Construction of expression vector

To determine the P6H activity of CYP82N2v2, a yeast expression vector was constructed in pGYR-SpeI with the *S. cerevisiae* NADPH-P450 reductase gene and an *SpeI* cloning site (Ikezawa et al., 2008). Full-length CYP82N2v2 cDNA was amplified by PCR with the following primers to introduce an *XbaI* site (underlined): forward primer (5′-CTACAAAATCTAGAAATGGATTCCTTAATC-3′) and reverse primer (5′-AACCACATTCTAGACTATTCGTACAACTTG-3′). After subcloning in pGEM T-easy vector and confirmation of the nucleotide sequence, full-length cDNA of CYP82N2v2 was cut with *XbaI*, and then ligated into the *SpeI* site of pGYR-*SpeI* to generate yeast expression vector, pGS-CYP82N2v2.

5.6. Heterologous expression of CYP82N2v2 in S. cerevisiae

pGS-CYP82N2v2 was introduced into *S. cerevisiae* strain AH22 (a *leu*2–3 *leu*2–112 *his*4–519 *can*1 (*cir*+)) (Oeda et al., 1985) by the LiCl method (Ito et al., 1983), and transgenic *S. cerevisiae* cells were cultivated in concentrated SD medium at 30 °C and 220 rpm (Sakaki et al., 1990). A microsomal fraction was prepared in the buffer (100 mM Hepes/NaOH pH 7.6) as described previously (Ikezawa et al., 2003), and used for the enzyme assay.

5.7. CO-difference spectra measurement

To determine the P450 nature of CYP82N2v2, reduced CO-difference spectra were measured with a Shimadzu UV-3101 spectrophotometer (Kyoto, Japan). The spectrum of the microsomal fraction (protein concentration of 1.65 mg/mL) in 50 mM Hepes/NaOH (pH 7.6) was measured before and after the addition of sodium dithionite, with the bubbling of CO, to obtain the reduced CO-bound spectrum of CYP82N2v2. The P450 hemoprotein content in the microsomal fraction was then determined using a difference of 91 mM^{-1} cm^{-1} between the extinction coefficients at 450 and 490 nm (Omura and Sato, 1964).

5.8. P6H enzyme assay

P6H activity was determined in a reaction mixture that consisted of 50 mM Hepes/NaOH (pH 7.6), 500 μM NADPH, 10 μM substrate, and the enzyme preparation (8.5 ng P450). Protopine (**5**) and allocryptopine (**11**) were used as standard substrates in the enzymological characterization of CYP82N2v2, and corycavine (**15**), 13-oxoprotopine (**17**), protopine *N*-oxide (**18**), reticuline (**1**), scoulerine (**2**), and *N*-methyl stylopine (**19**) were used for the characterization of substrate-specificity. The assay mixture was incubated at 30 °C for 30 min, except for the determination of kinetic parameters (incubation time; 5–10 min), and the reaction was then terminated by the addition of Cl_3CCO_2H (final concentration of 2%) and MeOH (final concentration of 40%). After protein precipitation, the reaction products were determined quantitatively by reversed-phase HPLC with a Shimadzu LC-10 A system: column, COSMOSIL πNAP column (4.6 × 250 mm; Nacalai Tesque); solvent system, 0.05% CF_3CO_2H/CH_3CN containing 0.05% CF_3CO_2H (1:1); flow rate, 0.5 mL/min; detection, absorbance measurement at 280 nm with a SPD6A photodiode array detector. Product formation was confirmed by LC–MS (LCMS-2020, Shimadzu; ESI-MS at 70 eV, positive ion mode) with the same conditions as in the HPLC analysis except for the solvent system: CH_3CN/H_2O/AcOH (99:99:2, v/v/v) and flow rate 0.5 mL/min.

To determine the kinetic parameters of P6H, the amount of dihydrosanguinarine (**7**) produced was estimated using a calibration curve of standard sample at 280 nm. Kinetic parameters were obtained from three independent experiments and then averaged to yield final estimates with a standard deviation.

Acknowledgments

We thank Drs. Y. Yabusaki and M. Mizutani for providing the expression vector, pGYR, and for the reduced CO-difference spectra measurement, respectively. We also thank Mitsui Chemicals, Inc. for generous gifts of the alkaloids. This research was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan [Grant-in-Aid (Nos. 21248013 and 23108511 to F.S.)]; the Japan Society for the Promotion of Science [fellowship to T.T.].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2012.02.013.

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