Molecular Cloning and Characterization of CYP719, a Methylenedioxy Bridge-forming Enzyme That Belongs to a Novel P450 Family, from cultured Coptis japonica Cells*

Received for publication, March 11, 2003, and in revised form, April 30, 2003
Published, JBC Papers in Press, May 5, 2003, DOI 10.1074/jbc.M302470200

Nobuhiro Ikezawa‡, Masaru Tanaka§, Masanori Nagayoshi, Raku Shinkyo¶, Toshiyuki Sakaki†, Kuniyo Inouye*, and Fumihiko Sato‡§¶¶

From the ‡Division of Integrated Life Sciences, Graduate School of Biostudies, and §Division of Applied Life Science and ¶¶Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

Two cytochrome P450 (P450) cDNAs involved in the biosynthesis of berberine, an antimicrobial benzylisoquinoline alkaloid, were isolated from cultured Coptis japonica cells and characterized. A sequence analysis showed that one C. japonica P450 (designated CYP719) belonged to a novel P450 family. Further, heterologous expression in yeast confirmed that it had the same activity as a methylenedioxy bridge-forming enzyme (canadine synthase), which catalyzes the conversion of (S)-tetrahydrocolumbamine ((S)-THC) to (S)-tetrahydrodroberberine ((S)-THB, (S)-canadine). The other P450 (designated CYP80B2) showed high homology to California poppy (S)-N-methylcoclaurine-3′-hydroxylation (CYP80B1), which converts (S)-N-methylcoclaurine to (S)-3′-hydroxy-N-methylcoclaurine. Recombinant CYP719 showed typical P450 properties as well as high substrate affinity and specificity for (S)-THC. (S)-Scoulerine was not a substrate of CYP719, indicating that some other P450, e.g. (S)-cheilanthifoline synthase, is needed in (S)-styrilone biosynthesis. All of the berberine biosynthetic genes, including CYP719 and CYP80B2, were highly expressed in selected cultured C. japonica cells and moderately expressed in root, which suggests coordinated regulation of the expression of biosynthetic genes.

Cells of higher plants produce structurally divergent chemicals such as terpenoids, phenylpropanoids, and alkaloids. Although these chemicals have been used as medicines, aromatic chemicals, insecticides, etc., the biochemical potential of plant cells themselves has recently attracted the attention of researchers who hope to use their biocatalytic activities for the conversion and/or production of chemicals.

Cytochrome P450 (P450)* is one of the most studied groups of enzymes because of the large number of compounds represented and their reaction diversity, e.g. hydroxylation, alken epoxidation, dealkylation, and oxidative deamination (1). For example, liver microsomal P450 is well known to exist in multiple forms and to play important roles in the detoxification of xenobiotics (2). Although the reactions in plants that involve P450s have not been well studied because of their low abundance and the difficulty of biochemical characterization, recent studies have shown that many biochemical reactions in plants are catalyzed by P450s (3).

In the biosynthesis of antimicrobial benzylisoquinoline (berberine) or benzophenanthridine (sanguinarine) alkaloids, several P450 reactions have been reported (Fig. 1) (4–8). A P450 (CYP80B1) catalyzes hydroxylation, i.e. the conversion of (S)-N-methylcoclaurine to (S)-3′-hydroxy-N-methylcoclaurine, and the cDNA has been isolated from California poppy (Eschscholzia californica) (5). Other P450 reactions include methylenedioxy bridge formation (6–8); e.g. the conversion of (S)-tetrahydrocolumbamine ((S)-THC) to (S)-tetrahydrodroberberine ((S)-THB, (S)-canadine) has been studied in part by using microsomal fractions of Thalictrum tuberosum cells (7). Two other methylenedioxy bridge-forming reactions from (S)-scoulerine via (S)-cheilanthifoline to (S)-styrilone have been partially characterized in California poppy (8). Because Coptis japonica cells produce berberine and coptisine, which is the oxidized form of (S)-styrilone, we speculated that the cells of this plant may also exhibit these methylenedioxy bridge-forming reactions along with the CYP80B hydroxylation reaction (Fig. 1).

Whereas previous reports have indicated that biosynthetic enzymes involved in berberine/(S)-styrilone biosynthesis have relatively strict substrate specificity (5, 7–12), many P450s, especially those involved in the detoxification of xenobiotics, are known to have relatively broad substrate specificity (2). Therefore, we hypothesized that all of the methylenedioxy bridge-forming reactions in berberine/coptisine biosynthesis in C. japonica might be catalyzed by a single P450, although with different reactivities. To clarify this point, we tried to isolate a methylenedioxy bridge-forming P450 cDNA from cultured C. japonica 156-1 cells.

Because cultured C. japonica 156-1 cells produce large amounts of berberine and coptisine (13), we speculated that the desired P450 involved in berberine/coptisine biosynthesis would be highly expressed in these cells. Based on this idea, we

---

* This research was supported in part by Research for the Future Program Grant JSPS-RFTF00L01606 from the Japan Society for the Promotion of Science (to F. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB025030 (CYP80B2) and AB026122 (CYP719).

To whom correspondence should be addressed: Division of Integrated Life Sciences, Graduate School of Biostudies, Kyoto University, Kyoto 606-8502, Japan. Tel.: 81-75-753-6381; Fax: 81-75-753-6398; E-mail: fumihiko@kais.kyoto-u.ac.jp.

† The abbreviations used are: P450, cytochrome P450; (S)-THC, (S)-tetrahydrocolumbamine; (S)-THB, (S)-tetrahydrodroberberine; SMT, S-adenosyl-L-methionine:scoulerine 9-O-methyltransferase; 6-OMT, S-adenosyl-l-methionine:norcoclaurine 6-O-methyltransferase; 4′-OMT, S-adenosyl-l-methionine:3′-hydroxy-N-methylcoclaurine 4′-O-methyltransferase; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectroscopy; (S)-CHS, (S)-cheilanthifoline synthase.
amplified cDNA fragments specific to eukaryotic P450s from a cDNA library prepared from cultured *C. japonica* 156-1 cells. Next, using these cDNA fragments, we isolated full-length cDNAs, determined the primary structures, and produced recombinant proteins in a yeast expression system to identify their biochemical activity. The data indicated that an isolated cDNA encoded a novel P450 that showed methylenedioxy bridge-forming activity to convert (S)-THC to (S)-THB. The kinetic parameters and substrate specificity were determined to clarify whether this P450 may function in other biosynthetic steps and to evaluate the metabolic regulation of berberine biosynthesis by this enzyme.

**FIG. 1.** Schematic biosynthetic pathway for a variety of isoquinoline alkaloids. (S)-Scoulerine is an intermediate at the branch point leading to berberine or (S)-stylopine.

(S)-N-Methylcoclaurine

(S)-Reticuline

(S)-3'-Hydroxy-N-methylcoclaurine

(S)-Scoulerine

(S)-Cheilanthifoline

(S)-Tetrahydrcolumbamine

(C) CYP719

(S)-Tetrahydroberberine (C)-Canadine

Berberine

(C) CYP80B2

(S)-Norcoellaurine

L-Tyrosine

 Californiapatpy

Co-optisine

(Coptis japonica)

(S)-Stylopine

(S)-Sanguinarine

Downloaded from http://www.jbc.org/ by guest on August 31, 2017
EXPERIMENTAL PROCEDURES

Plant Material—Coptis (C. japonica Makino var. Dissecta (Yatabe) Nakai) plants were obtained from the herbal garden of Takeda Chemical Industries. The original cultured cells were induced from rootlets of C. japonica, and a cell line (156-1) with high berberine productivity was described previously (13). Fourteen-day-old cultured cells were harvested and used for the extraction of mRNA.

Chemicals—Berberine sulfate was purchased from Tokyo Kasei Kogyo Co., Ltd. (S)-Coclaurine was a gift from Dr. Y. Sugimoto of Tottori University, Japan. (S)-Scoulerine, (R,S)-reticuline, (R,S)-norreticuline, and (R,S)-6-O-methylnorpseudodolosine were gifts from Mitsui Petrochemical Industries, Ltd. (S)-Tetrahydrocolumbamine was prepared from (S)-scoulerine by using recombinant S-adenosyl-l-methionine:scoulerine 9-O-methyltransferase (SMT) expressed in Escherichia coli as described previously (14). (R,S)-Tetrahydroberbine was prepared from berberine as described previously (15). Helix-K was synthesized and used for the extraction of mRNA.

Amplification of Cytochrome P450 cDNA Fragments from a cDNA Library—The above cDNA library was used to screen full-length cDNAs of P450. The second PCR products at about 200 bp were used as template for the second PCR using the F2 and R2 primers under the same PCR conditions as in the first PCR. The resultant PCR products at about 200 bp were subcloned into pBluescript II SK− (Stratagene) and sequenced in a DSQ-1000 automated sequencer (Shimadzu, Kyoto, Japan) with a Thermo Sequenase® fluorescent labeled primer cycle sequencing kit (Amersham Biosciences). Sequenced clones showed two different sequences that were obviously homologous to known P450s.
GAGGAAGTACTGTCC-3'. The amplified fragments at about 900 bp were subcloned into pT7Blue T-Vector (Novagen, Madison, WI) and sequenced as described above.

Alignment Analysis—The predicted protein sequences were aligned using ClustalW (19, 20) and Boxshade (www.ch.embnet.org/software/BOX_form.html). ClustalW was also used to calculate the phylogenetic tree using the neighbor-joining method (21).

RNA Blot Analysis—RNA was isolated from cultured C. japonica 156-1 cells and C. japonica plant tissues. Cultured C. japonica cells were harvested after 12 days of culture, when biosynthetic activity was high, to extract total RNA. Freshly harvested C. japonica plants were separated into leaves, petioles, rhizomes, and roots and then stored in liquid nitrogen until the extraction of total RNA. Total RNA was prepared as described previously (22). Ten μg of total RNA was separated on a 1.2% denaturing formaldehyde-agarose gel and blotted onto a Hybond-N+ nylon membrane (Amersham Biosciences) with 20× SSC (333 mM sodium chloride/sodium citrate) as a transfer buffer according to the standard procedure (23). The 32P-labeled probes were synthesized by random priming using a Random Primed DNA Labeling Kit (Roche Applied Science). Four types of probes were synthesized, corresponding to the following fragments: 3'-fragment of CYP719 cDNA, 3'-fragment of CYP80B2 cDNA, 5'-fragment of S-adenosyl-L-methionine:norcooclaurine 6-O-methyltransferase (6-OMT) cDNA (11), and 5'-fragment of S-adenosyl-L-methionine:3'-hydroxy-N-methylcoclaurine 4'-O-methyltransferase (4'-OMT) cDNA (11). Hybridization was carried out for 16 h at 60 °C with 6× SSC, 5× Denhardt's reagent, 0.1% SDS, 100 μg/ml of denatured salmon sperm DNA, and the labeled probes. After hybridization, the membrane was washed once for 10 min with 1× SSC, 0.1% SDS at room temperature and then twice for 30 min with 1× SSC, 0.1% SDS at 65 °C. The hybridization signal was recorded by autoradiography. RNA amounts were standardized by hybridization to an 18 S rRNA probe at 60 °C.

Construction of Yeast Expression Vector—The coexpression plasmid pAMR2 for rat CYP1A1 and yeast NADPH-P450 reductase has been constructed previously (24). The coexpression vector pGYR for P450 and yeast NADPH-P450 reductase, constructed in a similar manner, was kindly provided by Dr. Y. Yabusaki of Sumitomo Chemical Co., Ltd. The resulting vector contained a glyceraldehyde-3-phosphate dehydrogenase promoter and terminator (25) instead of an alcohol dehydrogenase promoter and terminator without CYP1A1. The cloning site of pGYR

FIG. 3. Phylogenetic tree of plant cytochrome P450 families. Plant cytochrome P450 amino acid sequences obtained from GenBank™ or SwissProt were used for tree building. Accession numbers are: M32885, avocado CYP71A1 (terpenoid metabolizing); L10081, Catharanthus roseus CYP72A1 (secologanin synthase); Z17369, Helianthus tuberosus CYP73A1 (cinnamate 4-hydroxylase); Z22544, petunia CYP75A1 (flavonoid 3',5'-hydroxylase); X71658, Solanum melongena CYP76A1 (function unknown); X7156, S. melongena CYP77A1 (hydroxylase); P48420, Zea mays CYP78A1 (lauric acid 12-monooxygenase); U32524, Sorghum bicolor CYP78A1 (tyrosine N-hydroxylase); P47195, Berberis stolonifera CYP80A1 (berhammine synthase); O64899, Eschscholzia californica CYP80B1 (S)-N-methylcoclaurine-S'-hydroxylase); Q43068, pea CYP82A1 (phenylpropanoid-metabolizing); P48422, Arabidopsis thaliana CYP86A1 (fatty acid ω-hydroxylase); Q42569, A. thaliana CYP90A1 (22α-hydroxylase for 6-αxocathaster- one). The branch length is proportional to the estimated divergence distance of each protein. The scale bar (0.05) corresponds to a 5% change.
was further modified to contain an NcoI site and an XhoI site to construct pGVR-NX.

Full-length CYP719 cDNA was amplified by PCR using single-stranded cDNAs synthesized from 1.3 μg of total RNA of cultured C. japonica cells with oligo(dT) primer and SuperScript II RNase H reverse transcriptase (Invitrogen). The following primers were used for CYP719: the forward primer (5′-ATATCTCGACCATGGAGATGAATCCACCTGC-3′) was designed to introduce a PstI site (CTGCAG) and an NcoI site (underlined, CCGTGG) at the ATG start codon, and the reverse primer (5′-ATCCAACCTCAAGATATTCTACCCAGG-3′) was designed to contain a stop codon (underlined).

PCR products for CYP719 were digested with PstI and introduced into pBluescript SK– with PstI and EcoRV sites. The resulting plasmid was then digested with NcoI and XhoI, which produced CYP719 coding fragment. This coding fragment was ligated into the NcoI/XhoI site of pGVR-NX to generate yeast CYP719 expression vector, pGN-CYP719. The resulting construct was completely sequenced to confirm that no changes were introduced by subcloning processes.

Heterologous Expression of CYP719 in Yeast—The expression plasmid for CYP719 (pGN-CYP719) was transformed into yeast strain AH22 (26) by the LiCl method (27). These recombinant yeast cells were cultivated in concentrated SD medium at 30 °C, 220 rpm (28). To suppress the expression of endogenous P450s in yeast, cultivation was carried out under aerobic conditions (29). Yeast microsomal fractions were prepared as described previously (26) with the following modification: a Dounce glass homogenizer was used to fracture yeasts instead of a sonicator, and the microsomal fractions that were finally obtained were suspended in 50 mM HEPES/NaOH (pH 7.6), 500 mM NADPH, 5 μM substrate, and the enzyme preparation. The assay mixture was incubated at 30 °C for 30 min, except for the determination of kinetic parameters (for 5–10 min), and then the reaction was terminated by the addition of trichloroacetic acid (final concentration 2%) and methanol (final concentration 15%). After protein precipitation, the reaction product was determined quantitatively by reversed-phase HPLC with a Shimadzu LC-10A system: column, TSKgel ODS-80TM (4.6 × 250 mm; Tosoh); solvent system, acetonitrile/H2O/acetic acid (35:64:1); flow rate, 250 μl/min; detection, absorbance measurement at 280 nm with a SPD6A photodiode array detector. Product formation was confirmed by mass chromatography of the fragments subcloned into pBluescript II SK– and the microsomal fraction of yeast CYP719 activity was determined by measuring the reduced CO-difference spectra with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) as described previously (26). The reduced CO-difference spectra of CYP719 were measured with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) as described previously (26).

Measurement of P450 Hemoprotein—The reduced CO-difference spectra were measured with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) as described previously (26). The P450 hemoprotein content in the microsomal fraction was determined from the reduced CO-difference spectrum using a difference of 91 nm−1 cm−1 between the extinction coefficients at 446 and 490 nm (30).

Assay of Enzymatic Activity—CYP719 activity was determined by high performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS). The standard enzyme reaction mixture consisted of 50 mM HEPES/NaOH (pH 7.6), 500 μM NADPH, 5 μM substrate, and the enzyme preparation. The assay mixture was incubated at 30 °C for 30 min, except for the determination of kinetic parameters (for 5–10 min), and then the reaction was terminated by the addition of trichloroacetic acid (final concentration 2%) and methanol (final concentration 15%). After protein precipitation, the reaction product was determined quantitatively by reversed-phase HPLC with a Shimadzu LC-10A system: column, TSKgel ODS-80TM (4.6 × 250 mm; Tosoh); solvent system, acetonitrile/H2O/acetic acid (35:64:1); flow rate, 0.8 ml/min; detection, absorbance measurement at 280 nm with a SPD6A photodiode array detector. Product formation was confirmed by mass chromatography of the fragments subcloned into pBluescript II SK– and the microsomal fraction of yeast CYP719 activity was determined by measuring the reduced CO-difference spectra with a Shimadzu UV-2200 spectrophotometer (Kyoto, Japan) as described previously (26). The P450 hemoprotein content in the microsomal fraction was determined from the reduced CO-difference spectrum using a difference of 91 nm−1 cm−1 between the extinction coefficients at 446 and 490 nm (30).

RESULTS

Isolation of Cytochrome P450 cDNAs—Cytochrome P450 (P450) cDNA fragments, amplified from a cDNA library, were prepared from high berberine-producing cultured C. japonica cells using degenerated primers designed from the conserved region of eukaryotic P450s (18). After nested PCR, clear PCR products at about 200 bp were obtained. Sequence analysis of the fragments subcloned into pBluescript II SK– showed that these PCR products contained two different cDNA sequences, both of which were homologous to plant P450s.

Thus, we used the PCR products as a probe to screen C. japonica P450s from the cDNA library. From ~200,000 plaque-forming units, 40 positive plaques were isolated, and their cDNAs were subcloned into pBluescript SK– by in vitro excision. PCR amplification of cDNAs in pBluescript SK– revealed that cDNAs consisted of two inserts of different lengths, ~1.7 and 0.8 kb. Preliminary sequence analysis of the 3’ untranslatable region of all cDNAs showed that the ~1.7-kb and ~0.8-kb fragments were basically distinguishable, and these were designated CYPCJK and CYPCKB, respectively.

Nucleotide Sequences and Predicted Amino Acid Sequences—The two longest cDNA clones, which represented CYPCJK and CYPCKB, were selected and sequenced. A representative CYPCJK clone carried 1,671 nucleotides with an open reading frame that encoded 488 amino acids (DDBJ/GenBank®/EMBL accession number AB025030). The predicted amino acid sequence had motifs characteristic of eukaryotic P450s and was sufficient to encode a full-length P450 (Fig. 2).

On the other hand, the sequenced CYPCKB had only 806 nucleotides and encoded a partial 3’-fragment of P450 cDNA. To isolate a full-length clone of CYPCKB, 5’-rapid amplification of cDNA end was conducted. Based on the sequence of the 5’-end obtained, full-length cDNA was amplified, and the nucleotide sequence was determined. Full-length CYPCKB contained 1,658 nucleotides with an open reading frame for 492 amino acids, which encoded a characteristic plant P450, indicating that it encoded a full-length cDNA of P450 (DDBJ/GenBank®/EMBL accession number AB026122) (Fig. 2).

The amino acid sequence of CYPCKB is quite similar (68.4% identity) to that of CYP80B1, (S)-N-methylcoclaurine-3'-hydroxylase in reticuline biosynthesis, from the California poppy, E. californica (5), suggesting that it is an ortholog of C. japonica (Fig. 3). On the other hand, the amino acid sequence of full-length CYPCKB is not particularly similar to any known P450, suggesting that it may belong in a novel P450 family.
These predicted amino acid sequences were classified by the P450 nomenclature committee, and CYPCJA and CYPCJB were named CYP80B2 and CYP719, respectively.

Although the identity between the amino acid sequences of CYP80B2 and CYP719 was rather low (23.5%), they had conserved eukaryotic P450 regions: a helix K region, an aromatic region, and a heme-binding region at the C-terminal end (Fig. 2). In addition, their N-terminal regions contained hydrophobic domains corresponding to the membrane anchor sequences of microsomal P450 species, suggesting that CYP80B2 and CYP719 are localized in the endoplasmic reticulum. On the other hand, CYP719 did not have a conserved threonine (corresponding to Thr-252 of P450cam), which plays a significant role in oxygen-activation (32), but had serine instead (Ser-296). This substitution is not seen in most P450s but is found in a few species such as Zea mays CYP88A1 (GenBank™ accession number U32579) and Nicotiana tabacum CYP92A2 (GenBank™ accession number X95342).

Expression of CYP719 Gene in Cultured C. japonica Cells and Plant Tissues of C. japonica—Because the biochemical

Fig. 5. LC-MS analysis of CYP719 reaction product (A), authentic (R,S)-tetrahydroberberine (B), and vector control reaction (C). TIC, total ion chromatogram. (S)-THC and (R,S)-THB could not be fragmented under our experimental conditions, and therefore their parent ions were detected.
activity of CYP719 was unknown, we determined the expression of CYP719 in cultured C. japonica cells and C. japonica plant tissues (Fig. 4). A previous study of SMT expression in the root, which is the primary biosynthetic organ in C. japonica (33). When the cDNA expression for CYP719 was compared with three biosynthetic genes (CYP80B2, 6-OMT, and 4’-OMT), all four genes showed similar expression profiles: very high expression in cultured cells, moderate expression in root, and scarce expression in other tissues. This result indicated that CYP719 is likely involved in berberine biosynthesis.

Heterologous Expression of CYP719 in Yeast and Its Activity—To confirm the function of CYP719, it was expressed heterologously in yeast. Yeast expression plasmid for CYP719 was constructed and transformed to yeast AH22. Because CYP719 had a putative endoplasmic reticulum-localizing signal, microsomal fractions were prepared from recombinant yeast cells, and its enzymatic activity was determined using LC-MS analysis.

Microsomal fractions of CYP719-expressing yeast showed canadine synthase activity, i.e. the formation of (S)-THB from (S)-THC was detected (Fig. 5). This result clearly indicated that CYP719 was a methylenedioxy bridge-forming enzyme, which produces (S)-THB from (S)-THC in berberine biosynthesis. Because the enzymologic properties of CYP719 were not clear, we used this recombinant CYP719 for further analysis.

Enzymatic Characterization of CYP719—First, the P450 nature of CYP719 was confirmed using reduced CO-difference spectra (Fig. 6). A clear peak at 446 nm showed that CYP719 was expressed as an active P450. This spectrum also showed that a recombinant microsomal fraction contained CYP719 at 48.8 pmol/mg of protein. CYP719 activity was also dependent on NADPH and oxygen; the absence of NADPH or the removal of O2 by the glucose/glucose oxidase/catalase system (8) clearly inactivated the CYP719 activity for (S)-THC (Table I). A synthetic fungicide, ketoconazole, which is a typical P450 inhibitor that interacts with the protemic heme group (34), also inhibited methylenedioxy bridge-forming activity (Table II).

Substrate Specificity and Affinity of CYP719—To examine the possibility of whether CYP719 could catalyze three different steps in berberine/coptisine biosynthesis, the substrate specificity of CYP719 was examined using LC-MS analysis in comparison with (S)-THC as a reference substrate. When seven structurally related substrates were used as the substrate (Fig. 7), none was converted by CYP719 to the corresponding products with a methylenedioxy bridge, indicating that CYP719 had high substrate specificity.

Next, the substrate affinity of CYP719 was determined using HPLC analysis with (S)-THC as the substrate. When the substrate concentration was varied, the reaction followed Michaelis-Menten-type kinetics. The kinetic parameters K_m and V_max values were estimated at 0.269 ± 0.003 μM and 1.86 ± 0.10 pmol of product/min/pmol of P450, respectively (Fig. 8).

Inhibition of CYP719 Activity by Structurally Related Isoquinoline Alkaloids—To understand the regulation of berberine biosynthesis, the effects of various structurally related isoquinoline alkaloids on CYP719 activity were examined. Interestingly, (S)-scoulerine, a precursor of (S)-THC, inhibited the methylenedioxy bridge-forming activity of CYP719 in a competitive manner with a K_i value of 12.1 ± 2.2 μM, whereas it was not a substrate for CYP719 (data not shown). Further, columbamine, an oxidized form of (S)-THC, also inhibited CYP719 activity and was not a substrate for CYP719 (Table II). Berberine, the end product of the berberine biosynthetic pathway, did not inhibit the methylenedioxy bridge-forming activity of CYP719.

DISCUSSION

In this study, we isolated two full-length cytochrome P450 cDNAs (CYP80B2 and CYP719) from high berberine-producing cultured C. japonica cells. Structural analysis and heterologous expression in yeast indicated that cultured plant tissues (Fig. 4). A previous study of SMT expression showed a high expression of SMT, and moderate expression profiles: very high expression in cultured cells, moderate expression in root, and scarce expression in other tissues. This result indicated that CYP719 is likely involved in berberine biosynthesis.
expression in yeast (data not shown) showed that CYP80B2 was an ortholog of CYP80B1, which is (S)-N-methylcoclaurine-3′-hydroxylase isolated from the California poppy (Fig. 2) (5). On the other hand, CYP719 was placed in a new P450 family and showed methylenedioxy bridge-forming activity for (S)-THC as a substrate, i.e. canadine synthase activity, which converts (S)-THC to (S)-THB (Fig. 5). CYP719 is the first P450 to be identified as a methylenedioxy bridge-forming enzyme.

Methylenedioxy bridge formation, which is the cyclization of an ortho-methoxyphenol, is commonly found in many secondary metabolites, including lignans. This reaction is difficult to mimic in organic chemistry. A previous study showed that the formation of two methylenedioxy bridges in (S)-stylopine biosynthesis in California poppy was catalyzed by P450s (8). These P450s (S)-cheilanthifoline synthase (S)-CHS and (S)-stylopine synthase catalyze the reaction of (S)-scoulerine to (S)-stylopine. These two steps are also found in C. japonica cells to produce coptisine (Fig. 1). Whereas we had speculated that a methylenedioxy bridge-forming enzyme would catalyze all conversions from (S)-scoulerine to (S)-cheilanthifoline, indicating that there are likely other methylenedioxy bridge-forming enzyme(s) in coptisine biosynthesis. On the other hand, CYP719 might function as (S)-stylopine synthase. When (S)-cheilanthifoline or (S)-CHS is available, we will examine this possibility regarding CYP719.

Analysis of the substrate specificity of CYP719 indicated that berberine was produced from (S)-THC via (S)-THB and not via columbamine in C. japonica. Interestingly, columbamine slightly inhibited the methylenedioxy bridge-forming reaction of CYP719. Our study as well as a previous report showed that methylenedioxy bridge-forming enzymes such as CYP719, (S)-CHS, and (S)-stylopine synthase have low $K_m$ values (0.269, 0.9, and 0.4 $\mu$M, respectively) for their corresponding substrates and high substrate specificities (8), indicating that their substrate recognition is very strict and controls the biosynthetic pathway.

(S)-Scoulerine was not used as a substrate but showed inhibitory activity for CYP719 with a $K_i$ value of 12.1 $\mu$M. The SMT reaction has been shown to be inhibited by the end-product, berberine (12). Modification of the berberine/coptisine ratio by the ectopic expression of SMT in C. japonica cells suggested that the balance of SMT and (S)-CHS regulates metabolic flow (35). The accumulation of (S)-scoulerine induced
by berberine may inhibit CYP719 activity and regulate the flow of substrate to coptisine in vivo.

Along with the dynamic regulation of secondary metabolism, the evolution of these divergent P450s is an interesting subject. (S)-CHS catalyzes the cyclization of an ortho-methoxyphenol of (S)-scoulerine in ring D (9,10-position), not in ring A (2,3-position). On the other hand, CYP719 catalyzes the cyclization of an ortho-methoxyphenol of (S)-THC in ring A (2,3-position) but not ring D (9,10-position). Despite this difference, CYP719 and (S)-CHS recognize their own substrates based solely on whether or not (S)-THC has a 9-methoxy moiety. It is interesting that (S)-scoulerine was inhibitory for CYP719, whereas the \( K_v \) value (12.1 \( \mu \)M) was about 45 times higher than the \( K_v \) value (0.269 \( \mu \)M) for (S)-THC. CYP719 and (S)-CHS might have a similar ancestral origin.

Isolation of the remaining methylenedioxy bridge-forming enzyme cDNAs in coptisine biosynthesis will promote our understanding of P450 evolution in the isoquinoline alkaloid biosynthetic pathway. Recently, we isolated the cDNA of (S)-adenosyl-l-methionine:columbamine O-methyltransferase using an expressed sequence tag library prepared from cultured C. japonica cells, whereas the biosynthetic activities of palmitine and other berberine-related alkaloids were relatively low in our cultured C. japonica cells. This success suggests that our expressed sequence tag library could be useful for isolating other isoquinoline biosynthetic enzymes including methylenedioxy bridge-forming P450s in coptisine biosynthesis. Further characterization has identified some expressed sequence tag clones, which showed some sequence similarity to plant P450 cDNA sequences different from those for CYP719 and CYP80B2. The isolation of full-length P450 cDNAs and their further characterization should add to our understanding of the structure/function relationship and evolution of P450s in isoquinoline alkaloid biosynthesis.

Acknowledgments—We thank the Herbal Garden of Takeda Chemical Industries Ltd. for the kind gift of plant materials. We also thank Dr. N. Nagakura, Dr. Y. Sugimoto, and Mitsu Petrochemical Industries Ltd. for their generous gifts of the alkaloids. We are grateful to L. Huang for technical assistance with preparing columbamine. We thank Dr. Y. Yabusaki of Sumitomo Chemical Co., Ltd. for providing the expression vector, pGyr. We also thank Dr. Y. Aoyama of Sohka University for providing ketoconazole.

REFERENCES

Molecular Cloning and Characterization of CYP719, a Methyleneedioxy Bridge-forming Enzyme That Belongs to a Novel P450 Family, from cultured Coptis japonica Cells

Nobuhiro Ikezawa, Masaru Tanaka, Masanori Nagayoshi, Raku Shinkyo, Toshiyuki Sakaki, Kuniyo Inouye and Fumihiko Sato

doi: 10.1074/jbc.M302470200 originally published online May 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302470200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 7 of which can be accessed free at http://www.jbc.org/content/278/40/38557.full.html#ref-list-1