Archives of Biochemistry and Biophysics 507 (2011) 186–193

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

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

Characterization of two methylenedioxy bridge-forming cytochrome P450-dependent enzymes of alkaloid formation in the Mexican prickly poppy *Argemone mexicana* ☆

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ARTICLE INFO

Article history:
Available online 20 November 2010

Keywords:
Cytochrome P450
Benzophenanthridine alkaloid
Mexican prickly poppy
Argemone mexicana
CYP719A

ABSTRACT

Formation of the methylenedioxy bridge is an integral step in the biosynthesis of benzo[*c*]phenanthridine and protoberberine alkaloids in the Papaveraceae family of plants. This reaction in plants is catalyzed by cytochrome P450-dependent enzymes. Two cDNAs that encode cytochrome P450 enzymes belonging to the CYP719 family were identified upon interrogation of an EST dataset prepared from 2-month-old plantlets of the Mexican prickly poppy *Argemone mexicana* that accumulated the benzo[*c*]phenanthridine alkaloid sanguinarine and the protoberberine alkaloid berberine. CYP719A13 and CYP719A14 are 58% identical to each other and 77% and 60% identical, respectively, to stylopine synthase CYP719A2 of benzo[*c*]phenanthridine biosynthesis in *Eschscholzia californica*. Functional heterologous expression of *CYP719A14* and *CYP719A13* in *Spodoptera frugiperda* Sf9 cells produced recombinant enzymes that catalyzed the formation of the methylenedioxy bridge of (*S*)-cheilanthifoline from (*S*)-scoulerine and of (*S*)-stylopine from (*S*)-cheilanthifoline, respectively. Twenty-seven potential substrates were tested with each enzyme. Whereas CYP719A14 transformed only (*S*)-scoulerine to (*S*)-cheilanthifoline (K_m 1.9 ± 0.3; k_{cat}/K_m 1.7), CYP719A13 converted (*S*)-tetrahydrocolumbamine to (*S*)-canadine (K_m 2.7 ± 1.3; k_{cat}/K_m 12.8), (*S*)-cheilanthifoline to (*S*)-stylopine (K_m 5.2 ± 3.0; k_{cat}/K_m 2.6) and (*S*)-scoulerine to (*S*)-nandinine (K_m 8.1 ± 1.9; k_{cat}/K_m 0.7). These results indicate that although CYP719A14 participates in only sanguinarine biosynthesis, CYP719A13 can be involved in both sanguinarine *and* berberine formation in *A. mexicana*.

Introduction

Berberine and sanguinarine are quaternary alkaloids that belong to the protoberberine- and benzo[*c*]phenanthridine class of alkaloids, respectively. Each has antibacterial properties [1,2] that are commercially exploited. Berberine has been used against eye infection in Asia [3]; sanguinarine has been used against oral bacterial plaque formation in Europe and America [4]. Investigations into new potential uses of each alkaloid in the treatment of infection and cancer indicate that interest in these plant natural products remains high [5,6].

The two classes of alkaloids accumulate broadly in species of the Berberidaceae, Ranunculaceae and Papaveraceae. The Mexican prickly poppy *Argemone mexicana*, a traditional plant used in native medicine in Mexico, produces both berberine and sanguinarine. Derived from L-tyrosine, the immediate biosynthetic precursor of protoberberine and benzo[*c*]phenanthridine alkaloids is (*S*)-reticuline (Fig. 1) [7]. Conversion of (*S*)-reticuline into berberine and sanguinarine requires the formation of methylenedioxy bridges from the guaiacol portion of the benzyl- and/or isoquinoline moieties of the carbon skeleton. Whereas the conversion of (*S*)-reticuline into berberine results in the formation of the one methylenedioxy bridge of (*S*)-canadine from (*S*)-tetrahydrocolumbamine, sanguinarine results from (*S*)-reticuline after two methylenedioxy bridges have been introduced into the molecule.

Formation of these methylenedioxy bridges has been shown to be catalyzed by cytochrome P450-dependent enzymes [8,9] and the stereochemistry of formation has been studied in detail [10]. Successful isolation of a cDNA encoding the cytochrome P450 that

☆ The sequences reported herein have been deposited in the GenBank with the Accession Codes EF451152 (CYP719A14) and EF451151 (CYP719A13).

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doi:10.1016/j.abb.2010.11.016

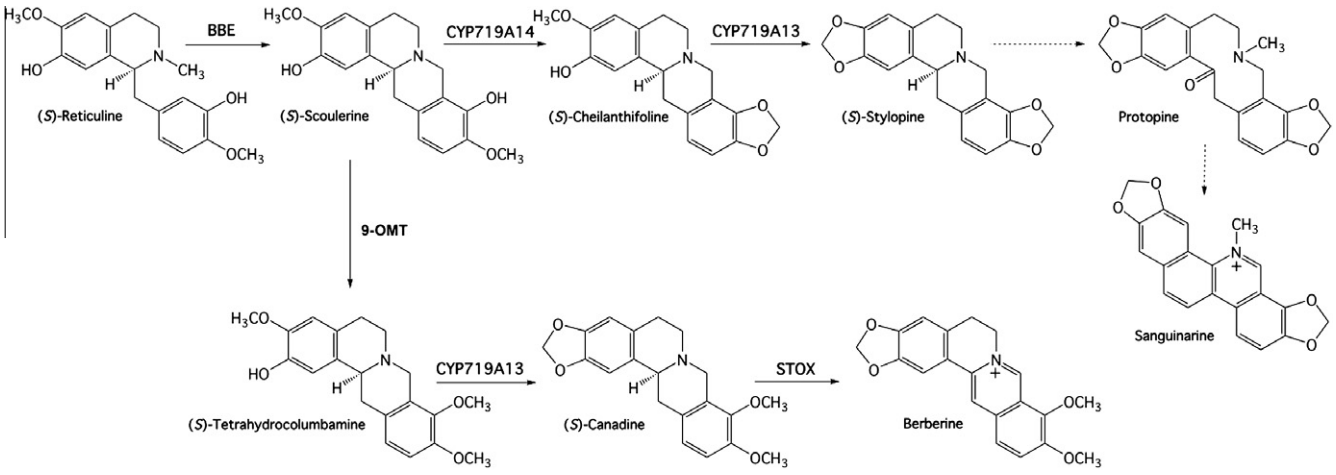

Fig. 1. Schematic presentation of the biosynthetic pathway leading from (*S*)-reticuline to sanguinarine and to berberine in the Mexican prickly poppy *A. mexicana*. Along the biosynthetic pathway to sanguinarine, the berberine bridge enzyme (BBE) oxidatively cyclizes the *N*-methyl group of (*S*)-reticuline into the berberine bridge carbon of (*S*)-scoulerine. CYP719A14 forms the first methylenedioxy bridge of (*S*)-cheilanthifoline from (*S*)-scoulerine. CYP719A13 then forms the second methylenedioxy bridge of (*S*)-stylopine from (*S*)-cheilanthifoline. Along the biosynthetic pathway to berberine, CYP719A13 catalyzes the formation of the methylenedioxy bridge of (*S*)-canadine from (*S*)-tetrahydrocolumbamine. 9-OMT, scoulerine 9-*O*-methyltransferase; STOX, (*S*)-tetrahydroprotoberberine oxidase.

converts (*S*)-tetrahydrocolumbamine to (*S*)-canadine in *Coptis japonica* revealed that this enzyme defined the CYP719A class of cytochromes [11]. Subsequent characterization indicated that CYP719A2 catalyzed the formation of (*S*)-stylopine from (*S*)-cheilanthifoline in the California poppy *Eschscholzia californica* [12]. In a likely mechanistically related reaction, CYP719B1 was found to catalyze formation of the C–C phenol couple in morphine biosynthesis in the opium poppy *Papaver somniferum* [13].

Investigation of the CYP719 family in *A. mexicana* was undertaken to establish the relationship between berberine and sanguinarine biosynthesis in a single species. An EST dataset prepared from 2-month-old plants of *A. mexicana* that accumulated both berberine and sanguinarine was queried for candidate cDNAs homologous to *CYP719* family members. CYP719A13 and CYP719A14 were identified and further characterized.

Materials and methods

Alkaloid extraction and analysis

In a pre-chilled mortar and pestle, 0.5 g plant tissue were ground to a fine powder in liquid nitrogen and transferred to a 2 ml tube. Alkaloids were extracted with 1 ml 80% (v/v) EtOH with shaking for 30 min. Cellular debris was removed by centrifugation at 14,000*g* for 5 min at room temperature. After evaporation of the solvent under nitrogen, the resulting extract was dissolved in 1 ml H_2O and the pH was adjusted to 9.0 with 1 M Na_2CO_3. Alkaloids were extracted twice with 500 µl ethylacetate and chloroform and the organic phase evaporated under nitrogen. The extracts were then dissolved in 100 µl 70% (v/v) EtOH and analyzed by HPLC or LC–MS.

HPLC sample analysis was performed using an LC 1100 series Agilent system equipped with a LiChrospher 60 RP-select B column with a flow rate of 1.0 ml/min and wavelength detection at 210, 255 and 285 nm. The solvent systems were: solvent A CH_3CN–H_2O (2:98; v/v) and solvent B CH_3CN–H_2O (98:2; v/v); 0.01% (v/v) H_3PO_4 was present in both solvents. The gradient increased from 0–46% B from 0 to 25 min, 46–60% B from 25 to 30 min, 60% B from 30 to 35 min, 60–100% B from 35 to 37 min and was held at 100% B for 3 min.

Alkaloid LC–MS analysis was carried out on a Mariner TOF mass spectrometer equipped with a Turbulon Spray source using a LC 1100 series Agilent system and a Superspher 60 RP-select B column. A flow rate of 0.2 ml/min was used with the following solvent and gradient system: solvent A CH_3CN–H_2O (2:98; v/v) and solvent B CH_3CN–H_2O (98:2; v/v); 0.2% (v/v) formic acid was present in both solvents. The gradient increased from 0% to 46% B in 25 min, to 90% in 1 min and was held at 90% B for 7 min.

EST database preparation

RNA was isolated from 2-month-old *A. mexicana* L. plants grown in a greenhouse facility without climate control in Saxony-Anhalt, Germany, using the Trizol method of Chomczynski and Sacchi [14]. cDNA was prepared from poly (A)+ RNA using a ZAP express™ cDNA Synthesis Kit (Stratagene, La Jolla, CA) and was packaged into phage lambda with Stratagene's Gigapack III gold packaging extract (Stratagene). Randomly selected cDNAs from *A. mexicana* were sequenced using the T3 primer and a Big-Dye Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) in an automated DNA sequencer ABI 3100 Avant Genetic Analyzer. The EST sequences were clustered and assembled using the SeqMan™ II (DNASTAR Inc., Madison, WI, USA) application with a minimum match size of 20 bp and a minimum match percentage of 80. Clusters were compared with the non-redundant database at the National Center for Biotechnology Information (NCBI) using the BLASTX algorithm with default parameters.

cDNA isolation, construction of viral expression vectors and generation of recombinant baculovirus

CYP719A13 and CYP719A14 full length cDNA were generated by RT-PCR using mRNA derived from *A. mexicana* roots with gene-specific primers: CYP719A13f: (5′-ATGGAGGAAAAAATCAT GACTAAC-3′), CYP719A13r: (5′-TTACATACGAGGAACAATACCAG-3′), CYP719A14f: (5′-ATGGATGAGACTATTTGGTTAATAATTA-3′) and CYP719A14r: (5′-TCAATGGATGCGACAAGTA-3′). Amplification was achieved with *Pfu* Hotstart polymerase (Stratagene) using the following cycle parameters: 2 min at 95 °C, succeeded by 30 cycles of amplification (30 s at 95 °C, 30 s at 52 °C, 2 min 30 s at 72 °C), and a final extension of 5 min at 72 °C in a total reaction volume of 50 µl. For subcloning into transfection vectors, cDNAs were excised from pCR2.1 vectors (Invitrogen, Carlsbad, CA, USA) with *Not* I and *Bam* HI enzymes and inserted into the equivalent

sites of the transfer vector pVL1392 (BD Bioscience, Franklin Lakes, NJ, USA). Recombinant baculovirus was generated by cotransfection with linearized baculovirus DNA using the BaculoGold Transfection Kit (BD Biosciences) and *Spodoptera frugiperda* Sf9 cells according to the manufacturer's instructions. Viral amplification proceeded according to Ref. [13]. Sf9 cells were propagated in TC-100 medium (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT, USA) and were regularly maintained as previously described [15]. Full length berberine bridge enzyme cDNA from *E. californica* (in bacterial vector pUC19) was generated by PCR with the following primers that incorporated *Pst* I and *Bam* HI restriction sites into the 5′- and 3′-ends (respectively): forward (5′-GGGCTGCAGATGGAAAACAAAA CTCCCATC-3′) and reverse (5′-GGGGGATCCCTATATTACAACTTCTCC ACC-3′). Amplification was achieved with *Pfu* Hotstart polymerase using the following cycle parameters: 2 min at 95 °C, succeeded by 30 cycles of amplification (30 s at 95 °C, 30 s at 52 °C, 2 min at 72 °C), and a final extension of 5 min at 72 °C in a total reaction volume of 50 μl. Recombination in baculovirus and viral amplification was identical to CYP719A13 and CYP719A14.

Heterologous expression in S. frugiperda Sf9 cells

Sf9 cells were grown in 50 ml of suspension media (TC-100 supplemented with 10% fetal bovine serum and 0.1% pluronic) to a density of 2×10^6 cells/ml (27 °C, 140 rpm). Cells were collected by centrifugation (900*g* for 10 min at room temperature), resuspended in 7.5 ml of suspension media and moved to a sterile 50 ml flask with foam plug. For double infections, 1.25 ml (MOI 5) of virus containing *CYP719A13* or *CYP719A14* and 1.25 ml (MOI 5) of virus containing full-length *E. californica* cytochrome P450 reductase (*CPR*)[3] were added. Each P450 was also coexpressed with petunia *CPR* and *Arabidopsis thaliana CPR* as previously described [13]. For triple infections, the 7.5 ml of cells were supplemented with 0.83 ml (MOI 3.3) of each recombinant virus (*CYP719A13*, *CYP719A14*, and *CPR*). Quadruple infections were carried out by the addition of 0.625 ml (MOI 2.5) of virus containing *BBE1* (berberine bridge enzyme), *CYP719A13*, *CYP719A14*, and *CPR*. Protein collection for each infection proceeded as previously described [13]. For large scale infection of cells coexpressing CYP719A13 or CYP719A14 and CPR, Sf9 cells were grown in 750 ml of suspension media in a 1 L flask to a density of 2×10^6 cells/ml (27 °C, 140 rpm). Infection proceeded as described above with viral volumes proportional to the culture volume.

Microsome preparation and reduced carbon monoxide difference spectrum

Cells coexpressing *CYP719A13* or *CYP719A14* and *CPR* were harvested from a large scale infection by centrifugation (1000*g* for 5 min at room temperature) and washed twice with 40 ml of 1× PBS buffer (130 mM NaCl, 3 mM NaH_2PO_4, 7 mM Na_2HPO_4). The pellet was resuspended in 40 ml of ice-cold suspension buffer. Half of the cells were immediately frozen in liquid nitrogen and placed in −80 °C and the remaining 20 ml were subjected to sonication (Fisher Scientific Sonic Dismembrator Model 100; four times on ice, 10× pulsed, 50 W). The remainder of each microsome preparation and reduced CO difference spectrum assays were carried out exactly according to Refs. [13,16] using a Cary 300 UV–Visible spectrophotometer. The resulting spectrum was used to determine microsomal P450 protein content using an extinction coefficient of 91 cm^{-1} mM^{-1} (data not shown). Cytochrome P450 protein content in whole cells used for enzyme assays was calculated from a comparison of enzyme activity obtained with hypotonically lysed whole cells and microsomes for which the cytochrome P450 content had been measured.

Enzyme assays

Triple infections that resulted in the production of CYP719A13, CYP719A14 and CPR were done to initially screen large numbers of substrates for enzymatic conversion by either cytochrome P450. The screen was done in this manner in order to conserve on substrate used, since many of these chemicals are not commercially available. Double infections were then carried out to produce CYP719A13 and CPR, and CYP719A14 and CPR for kinetic analysis. The quadruple infection was a readily achievable test for channeling in the pathway. Standard assay conditions contained 30 mM potassium phosphate buffer (pH 8.0), 1.25 mM NADPH, 5 μM substrate, and 70 μl of Sf9 cell suspension in a final volume of 200 μl. Control assays were run alongside each reaction without NADPH and without enzyme. Reactions incubated for 2 h at 30 °C and were terminated by the addition of 400 μl sodium carbonate buffer (pH 9.5) and 400 μl chloroform followed by rapid vortexing for 1 min and centrifugation (2 min at 13,000 rpm). Extraction was repeated on the organic layer once and dried with N_2. The final product was dissolved in 200 μl of 80% methanol.

Kinetic constants for CYP719A13 were determined for (*S*)-cheilanthifoline, (*S*)-scoulerine, and (*S*)-tetrahydrocolumbamine and (*S*)-scoulerine for CYP719A14 using similar assay conditions as above with the following amendments. The reaction mixtures with CYP719A14 contained 70 μl of cell suspension (containing 6.6–25.7 pmol cytochrome P450 protein) and were stopped after 15 min. Assays with CYP719A13 contained 35 μl of cell suspension (containing 1.9–6.0 pmol cytochrome P450 protein) and were stopped after 10 min. All reactions contained increasing substrate concentrations (0, 0.5, 1, 5, 10, 15, 20, 30, and 50 μM), proceeded at 30 °C and were terminated by freezing in liquid nitrogen followed by chloroform extraction (described above). The enzymatic products were quantified by LC–MS/MS analysis and the kinetic parameters (K_m and V_{max}) were estimated by non-linear regression with GraphPad Prism in three independent experiments. Due to high variation in protein content between different viral infections, kinetic data were normalized using protein concentration determined from CO difference spectrum assays. Conversion rates of product formation for CYP719A13 were determined for (*S*)-scoulerine, (*S*)-tetrahydrocolumbamine, and (*S*)-coreximine using the conversion of (*S*)-cheilanthifoline to (*S*)-stylopine as 100% with reaction conditions stated above.

LC–MS/MS analysis of enzyme assays

Product identification and alkaloid quantitation was performed using LC–MS/MS with Analyst 1.4.2 on a 4000 QTrap (AB Sciex Instruments) for mass spectroscopic analysis with chromatographic separation on an LC-20AD (Shimadzu). Program parameters included a TurboIonSpray ionization source temperature of 500 °C and low resolution for Q1 and Q3 done with EPI and MRM scans in the positive ion mode. Fragmentation patterns for (*S*)-scoulerine, (*S*)-cheilanthifoline, and (*S*)-stylopine were identified with EPI scans for *m/z* 328, *m/z* 326, and *m/z* 324 ions, respectively. Similar programs for (*S*)-coreximine, (*S*)-coreximine product, (*S*)-tetrahydrocolumbamine, (*S*)-canadine, and (*S*)-nandinine were identified with EPI scans for *m/z* 328, *m/z* 326, *m/z* 342, *m/z* 340, and *m/z* 326, respectively. Specific parameters for each method

[3] *Abbreviations used:* CPR, cytochrome P450 reductase; RT-PCR, reverse transcriptase-polymerase chain reaction; PCR, polymerase chain reaction; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MRM, multiple reaction monitoring; EPI, enhanced product ion; BBE, berberine bridge enzyme; MOI, multiplicity of infection.

can be found in Table S1. EPI scans were eliminated during quantitation to increase sensitivity and accuracy.

All samples were diluted 50-fold with the exception of assays containing (*S*)-coreximine that were diluted 10-fold prior to a 10 μl injection onto a Phenomenex Gemini C6-phenyl (150 × 2.00 mm, 5 μm) column with a flow rate of 0.6 ml/min. Alkaloid separation was performed with a solvent system consisting of A (40% CH_3CN/2.5 mM NH_4OH/5 mM ammonium acetate) and B (90% CH_3CN/15 mM NH_4OH/10 mM ammonium acetate) with a gradient of 0–1.0 min 0% solvent B, 1.0–5.0 min 0–100% solvent B, 5.0–7.0 min 100% solvent B, and 100–0% solvent B from 7.0 to 8.0 min and held at 0% B for an additional 2 min.

Retention times for each compound were as follows: (*S*)-scoulerine: 2.00 min; *m/z* 328 $[M+H]^+$, (*S*)-cheilanthifoline: 3.00 min; *m/z* 326 $[M+H]^+$, (*S*)-stylopine: 4.05 min; *m/z* 324 $[M+H]^+$, (*S*)-coreximine: 1.70 min; *m/z* 328 $[M+H]^+$, (*S*)-coreximine product: 2.85 min; *m/z* 326 $[M+H]^+$, (*S*)-tetrahydrocolumbamine: 2.80 min; *m/z* 342 $[M+H]^+$, (*S*)-canadine: 3.8 min; *m/z* 340 $[M+H]^+$, and (*S*)-nandinine: 3.25 min; *m/z* 326 $[M+H]^+$. Authentic alkaloids were used for compound identification by comparison of retention times and fragmentation patterns and quantitation by plotting peak area versus pmol standard. Unknown products were quantitated by comparing the compound with the most similar fragmentation pattern [(*S*)-stylopine]. Monitored mass transitions used in quantification for (*S*)-scoulerine and (*S*)-coreximine were: *m/z* 328 to *m/z* 178 $[M+H]^+$, (*S*)-cheilanthifoline: *m/z* 326 to *m/z* 178 $[M+H]^+$, (*S*)-stylopine: *m/z* 324 to *m/z* 176 $[M+H]^+$, (*S*)-coreximine product: *m/z* 326 to *m/z* 176 $[M+H]^+$, (*S*)-tetrahydrocolumbamine: *m/z* 342 to *m/z* 178 $[M+H]^+$, (*S*)-canadine: *m/z* 340 to *m/z* 176 $[M+H]^+$, and (*S*)-nandinine: *m/z* 328 to *m/z* 176 $[M+H]^+$.

HPLC analysis of enzyme assays

HPLC detection was used for several compounds (guaiacol, (*R*)-4′-*O*-methyllaudanosoline, vanillin, 6-*O*-methyl-*N*-deacetylipecoside, and 7-*O*-methyl-*N*-deacetylipecoside) when testing for possible enzyme activity on a Hitachi D700 system. Samples were injected onto a Nova Pak C-18 column (3.9 × 300 mm, 4 μm, Waters) with a flow rate of 0.8 ml/min and the following solvent system: A (0.1% trifluoroacetic acid) and B (CH_3CN) where the gradient increased from 0.0 to 21.0 min 20–48% solvent B. Compounds were detected spectrophotometrically using an absorbance of 230 nm.

Results and discussion

Argemone mexicana contains homologs of CYP719A1

Two-month-old greenhouse grown plants of *A. mexicana* were extracted in 80% (v/v) ethanol and analyzed for alkaloid content by LC–MS. The extracts were found to contain reticuline, protopine, allocryptopine, cryptopine, berberine, chelerythrine and sanguinarine. Except for sanguinarine that was found mainly in root, the remaining alkaloids were detected in leaf, stem, capsule, latex and root. The sanguinarine biosynthetic precursor protopine (Fig. 1) and allocryptopine, which contain methylenedioxy bridges, were the main alkaloids accumulated in the plants and were therefore quantitated by HPLC in a time course analysis of developing plants. Protopine and allocryptopine were present in leaf (Fig. 2A) and root (Fig. 2B) tissue in 3-week-old plants. Levels of allocryptopine then decreased slightly in leaf, but increased in root. Protopine continued to accumulate in leaf up to 12 weeks. Since increasing amounts of protopine and allocryptopine were indicative of alkaloid metabolizing enzyme activity and alkaloid quantities were higher in root than in leaf tissue, roots from 3- to 4-week-old plants were used for RNA isolation and cDNA library preparation.

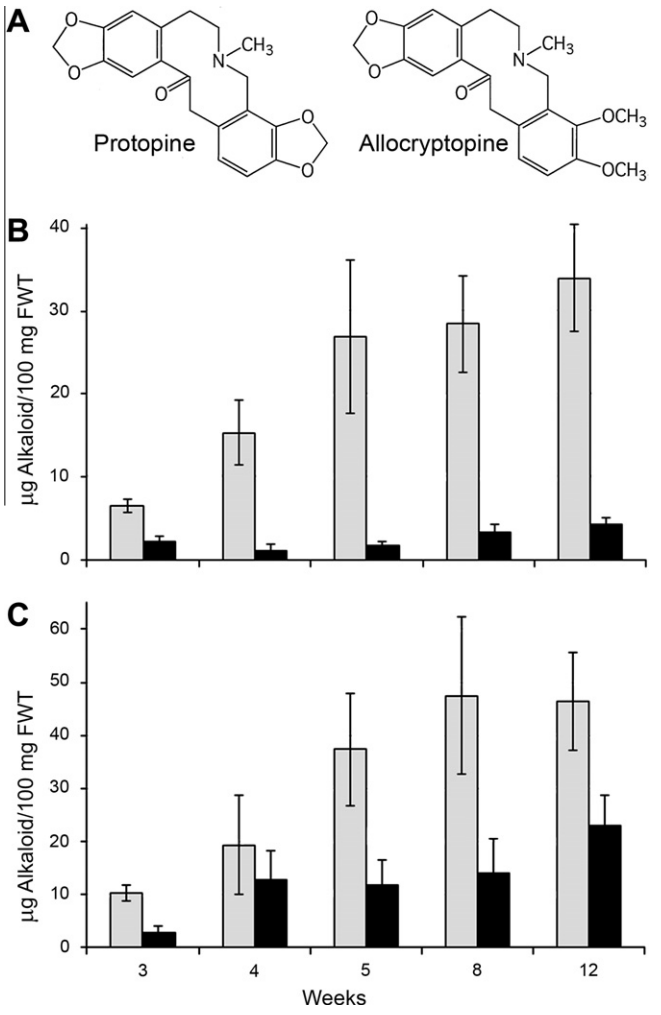

Fig. 2. Quantification of (A) protopine and allocryptopine in extracts of (B) leaf and (C) root of developing *A. mexicana* plants. Fresh tissue samples (0.5 g) were extracted with ethanol and were analyzed by HPLC. Grey bar, protopine; black bar, allocryptopine.

The directed *A. mexicana* root cDNA library in phage λ contained approximately 160,000 clones. The insert size of randomly picked clones ranged from about 0.5 to 3.0 kb with an average of 0.7 kb. Randomly selected cDNA clones were sequenced from the 5′-end with standard T3 primer to avoid sequencing 3′ untranslated regions that tend to have less cross-species conservation than do coding sequences. Approximately 1600 expressed sequence tags (ESTs) were generated. The sequences generated were analyzed with the SeqMan program (DNASTAR); clones with more than 80% identity for more than 20 bp were grouped together. Leading vector or poor quality sequences were removed from each file. Each clustered EST was compared with the non-redundant database at the NCBI. Putative identification of the ESTs was assigned based on the annotations of genes with similarities to those ESTs. Sequences deemed to be of phage origin were removed from the collection. After screening and editing, 1255 independent ESTs were generated. The average length of the EST clusters was 486 bp. Forty-three percent of the independent ESTs had significant homology to sequences of known genes, 36% displayed homology to uncharacterized or hypothetical proteins and 21% displayed no significant homology to known or hypothetical proteins.

ESTs were classified into seven different functional categories according to the top match against the Munich Information Center for Protein Sequences (MIPS) protein entry codes. The two largest

predominant categories of *A. mexicana* ESTs according to this functional categorization were: metabolism (30%) and cell cycle, DNA processing and protein synthesis (29%). From the ESTs involved in metabolism, 28% were related to secondary pathways such as phenylpropanoid, flavonoid and alkaloid biosynthesis. Among them, ESTs similar to proteins presumably involved in the biosynthesis of alkaloids were the most representative with 21 unique sequences representing 13% of this category. Seven encoded functionally characterized enzymes of alkaloid biosynthesis, which included norcoclaurine synthase, (*S*)-adenosyl-L-methionine: norcoclaurine 6-*O*-methyltransferase, (*S*)-adenosyl-L-methionine: coclaurine *N*-methyltransferase, (*S*)-*N*-methylcoclaurine 3′-hydroxlyase, (*S*)-adenosyl-L-methionine: 3′-hydroxy-*N*-methylcoclaurine 4′-*O*-methyltransferase, berberine bridge enzyme and (*S*)-adenosyl-L-methionine: scoulerine 9-*O*-methyltransferase [7]. In addition, two of these unique sequences were homologous to the methylenedioxy bridge-forming enzyme CYP719A1 canadine synthase from *C. japonica* [11].

The open reading frame from each CYP719 homolog was sequenced. The nucleotide and predicted amino acid sequence of both cytochrome CYP719 encoding cDNAs were analyzed using BLASTX and MegAlign software with CLUSTALW algorithms. CYP719A13 possessed an open reading frame of 1515 bp encoding for a putative enzyme of 505 amino acids with a predicted molecular mass of 57.45 kDa. The second cytochrome isolated, CYP719A14, contained and open reading frame of 1485 bp encoding for a putative protein of 495 amino acids with a predicted molecular mass of 55.7 kDa. Analysis of both cDNAs with the SignalP 3.0 software indicated the presence of an N-terminal signal peptide (the first 35 amino acids in CYP719A13 and the first 24 amino acids in CYP719A14) necessary for anchoring the cytochromes in the endoplasmic reticulum. The predicted amino acid sequences displayed 58.2% identity between CYP719A13 and CYP719A14 and 65.2% and 62.1% homology to the cytochrome CYP719A1 canadine synthase, respectively. CYP719A14 clusters on a phylogenetic tree mostly closely with CYP719A5 ((*S*)-cheilanthifoline synthase) of *E. californica* (Fig. 3). CYP719A13 is more distantly related to CYP719A2 and CYP719A3 ((*S*)-stylopine synthase) and CYP719A9 (forming a methylenedioxy bridge on (*R*,*S*)-reticuline) of *E. californica*.

CYP719A13 and CYP719A14 are methylenedioxy bridge-forming enzymes

The reading frames of *CYP719A13* and *CYP719A14* were generated by PCR amplification out of pCR2.1 and were ligated into the baculovirus transfer vector pVL1392. An insect cell culture/baculovirus heterologous expression system was chosen because a number of alkaloid biosynthetic cytochrome P450-dependent enzymes have been successfully functionally expressed in this manner in our hands (CYP80A1, [17]; CYP80B1, [18]; CYP719B1, [13]). Selected cytochrome P450-dependent enzymes can require the presence of a plant cytochrome P450 reductase (CPR) for either optimal enzyme activity, such as (*S*)-*N*-methylcoclaurine 3′-hydroxylase (CYP80B1) from the California poppy *E. californica* [18], or for activity *per se*, such as (*S*)-*N*-methylcoclaurine 3′-hydroxylase CYP80B3 [19] and salutaridine synthase CYP719B1 [13], both from the opium poppy *P. somniferum*. To provide a plant cytochrome P450 reductase to the enzyme assays, the *CPR* cDNA from *E. californica* ([20]; Accession No. O24425), *A. thaliana* ([21]; Accession No. X66016) and petunia (Accession No. DQ099545) were also individually introduced into pVL1392. *CYP719A13*, *CYP719A14* and *CPR* in pVL1392 were brought into the linearized baculovirus by homologous recombination and the *S. frugiperda* Sf9 cells were then transfected with the viruses in various combinations. After several rounds of virus amplification [15], recombinant CYP719A13 and CYP719A14 were tested for enzyme activity. Sf9 cells were infected with baculovirus containing either

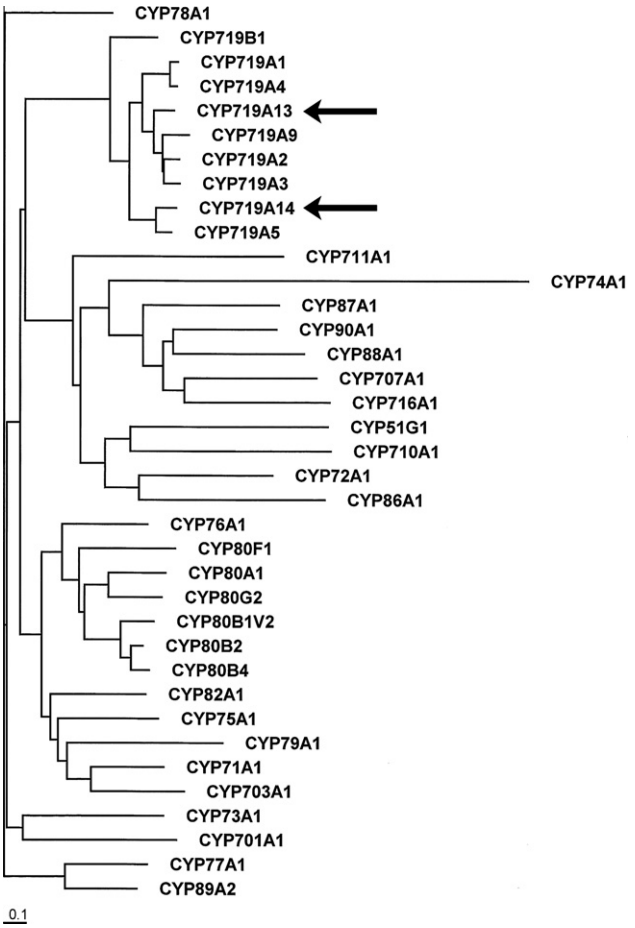

Fig. 3. Phylogenetic comparison of selected plant cytochrome P450-dependent enzymes. Amino acid sequences used for the tree were obtained from GenBank or SwissProt with the following Accession Nos. AB014459, CYP51G1 (obtusifoliol 14-demethylase), *Arabidopsis thaliana*; AF212990, CYP701A1 (ent-kaurene oxidase), *Cucurbita maxima*; AB006790, CYP703A1 (lauric acid monooxygenase), petunia; NM_202845, CYP707A1 (Abscisic acid 8′-hydroxylase), *Arabidopsis thaliana*; M32885, CYP71A1, *Persea americana;* NM_129002, CYP710A1 (C22-sterol desaturase), *Arabidopsis thaliana*; NP_850074, CYP711A1, *Arabidopsis thaliana*; NM_123002, CYP716A1, *Arabidopsis thaliana*; AB026122, CYP719A1 (canadine synthase), *Coptis japonica*; AB126257, CYP719A2 and AB126256, CYP719A3 (stylopine synthase), *Eschscholzia californica*; AY610513, CYP719A4 (canadine synthase), *Thalictrum flavum*; AB434654, CYP719A5 (cheilanthifoline synthase), *Eschscholzia californica*; AB434655, CYP719A9 (accepts (*R*,*S*)-reticuline as substrate), *Eschscholzia californica*; EF451151, CYP719A13 (stylopine synthase), *Argemone mexicana*; EF451152, CYP719A14 (cheilanthifoline synthase), *Argemone mexicana*; EF451150, CYP719B1 (salutaridine synthase), *Papaver somniferum*; L10081, CYP72A1 (secologanin synthase), *Catharanthus roseus*; Z17369, CYP73A1 (cinnamate 4-hydroxylase), *Helianthus tuberosus*; U00428, CYP74A1 (allene oxide synthase), *Linum usitatissimum*; Z22545, CYP75A1 (flavonoid 3′,5′-hydroxylase), petunia; X71658, CYP76A1, *Solanum melongena*; X71656, CYP77A1 (hydroxylase), *Solanum melongena*; P48420, CYP78A1, *Zea mays*; U32624, CYP79A1 (tyrosine *N*-hydroxylase), *Sorghum bicolor*; U09610, CYP80A1 (berbamunine synthase), *Berberis stolonifera*; AF014801, CYP80B1V2 ((*S*)-*N*-methylcoclaurine 3′-hydroxylase), *Eschscholzia californica*; AB025030, CYP80B2 ((*S*)-*N*-methylcoclaurine 3′-hydroxylase), *Coptis japonica*; AY610509, CYP80B4 ((*S*)-*N*-methylcoclaurine 3′-hydroxylase), *Thalictrum flavum*; AB288053, CYP80G2 ((*S*)-corytuberine synthase), *Coptis japonica*; Q43068, CYP82A1, *Pisum sativum*; P48422, CYP86A1 (fatty acid omega-hydroxylase), *Arabidopsis thaliana*; AF216313, CYP87A1, *Helianthus annuus*; U32579, CYP88A1 (ent-kaurenoic acid oxidase), *Zea mays*; U61231, CYP89A2, *Arabidopsis thaliana*; Q42569, CYP90A1 (6-oxo-cathasterone 23a-hydroxylase), *Arabidopsis thaliana*. The alignment was obtained using CLUSTALW (version 1.83) based on the neighbor-joining method; TreeView (version 1.6.6; Yves van de Peer, University of Constance, Germany) was used to generate the image of the phylogenetic tree. A 10% change is indicated by the scale bar. Arrows point to the enzymes CYP719A13 and CYP719A14 that are the topic of this study.

CYP719A13 or *CYP719A14* alone, *CPR* alone or a 1:1 mixture of *CYP719A13* or *CYP719A14* and *CPR* as was performed for CYP80B1 from *E. californica* [18].

Microsomes were isolated from Sf9 cells coinfected with baculoviruses containing *CYP719A13* or *CYP719A14* and *E. californica CPR* and the reduced CO difference spectrum of the microsomes was measured (data not shown). Quantitation of recombinant CYP719A13 and CYP719A14 as determined by reduced CO difference spectrum determined the amount of cytochrome to be 264 and 512 nmol/750 ml Sf9 cell culture, respectively. Since the enzymes were efficiently expressed in the Sf9 cells, the cells were hypotonically lysed and directly used for enzymes assays. Twenty-seven alkaloidal and non-alkaloidal compounds were tested as substrate for methylenedioxy bridge formation (Table 1). The chemical structures are given in Fig. S1. Whereas CYP719A14 transformed only (*S*)-scoulerine to (*S*)-cheilanthifoline (K_m 1.9 ± 0.3; k_{cat}/K_m 1.7), CYP719A13 converted (*S*)-tetrahydrocolumbamine to (*S*)-canadine (K_m 2.7 ± 1.3; k_{cat}/K_m 12.8), (*S*)-cheilanthifoline to (*S*)-stylopine (K_m 5.2 ± 3.0; k_{cat}/K_m 2.6) and (*S*)-scoulerine to (*S*)-nandinine (K_m 8.1 ± 1.9; k_{cat}/K_m 0.7). These results importantly indicate that although CYP719A14 participates in only sanguinarine biosynthesis, CYP719A13 can be involved in both sanguinarine *and* berberine formation in *A. mexicana*, although knock-down experiments will be necessary to prove the dual function of this enzyme *in planta*. The robust activity of CYP719A13 with both (*S*)-cheilanthifoline and (*S*)-tetrahydrocolumbamine as substrate differs from that observed with the homologous enzyme from *E. californica* cell culture, which does not accumulate berberine [12]. All enzyme assays were analyzed by LC–MS/MS and the enzyme assay results obtained with (*S*)-scoulerine, (*S*)-cheilanthifoline and (*S*)-tetrahydrocolumbamine as substrate are shown in Fig. 4. Enzyme activity was obtained when coexpressed with either an *E. californica*, *Arabidopsis* or petunia CPR.

The pH optimum for CYP719A14 activity was determined by incubating cell extracts of insect cell expressing *CYP719A14* with (*S*)-scoulerine, over a range of pH from 6.0 to 10.0 (sodium phosphate buffer pH 6.0–8.5, tricine buffer pH 7.4–8.8 and glycine buffer pH 9.0–10.0). The pH optimum for CYP719A14 activity was 7.5 with half optimal activity at pH 6.5 and 8.5. The optimum temperature for CYP719A14 activity with (*S*)-scoulerine was observed between 30 and 35 °C. CYP719A13 activity was measured over a pH range from 6.0 to 10.0 using (*S*)-cheilanthifoline as substrate. Optimal enzyme activity was observed at pH 8.0 with half optimal activity at pH 6.5 and 9.0. The optimum temperature for CYP719A13 activity with (*S*)-cheilanthifoline was between 30 and 35 °C.

The full-length clones of *CYP719A13* and *CYP719A14* were used as hybridization probes for northern blot analysis using RNA isolated from different tissues of *A. mexicana*. Results displayed in Fig. 5 showed that the transcripts of both genes *CYP719A13* (Fig. 5A) and *CYP719A14* (Fig. 5B) were highly expressed in mature roots and to a lesser extent in stem, leaf and 3-week-old plantlets. This is in accordance with the fact that alkaloids mainly accumulated in the roots of the mature plant.

Table 1
Substrate specificity of recombinant CYP719A13 and CYP719A14. Enzyme assays were analyzed by LC–MS/MS. The chemical structures are provided in Fig. S1. The reaction mixtures with CYP719A14 contained 6.6–25.7 pmol cytochrome P450 protein. Assays with CYP719A13 contained 1.9–6.0 pmol cytochrome P450 protein.

Substrate	CYP719A13 k_{cat} [%]	CYP719A14 k_{cat} [%]
Isoquinoline alkaloids		
(*S*)-Cheilanthifoline	100[a]	nd[c]
(*S*)-Coclaurine	nd	nd
(*S*)-Coreximine	1	nd
(*R,S*)-Isococlaurine	nd	nd
(*R,S*)-Isoorientaline	nd	nd
(*R,S*)-*N*-Methylcoclaurine	nd	nd
(*R,S*)-4′-*O*-Methylcoclaurine	nd	nd
(*R,S*)-4′-*O*-Methyllaudanosoline	nd	nd
(*S*)-4′-*O*-Methyllaudanosoline	nd	nd
(*R,S*)-6-*O*-Methyllaudanosoline	nd	nd
(*R,S*)-Nororientaline	nd	nd
(*R*)-Norprotosinomenine	nd	nd
(*S*)-Norprotosinomenine	nd	nd
(*R*)-Norreticuline	nd	nd
(*S*)-Norreticuline	nd	nd
(*R,S*)-Protosinomenine	nd	nd
(*R*)-Reticuline	nd	nd
(*S*)-Reticuline	nd	nd
Salutaridine	nd	nd
Salutaridinol	nd	nd
(*S*)-Scoulerine	29	100[b]
(*S*)-Tetrahydrocolumbamine	135	nd
Terpenoid-isoquinoline alkaloids		
6-*O*-Methyl-*N*-deacetylipecoside	nd	nd
7-*O*-Methyl-*N*-deacetylipecoside	nd	nd
Simple phenols		
Guaiacol	nd	nd
Vanillic acid	nd	nd
Vanillin	nd	nd

[a] 100% activity is 13.8 min^{-1}.
[b] 100% activity is 3.3 min^{-1}.
[c] nd, not detected.

Conversion of (S)-reticuline to (S)-stylopine

The conversion of (*S*)-reticuline is a three-step process that requires the four enzymes the berberine bridge enzyme (BBE) [22], CYP719A14, CPR and CYP719A13. There is indication in plant natural product biosynthetic pathways that consecutive enzymes in a given pathway act in a coordinated fashion. In the 1970s, interaction between cucumber phenylalanine ammonia lyase and cinnamate 4-hydroxylase was suspected because of the metabolism of L-phenylalanine into phenolics in cotyledons [23]. Macromolecular organization of the enzymes of the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* that was suggested in 1980 [24] is today understood to be a channeled pathway that is catalyzed by two multifunctional cytochrome P450-dependent enzymes (CYP79A1 and CYP71E1) and a soluble UDP-glucosyltransferase (UGT85B1) that are associated into a macromolecular complex on the cytosolic side of the endoplasmic reticulum [25,26]. Evidence has also been obtained that two consecutive enzymes of morphine biosynthesis, salutaridine reductase and salutaridinol 7-*O*-acetyltransferase, interact in *P. somniferum* [27]. Since BBE, CYP719A14 and CYP719A13 act consecutively in *A. mexicana* in the formation of (*S*)-stylopine and are associated with the plant endomembrane, a quadruple infection (together with CPR) was undertaken in an attempt to generate assembled pathway enzymes, should such a complex exist. (*S*)-Reticuline was added to harvested insect cells that contained recombinant BBE, CYP719A14, CYP719A13 and CPR. Aliquots were removed up to 60 min and analyzed for the alkaloids (*S*)-reticuline, (*S*)-scoulerine, (*S*)-cheilanthifoline and the end product of this minipathway, (*S*)-stylopine by LC–MS/MS. As shown in Fig. 6, (*S*)-reticuline is immediately 100% converted to (*S*)-scoulerine. It is known that BBE is inhibited by the reaction product (*S*)-scoulerine [22]. Conversion rates of 50% were normally observed in single enzyme assays [28,29]. (*S*)-Scoulerine is depleted as (*S*)-cheilanthifoline accumulates. (*S*)-Stylopine accumulates with a lag phase that is much slower than the rapid accumulation of (*S*)-cheilanthifoline. If CYP719A14 and CYP719A13 did indeed interact, the observed accumulation of (*S*)-cheilanthifoline and (*S*)-nandinine would not be expected. In fact, only traces of (*S*)-nandinine were produced in this experiment. In addition, in this heterologous system, the ratios of the various enzymes have not been titrated; the results obtained herein may suggest an interaction of the biosynthetic enzymes *in planta*.

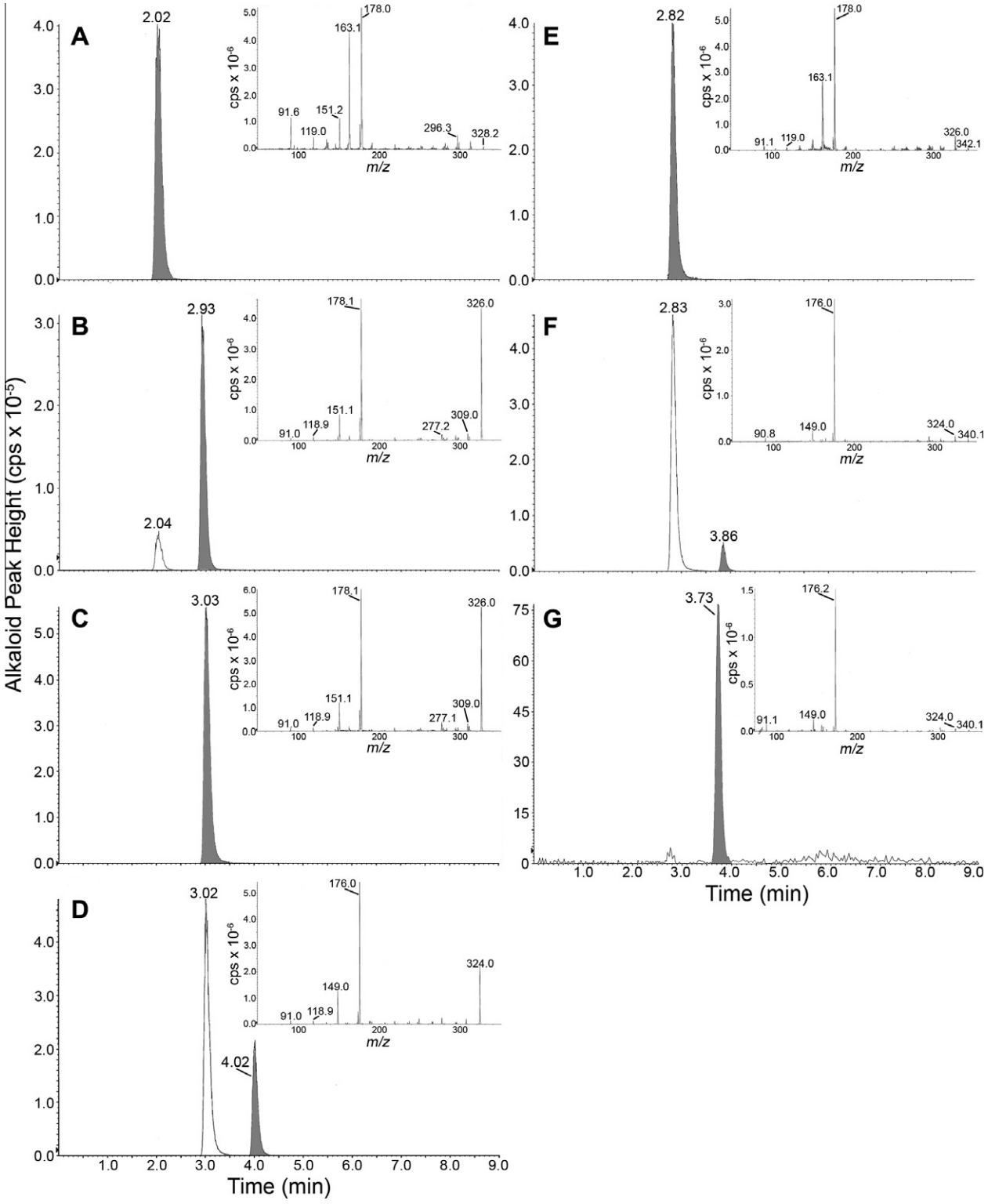

Fig. 4. Mass spectrometric analyses of reaction products produced by CYP719A13 and CYP719A14. MRM spectra (*m*/*z* 328 → 178 for (*S*)-scoulerine, *m*/*z* 326 → 178 for (*S*)-cheilanthifoline, *m*/*z* 324 → 176 for (*S*)-stylopine, *m*/*z* 342 → 178 for (*S*)-tetrahydrocolumbamine and *m*/*z* 340 → 176 for (*S*)-canadine) are shown. (A) (*S*)-Scoulerine no enzyme control; (B) product of the enzyme assay containing (*S*)-scoulerine and recombinant CYP719A14; (C) (*S*)-cheilanthifoline no enzyme control; (D) product of the enzyme assay containing (*S*)-cheilanthifoline and recombinant CYP719A13; (E) (*S*)-tetrahydrocolumbamine no enzyme control; (F) product of the enzyme assay containing (*S*)-tetrahydrocolumbamine and recombinant CYP719A13; (G) standard (*S*)-canadine. Insets, EPI spectra of shaded peaks.

Conclusions

An *A. mexicana* EST dataset was created from alkaloid-rich root tissue. Twenty-one unigenes with homology to alkaloid biosynthesis genes could be identified. A query of the EST dataset for CYP719A homologs resulted in the identification of two cytochrome P450-dependent enzymes that are sufficient to catalyze formation of all methylenedioxy bridges of the alkaloids berberine and sanguinarine in that plant species. One CYP719A14 was newly assigned as cheilanthifoline synthase, the other CYP719A13 is a

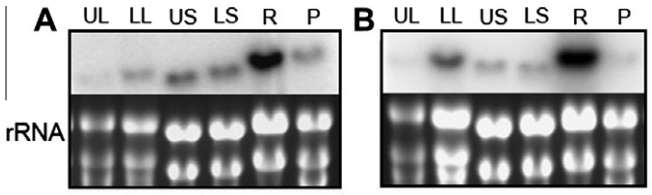

Fig. 5. RNA gel blot analysis of (A) *CYP719A13* and (B) *CYP719A14* gene transcript in tissue of *A. mexicana*. Total RNA (10 μg) was isolated from stem, leaf, root and whole 3-week-old plantlet. Gel blots were hybridized to radiolabeled *CYP719A13* or *CYP719A14* cDNA. Lanes: UL, upper leaf; LL, lower leaf; US, upper stem; LS, lower stem; R, root; P, plantlet.

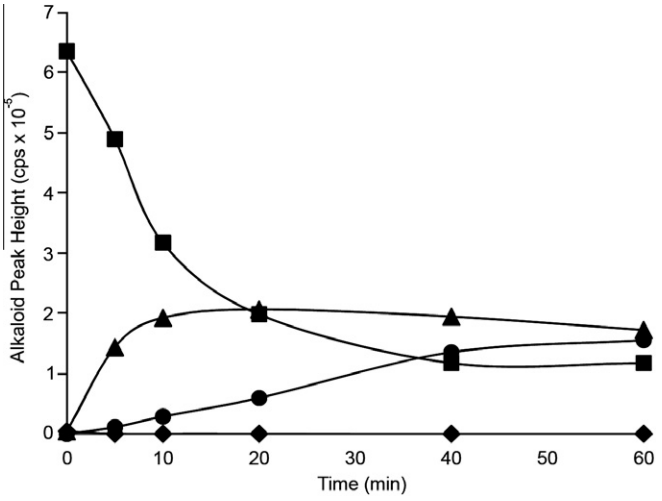

Fig. 6. Metabolism of insect cells that produced recombinant BBE, CYP719A14, CYP719A13 and CPR. (*S*)-Reticuline (5 μM) was added to harvested Sf9 cells and aliquots were removed for alkaloid analysis by LC–MS/MS. Diamond, (*S*)-reticuline; square, (*S*)-scoulerine; triangle, (*S*)-cheilanthifoline; circle, (*S*)-stylopine; cps, counts per second.

homolog of the *E. californica* stylopine synthase CYP719A2 [12]. The robust activity of CYP719A13 with either (*S*)-cheilanthifoline or (*S*)-tetrahydrocolumbamine as substrate suggests that CYP719 A13 participates in both sanguinarine and berberine biosynthesis in *A. mexicana*. Furthermore, CYP719A13 introduces a methylenedioxy bridge onto the isoquinoline moiety of (*S*)-scoulerine, albeit with lower activity than with either (*S*)-cheilanthifoline or (*S*)-tetrahydrocolumbamine. In contrast, CYP719A14 forms a methylenedioxy bridge exclusively on (*S*)-scoulerine.

Acknowledgments

The authors thank Dr. Leslie Hicks and Dr. Baichen Zhang of the Proteomics & Mass Spectrometry Facility of the Donald Danforth Plant Science Center and Dr. Jürgen Schmidt of the Institut für Pflanzenbiochemie, Halle (Saale), Germany for help with the mass spectrometric analyses. The authors also thank Dr. Jan Jaworski of the Donald Danforth Plant Science Center for the Arabidopsis and petunia *CPR* cDNAs. Alkaloids used in this study were from the natural product collection of Prof. M.H. Zenk of the Donald Danforth Plant Science Center. This work was supported by the Mallinckrodt Foundation, St. Louis (to T.M.K.) and by doctoral stipends (to M.L.D.C.) from the German Academic Exchange Service (DAAD), Bonn and Consejo Nacional de Ciencia y Tecnología (CONACyT) and Programa de Mejoramiento del Profesorado/Universidad Autónoma del Estado de Morelos (PROMEP/UAEM), Mexico.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.abb.2010.11.016.

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