

**Isolation and characterization of (*S*)-chelianthifoline synthase,
(*S*)-stylopine synthase and two FAD oxidases from a cDNA
library from *Argemone mexicana***



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Abbreviations

a.a.	Amino acid
AcNPV	<i>Autographa californica</i> nuclear polyhedrosis virus
Amp	Ampicillin
APS	Ammonium persulfate
BBE	Berberine bridge enzyme
bp	Base pair
BEVS	Baculovirus expression vector system
BPB	Bromophenol blue
BSA	Bovine serum albumin
cDNA	Complementary DNA
CIAP	Calf intestinal alkaline phosphatase
CYP450	Cytochrome P450
dNTP	2'-deoxynucleoside 5'-triphosphate
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
EtBr	Ethidium bromide
FBS	Foetal bovine serum
GSP	Gene specific primers
h	Hour
kb	Kilobases
kD	Kilodalton
LB	Luria-bertani (broth)
MCS	Multiple cloning site
min	Minute
MMLV-RT	Moloney murine leukemia virus-reverse transcriptase
mRNA	Messenger ribonucleic acid
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pfu	Plaque-forming units
RT	Room temperature
<i>Sf</i>	<i>Spodoptera frugiperda</i>
SDS	Sodium dodecyl sulfate
STOX	(S)-tetrahydroprotoberberine oxidase
TBE	Tris borate/EDTA
TC100/FBS	TC-100 medium containing 10% FBS
TC100/FBS/P	TC-100 medium containing 10% FBS and 0.1% v/v Pluronic F-68
TE	Tris/EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TGA	Thioglycolitic acid
U	Unit
V	Volts
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

1. Introduction

1.1. Alkaloids

The term alkaloid is derived from Arabic word *al-qali*, the plant from which “soda” was first obtained (Kutchan, 1995). Alkaloids are a group of naturally occurring low-molecular weight nitrogenous compounds found in about 20% of plant species. The majority of alkaloids in plants are derived from the amino acids tyrosine, tryptophan and phenylalanine. They are often basic and contain nitrogen in a heterocyclic ring. The classification of alkaloids is based on their carbon-nitrogen skeletons; common alkaloid ring structures include the pyridines, pyrroles, indoles, pyrrolidines, isoquinolines and piperidines (Pettersen *et al.*, 1991; Bennett *et al.*, 1994).

In nature, plant alkaloids are mainly involved in plant defense against herbivores and pathogens. Many of these compounds have biological activity which makes them suitable for use as stimulants (nicotine, caffeine), pharmaceuticals (vinblastine), narcotics (cocaine, morphine) and poisons (tubocurarine). The discovery of morphine by the German pharmacist Friedrich W. Sertürner in 1806 began the field of plant alkaloid biochemistry. However, the structure of morphine was not determined until 1952 due to its stereochemical complexity. Major technical advances occurred in this field allowing for the elucidation of selected alkaloid biosynthetic pathways. Among these were the introduction of radiolabeled precursors in the 1950s and the establishment in the 1970s of plant cell suspension cultures as an abundant source of enzymes that could be isolated, purified and characterized. Finally, the introduction of molecular techniques has made possible the isolation of genes involved in alkaloid secondary pathways (Croteau *et al.*, 2000; Facchini, 2001).

1.1.1. Benzyloisoquinoline alkaloids

Isoquinoline alkaloids represent a large and varied group of physiologically active natural products. The isoquinoline skeleton is a basic building block of various types of alkaloids which can be divided into approximately 20 categories including benzophenanthridine, protoptines, protoberberines or morphinans. Most of these alkaloids are derived from a common intermediate, (*S*)-reticuline. In the literature, the biosynthesis of isoquinoline alkaloids has been reviewed by Kutchan (1998).

The biosynthesis of (*S*)-reticuline (Figure 1.1) begins with the condensation of two L-Tyrosine derivatives (dopamine and *p*-hydroxyphenylacetaldehyde) by (*S*)-norcoclaurine synthase to produce the central precursor (*S*)-norcoclaurine (NCS) (Rüffer *et al.*, 1981; Sato *et al.*, 2007). The enzyme 6-*O*-methyltransferase (6OMT) methylates (*S*)-norcoclaurine at the 6-hydroxy position synthesizing (*S*)-coclaurine (Rüffer *et al.*, 1983; Sato *et al.*, 1994). A second methylation at the N-position of the isoquinoline moiety in (*S*)-coclaurine is catalyzed by (*S*)-coclaurine *N*-methyltransferase (CNMT) (Choi *et al.*, 2001, 2002). The P450-dependent monooxygenase (*S*)-*N*-methylcoclaurine 3'-hydroxylase (NMCH) catalyzes the 3'-hydroxylation of (*S*)-*N*-methylcoclaurine (Pauli and Kutchan, 1998). Finally, the 4'-*O*-methylation of (*S*)-3'-hydroxy-*N*-methylcoclaurine by 4'-*O*-methyltransferase (4OMT) leads to the central intermediate (*S*)-reticuline (Frenzel and Zenk, 1990; Morishige *et al.*, 2000; Ziegler *et al.*, 2005). From here, multiple biosynthetic pathways lead to the various structural types of benzyloisoquinoline alkaloids.

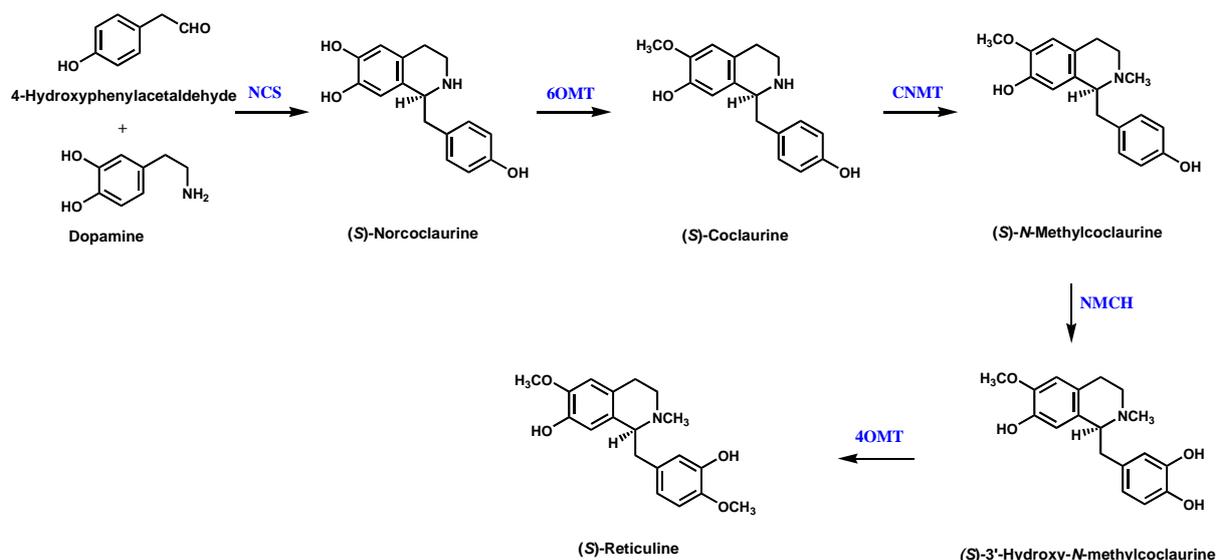


Figure 1.1 Biosynthetic pathway leading to (*S*)-reticuline. NCS, norcoclaurine synthase; 6OMT, 6-*O*-methyltransferase; CNMT, (*S*)-coclaurine *N*-methyltransferase; NMCH, (*S*)-*N*-methylcoclaurine 3'-hydroxylase; 4OMT, 3'-hydroxy-*N*-methylcoclaurine 4'-*O*-methyltransferase.

1.1.2. Biosynthesis of berberine and sanguinarine

After (*S*)-reticuline is synthesized, the berberine bridge enzyme (BBE) catalyzes the oxidative conversion of this compound to (*S*)-scoulerine (Dittrich and Kutchan, 1991; Kutchan and Dittrich, 1995; Facchini *et al.*, 1996; Huang and Kutchan, 2000; Steffens *et al.*, 1985), the first committed step in the sanguinarine and berberine pathway. In both pathways, only methyltransferases and oxidoreductases are involved. In the biosynthesis to berberine (Figure 1.2), (*S*)-scoulerine is first methylated to (*S*)-tetrahydrocolumbamine. This reaction is catalyzed by (*S*)-scoulerine 9-*O*-methyltransferase (SOMT) which was purified and isolated from *C. japonica cells* (Sato *et al.*, 1993; Takeshita *et al.*, 1995). The oxidation of (*S*)-tetrahydrocolumbamine to (*S*)-canadine is due to the action of the cytochrome P450 (*S*)-canadine oxidase (SCO) a methylenedioxy bridge-forming enzyme (Rueffer and Zenk, 1994; Ikezawa *et al.*, 2003). Finally, a flavoprotein (*S*)-tetrahydroberberine oxidase (THB) is involved in the conversion of (*S*)-canadine to berberine (Amann *et al.*, 1984; Okada *et al.*, 1988).

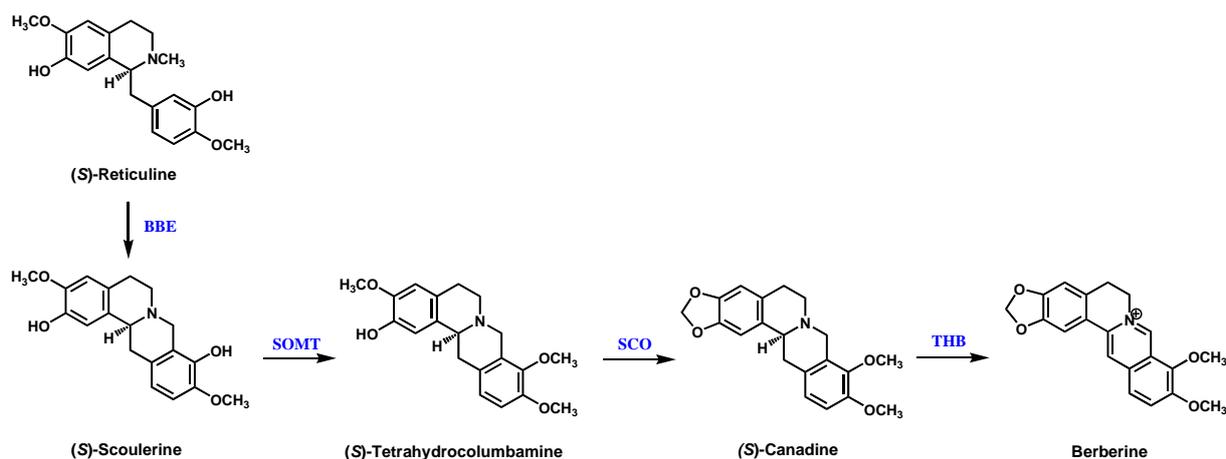


Figure 1.2. Enzymatic biosynthetic pathway from (*S*)-reticuline to berberine. BBE, berberine bridge enzyme; SOMT, (*S*)-9-*O*-methyltransferase; SCO, (*S*)-canadine oxidase; THB, (*S*)-tetrahydroberberine oxidase.

The biosynthetic pathway leading to sanguinarine (Figure 1.3) begins with the oxidation of (*S*)-scoulerine by two consecutive cytochromes specific to this pathway (*S*)-cheilanthifoline synthase (SCHS) and (*S*)-stylophine synthase (STS) (Bauer and Zenk, 1989, 1991). (*S*)-stylophine is then *N*-methylated by (*S*)-tetrahydroprotoberberine *cis-N*-methyltransferase (TPNMT) to (*S*)-*cys-N*-methylstylophine (O'Keefe und Beecher, 1994; Ruffer und Zenk, 1986; Ruffer *et al.*, 1990). The next step is catalyzed by the cytochrome (*S*)-*cis-N*-methyltetrahydroprotoberberine 14-hydroxylase (NMTOH) resulting in the formation of

protopine. The hydroxylation of protopine at the C-6 position occurs by the last cytochrome of the pathway protopine 6-hydroxylase (P6OH). The transformation from 6-hydroxy-protopine into the first benzophenanthridine, dihydrosanguinarine, occurs spontaneously (Tanahashi and Zenk, 1990). The last step is the oxidation of dihydrosanguinarine to sanguinarine catalyzed by dihydrobenzophenanthridine oxidase (DHBO).

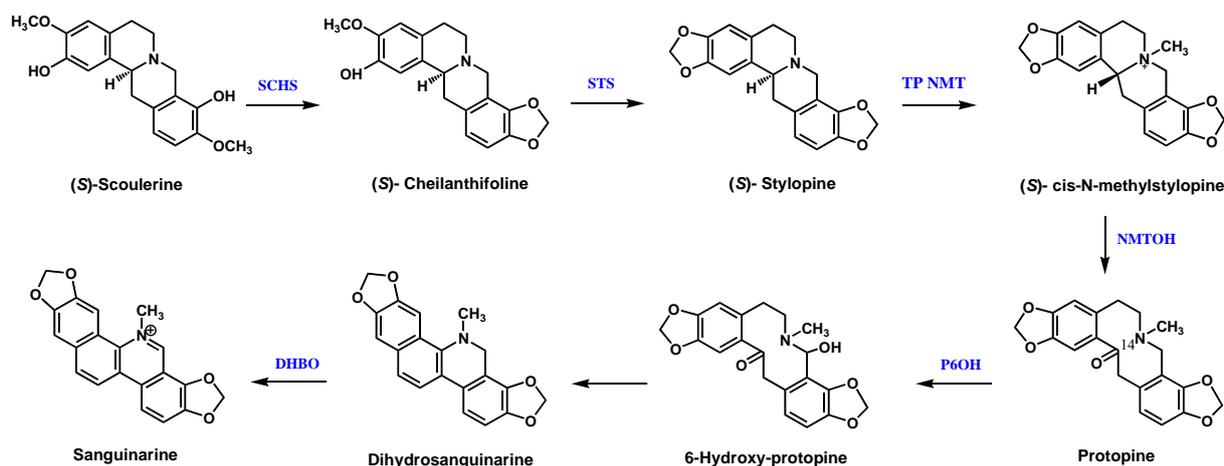


Figure 1.3. Biosynthetic pathway from (S)-scoulerine to sanguinarine. SCHS, (S)-cheilanthifoline synthase; (STS), (S)-stylopine synthase; TPNMT, (S)-tetrahydroprotoberberine cis-N-methyltransferase; (NMTOH) (S)-cis-N-methyl-tetrahydroprotoberberine 14-hydroxylase; (P6OH) protopine 6-hydroxylase; (DHBO) dihydrobenzophenanthridine oxidase.

1.2. Cytochrome P450

Cytochromes P450, one of the largest families of enzymes, are heme-containing proteins involved in a series of oxidative reactions. They are present in bacteria, insects, animals, humans, plants and fungi. Most of the eukaryotic CYP450s are anchored to the endoplasmic reticulum by a hydrophobic helix near to N-terminus with most of the protein residing on the cytosolic face of the membrane (Chapple *et al.*, 1998). Cytochrome P450s depend for their activity on associated proteins such as NADPH:Cyt P450 reductase, which catalyzes the transfer of electrons from NADPH *via* FAD and FMN to the prosthetic heme group of the CYP450 protein (Donaldson R. and Douglas G., 1991; Yeom *et al.*, 1995). Cytochromes P450 possess the property to bind carbon monoxide (CO) instead of oxygen thereby exhibiting a characteristic reduced difference spectra with an absorption maximum at 450 nm (Omura and

The CYP719A subfamily corresponds to methylenedioxy bridge-forming enzymes associated with the late stage of benzyloquinoline alkaloid biosynthesis leading to berberine and sanguinarine. Methylenedioxy bridge formation is the cyclization of an ortho-methoxyphenol and is difficult to mimic in organic chemistry. Canadine synthase (CYP719A1) catalyzes the conversion of (*S*)-tetrahydrocolumbamine to (*S*)-tetrahydroberberine (canadine). This reaction was studied for the first time using microsomal fractions of *Thalictrum tuberosum* cells (Rüffer and Zenk, 1994) and later isolated and characterized from a cDNA library prepared from *Coptis japonica* cells (Ikezawa *et al.*, 2003). The enzyme displayed a high substrate specificity for (*S*)-tetrahydrocolumbamine. Two other methylenedioxy bridge-forming enzymes were detected to be involved in the biosynthesis of (*S*)-stylophine from (*S*)-scoulerine via (*S*)-cheilanthifoline (Figure 1.5). (*S*)-stylophine is a common branch point intermediate for protoberberine and benzophenanthridine alkaloids. The enzyme activities were partially characterized using microsomal fractions isolated from *Eschscholzia californica* cells (Bauer and Zenk, 1989). Experiments with radio labeled substrates showed that the methylenedioxy bridge group is formed first in ring D before that in ring A and the biosynthesis to stylophine occurs via cheilanthifoline and not nandinine as originally was thought. The first enzyme (*S*)-cheilanthifoline synthase [(*S*)-CHS, EC 1.14.21.2], catalyses the formation of the 9,10-methylenedioxy group in (*S*)-scoulerine (ring D) leading to (*S*)-cheilanthifoline. The second P450 (*S*)-stylophine synthase [(*S*)-STS, EC 1.14.21.1] forms the 2,3-methylenedioxy group in (*S*)-cheilanthifoline (ring A) to form (*S*)-stylophine. The existence of two different enzymes for this similar reaction is supported by the differential inhibition of the two reactions by cytochrome P450 inhibitors and difference in induction after elicitor treatment (Bauer and Zenk, 1989).

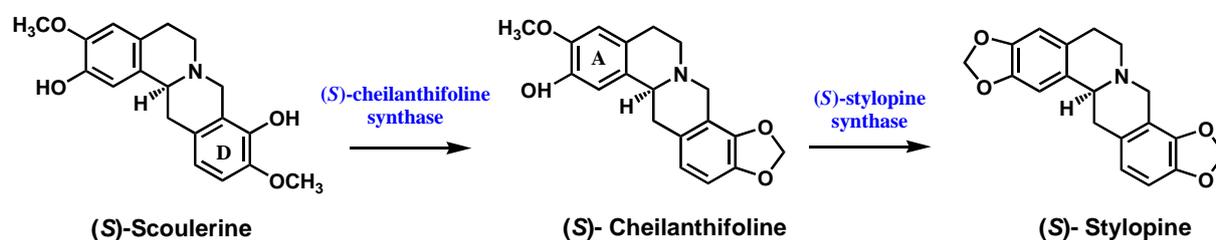


Figure 1.5. Enzymatic synthesis of (*S*)-stylophine via (*S*)-cheilanthifoline.

Recently, two cytochrome P450 cDNAs involved in stylopine biosynthesis were isolated from *Eschscholzia californica* cells (Ikezawa *et al.*, 2007). The primary structures of CYP719A2 and CYP719A3 showed high similarity to *C. japonica* CYP719A1 (65.1 and 64.8% identity, respectively). Both enzymes catalyzed methylenedioxy bridge-formation from (*S*)-cheilanthifoline to (*S*)-stylopine. Neither CYP719A2 nor CYP719A3 converted (*S*)-scoulerine to (*S*)-cheilanthifoline but, transformed (*S*)-scoulerine to (*S*)-nandinine which has a methylenedioxy bridge in ring A (2,3-position). Interestingly, CYP719A3 accepted also (*S*)-tetrahydrocolumbamine as substrate to form (*S*)-canadine (2,3-methylenedioxy bridge in ring A) like CYP719A1 enzyme. (*S*)-Cheilanthifoline synthase remains unknown.

This type of reaction is not exclusive to alkaloids; methylenedioxy bridge formation is commonly found in many secondary metabolites including lignans and isoflavonoids (Jiao *et al.*, 1998, Clemens S. and Barz W., 1996). CYP81Q1 isolated from sesame (*Sesamum indicum* L.) catalyzes dual methylenedioxy bridge formation on (+)-piperitol. Although CYP81Q1 is a methylenedioxy bridge enzyme like CYP719A1, they show only 24% sequence identity to each other (Ono *et al.*, 2006).

1.3. Berberine bridge enzyme (BBE) and BBE-like proteins

The berberine bridge enzyme ([*S*]-reticuline: oxygen oxidoreductase [methylene bridge forming], EC 1.5.3.9), catalyzes the oxidative cyclization of the *N*-methyl moiety of (*S*)-reticuline into the berberine bridge carbon, C-8 of (*S*)-scoulerine, a branch point intermediate in the biosynthesis of protopine, protoberberine and benzophenanthridine alkaloids (Figure 1.6). This conversion was originally achieved by Rink and Böhm (1975) with cell free preparations from *Macleaya microcarpa* suspension culture, and later purified and characterized from *Berberis beaniana* (Steffens *et al.*, 1985). Its cDNA was isolated from elicited cell suspension culture of *Eschscholzia californica* (Dittrich and Kutchan, 1991) and overexpressed in insect cell culture (Kutchan and Dittrich, 1995). This allowed to the identification of a FAD cofactor covalent bound to histidine (H104) in a consensus sequence comprising amino acids 100-110 of the protein. The first 22 amino acids of the deduced amino acid sequence of BBE correspond to an N-terminal signal peptide that directs the preprotein into the specific alkaloid biosynthesis vesicles in which it accumulates (Dittrich and Kutchan, 1991; Amman *et al.*, 1986).

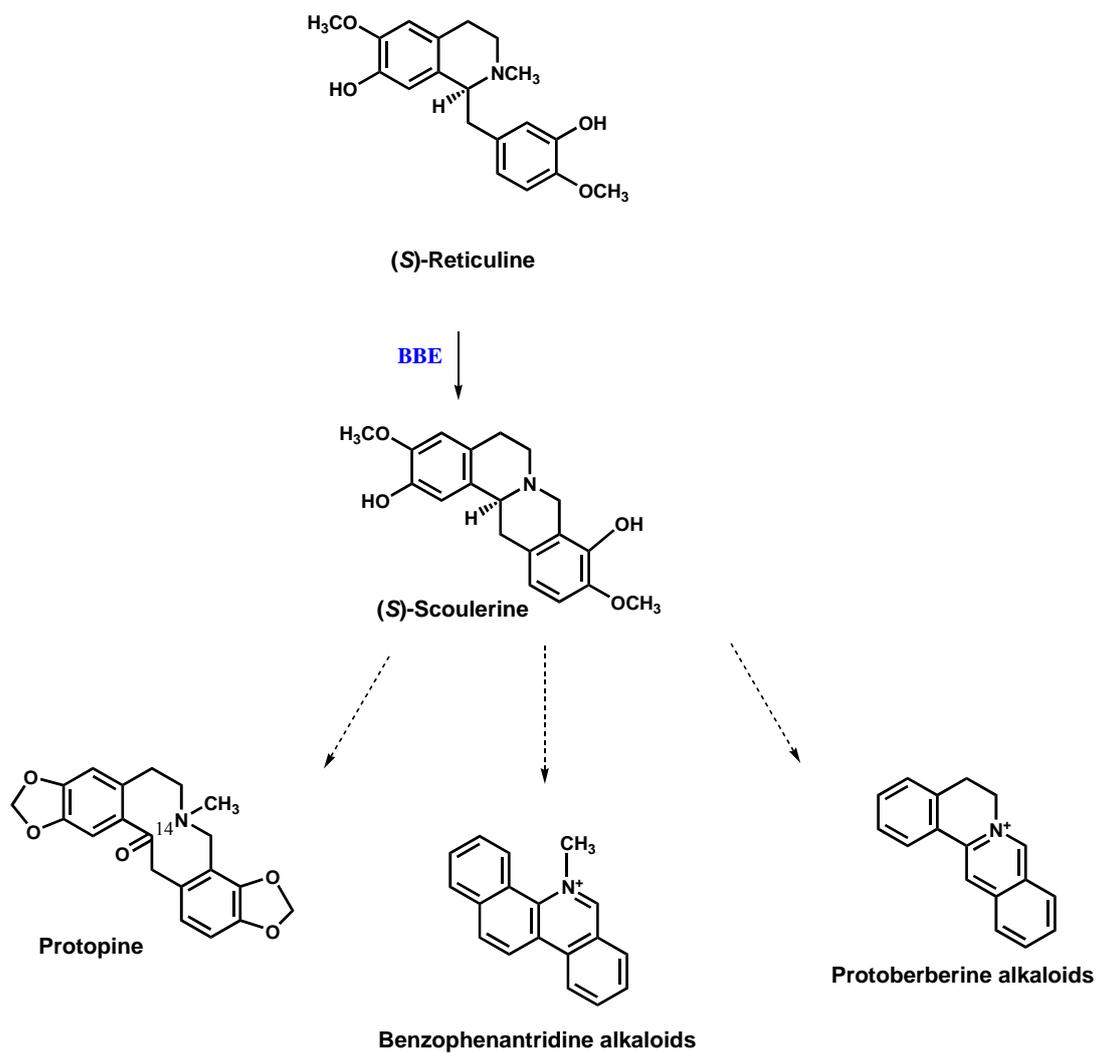


Figure 1.6. Biosynthesis of (S)-scoulerine the central intermediate to protopines, benzophenanthridines and protoberberine type alkaloids. BBE, berberine bridge enzyme.

The elucidation of the crystal structure of a glucooligosaccharide oxidase (AcstGOOX) from *Acremonium strictum* identified a second residue (cysteine) besides histidine required for the attachment of FAD to the protein (Huang *et al.*, 2005). These residues were found to be conserved in several oxidases BBE-like proteins (Lee *et al.*, 2005). In BBE from *E. californica* this residues correspond to H104 and C166 (Figure 1.7; Winkler *et al.*, 2006).

```

EscaBBE  97 IRLRSGGHSY (55) TAGWCPTVGTGGHISGGGFGMMSR 185
          *  :.*****      : * **:**.***: ***:*: ::
AcstGOOX 63 ISAKGGGHSY (53) SHGTCCPAVGVGGHVLGGGYGFATH 149

```

Figure 1.7. Alignment of the active site residues of BBE from *Eschscholzia californica* (EscaBBE) with glucooligosaccharide oxidase from *Acremonium strictum* (AcstGOOX). A (*) indicates invariant amino acids, (:) are conserved residues and (.) semiconservative amino acids. The underlined residues (H104 and C166 for EscaBBE) represent the sites of covalent attachment (from Winkler *et al.*, 2006).

1.4. (S)-tetrahydroprotoberberine oxidase (STOX)

(S)-tetrahydroprotoberberine oxidase [EC 1.3.3.8] (STOX), identified as a flavoprotein, was purified from suspension cells of *Berberis wilsoniae*. STOX oxidizes a series of tetrahydroprotoberberine and isoquinoline alkaloids exclusively with (S)-configuration. This is a dimeric protein with a molecular weight for the monomeric protein of 53kD and a pH optimum of 8.9 like that for BBE. STOX is compartmentalized together with BBE in smooth vesicles, probably derived from the smooth endoplasmic reticulum (ER), where protoberberine alkaloids (columbamine, jatrorrhizine, palmatine and berberine) are accumulated. These vesicles migrate to, and fuse with, the tonoplast membrane, releasing their content into the vacuole (Amann *et al.*, 1986). The STOX enzyme activity was detected in several plants species containing protoberberine alkaloids (Amann *et al.*, 1984, 1988), but the cDNA sequence has not been published.

1.5. *Argemone mexicana* L.

The family Papaveraceae is a famous family of plants because of the secondary compounds contained in its tissue. The best known or studied plant from this family is *Papaver somniferum* “opium poppy,” the source of the narcotic analgesics morphine and codeine.



Argemone mexicana L. (Papaveraceae) commonly known as prickly poppy, is a spiny herbaceous annual plant native to Central America and south-western North America. Now-a-days- it is wide spread around the world to such an extent that it has become a troublesome weed in some localities by contaminating crops. Stems, leaves and capsules have prickles and when bruised, exude a yellow, milky juice which is acrid and of penetrating odor. The seeds are black and spherical with a diameter ca. 1.5–2.5 mm very similar to that of the mustard (Schwarzbach and Kadereit 1999; Karlsson *et al.*, 2003). Different parts of the plant have been used in the traditional medicine for the treatment of fever, pain, diarrhea, cutaneous infections, itches, conjunctivitis and cancer (Chopra 1986, Monroy 2000; Chang *et al.*, 2003a, 2003b). The word *Argemone* is derived from the Greek *argema* meaning eye cataract, as the juice of this plant was used as a remedy in the treatment of this disease. The oil from the seed is purgative, but prolonged ingestion produces toxic effects and even death. In some regions of India, epidemic dropsy which is characterized by edema, cardiac insufficiency and renal failure, results from the ingestion of mustard oil contaminated with *Argemone* oil. (Sharma *et al.*, 1999; Sanghvi *et al.*, 1960). Pharmacological studies on this plant using crude extracts showed *in vitro* bactericidal effect against some pathogenic bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*), growth inhibition against human nasopharyngeal carcinoma and human gastric cancer, and anti-HIV activity (Bhattacharjee, 2006; Chang *et al.*, 2003a, 2003b).

The alkaloid contents of *Argemone* have been studied in detail by several authors (Hussain *et al.*, 1983; Dalvi *et al.*, 1985; Capasso *et al.*, 1997; Chang *et al.*, 2003a, 2003b) and their structures elucidated by HNMR, CNMR, *etc.* Among them, protopine, protoberberine-type alkaloids and benzophenanthridine alkaloids were identified (Table 1.1).

Table 1.1. Benzophenanthridine alkaloids identified in *Argemone mexicana*.

Alkaloids types	Compound
Benzylisoquinoline	(+)-argenaxine, norcoclaurine, reticuline
Protopines	argemexicaine A, argemexicaine B, protopine, allocryptopine, coptisine
Protoberberine	tetrahydroberberine, berberine, dehydrocheilantifoline
Benzophenanthridine	(±)-6-acetyldihydrochelerythrine, norchelerythrine, methylzanthoxyline, sanguinarine, arnottianamide, angoline, chelerythrine, (±)-6-acetyldihydrosanguinarine, <i>N</i> -demethoxy-sanguinarine, pancorine

Considering the type of alkaloids reported for this plant, for example those with a methylenedioxy bridge or protoberberine, we expected that one or several P450 enzymes with methylenedioxy bridge activity like CYP719A1 and FAD-dependent oxygenases like BBE and STOX may play a role in alkaloid biosynthesis of *A. mexicana*.

In this work, the first cDNA library from *Argemone mexicana* was constructed with the aim to identify and characterize genes specially involved in the biosynthesis of benzophenanthridine and protoberberine alkaloids by analysis of expressed sequence tags (ESTs). Partial sequences, database comparisons and functional categorization of 1,265 randomly collected cDNA clones of *A. mexicana* are reported. These results led us to the identification and characterization of two methylenedioxy bridge-forming enzymes and two FAD-oxidases.

2. Material

2.1. Enzymes

	Company
BD Advantage™ 2 PCR Enzyme System	BD Biosciences
Restriction enzymes, <i>EcoRI</i>	New England Biolabs
Restriction enzymes, <i>BamHI</i> , <i>Not I</i>	Invitrogen
MMLV-RT superscript	GIBCO
<i>Taq</i> DNA Polymerase	Promega
<i>Pfu</i> -Polymerase	Fermentas
T4-DNA Ligase (3 U/μl)	Promega

2.2. Proteins

Protein Molecular Weight Marker	Fermentas
Bovine serum albumin (BSA)	Roth

2.3. Nucleotides

dATP, dTTP, dCTP, dGTP	Promega
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2.4. DNA fragments

O'GeneRuler™ 100bp DNA Ladder Plus	Fermentas
1 kb base pair ladder	New England Biolabs

2.5. Cloning vectors

TA cloning vector	
pCR® 2.1	Invitrogen
pGEM®-T easy vector	Promega
Transfer vector	
pVL1392	BD Bioscience
pFastBac™1	Invitrogen

2.6. Synthetic Oligonucleotides

General Oligonucleotides	(MWG-Biotech AG)	
Primer	Sequence 5' → 3'	T _m
M13 rev	CAG GAA ACA GCT ATG ACC	53.7 °C
M13 for (-20)	GTAAAACGACGGCCAG	52.0 °C
T3	GCT CGA AAT TAA CCC TCA CTA AAG	59.3 °C
T7	GAA TTG TAA TAC GAC TCA CTA TAG	55.9 °C
dT20VN	(T) ₂₀ VN	

2.7. Organisms

2.7.1. Plants

Plants from *Argemone mexicana* were grown in the green house at the Institute of Plant Biochemistry without control of humidity or temperature. Plants germinated twice a year in June and October with a period of dormancy between 1 to 3 months.

2.7.2. Bacteria

<i>Escherichia coli</i> strains	Genotype
TOP10 (Invitrogen)	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139 Δ(<i>araleu</i>)7697 <i>galU galK rpsL</i> (StrR) <i>endA1 nupG</i></i>
XL1-Blue MRF' (Stratagene)	Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> [<i>F'</i> <i>proAB lacI</i> ^q <i>Z</i> Δ .M15 <i>Tn 10</i> (<i>Tet</i> ^r)]
DH10Bac™ (Invitrogen)	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 endA1 araD139 Δ(<i>ara, leu</i>)7697 <i>galU galK</i> λ- <i>rpsL nupG</i> /pMON14272 / pMON7124</i>

2.7.3. Insect cells work

<i>Sf9</i> (<i>Spodoptera frugiperda</i>)	Invitrogen
TC100 Insect Medium	Invitrogen
Foetal Bovine Serum	Invitrogen
Recombinant NADPH-cytochrome P450 reductase viral stock as described by Hubert Pauli (1998)	
Alkaloids were from the collection of the department of Natural Product Biotechnology, IPB and from Prof. M. H. Zenk (Biozentrum)	

2.8. Antibiotics

Ampicillin, Kanamycin	Sigma-Aldrich
Gentamycin, Streptomycin	Serva
Tetracycline	Roche
Antibiotic-antimycotic 100X (penicillin G, streptomycin sulfate, and amphotericin B)	GIBCO

2.9. Internet searches and alignments

Sequence searches were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1997) available on the National Center for Biotechnology Information (NCBI) web site (<http://www.ncbi.nlm.nih.gov/BLAST/>). Alignments were done with the DNASTAR software (Lasergene) using the ClustalW algorithm method. BLAST from the Munich Information Center for Protein Sequences (MIPS) - *Arabidopsis thaliana* Database (MAtdB) was also used to visualize the distribution of the clone homologies.

2.10. Chemicals

	Company
[α - ³² P]-dATP, 3000 Ci/mmol	Biomedicals, ICN
Acetic acid	Roth
Agar	Serva
Agarose	Roth
Bromophenol blue	Sigma-Aldrich
Chloroform	Roth
Coomassie Brilliant Blue G-250	Serva
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
DTT (Dithiothreitol)	Roth
EDTA (Ethylenediaminetetraacetic acid)	Roth
Emulgen 913	Karlau
Ethanol	Merck
Ethidium bromide	Sigma-Aldrich
Formaldehyde	Merck
Formamide	Fluka
Glycerol	Roth
Glycine	Roth
IPTG (Isopropyl- β -D-1-thiogalactopyranoside)	Fluka
Isopropanol (2-Propanol)	Merck
Methanol	Merck
MgCl ₂	Roth
MgSO ₄	Sigma-Aldrich
3-(N-morpholino)propanesulfonic acid (MOPS)	Roth
NaOH	Roth
α -Naphthalene acetic acid (NAA)	DuchefaBiochemie
NADPH	SIGMA
Scoulerine	Roth
Sodium acetate	Merck
Sodium citrate	Roth
Phenol-Chloroform	Roth
Pluronic F-68	GIBCO
Polyacrylamine Gel 30	Roth
PVP (Polyvinylpyrrolidon)	Sigma-Aldrich
SDS (Sodium lauryl sulphate)	Roth
Sephadex G-50 Superfine	Amersham Pharmacia
N,N,N',N'-tetramethylethylenediamine (TEMED)	Roth
Tris (Tris-hydroxymethylaminoethane)	Roth
Tricine	Roth
Tween 20	Roth
5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside	Roth
β -Mercaptoethanol	Roth

2.11. Kits

BD SMART™ RACE cDNA amplification kit	BD Biosciences
Big Dye terminators version 1.1	Applied Biosystems
BD BaculoGold™ expression system	BD Bioscience Pharmigen
Bac-to-Bac® expression system	Invitrogen-GIBCO
Megaprime™ DNA labelling system	GE Healthcare
Oligotex® mRNA mini kit	QIAGEN
pGEM®-T Easy vector system	Promega
Plasmid purification mini kit	QIAGEN
QIAquick® gel extraction kit	QIAGEN
QIAprep® spin miniprep kit	QIAGEN
Superscript first-strand synthesis system	Invitrogen
TA cloning kit with pCR®2.1	Invitrogen
ZAP Express® cDNA synthesis kit	Stratagene
ZAP Express® cDNA Gigapack® III gold cloning kit	Stratagene

2.12. Consumables

Bodyne Membrane	Pall
Filter paper GB 004 gel blotting paper	Schleicher and Schuell
Phosphor-image screen	Molecular Dynamics
ProbeQuant™ G-50 micro columns	Amersham Biosciences
T-25 flask	NUNC
CellStar, 6W Plate, TC	Greiner bio-one
LiChrospher 60 RP-select B (250x4 mm, 5 µm) column	Merck
Superspher 60 RP-select B (125x2 mm, 5µm) column	Merck

2.13. Instruments

Centrifuge	Centrifuge 5810R and 5415D (Eppendorf), Sorvall RC 26 Plus (DuPont), LE-80 ultracentrifuge (Beckman)
Electrophoretic	Vertical und horizontal gel apparatus (Biometra), microcomputer electrophoresis power supply E455 (Consort), GeneGenius Bio Imaging Systems (Syngene)
LC/MS	1100 Series (Agilent); MS-TOF (Applied Biosystems), Turbulon Spray source (PE-Sciex)
HPLC	1100 Series (Agilent)
PCR-Machine	Thermal cycler GenAmp PCR system 9700 (PE Applied Biosystems), PTC 200 Peltier thermal cycler (MJ Research)
Radioactivity measure	Storage phosphor screen, storm phosphor imager 860 and image eraser (Molecular Dynamics)
Sequencer	ABI 310 and ABI 3100 Avant Genetic Analyzer (Applied Biosystems)
Spectrophotometer	PerkinElmer Lambda 800 UV/Visible, ultrospec 3000 UV/Visible spectrophotometer (Pharmacia Biotech)
Various	Incubator shaker innova 4330 (New Brunswick Scientific) Hybridising oven 7601 (GFL), cold water bad K15 (Haake), water bad 13 A (Julabo), orbital shaker RCT basic (IKA Werke), SC100 speed vac (Savant), Thermo mixer 5436 (Eppendorf), UV Stratalinker 1800 (Stratagene).

3. Methods

3.1. Alkaloids

3.1.1. Alkaloid extraction from plant tissues

In a pre-chilled mortar and pestle, 0.5 g plant tissue was ground with liquid nitrogen and transferred to a 2 ml tube. Alkaloids were extracted with 1 ml 80% (v/v) EtOH with shaking for 30 min, followed by centrifugation at max speed for 5 min in a bench centrifuge (16,000 x g). After evaporation of the solvent, the resulting extract was resuspended in 1 ml H₂O and the pH made basic (pH 9.0) with 1 M Na₂CO₃. Alkaloids were extracted twice with 500 µl ethylacetate and chloroform and the organic phases evaporated. Finally, the extracts were resuspended in 100 µl 70% v/v EtOH and analyzed by HPLC and LC-MS.

3.1.2. Analyses by High Performance Liquid Chromatography (HPLC)

HPLC sample analyses 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 system and gradient was as follows:

Solvent system	A	98% H ₂ O, 2.0 % CH ₃ CN, 0.01 % H ₃ PO ₄							
	B	90% CH ₃ CN, 2.0% H ₂ O, 0.01% H ₃ PO ₄							
Gradient	Time (min)	0	25	30	35	37	40	43	46
	% B	0	46	60	60	100	100	0	0

3.1.3. Analyses by Liquid Chromatography- Mass Spectrometry (LC-MS, TOF)

Alkaloids LC-MS analyses were carried out on a Mariner TOF mass spectrometer equipped with a Turbulon Spray source using an 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 CH₃CN-H₂O (2:98; v/v) and solvent B CH₃CN-H₂O (98:2; v/v), 0.2% (v/v) formic acid in both solvents. The gradient increased from 0% to 46% B in 25 min, to 90% in 1 min and was held at 90% for 7 min, post time was 5 min.

I would like to thank Dr. Robert Kramell (IPB) for alkaloid analyses by LC-MS.

3.2. Isolation of RNA

3.2.1. RNA Isolation

Extraction buffer (EB)	0.8 M guanidine thiocyanate, 0.4 M Ammonium thiocyanate, 0.1 M Sodium acetate pH 5.0, 5% Glycerol, 38% Phenol (pH 4.5-5.0)
	Chloroform, isopropanol, ethanol

Plant tissue-RNA isolation was based on the Trizol® method described by Chomczynski (1987). In a pre-chilled mortar and pestle, plant tissue was ground with liquid nitrogen and transferred to a 50 ml conical tube containing extraction buffer EB [10 ml EB/ g tissue], followed by 1 min vortexing. After incubation for 5 min RT, 2 ml chloroform/ g tissue were added and the sample mixed vigorously for 20 sec. By centrifugation at 3220 x g for 20 min at 4°C, RNA contained in the aqueous phase was separated from DNA and proteins which remained in the phenol-chloroform organic phase. One volume isopropanol was added to the aqueous phase to precipitate the RNA and mixed by inverting the tube gently. The sample was incubated for 10 min at RT and centrifuged at 3220 x g for 10 min at 4°C. The supernatant was removed and the RNA pellet washed twice with 70% v/v ethanol with a centrifugation step in between (3220 x g for 2 min at 4°C). After air drying for 5 min, the pellet was dissolved in ddH₂O. To increase the solubility of the RNA pellet, the tube was heated at 65°C for 10 min, followed by 2 min centrifugation in a microcentrifuge at max speed (16,000 x g). Quality of the RNA was analyzed by gel electrophoresis (3.6.2) and quantified by measuring the absorbance of a dilute RNA solution at 260 nm (A_{260}). As an absorbance of 1 unit at 260nm corresponds to 40 µg of RNA, the concentration of each sample can be calculated using the equation:

$$[\text{RNA}] \text{ in } \mu\text{g}/\mu\text{l} = (40 \times \text{dilution factor} \times \text{absorbance at } A_{260}) / 1000$$

Purity levels of RNA can be estimated by calculating the ratio between the absorbance value at 260 nm and 280 nm $A_{260}:A_{280}$. An acceptable ratio is between 1.5 and 2.0.

3.2.2. Poly-(A)⁺ RNA isolation

Poly (A)⁺ RNA was isolated from total RNA obtained from *Argemone mexicana* roots using an Oligotex® kit (QIAGEN). This method is based on the hybridization of poly-(A)⁺ RNA to oligonucleotides (dC₁₀T₃₀) immobilized on polystyrene-latex particles. In contrast, rRNA and tRNAs are not polyadenylated and therefore will not bind to the oligo-matrix. The hybridization of mRNA to the oligotex resin occurs at high salt conditions and is then recovered from the column with a low salt buffer.

3.3. Isolation of DNA

3.3.1. Plasmid DNA purification

The QIAprep® miniprep kit (QIAGEN) was used for the isolation of plasmid DNA from bacterial cells < 10 kb and a plasmid purification mini Kit (QIAGEN) for plasmid (Bacmid) > 50 kb. This method is based on the alkaline extraction method for plasmid DNA developed by Birnboim and Doly (1979). Basically, bacterial cells are lysed under alkaline conditions followed by the neutralization of the lysate in presence of high salt concentrations. In this way, chromosomal DNA and other contaminants that precipitate are removed by centrifugation. Plasmid DNA is then absorbed onto a silica membrane, washed and finally eluted from the column under low-salt concentrations.

3.3.2. Purification of DNA fragments from agarose gel

The MinElute Gel kit (QIAGEN) was used to purify the DNA fragments separated by agarose gel electrophoresis (3.5.3) following the manufacture's instructions. To an excised agarose gel slice, a specified amount of binding buffer was added and the gel slice melted at 50°C. The molten solution was applied to a column in which the DNA bound to a membrane and contaminants such as dNTP's, enzyme and primers were washed out. DNA was eluted from the column with 10 µl sterile water.

3.3.3. Baculovirus DNA from infected Sf9 cells

Recombinant baculovirus DNA obtained by infection of insect cells was isolated by the phenol-chloroform method.

Phenol: Chloroform: Isoamylalcohol (25: 24: 1)

3 M sodium acetate pH 5.5

To 300 μ l of recombinant viral stock (3.18.6), an equal volume of phenol-chloroform was added. The sample was vortexed and centrifuged for 1 minute at 10,000 rpm in a bench centrifuge (9,000 x g). The upper phase was transferred to a new tube and 1/10 volume of 3 M sodium acetate pH 5.5 (30 μ l) and 2 volumes of 100% ethanol (600 μ l) were added. The sample was centrifuged at maximum speed (16,000 x g) for 5 minutes at RT to precipitate the DNA. The supernatant was removed and the pellet rinsed twice with 80% ethanol. The pellet was air dried for 5 min and finally resuspended in 50 μ l TE buffer or water. One microliter of the sample was used as a template for a PCR reaction using GSP and vector primers.

3.4. Insect cell culture

3.4.1. Maintenance

Cells of the fall army worm *Spodoptera frugiperda Sf9* were routinely incubated at 27°C in T-25 flasks containing 5 ml of growth media [TC100 medium, 10% v/v foetal bovine serum (TC100/FBS)]. After confluence was reached (every 2 days), cells were subcultured 2:1 into a new flask containing fresh medium. In order to scale up the insect cells cultures, cells were transferred into a 200 ml conical flask containing 45 ml of TC100/FBS media supplemented with 0.1% v/v pluronic and incubated at 27°C, 150 rpm,. Cells were subcultured 3:1 after cells reaches 2×10^6 cells/ml.

3.5. Electrophoresis

3.5.1. Protein polyacrylamide gel electrophoresis (PAGE)

Destaining solution	30% methanol (v/v), 20% (v/v) glacial acetic acid, water to 1 L
Stain solution	0.2% w/v Coomassie Brilliant Blue R-250 in destaining solution
Running buffer 1X	25 mM Tris base, 250 mM glycine, 0.1% SDS
5X sample buffer	3.12 ml 1 M Tris/HCl (pH 6.8), 1.0 g SDS powder, 2.5 ml glycerol, 75 μ l BPB (2% in ethanol), 0.5 μ l 2-mercaptoethanol, water to 10 ml

Expressed proteins were analyzed by SDS polyacrylamide gel electrophoresis according to the protocol described by Sambrook (1989). The components of the separating gel solution (Table 3.1) were mixed and then poured between two glass plates to the desired level. The top of the gel was overlaid with isopropanol to create a barrier between the gel and the air. The gel was allowed to polymerize 30-60 minutes at RT or until an interface appeared. After removal of the isopropanol, the stacking gel solution was poured on top of the separating gel in the presence of a tooth comb (The stacking gel depth was 1/2 well below the deepest level that the comb teeth reached). After polymerization, the comb was removed and the slab gel was placed on a vertical electrophoresis chamber. Protein samples were mixed with loading buffer and heated at 95°C for 5 minutes, then loaded into the wells. A protein molecular weight marker (Fermentas) was used as reference. The gel ran at 100 V until dye front reached the bottom of the gel. The protein gel was stained for 2 h and after that destained for 2-3 hours.

Table 3.1 Volume of reagents used for SDS-PAGE

Reagent	Separating gel 12% (ml)	Stacking gel 4% (ml)
Acrylamide (30%)	4.0	0.62
1 M Tris/HCl pH=8.8	2.5	----
1 M Tris/HCl pH=6.8	----	1.25
SDS 10% w/v	0.10	0.05
APS 10% w/v	0.05	0.04
Distilled water	3.30	3.00
TEMED	0.01	0.01

3.5.2. RNA agarose gel

10X MOPS	200 mM MOPS, 50 mM Sodium acetate, 10 mM EDTA pH 7.0 (when autoclaved, it may turn yellow).
Denaturing buffer (100 µl)	13 µl 10X MOPS, 23 µl formaldehyde 37% w/v, 64 µl formamide, 20 µl 10x loading buffer.
10x loading buffer	50% v/v glycerol, 0.1 M EDTA pH 8, 0.25% w/v BPB, 0.25% w/v xylene cyanol, 100 µg/ ml EtBr.

The quality of the isolated RNA was assessed by denaturing formaldehyde gel electrophoresis. For 100 ml of a 1.2% denaturing gel, 1.2 g agarose in 72 ml sterile water was heated in a microwave oven until completely melted. After cooling the solution to about 60°C, 10 ml 10X MOPS, 5.5 ml formaldehyde 37% w/v and EtBr (0.4 µg/ ml) were added. The molten gel solution was poured into a tray and allowed to solidify at RT. The samples were

prepared with RNA loading buffer and were denatured for 5 min at 65°C. Electrophoresis was carried out at 50-80 V in 1X MOPS. RNA was detected by visualization under UV light and the image recorded using the gel documentation system Gene Genius.

3.5.3. DNA agarose gel

Buffer	Working solution	Stock solution
TAE	1X	50X
	40 mM Tris-acetate, 1 mM EDTA	242 g Tris-base, 57.1 ml acetic acid, 100 ml 0.5 M EDTA (pH 8.0), volume adjusted to 1 L

DNA fragment analysis was done in a 1% w/v agarose gel with 0.4 µg/ml EtBr in 1X TAE. The sample was loaded in 6X DNA loading buffer (Fermentas) and run in 1X TAE at 70-90 V. The size of the DNA fragments were determined by comparison with molecular markers. Purification of DNA fragments was performed with a MinElute Gel kit (3.3.2)

3.6. cDNA library

3.6.1. λ-cDNA library construction

A lambda ZAP express cDNA library from *Argemone mexicana* was constructed by using a ZAP Express® cDNA Synthesis Kit and mRNA isolated from roots of two-month old plants. According to the manufacturer's instructions, the first-strand cDNA was synthesized using MMLV reverse transcriptase, 7 µg mRNA, oligo(dT) linker-primer containing an XhoI restriction site and a nucleotide mix containing dATP, dTTP, dGTP and 5-methyl dCTP. 5-Methyl dCTP hemimethylated cDNA protected the cdNA from digestion with XhoI restriction enzyme at a later step. Second-strand cDNA started after the addition of Rnase H and DNA polymerase I. In this step, dCTP was used instead of 5-methyl dCTP to reduce the probability of 5-methyl dCTP becoming incorporated in the second strand. After second-strand cDNA synthesis, the uneven termini of the double-stranded cDNA were filled in with *Pfu* DNA polymerase. EcoR I adapters as below were ligated to the blunt ends and the double-strand cDNA was digested with Xho I restriction enzyme. The cDNA was size fractionated by agarose gel electrophoresis. cDNA fragments less than 400 bp were removed and the remaining cDNA was ligated into the λ-ZAP Express vector.

5'- AATTCGGCACGAGG-3'
3'- GCCGTGCTCC-5'

Packaging extracts were used to package the recombinant λ -phage following the instructions of the manufacturer (Gigapack III Gold Packaging Extract; Stratagene). The recombinant packaged phage was used for titering and library screening.

3.7. Preparation of plating cells for library amplification

One single colony of XL1-Blue MRF['] was inoculated into 20 ml of LB broth (supplemented with 10 μ g/ml tetracycline, 0.2% (w/v) maltose, and 10 mM MgSO₄), and incubated at 37°C with shaking until an OD₆₀₀ of 9.0-1.0 was reached. Cells were collected by centrifugation (1,000 x g, 10 min, 4°C) and resuspended to an OD₆₀₀ of 0.5 in 10 mM MgSO₄.

3.8. Plating bacteriophage λ

This procedure was used to isolate pure populations of λ -phage from a single plaque for screening or providing the titer of the λ -library stock. Following the manufacture's protocol, serial dilutions of the packaged phage were prepared in SM buffer. One microliter of the appropriate serial dilutions was added to 200 μ l of prepared plating bacteria (3.7) and incubated for 15 min at 37°C to allow the phage to attach to the cells. After that, 3 ml of NZY top agar (melted and cooled to ~ 48°C) supplemented with 7.5 μ l 1M IPTG and 50 μ l of X-gal [250 mg/ ml in DMF] were added to the cells and poured onto a LB plate. After the NZY top agar solidified, the plate was incubated for 8-10 h at 37°C.

3.9. Picking bacteriophage λ plaques

After recombinant λ virions formed plaques on the lawn of *E. coli*, each plaque was picked up using a sterile pipette tip and transferred to a 96-well plate containing 25 μ l of SM buffer. The pipette tip was allowed to stand for 30 min at RT to facilitate bacteriophage diffusion from the agar into the medium. The phage suspension was used for analysis or stored at -80°C in SM buffer containing 20% v/v DMSO.

3.10. Library screening

The cDNA insert from a positive recombinant phage was amplified by PCR (3.14.1) using 2 μ l of the phage suspension (3.9) and primers T7 and T3. Both primers were complementary to vector sequences flanking the cloning site. PCR products were analyzed by agarose gel electrophoresis (3.5.3) and those cDNA fragments >400 bp were sequenced further (3.14.3).

The resultant cDNA partial sequences were analyzed with the BLAST network service of the NCBI (<http://www.ncbi.nlm.nih.gov/>).

Primer	Sequence 5' → 3'	T _m
T3	GCT CGA AAT TAA CCC TCA CTA AAG	59.3 °C
T7	GAA TTG TAA TAC GAC TCA CTA TAG	55.9 °C

3.11. Northern Blot

Northern blotting or northern hybridization is a method developed by Alwine (1977) used for the analysis of mRNA expression in tissues or cell culture. The steps involved in northern blot analyzes include:

- RNA isolation
- RNA fractionation according to size through a denaturing agarose gel
- Transfer to a solid support (blotting) and immobilization
- Hybridization with DNA or RNA probes

Required solutions for northern blot

Required solution	Working solution	Stock solution
SSC	2X 0.3 M NaCl, 3 mM Sodium citrate	20X 175.3 g NaCl, 88.2 g sodium citrate, adjust the volume to 1 L
Dehnhardt		100X 2 g BSA, 2 g PVP and 2 g ficoll dissolved in 100 ml water
Prehybridization buffer	4X SSC, 0.1% SDS, 5X Dehnhardt, 126 µg/ml Salmon sperm	1 ml 20X SSC, 50 µl 10% SDS, 0.250 ml 100X Dehnhardt, 63 µl Salmon-sperm* (10 mg/ml). Volume adjusted to 5 ml * Heat for 5 min at 95°C, then chill on ice for 5 min.
Wash solution	2X SSC with 0.1% SDS	

3.11.1. Blotting

Electrophoresis of total or mRNA was carried out on 1.2% w/v formaldehyde containing gels as described in section 3.5.2. Subsequently, the gel was soaked in a tray containing 10X SSC

buffer and agitated for 20 minutes to remove the excess formaldehyde. The capillary transfer system (Figure 3.1) was set up as follows: A piece of Whatman filter paper (3 mm) was placed over a glass plate placed between two reservoirs filled with 10X SSC; the solution was allowed to transfer to the paper. All air bubbles between paper and glass were removed by rolling a pipette across. The gel was placed face down, removing air bubbles between the paper and gel. A nylon membrane cut to the same size as the gel was soaked in 10X SSC for 5 min and placed on the top of the gel, followed by three pieces of 3 mm Whatman filter paper presoaked in 10X SSC. Paper towels were stacked over the Whatman paper to absorb the buffer and the capillary transfer was allowed to proceed overnight. Once completed, the blot was disassembled and the wells were marked with a pencil. Successful transfer of RNA was detected by visualization of EtBr stained rRNAs on the membrane under UV light. The membrane was rinsed in wash solution, and exposed to UV light to crosslink the mRNA to the membrane. The blot was stored in wash solution at RT or used directly for hybridization.

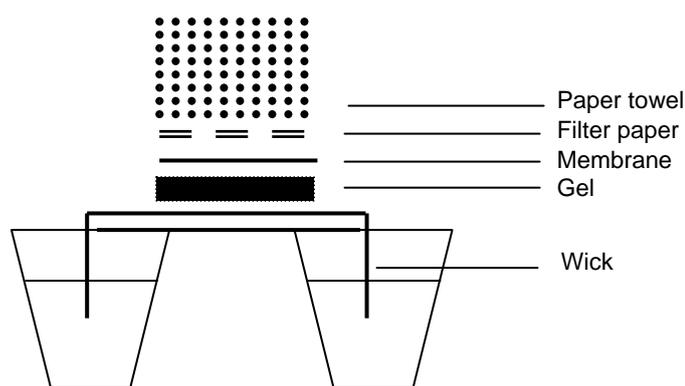


Figure 3.1. Upward capillary transfer of RNA from agarose gel.

3.11.2. Prehybridization

In a hybridization tube, 5 ml of prehybridization buffer was added to the membrane and incubated for 3 h at 65°C.

3.11.3. Random primer labelling of DNA

DNA labelling was achieved using a Megaprime™ DNA Labelling Kit (Amersham). Basically, random primers are annealed to a denatured DNA template and extended by Klenow fragment in presence of [α -³²P]-dATP. The reaction was set up as follows: Approximately 50 ng dsDNA in a volume of 21 μ l water were denatured by heating to 95°C for 5 min together with 5 μ l of primer mix. Random primer labeling proceeded by adding 4 μ l

each of unlabelled dNTPs (dCTP, dGTP, dTTP), 5 μ l [α -³²P]-dATP (3000 Ci/mMol) and 2 μ l of the enzyme DNA polymerase 1 Klenow fragment. The content of the tube was gently mixed and incubated at 37°C for 1 h. Unincorporated nucleotides were removed using a Sephadex G-50 spin-column.

3.11.4. Hybridization

Labeled DNA was added to the prehybridized membrane and the blot was incubated at 65°C overnight. Following hybridization, unbound and nonspecifically bound probe were removed from the membrane by washing 3 times for 15 min at 65°C in wash solution or until getting a low signal background. The blot was wrapped with Saran wrap and exposed to a phosphorimager screen overnight. The hybridization incidence was detected with a Phosphorimager Storm 860.

3.12. First-strand cDNA synthesis

First-strand cDNA synthesis was performed by Moloney Murine Leukemia Virus (MMLV) reverse transcriptase using mRNA isolated from roots as template.

	RACE- cDNA	Standard
mRNA	0.5 μ g	0.5 μ g
5'-oligo(dT) ₂₀ VN-3'	12 pmol	200 pmol
BD SMART II A oligo (12 μ M)	1.0 μ l	-
Final volume	10 μ l	20 μ l

In a microcentrifuge tube, mRNA was denatured for 5 min at 70°C, together with oligo(dT)₂₀VN, and in the case of 5'-RACE, the BD SMART II A oligo, then chilled on ice. The reverse transcription reaction was started by addition of 5X reaction buffer, 1 μ l dNTPs mix (10 mM each), and 200 U MMLV-RT. The reaction was incubated at 42°C for 1.5 h and terminated by heating the sample at 72°C for 7 min. Aliquots of the cDNA were stored at -20°C or used for PCR amplifications. 5'-RACE- cDNA reaction was diluted with 250 μ l Tricine-EDTA Buffer before use or storage.

3.13. Rapid amplification of 5'-cDNA ends (5'-RACE)

According to the kit specifications (BD SMART™ RACE cDNA amplification Kit), two sets of primers (GSP and NGSP) were designed for 5'-RACE PCR reactions, based on the partial sequences of the gene of interest. A reactions was composed of 2.5 µl 5'-RACE-Ready cDNA, 5 µl UPM (10X), 1 µl GSP1 (10 µM), 5 µl 10X BD Advantage2 PCR Buffer, 1 µl dNTP Mix (10 mM), 1 µl 50X BD Advantage2 polymerase mix, 34.5 µl PCR-grade water. The PCR program was 20 cycles composed of 30 sec denaturation at 94°C, 30 sec annealing at 68°C and 3 min extension at 72°C. PCR products were analyzed by agarose gel electrophoresis (3.5.3) and positive fragments were cloned into the PCR2.1 vector prior to sequencing. Nested PCR was performed for those RACE reactions that resulted in a smear of DNA rather than discrete bands on a gel. The procedure was as above, substituting 5'-RACE-Ready cDNA, UPM and GSP1 for 5 µl of diluted primary PCR product (1:50 in Tricine-EDTA buffer), 1 µl of the NUP primer and 1 µl of NGSP using the same PCR program as above.

3.14. Polymerase chain reaction (PCR)

3.14.1. Standard PCR reaction

The Polymerase Chain Reaction method developed by Mullis and Faloona (1987) is a cycling reaction in which a specific DNA fragment is denatured by heating to separate dsDNA, followed by hybridization of the denatured DNA with specific primers (annealing). The cycle ends as the primer molecules are elongated by the action of DNA polymerase to produce a new strand of DNA. Repeating the cycle (usually 25 to 35 times) produces sufficient amounts of the specific DNA fragment for analyzes. A standard PCR reaction was set up as below:

PCR mix	amount
10X PCR reaction buffer	3.0 µl
dNTP's (10 mM)	0.5 µl
5' and 3' primer (10 µM)	2.0 µl
DNA template ¹	100-200 ng**
DNA polymerase ²	0.5 U
H ₂ O	adjust to 30 µl

¹ For full-length amplifications, 2 µl cDNA (3.12) was used as template.

Thermal cycling conditions

Step	Temp (°C)	Time	cycles
Initial denaturation	95	1-3 min	1
Denaturation	95	0.5-2 min	
Annealing	45-65	30 sec	25-35
Extension	72	2-4 min	
Final extension	72	5 min	1
Stop	4	∞	

² *Taq* polymerase was used for routine PCR reactions and *Pfu* polymerase was used for full-length amplifications.

3.14.2. Screening bacterial colonies by PCR

This method was used as a preliminary step to identify bacterial colonies containing recombinant plasmid. Using a white tip, a single colony at random (or white in blue/white screening) was picked up and streaked onto a fresh LB plate used as a replica (about 20 colonies on a single plate). The plate was incubated at 37°C, and then stored at 4°C until needed. After streaked onto a plate, the tip was then dipped into a PCR tube containing PCR mix and a PCR reaction was run as described in (3.14.1) using vector primers or GSP. The amplified PCR fragments were analyzed by agarose gel electrophoresis (3.5.3). Colonies yielding products of the expected size were selected for plasmid isolation (3.3.1) and DNA sequencing.

3.14.3. Sequencing of DNA

Nucleotides sequences were determined using a BigDye™ Terminator cycle sequencing kit in an automated DNA sequencer ABI 3100 Avant Genetic Analyzer (Applied Biosystems). The standard reaction conditions were as follows:

Sequencing mix	Volume (µl)
BigDyeMix V 1.1	4.0
Primer (5 µM)	1.0
Plasmid-DNA**	1-5
H2O	Adjust to 10 µl

** 600 ng plasmid; 20 ng PCR product

Cycle sequencing		
Step	Temp (°C)	Time
25 cycles		
Denaturation	96	10 sec
Annealing	50	5 sec
Elongation	60	4 min
Stop	4	∞

Once the sequencing reaction was completed, samples were purified by gel filtration with sephadex G-50 superfine (20-80 μ m) columns.

Sequences were compared using the BLAST program provided by the NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>).

3.15. DNA modifications

3.15.1. Addition of a 3'-A Overhang

Pfu DNA polymerase generates blunt-ended PCR products due to its 3' and 5' exonuclease proofreading activity that removes the 3' overhangs necessary for TA cloning. However 3'-A overhang can be added to blunt-end fragments using Taq DNA polymerase after PCR amplifications. In a vial on ice, 7 μ l of PCR reaction were mixed with 0.2 unit of Taq polymerase, 2 μ l of dATP 1 μ M and 1 μ l 10X Taq Buffer and incubated at 72°C for 15 minutes. After agarose gel purification (3.3.2), fragments were ligated into a T-vector (3.16.1).

3.15.2. Dephosphorylation of DNA fragments

In order to prevent self-enclosure of a vector in ligation reactions, those vectors digested with a single restriction enzyme were dephosphorylated (hydrolysis of the 5'-terminal phosphate residue) using calf intestine alkaline phosphatase (CIAP) enzyme. After vector digestion with a restriction endonuclease, 3 μ l of 10X buffer, 1 μ l CIAP were added to the reaction and the volume adjusted to 30 μ l with sterile water. The sample was incubated for 30 min at 37°C followed by DNA purification on agarose gel (3.3.2).

3.15.3. Restriction enzyme digestion

Restriction endonucleases recognize specific sequences in the DNA and cleave a phosphodiester bond on each strand at that sequence. A general procedure for conducting restriction digestion consisted of 1X reaction buffer, 1X BSA (if required), 0.5-1.0 µg DNA, and 1 U enzyme per µg DNA in a total volume of 20 µl. The sample was incubated at 37°C for 3 hr and heat inactivated before adjusting conditions for a second enzyme (if required). The resultant DNA fragments were separated by agarose gel electrophoresis (3.5.3) and purified using a minelute kit (3.3.2).

3.16. DNA cloning

The ligation of a DNA fragment into a vector was catalyzed by T4 DNA ligase. This enzyme catalyzes the formation of a phosphodiester bond by the condensation of a 5' phosphate and 3' hydroxyl group of adjacent nucleotides occurring in a nick or between cohesive or blunt termini of DNA.

3.16.1. TA cloning

PCR products with a 3'-A overhang can be directly cloned into a linearized vector with a complementary 3'-T overhang. The amount of PCR product needed to ligate with 50 ng T-vector (pCR[®]2.1 or pGEM[®]-T easy) was calculated as below. The ligation reaction was set up by mixing the calculated fresh PCR product, 1X ligation buffer, 50 ng vector, 1 µl of the T4 DNA ligase (3-4 Weiss units/ µl) in a total volume of 10 µl. The sample was briefly centrifuged and incubated overnight at 14°C. The recombinant plasmid was transformed into the appropriate competent cells and purified for further subcloning or sequencing. A ratio 3:1 insert to vector was normally used.

$$\text{ng of insert} = \frac{(\text{ng of vector}) (\text{kb size of insert})}{\text{kb size of vector}} * \text{molar ratio} \frac{\text{insert}}{\text{vector}}$$

3.16.2. Subcloning

The gene to be expressed in *Sf9* insect cells was first excised from a T-vector with specific restriction enzymes (3.15.3) and inserted into the same sites of a baculovirus transfer vector

(pVL1392 or pFastBac1) pre-digested and dephosphorylated (if required). Both vectors contain a multiple cloning site downstream of a baculovirus promoter required for expression of proteins in insect cells. The amount of insert and the conditions required to ligate with 100 ng transfer vector were calculated as in (3.16.1). The reaction was carried out at 4°C overnight in a 20 µl ligation mixture containing 2µl 10X ligase buffer, 1 µl T4 DNA ligase (3 U/µl), 100 ng Vector and the insert.

3.17. Transformation of competent cells

Once the ligation reaction was completed, a standard procedure for transformation of bacteria was performed as follows: In an Eppendorf tube on ice, 2 µl of ligation product were added to 50 µl competent cells and left on ice for 30 min. After that, cells were heat shocked for 30 sec in a water bath at 42°C and placed back on ice for 2 min. After addition of 250 µl SOC medium, cells were grown at 37°C for 1 h with shaking (225 rpm). Twenty to 100 µl from each transformation were plated onto LB plates containing the appropriate supplement (below) and incubated overnight at 37°C.

Supplements required for LB plates

Cells/vector	Supplements
TOP10 cells/pCR2.1	50 µg/ ml kanamycin
XL1-Blue MRF ⁺ cells/ pGEM-T	100 µg/ ml ampicillin, 0.5M IPTG, 80 µg/ ml X-Gal

After transformation, plasmid was purified and analyzed for correct insertion of the gene by restriction endonuclease digestion (3.15.3), PCR analysis (3.14.1) or sequencing (3.14.3).

3.18. Protein Expression

3.18.1. BaculoGold Expression Vector System

The Baculovirus Expression Vector System (BEVS) from BD-PharMingen uses the *Autographa californica* nuclear polyhedrosis virus (AcNPV) for the expression of foreign genes in insect cells. The gene of interest is cloned in a transfer vector within flanking sites homologous to the AcNPV DNA. Recombination between both sites occurs via co-transfection of the transfer vector and the AcNPV DNA into *Spodoptera frugiperda* (*Sf*) cells.

Infection and amplification of *Sf9* cells with the recombinant virus results in the expression of mRNA and protein production.

3.18.2. Co-transfection using BD Baculogold

Transfection buffer A: Grace's medium with 10% FBS

Transfection buffer B: 25 mM HEPES pH 7,1; 125 mM CaCl₂ ; 140 mM NaCl

Recombinant baculovirus was generated by cotransfection of linearized baculovirus DNA (BD Baculogold™) and pVL1392 vector containing the gene of interest by using BD Baculogold kit. Co-transfection in insect cells was developed as follows:

- (1) 5×10^5 *Sf9* cells/well was seeded onto a 37 mm 6 well plate. After 15 minutes, the culture medium was removed and replaced with 1 ml of transfection buffer A.
- (2) In a sterile Eppendorf tube, 0.05 µg baculovirus DNA was mixed with 2 µg of recombinant plasmid and after 5 min, 1 ml of transfection buffer B was added.

The mixture from the step 2 containing buffer B was added drop-by-drop to the insect cells in the step 1, rocking the plate back and forth to mix the newly added solution with the transfection buffer A. The plate was incubated at 27°C for 4 hr after that, the solution was removed and cells were washed with 3 ml of TC-100 medium. Finally, 3 ml of TC100/FBS medium were added to the plates and incubated at 27°C for 5 days. After 5 days the medium was harvested and centrifuge (700 x g) for 5 min at RT. The supernatant containing recombinant virus was used for further rounds of amplification (3.18.6) or stored at 4°C in a dark place.

3.18.3. BAC-to-BAC expression system

In the baculovirus expression system from Invitrogen, the gene of interest is cloned into a transfer vector (pFastBac™1) and then inserted into a bacmid (baculovirus shuttle vector) propagated in *E. coli* cells (DH10Bac™) by site-specific transposition. Bacterial colonies containing the recombinant bacmid DNA can be selected by white/blue selection and the purified recombinant bacmid DNA used for transfection in insect cells.

3.18.4. Bacmid transposition

Recombinant plasmid (pFastBac™1) was transformed into DH10Bac™ competent cells by mixing 3 µl of ligation mix with 40 µl of the cells. The sample was incubated on ice for 30 min, heat shocked for 45 sec in a water bath at 42°C and placed back on ice for 2 min. After addition of 900 µl SOC medium, cells were grown at 37°C for 4 h with gentle shaking. Fifty to 100 µl were plated onto LB plates containing 50 µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg/ml gentamycin, 10 µg/ml tetracycline, 80 µg/ml X-Gal, 40 µg/ml IPTG and incubated for 24 to 48 h at 37°C. White colonies were selected for recombinant bacmid DNA isolation (3.3.1). The insertion of the gene of interest into the bacmid was confirmed by PCR (3.14.1) using GSP and vector primers.

3.18.5. Transfection of *Sf9* insect cells with recombinant bacmid DNA

Transfection of recombinant bacmid was performed using the Cellfectin reagent (Invitrogen) according to the manufacturer's instructions. The procedure was as follows: In a 6-well tissue culture plate, 5×10^5 *Sf9* cells/well were seeded and allowed to attach to the bottom of the plate for 30 min at 27°C, then washed once with 2 ml of TC100 medium. For each transfection in insect cells, 5 µl of mini-prep bacmid DNA and 6 µl Cellfectin (lipid reagent) were diluted separately into 100 µl TC100 Medium. Both solutions were mixed together and incubated for 40 min at RT, then diluted to 1 ml with TC100 medium and added to the cells. The cells were incubated for 5 h at 27°C after which the transfection medium was removed and replaced with 2 ml TC100/FBS. Cells were incubated for 3-4 days at 27°C. The supernatant containing recombinant virus was used for further rounds of amplification (3.18.6) or stored at 4°C protected from light.

3.18.6. Baculovirus amplification

The recombinant baculovirus obtained after transfection of *Sf9* cells (3.19.2 or 3.19.5), was further amplified three times by infecting additional *Sf9* insect cells. 1 ml of the previous amplified virus was added to a T-25 flask containing 2.0×10^6 cells/ml in TC100/FBS, and incubated at 27°C for 4 or 5 days. After about 90% of the cells were lysed, the supernatant was harvested from the plate and the cellular debris spin down in a centrifuge at $700 \times g$; 5 min at RT. Supernatant was transferred into a sterile tube and stored at 4°C in a dark area. A high recombinant virus stock was preparing by infecting 50 ml insect cell culture supplemented with TC100/FBS/P, with the resultant 5 ml recombinant virus from the fourth

amplification. Cells were incubated for 4 to 5 days at 27°C with shaking (150 rpm) until about 90% of the cells were lysed. After that, cells were centrifuge at 700 x g for 5 min at RT and the supernatant containing virus stored at 4°C in a dark area. This viral stock was used to overexpress the protein of interest in insect cells.

3.18.7. CYP450 expression in *Sf9* cells

Resuspension buffer	100 mM Tricine/NaOH pH 8.0, 5 mM TGA, 10% v/v glycerol, 2 mM EDTA, 10 mM 2-mercaptoethanol.
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In a 200 ml conical flask, 50 ml *Sf9* cell cultures were grown to a density of 2.0×10^6 cells/ml in TC100/FBS/P medium and recovered by centrifugation at 700 x g for 5 min at RT. Cells were resuspended in 10 ml of TC100/FBS/P medium and mixed with 0.5 ml recombinant P450 viral stock and 0.5 ml of a viral stock containing cDNA that encodes NADPH-cytochrome P450 reductase (Huang 2000). *Sf9* cells were incubated at 27°C for 1 h with shaking at 150 rpm, after that, 40 ml of TC100/FBS/P medium were added. To compensate for the low endogenous level of hemin in insect cells, hemin was added to a final concentration of 2 µg/ml 24 h after infection. *Sf9* cells were harvested 72 h after infection or until the cells enlarged and about 10-20% of cells were disrupted. Infected cells were collected by centrifugation (700 x g, 5 min, RT) and resuspended in 3.5 ml of resuspension buffer.

3.18.8. Ctg9 and ctg11 expression in *Sf9* cells

	A) 100 mM Tricine/NaOH pH 7.5, 5 mM TGA, 10% v/v glycerol, 2 mM EDTA, 10 mM 2-mercaptoethanol.
Resuspension buffer	B) 50 mM phosphate buffer pH 7.5 or 9.0
	C) 50 mM phosphate buffer pH 7.5 or 9.0, 1.0 % emulgen 913

In a 200 ml conical flask, 50 ml *Sf9* cell cultures were grown to a density of 2.0×10^6 cells/ml in TC100/FBS/P medium and recovered by centrifugation at 700 x g for 5 min at RT. Cells were resuspended in 10 ml of TC100/FBS/P medium and mixed with 0.5 ml recombinant ctg9 or ctg11 stock. *Sf9* cells were incubated at 27°C for 1 hr with shaking at 150 rpm, after that, 40 ml of TC100/FBS/P medium were added. *Sf9* cells were harvested 72 h after infection or

until the cells enlarged and about 10-20% of cells were disrupted. Infected cells were collected by centrifugation (700 x g, 5 min, RT) and resuspended in 3.0 ml of resuspension buffer.

3.19. Enzymatic assay

3.19.1. CYP450 activity assays

The standard conditions for activity assays consisted of 60 µl cell suspension (3.18.7), 200 mM tricine/NaOH pH 8.0, 0.5 mM NADPH and 50 µM substrate in a total volume of 80 µl. The assay mixture was incubated for 30 min and then terminated by the addition of 1 volume methanol. After protein precipitation, the sample was centrifuge at maximum speed for 2 min in a centrifuge bench. The reaction products were analyzed by HPLC and LC-MS (3.1.2 and 3.1.3). For pH studies, the buffer was phosphate buffer pH 6.0 – 8.5, tricine buffer pH 7.4 – 8.8, glycine buffer pH 9.0 – 10.0.

3.19.2. Ctg9 and Ctg11 activity assays

The standard conditions for activity assays consisted of 150 µl cell suspension or supernatant (3.18.8) with 100 mM tricine/NaOH (pH 7.5) or 100 mM phosphate buffer (pH 7.5 or 9.0) and 50 µM substrate in a total volume of 200 µl. The assay mixture was incubated for 2 h at 30 and 37°C and then terminated by the addition of 1 volume methanol. After protein precipitation, the sample was centrifuge at maximum speed for 2 min in a centrifuge bench. The samples were concentrated and then analyzed by HPLC and LC-MS (3.1.2 and 3.1.3).

3.20. Preparation of microsomes

500 ml of *Sf9* suspension culture was grown to a density of 2×10^6 cells/ml in TC100/FBS/P medium and collected by centrifugation at 700 x g for 5 min. The pellet was resuspended in 100 ml fresh medium, followed by the addition of 6.5 ml of recombinant P450 viral stock. After 1 h incubation at 27°C and 150 rpm, 400 ml TC100/FBS/P medium was added and the suspension incubated for 72 h. Hemin was added to a final concentration of 2 µg/ml 24 h after infection. Cells were collected by centrifugation (1000 x g, 5 min, RT) and the pellet washed twice with 40 ml PBS buffer (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, pH 7.4). After homogenisation in 30 ml of 100 mM tricine (pH 7.5)/ 5 mM thioglycolic acid (TGA), half the sample was frozen with liquid nitrogen and stored at -80°C. The remaining sample

was sonicated four times (10 x pulsed, output 5, 50% duty cycle) and protected from the light. Cell debris was removed by centrifugation at 8,000 x g, 20 min, 4°C (10,000 rpm Beckman Le-80 ultracentrifuge). Microsomes (in supernatant) were further sedimented by centrifugation at 105,000 x g for 65 min, 4°C (40,000 rpm, Beckman Le-80 ultracentrifuge). The resulting microsomal pellet was resuspended in 1.5 ml of 100 mM tricine (pH 7.5)/ 5 mM TGA using a Pasteur pipette, and homogenized in a chilled glass homogenizer with a Teflon pestle. 600 µl of the microsomal suspension was transferred into a new Eppendorf tube for measurement of a CO difference spectrum, the rest brought to 20% glycerol and stored at -80°C.

3.21. Measurement of CO difference spectra

A difference spectrum of microsomal preparations was measured with a Perkin Elmer Lambda 800 UV/Visible spectrophotometer with 1 mm light path cuvettes. 600 µl microsomal suspension were homogenized with 900 µl of 100 mM tricine buffer pH 7.5/ 5 mM TGA and 0.5 % (v/v) emulgen 913 (detergent) and agitated for 15 min. Insoluble debris was removed by centrifugation (15,000 x g, 15 min, 4°C) and the supernatant was divided into two cuvettes. A small amount of sodium dithionite (about 1 mg) was added to each sample and mixed by inverting the cuvette. Microsomal preparations were placed in both the sample and reference cells of the spectrophotometer. After recording the base line, the content of the sample cell was carefully gassed with CO for about 1 min and the spectral shift from 420 to 450 nm was measured.

3.22. Protein determination

Protein concentration was determined according to the method of Bradford (1976) with known concentrations of bovine serum albumin as standards. This method is based on the binding of Coomassie brilliant blue G-250 dye (CBB) to proteins with an absorbance maximum at 595 nm (blue).

4. Results

4.1. Alkaloids analysis

Plants of *Argemone mexicana* used in these studies were grown in the green house of the Leibniz-Institute of Plant Biochemistry for a total period of 12 weeks. Plants germinated twice a year in June and October with a period of dormancy between 1 to 3 months. The plant material was harvested, frozen in liquid nitrogen and stored at -80°C until used. For alkaloids analysis, total alkaloids were extracted from the frozen material (leaves, stem, roots and capsules, latex) as described in methods section 3.1.1 and analyzed on a gradient HPLC system equipped with a C18 reversed-phase column with wavelength detection at 210, 255 and 285 nm (3.1.2). The HPLC chromatogram in figure 4.1 is a representation of the profile of alkaloids in extracts of *A. mexicana*. Protopine and allocryptopine were identified as the main alkaloids in all the extracts analyzed. In LC-MS analysis, reticuline, methylcoclaurine, berberine, cryptopine and chelerythrine were also identified in all extracts. Sanguinarine was mainly in roots, otherwise only traces where found. (+/-)-6-Acetyldihydrosanguinarine was detected only in root extracts. Protopine, allocryptopine and berberine where also identified by HPLC analysis in latex.

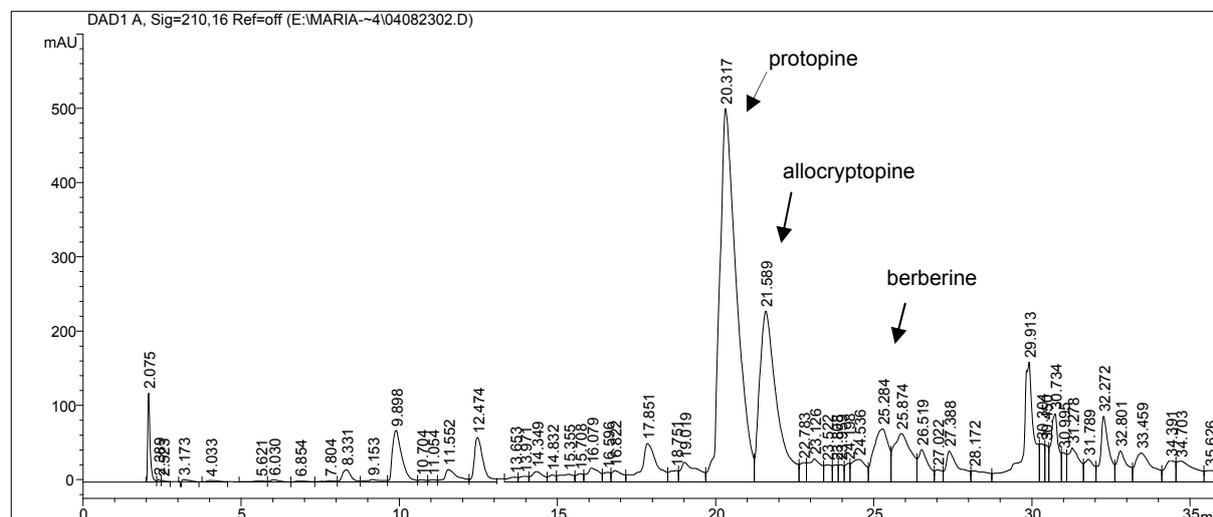


Figure 4.1. HPLC chromatogram of alkaloidal extracts from *Argemone mexicana* roots. Alkaloids were extracted with ethanol from a one month old plant.

Phytochemical studies using the whole plant have identified some other alkaloids especially those derived from the benzophenanthridine, protopine and protoberberine pathways. There are also examples of structures like argemexicaine A and B, which are structural isomers of allocryptopine, however, the lack of such standards hindered their identification by LC-MS.

4.2. Quantification of alkaloids

Protopine and allocryptopine accumulation was quantified in roots and leaves from *A. mexicana* for a period of 2 to 12 weeks. About 1.0 g of plant material was used for alkaloid extraction using the method previously described. Dihydrocodeine (dhc used as internal standard) was added prior to alkaloid extraction to correct for the loss of the analyte during extraction and alkaloid quantification. The data are reported as microgram of alkaloids per 100 mg fresh weight material. The results of the alkaloid quantification in leaves are displayed in figure 4.2. Protopine is accumulated during plant growth to a maximum of four times that amount present in the second week. After the third week, the amount of this alkaloid doubles and between the fifth and twelve week, its concentration was almost constant. In the case of allocryptopine, accumulation was not as evident as that of protopine, doubling only after 12 weeks. Comparing both alkaloids, protopine accumulation in leaves was nine times more than that of allocryptopine.

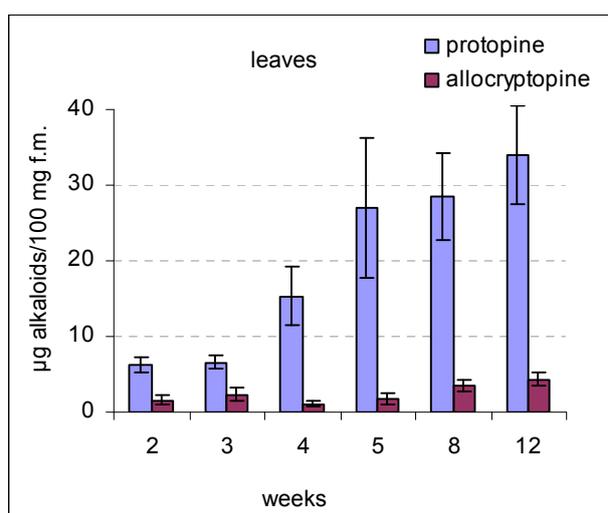


Figure 4.2. Quantification of protopine and allocryptopine in leaf extracts. About 1 g of leaves was used for alkaloid analysis. Samples were extracted with ethanol and analyzed by HPLC using dhc as internal standard.

The quantification of alkaloids extracted from roots is displayed in figure 4.3. Protopine and allocryptopine accumulation displayed a similar pattern as in leaves. The amounts of protopine doubled after the third week, reaching a maximum concentration in the eighth week.

In the case of allocryptopine, there was an increase after the third week reaching a maximum in the 12 week. Protopine and allocryptopine were each found in higher amounts in roots than in leaves, resulting in about 1.5 and 3 times more for protopine and allocryptopine, respectively.

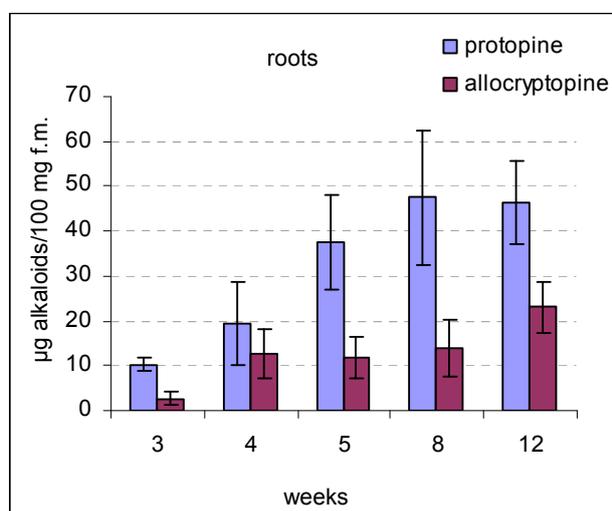


Figure 4.3. Quantification of protopine and allocryptopine in roots extracts. About 1 g of roots were used for alkaloid analysis. Samples were extracted with ethanol and analyzed by HPLC using dhc as internal standard.

These results showed that protopine and allocryptopine accumulate in higher amounts in roots than in leaves, but in both cases there was an increase in alkaloid production after the third week. These results could be associated with an increase in the production of enzymes involved in the protopine and benzophenanthridine pathways. Based on these results, a cDNA library from mRNA isolated from 3-4 weeks old roots of *Argemone mexicana* was constructed.

4.3. λ -cDNA library construction

A cDNA library was generated using 7 μ g Poly(A)⁺ RNA isolated from 3-4 week old roots of *Argemone mexicana*. Poly(A)⁺ RNA was primed with an oligo(dT)₁₈ primer carrying an XhoI site, and the first strand synthesis reaction was performed at 42°C. After second strand synthesis, the termini of cDNAs were converted to blunt-ends and EcoRI adaptors were ligated to both ends. Then the XhoI sites at the 3'-ends of cDNAs were generated by digestion with XhoI. After size fractionation, fractions greater than 0.4 kb were ligated into the λ -ZAP express vector. Finally, packaging extracts were used to package the recombinant λ -phage. The titer of the cDNA library was estimated to contain about 160 000 pfu. The λ -cDNA library was used to infect XL1-Blue MRF' *E. coli* cells followed by plating on LB plates with IPTG/X-gal and incubating overnight at 37°C. The white plaques containing recombinant

phages where picked up and transferred to SM buffer (3.9). Inserts were amplified with *Taq* polymerase, using 2 μ l of the phage suspension as template and T7 (5'-GAA TTG TAA TAC GAC TCA CTA TAG-3') and T3 (5'-GCT CGA AAT TAA CCC TCA CTA AAG-3') primers. The thermo cycling conditions were as follows: One cycle of 94°C/3 min// 5 cycles of 94°C/30 sec, 65°C/30 sec, 72°C/3:0 min//30 cycles of 94°C/30 sec, 58°C/30 sec, 72°C/3:0 min// and one cycle of 72°C/7 min and 4°C/ ∞ . A representation of PCR products analyzed by 1% agarose gel electrophoresis is showed in figure 4.4. The insert size of the total randomly picked clones ranged from about 0.5 kb to 3.0 kb with an average of 0.7 kb.

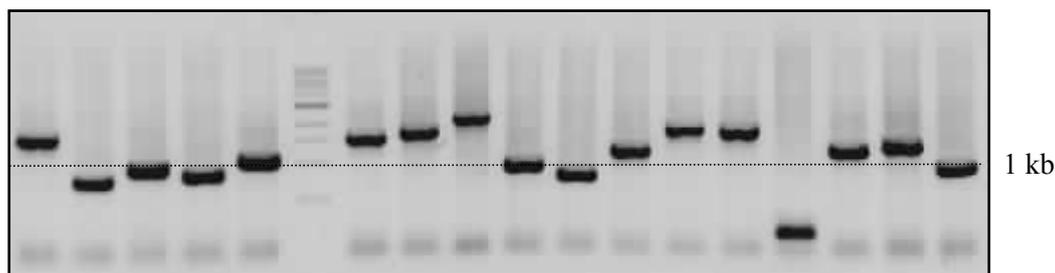


Figure 4.4. Agarose gel electrophoresis of PCR products amplified from a cDNA library of *Argemone mexicana*. Amplified cDNA was compared with a 1 kb ladder.

4.4. Relative abundance in ESTs

Randomly selected cDNA clones were sequenced from the 5'-end with standard T3 primer using a BigDye Terminator Cycle Sequencing Kit in an automated DNA sequencer ABI 3100 Avant Genetic Analyzer. About 1,600 expressed sequence tags (ESTs) were generated. This region (5'-EST) tends to be conserved across species and do not change much within a gene family. 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 nonredundant database at the National Center for Biotechnology Information (NCBI) using the BLASTX algorithm with default parameters. This program compares translated nucleotide sequences with protein sequences. 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 ESTs clusters was 486 bp. As shown in figure 4.5, 43% 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.

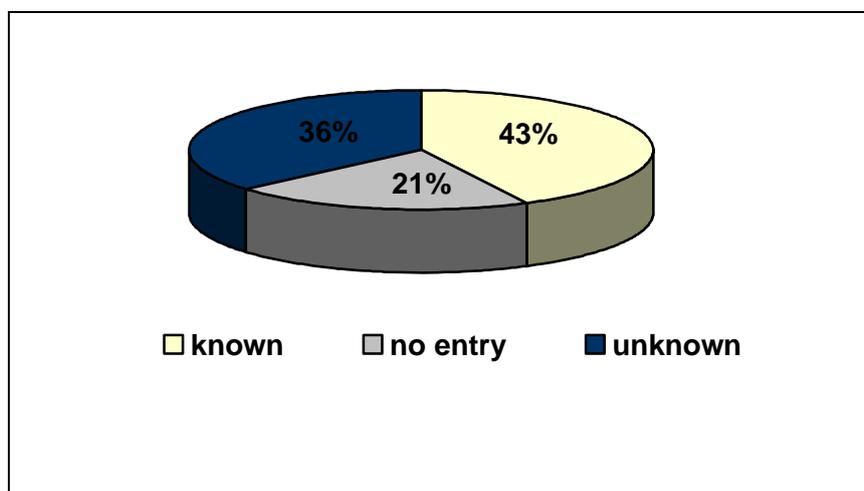


Figure 4.5. Classification of ESTs generated from *Argemone mexicana* roots. Each sequence was compared with the nonredundant database at the NCBI, using the BLASTX algorithm.

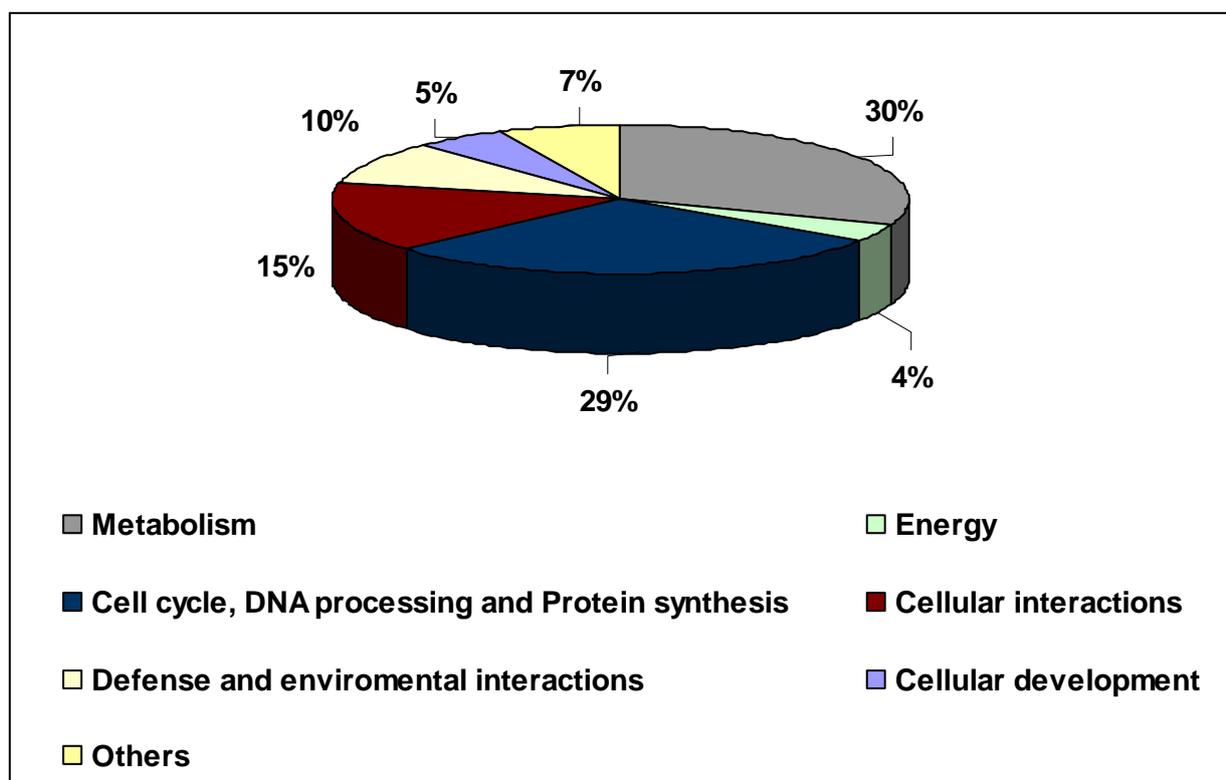


Figure 4.6. Representation of functional category of genes. Clustered ESTs were assigned to a functional category based on top BLASTP similarity scores. The analyses were performed at the Munich Information Center for Protein Sequences.

4.5. Annotation of *Argemone mexicana* ESTs

ESTs were classified into 7 different functional categories according to the top match against the Munich Information Center for Protein Sequences (MIPS) protein entry codes (http://mips.gsf.de/proj/thal/db/tables/tables_func_frame.html). As shown in figure 4.6, 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% are related to secondary pathways such as phenylpropanoid, flavonoid and alkaloid biosynthesis. Among them, ESTs similar to proteins presumably involved in the biosynthesis of alkaloids are the most representative with 21 unique sequences representing 13% of this category. Table 4.1 lists the enzymes which displayed identity to the sequences analyzed.

Table 4.1 Representation of genes involved in metabolism.

Homologues to alkaloid biosynthetic genes	
NCS	Norcochlorine synthase
6OMT	(<i>S</i>)-adenosyl-L-methionine:norcochlorine 6- <i>O</i> -methyltransferase
CNMT	(<i>S</i>)-adenosyl-L-methionine: coclorine <i>N</i> -methyltransferase
CYP80B1	(<i>S</i>)- <i>N</i> -methylcoclorine 3'-hydroxylase
4'OMT	(<i>S</i>)-adenosyl-L-methionine: 3'-hydroxy- <i>N</i> -methylcoclorine 4'- <i>O</i> -methyltransferase
BBE	Berberine bridge enzyme
S9OMT	(<i>S</i>)-adenosyl-L-methionine: scoulerine 9- <i>O</i> -methyltransferase
CYP719	Canadine synthase (methylenedioxy-bridge-forming)

The enzymes NCS to 4'OMT, are involved in the early steps of the biosynthesis of benzyloquinoline alkaloids leading to the intermediate (*S*)-reticuline. S9OMT methylates scoulerine at the OH group in position 9. All these enzymes have been already cloned and characterized in various species (Kutchan and Zenk, 1993).

BBE, a methylene bridge forming enzyme, catalyses the conversion of (*S*)-reticuline to (*S*)-scoulerine which is an important intermediate in the pathway leading to protoberberine and benzophenanthridine alkaloids. This enzyme was isolated and characterized from elicited cell suspension culture of *E. californica*. After Southern blot analysis, only one gene of BBE was found in the genome of *E. californica* (Dittrich and Kutchan, 1991). In this cDNA library, two independent clones displayed homology to this enzyme (Table 4.2), indicating the possibility of two BBE related enzymes. Further analysis could provide information of the relations of both cDNAs in alkaloid biosynthesis. CYP719A1 or canadine synthase is a methylenedioxy-bridge-forming enzyme involved in the biosynthesis of canadine leading to berberine. Besides this enzyme, evidence obtained from studies in *Eschscholzia californica* cell suspension cultures indicated the possibility that two different methylenedioxy-bridge-forming enzymes are involved in the biosynthesis of stylophine from (*S*)-scoulerine via (*S*)-cheilanthifoline (Bauer and Zenk, 1989). Considering that *Argemone mexicana* contains mainly protopine alkaloids, both enzyme activities could be present in this plant. To analyze their potential involvement in the biosynthesis of alkaloids, those ESTs of the cDNA library homologous to BBE and CYP719 were selected for further isolation and characterization. As displayed in Table 4.2, contig 5 was represented by 11 clones, which sizes varied between 1.0 and 2.0 kb. Contig 12 contained only one clone of about 1.6 kb. In contig 5, the first 553 nucleotides sequenced displayed the highest homology to the CYP719 enzyme isolated from *C. japonica* (Ikezawa *et al.*, 2003), but not to the 440 nucleotides sequenced from contig 12, which presented a low homology to the same. Both cDNAs sizes were in the range of the expected size. These cDNAs were sequenced in their entirety and compared to CYP719 of *C. japonica*. Two other ESTs clusters selected for homology to BBE were designated contig 9 and contig 11. The first one was represented by 2 cDNAs and the last one by one cDNA. The expected size was ~1.6 kb, but about 800 and 500 nucleotides from the N-terminal sequence in ctg 9 and ctg 11 were missing, respectively. To obtain the complete coding regions of both BBE homologues, a 5'-RACE strategy was undertaken.

Table 4.2. Clones selected from the cDNA library based on its homology to BBE and CYP9719 enzymes.

Clone ID	cDNA	BLASTX Homology	Score	E-value
Ctg 5	13C10, 21C4, 15F05, 15E02, 12E06, 14A05, 04E05, 14F04, 12H11, 11H03, 10G01	BAB68769.1 methylenedioxy bridge-forming enzyme [<i>Coptis japonica</i>]	390	1.0E-107
		O48928 cytochrome P450 77A3p – soybean [<i>Glycine max</i>]	91.7	3.0E-17
		CAB86008.1 cytochrome P450-like protein [<i>Arabidopsis thaliana</i>]	88.2	4.0E-16
		542070 cytochrome P450 77A1 - eggplant	87.4	6.0E-16
Ctg 9	7A01, 5E12	AAC61839.1 berberine bridge enzyme [<i>Papaver somniferum</i>]	210	5.0E-54
		AAC39358.1 berberine bridge enzyme [<i>Eschscholzia californica</i>]	199	1.0E-50
		AAD17487.1 berberine bridge enzyme [<i>Berberis stolonifera</i>]	172	2.0E-42
Ctg 11	18D11	BAB10121.1 berberine bridge enzyme [<i>Arabidopsis thaliana</i>]	108	3.0E-23
		AAC39358.1 berberine bridge enzyme [<i>Eschscholzia californica</i>]	105	4.0E-21
		AAO50720.1 putative FAD-linked oxidoreductase [<i>Arabidopsis thaliana</i>]	99	3.0E-20
		AAP30841.1 nectarin 5 [<i>Nicotiana langsdorffii</i> x <i>Nicotiana sanderae</i>]	94.7	5.0E-19
Ctg 12	14D01	BAB68769.1 methylenedioxy bridge-forming enzyme [<i>Coptis japonica</i>]	40.8	0.009
		AAM20137.1 Putative (CYP71A22) [<i>Arabidopsis thaliana</i>]	38.5	0.045

4.6. Isolation of cytochrome P450 encoding cDNAs homologues to CYP719A1

The open reading frame from both CYP719 homologues ctg5 and ctg12 was elucidated by sequencing the amplified fragments with primers designed from the deduced known sequences. In order to minimize errors due to nucleotide misincorporation by Taq DNA polymerase, these sequences were confirmed by comparing them with cDNA clones amplified by PCR with *Pfu* polymerase using 2 µl of cDNA produced by RT-PCR (3.12). Primers and thermal cycling conditions were as follows:

Name	Primer 5'→3'	Tm
contig5flf	ATGGAGGAAAAAATCATGACTAAC	56
contig5flrc	TTACATACGAGGAACAATACCAG	57
contig12flf	ATGGATGAGACTATTTGGTTAATAATTA	56
contig12flrc	TCAATGGATGCGACAAGTA	52

one cycle of 94°C/2 min// 30 cycles of 95°C/30 sec, 50°C/30 sec, 72°C/2:30 min// 1 cycle of 72°C/5 min and 4°C/ ∞. After A tailing each amplified cDNA was cloned into pCR2.1 (3.16.1) for sequencing and compared with the first one. Nucleotide and predicted protein sequence of both cytochrome CYP719 encoding genes were analyzed and compared by using BLASTX and MegAlign software with CLUSTALW algorithms. Contig 5 poses an ORF of 1515 bp encoding for a putative CYP719 enzyme of 505 amino acids with a predicted molecular mass of 57.45 KDa (Figure 4.7). The second cytochrome isolated, contig 12 contains an ORF of 1485 bp encoding for a putative protein of 495 aa with a predicted molecular mass of 55.7 KDa (Figure 4.8). Analysis of both cDNAs with the SignalP 3.0 software (<http://www.cbs.dtu.dk/services/SignalP/>) displayed the presence of an N-terminal signal peptide (the first 35 aa in ctg5 and the first 24 aa in ctg12) necessary for the incorporation of the cytochromes into the endoplasmic reticulum. Predicted amino acid sequence displayed 58.2% identity between ctg 5 and ctg12 and 65.2 and 62.1% homology to the cytochrome CYP719 canadine synthase, respectively. Parallel to the conclusion of the experimental part of this thesis, two new enzymes involved in stylophine synthesis were isolated (Ikezawa *et al.*, 2007). The identity between these two enzymes identified as CYP719A2 and CYP719A3 is 84%. Both cytochromes, displayed about 74% and 60% similarity to ctg5 and ctg12, respectively.

```

1  ATGGAGGAAAAAATCATGACTAACAATAGTCCATGGATTCTTACTTCATCAACAACAACAACAACAA
   M E E K I M T N N S P W I L T S S T T T T T T
71  TACTACTATCATTACTCTTACTATCTTCATAATTTAAGAAGAAACAATCATCATCAAAAATGGT
   I L L S L L F T I F I I L R R N K S S S S K M V
141 ATGGCCAACAGGGCCAAAAACCTTACCAATTATTGGTAATATGAATATTCTTGGTGGTACAGCTCTTCAT
   W P T G P K T L P I I G N M N I L G G T A L H
211 GTCGTTTTACATAACCTTGCCAAAACCTTATGGTAACGTTATGACTATATGGATTGGTAGTTGGAGACCCG
   V V L H N L A K T Y G N V M T I W I G S W R P
281 TGATCGTTGTTTTAGATATCGATCGAGCTTGGGAAGTTCTTGTTAATAAATCTTCAGATTATTCAGCTCG
   V I V V S D I D R A W E V L V N K S S D Y S A R
351 AGATATGCCCGAAATTACTAAACTTGCTACAGCTGATTGGAAAACAATTTCTAGTTCTGATTCAGGTCCT
   D M P E I T K L A T A D W K T I S S S D S G P
421 TTTTGGACTAACCTTCGAAAAGGTCTTCAAATGTAGCTTTATCACCTCAAATTTATCATCACAAAAGTA
   F W T N L R K G L Q N V A L S P Q N L S S Q S
491 AATTTCAAGAACGTGATATAATCAAACGATTCAAATTTGAAAGAAGAAGCTAAAATGAATAATGGAAT
   K F Q E R D I I K T I Q N L K E E A K M N N G I
561 TGTTAAACCACTTGATCATCTTAAGAAAGCTATGGTTAGATTAATTAGTCGTTTAATTTACGGTCAAGAT
   V K P L D H L K K A M V R L I S R L I Y G Q D
631 TTTGATAACGATGAGTACGTTGAAGAAATGCATCATACTATCGAAGAATTGATACGTTAGTGGTTATG
   F D N D E Y V E E M H H T I E E L I R V S G Y
701 CGCGTTTAGCTGAAGCATTATTTATTATGCTAAGTATTTACCAAGTCATAAAAAAGCTGTTAGAGAAGTCCT
   A R L A E A F Y Y A K Y L P S H K K A V R E V L
771 ACAAGCAAATCAAAGAGTTCAAATCTAGTACGTCGGCTTTTATCGTTAAATTCACCTACTAATACTTAC
   Q A N Q R V Q N L V R P L L S L N S P T N T Y
841 TTACATTTTCTTAGATCACAGAACTACGAAGATGAAGTTATTATATTTCGCGATATTTGAAGCTTATCTTT
   L H F L R S Q N Y E D E V I I F A I F E A Y L
911 TGGGTGTAGATAGTACTTCTTCAACTACAGCATGGGCACCTCGCGTATTTAATACGAGAACCAAATGTTCA
   L G V D S T S S T T A W A L A Y L I R E P N V Q
981 AGAGAAATTATATGAAGAACTTAAGAATTTACGAACGATAACGATCGTAAAATGGTGAAATTTGAAGAT
   E K L Y E E L K N F T N D N D R K M V K F E D
1051 TTAACAAGTTACAATATTTACAAGCTGTAGTTAAAGAAACAATGAGGATGAAACCAATTGCACCATTAG
   L N K L Q Y L Q A V V K E T M R M K P I A P L
1121 CAATACCTCATAAAGCTTGTAGAGAACTTCATTAATGGGGAGAAAAGTTAATCAAGGAACCTAGGGTTAT
   A I P H K A C R E T S L M G R K V N Q G T R V M
1191 GGTAAATATATATGCATTACATCATAATCAAATGTATGGAAAGAACCTTATAAATTTAATCCAGAGAGA
   V N I Y A L H H N Q N V W K E P Y K F N P E R
1261 TTCTTACAGAAAAATCAAGATGGTGTGATGGTAAAGCAATGGAGCAATCACTATTACCATTTAGTGCAG
   F L Q K N Q D G V D G K A M E Q S L L P F S A
1331 GTATGAGAAATTTGTGCAGGAATGGAATTAGGTAACCTTCAATTTAGTTTTGCTCTTGCTAATCTTGTTAA
   G M R I C A G M E L G K L Q F S F A L A N L V N
1401 TGCTTTTAAATGGTCTTGTGTTTCTGATGGAGTATTTCTGATATGAGTGATCAATTAGGGTTTTGTTTTG
   A F K W S C V S D G V F P D M S D Q L G F V L
1471 TTGATGAAAACACCACTCGAAGCTGGTATTGTTTCCTCGTATGTAA
   L M K T P L E A G I V P R M*

```

Figure 4.7. Nucleotide sequence and deduced amino acid sequence of contig 5 (canadine synthase homologue). *Ctg5* cDNA contains an ORF of 1515 bp encoding a putative protein of 505 aa with a predicted molecular mass of 57.45 KDa. Dotted lines show the positions of the gene specific primers designed for sequencing.

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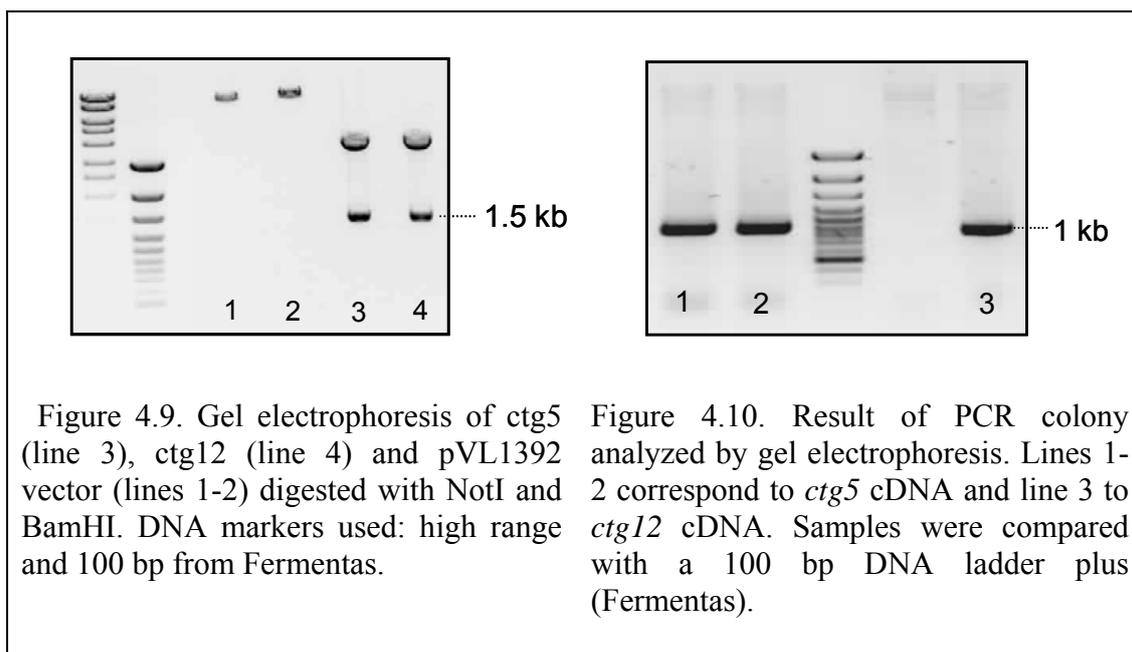
1  ATGGATGAGACTATTTGGTTAATAATTAGTACTGTAATTATAGTACTTGAATAGCAAAATTTCTATTAG
   M D E T I W L I I S T V I I V L G I A K F L L
71  GAAATCTTCTTCTTCTTCTCTCAACAATGGAATGGCCAGTCGGTCCAAAAAATTACCCATAATTGG
   G K S S S S S L S T M E W P V G P K K L P I I G
141 AAACCTTCATCAATTAGGAGGAGATGTTTTCCATGTTGTTTTAGCAAATTTAGCTAAAGTTTATGGGAGT
   N L H Q L G G D V F H V V L A N L A K V Y G S
211 GTTTTACAATTTGGGTAGGTAGTTGGCGTCCGATGATTATCGTAAGCGATATCGATAAAGCTTGGGAAG
   V F T I W V G S W R P M I I V S D I D K A W E
281 TTCTTGTTAATAAATCTTCAGATTATTCAGCTAGAGATATGCCTGATATTACTAAAATTTTTCAGCAAA
   V L V N K S S D Y S A R D M P D I T K I I S A N
351 TTGAAAAATATTTTCATGTTCTGATTGAGGTCATTTTGGCATAACTTAAGAAAAGGTCTTCAAGGTGTT
   W K N I S C S D S G P F W H N L R K G L Q G V
421 GCCCTAACTCCTCTTAATGTTGCATCTCAATATCATTTACAAGAAAGAGATATGAAAAATTTAATCAATT
   A L T P L N V A S Q Y H L Q E R D M K N L I N
491 CCATGTACAAAGACGCGTCGCGAAAAAATGGGATTTTAAAACCACTTGATTATCTTAAAGAAGAGACGGT
   S M Y K D A S R K N G I L K P L D Y L K E E T V
561 TCGATTACTAAGTCGTCTAATTTTCGGACAAGATTTTCAAGATGAGAAATTAGTTGTTGGTATGCATCAT
   R L L S R L I F G Q D F Q D E K L V V G M H H
631 GCCCTAGATGATTTGGTACGTATAAGTGGGTATGCTAGTTTACCTGATGCTTTTTAAATTTTTCGAAAAAT
   A L D D L V R I S G Y A S L A D A F K F C E N
701 TACCAAGTCATAAAAAATCTATTAGAGAAGTTCATGAAGTTAAAAAAGAGTCGAAAAATTTGATTTCGTCC
   L P S H K K S I R E V H E V K K R V E N L I R P
771 ACATATTGTTTCGAATCCTCCTACAAATACTTACTTATATTTTCTTAAAACCAAGATTTTAAACGAAGAT
   H I V S N P P T N T Y L Y F L K T Q D F N E D
841 ATTATAATATCTGCTATTCTTGAAGTTTATGACTTGGGTGTCGATAGTACTGCATCGACAACCTGTTTGGG
   I I I S A I L E V Y D L G V D S T A S T T V W
911 CATTAACGTTTTTATGTTTCGCGAACAAGAAATTCAGAAAACTTTATCGCGAAATTGTAATGTTACGGG
   A L T F L V R E Q E I Q E K L Y R E I V N V T G
981 CGGGAAAAGATCTGTTAAAGTTGAAGATGTGAATAAAATGCCATATTTACAAGCAGTAATGAAAGAAACA
   G K R S V K V E D V N K M P Y L Q A V M K E T
1051 ATGAGGATGAAACCCATTGCACCAATGGCAATACCACATAAAACTTCAAAGATACTTCATTAATGGGGA
   M R M K P I A P M A I P H K T S K D T S L M G
1121 AAAAAATTAATAAAGTTTCAGTGATAATGGTGAATCTTTATGCTATTCATCATAACCCTAAGGTATTTCC
   K K I N K G S V I M V N L Y A I H H N P K V F P
1191 TGAACCATATAAATTTATGCCAGAAAGATTTTTAAAGGATGTTAATAGTGATGAAAGTTTGGGTAATATT
   E P Y K F M P E R F L K D V N S D E S L G N I
1261 AAAACAATGGAAGTTCTTTATTAGCATTAGTGCTGGTATGAGAATTTGTCAGGTATGGAATTAGGTA
   K T M E S S L L A F S A G M R I C A G M E L G
1331 AACTTCAATTAGCTTTTGGTCTTGCAAGTTTGGTTCATGAATTCAAATGGAGTTGTTCTGTTGATGGAAA
   K L Q L A F G L A S L V H E F K W S C S V D G K
1401 ATTGCCTGATCTTAGTGAAGATCATTGTTTCATTCTCTTGATGAAAAACCACTCGAAGCGAAAATTACT
   L P D L S E D H C F I L L M K N P L E A K I T
1471 TGTCGCATCCATTGA
   C R I H *

```

Figure 4.8. Nucleotide sequence and deduced amino acid sequence of contig 12 (canadine synthase homologue). *Ctg12* cDNA contains an ORF of 1485 bp encoding a putative protein of 495 aa with a predicted molecular mass of 55.7 KDa. Dotted lines show the positions of the gene specific primers designed for sequencing.

4.7. Heterologous expression of cytochromes P450 *ctg5* and *ctg12*

CYP719 homologues were heterologously expressed in insect cells using the baculovirus expression system Baculogold from BD Bioscience with the transfer vector pVL1392. First, *ctg5* and *ctg12* genes were excised from the pCR 2.1 vector with NotI and BamHI enzymes (3.15.3). The digested products were subjected to 1% agarose gel electrophoresis (3.5.3) (Figure 4.9), purified (3.3.2) and then ligated into the same sites of the digested vector pVL1392 (3.16.2).



About 46 ng of digested insert were required to ligate with 100 ng linearized vector. The ligation reaction was transformed into bacteria then grown under ampicillin selection. Randomly selected colonies were analyzed by PCR (3.14.2) with the following primers: for *ctg5* (5'-GATGAGTACGTTGAAGAAATGC-3' and 5'-TTACATACGAGGAACAATACCAG-3') and for *ctg12* (5'-GTGGGTATGCTAGTTTAGCTG and 5'-TCAATGGATGCGACAAGTA-3'). PCR products analyzed by gel electrophoresis (Figure 4.10) displayed a band of about 900 bp that corresponded to the expected size of each fragment. Positive colonies designated pVL1392/*ctg5* and pVL1392/*ctg12* were sequenced again to confirm the sequence and orientation of the inserts.

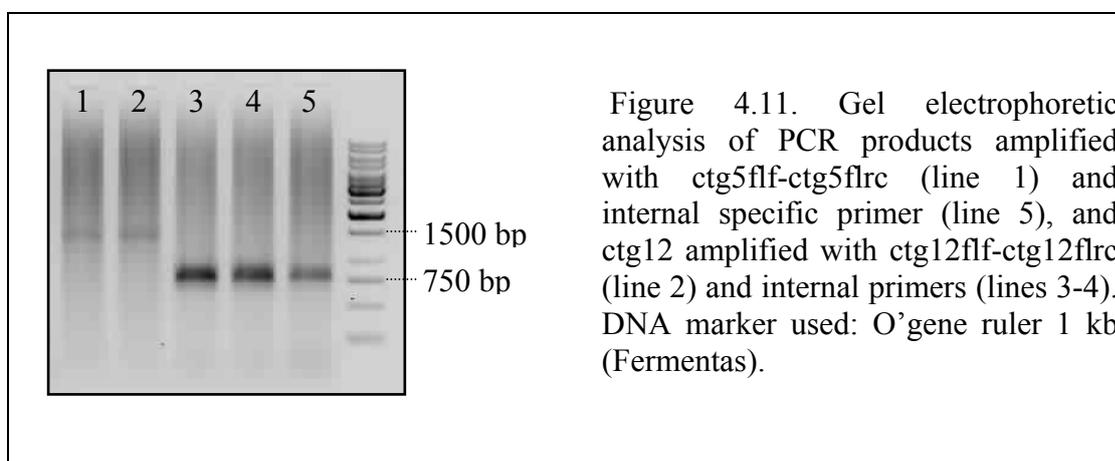
4.7.1. Cotransfection in insect cells

Recombinant baculovirus designated AcNPV /*ctg5* or AcNPV /*ctg12* was generated by cotransfection of linearized baculovirus DNA (BD Baculogold™) with pVL1392/*ctg5* or pVL1392/*ctg12* plasmids in insect cells *Sf9* as described in the methods section (3.18.2). After

cotransfection, a high titer recombinant baculovirus stock was prepared by serial infection of insect cells (3.18.6). The insertion of the gene of interest within the AcNPV DNA was confirmed by PCR amplification using 2 μ l of recombinant DNA baculovirus isolated as mentioned in methods section (3.3.3). Primers flanking the MCS pV_Lf and pV_Lr_c and gene specific primers for ctg12 and ctg5 were used.

Name	Primer 5'→3'
contig5p3	GATGAGTACGTTGAAGAAATGC
contig5flrc	TTACATACGAGGAACAATACCAG
contig12p3	GTGGGTATGCTAGTTTAGCTG
contig12flrc	TCAATGGATGCGACAAGTA
pV _L f	TATAGTTGCTGATATCATGGAG
pV _L r _c	GAGTTCTACAGGGAAACTTG

After gel electrophoretic analysis, PCR products of ctg5 and ctg12 amplified with vector primers appeared as a smear. A second PCR was done using 1 μ l of the first PCR as template and primers specific to ctg5 (ctg5p3-ctg5flrc and ctg5flf-ctg5flrc) and to ctg12 (ctg12p3-ctg12flrc and ctg12flf-ctg12flrc). The expected size for positive recombinant virus in both cases was about 1.5 kb and 800 bp as shown in figure 4.11.



4.7.2. Analyzing recombinant virus

In a 6-well plate, 5×10^5 *Sf9* cells/well were seeded. After 15 minutes, the culture medium was removed and replaced with 1 ml of fresh medium. One well was used as a control and to the other well 10 μ l of recombinant virus/ctg12 was added. Cells infected with recombinant virus displayed typical signs of infection in comparison with non-infected cell, as shown in figure 4.12. After 24 hrs of viral infection, cells infected with recombinant baculovirus

pVL1392/ctg12 displayed an increase in cell diameter and cessation of cell growth in comparison with non-infected cells and after 72 hrs of infection cells were lysed.

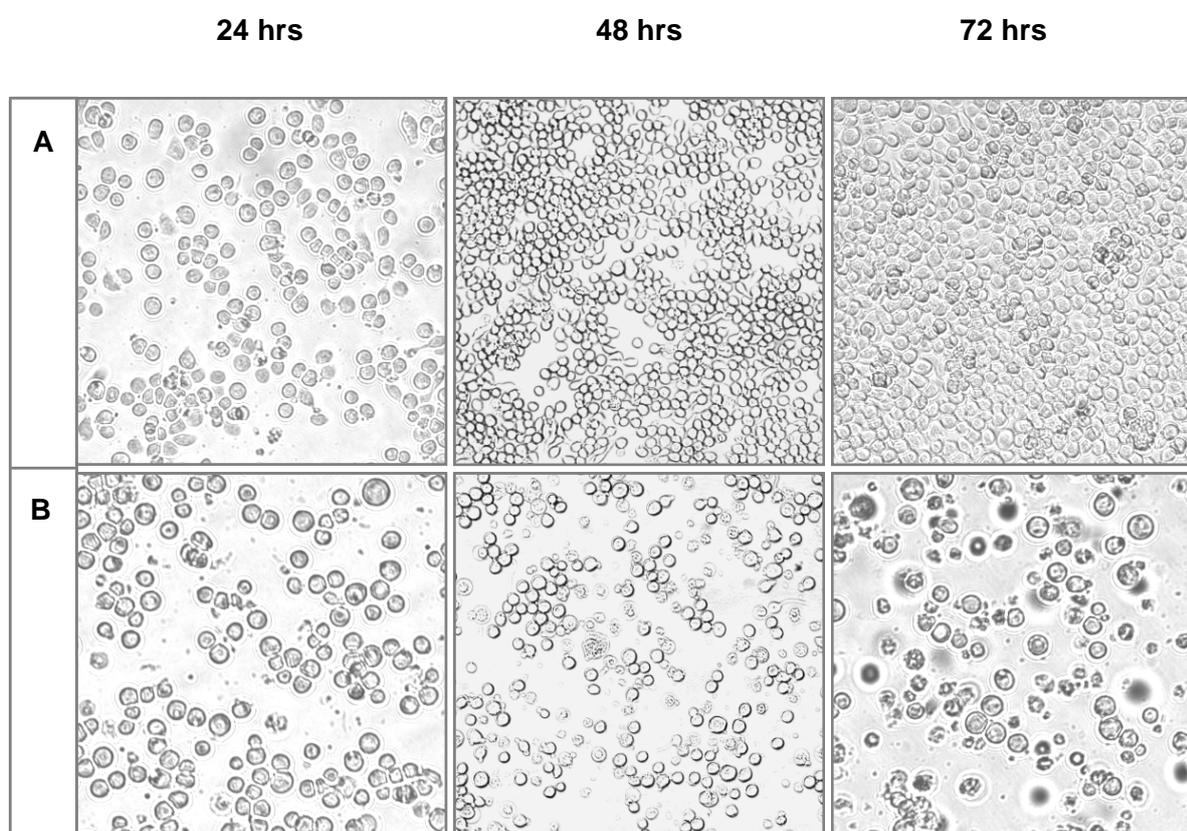


Figure 4.12. Panel (A) shows non-infected *Sf9* insect cells in comparison with panel (B) in which cells were infected with recombinant virus/ctg12. Pictures were taken at 24, 48 and 72 hrs after infection using inverted phase microscope at 30x magnification.

4.7.3. CO difference spectrum

Cytochromes P450 possess the property to bind carbon monoxide (CO) instead of oxygen exhibiting a characteristically reduced difference spectra with an absorption maximum at 450 nm (Omura and Sato, 1964). This binding avoids oxygen activation and consequently the enzyme activity is inhibited. It was observed that this inhibition can be reversed by irradiation with 450 nm light (Estabrook, 1963). This CO difference spectrum can be used as an indication that the protein was correctly expressed and folded and consequently has the property to bind CO. We used this property to determine if both recombinant cytochromes isolated from *Argemone mexicana* were correctly expressed in insect cells. Microsomal preparations of insect cells expressing AcNPV/ctg5 or AcNPV/ctg12 were prepared as described previously (3.20). A CO difference spectrum of reduced microsomal preparation

was recorded in a Perkin Elmer Lambda 800 UV/Visible spectrophotometer. As shown in figure 4.13, both cytochromes ctg5 (B) and ctg12 (C) exhibited a maximum absorbance at 450 nm, whereas microsomes from non-infected cells lacked the 450-nm peak (A).

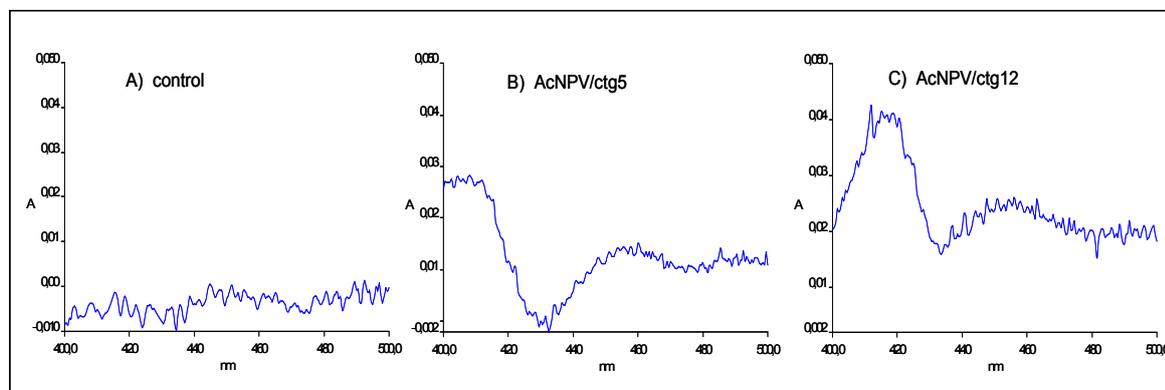


Figure 4.13. CO difference spectra of insect cells microsomes expressing recombinant ctg5 protein (B) and ctg12 (C) in comparison to control (A).

4.8. Catalytic activity of recombinant virus AcNPV/ctg5 or AcNPV/ctg12

CYP719 enzymes have been identified to be involved in the formation of a methylenedioxy-bridge group in THC, (*S*)-scoulerine and (*S*)-cheilanthifoline leading to (*S*)-canadine, (*S*)-cheilanthifoline and (*S*)-stylophine, respectively (Bauer and Zenk, 1991; Ikewaza *et al.*, 2003, 2007). In order to assay the recombinant enzymes ctg5 and ctg12 for methylenedioxy-bridge forming activity, THC, (*S*)-scoulerine and (*S*)-cheilanthifoline were tested. *Sf9* cells were infected with recombinant virus AcNPV/ctg5 or AcNPV/ctg12 as described previously (3.18.7). After 72 h, cells were harvested and resuspended in resuspension buffer (3.18.7). The standard conditions for activity assays consisted of 200 mM tricine/NaOH pH 8.0, 0.5 mM NADPH, 50 μ M substrate and 60 μ l of the cell suspension in a total volume of 80 μ l. The reaction mixture was incubated for 60 min at 30°C, and then terminated by the addition of 1 volume methanol. After centrifugation, reaction products were identified by HPLC and LC-MS (3.1.2 and 3.1.3), comparing them with those of authentic standards. Control studies were performed by incubating uninfected *Sf9* cell and baculovirus-infected *Sf9* cells expressing recombinant NADPH-cytochrome P450 reductase. Protein concentration of the suspension cells was measured using the method of Bradford with bovine serum albumin as standard.

4.8.1. AcNPV/ctg12 activity

Results of HPLC analyses (Figure 4.14) of the incubation of AcNPV/ctg12 with (*S*)-scoulerine gave one product peak at 23 min that comigrated with (*S*)-cheilanthifoline standard. To confirm the methylenedioxy-bridge formation, the product was analyzed by LC-MS. The results displayed native and derivatized mass spectra identical to (*S*)-cheilanthifoline. As shown in figure 4.15, there is a molecular ion at m/z 326 for (*S*)-cheilanthifoline and a fragment ion at m/z 178 that correspond to the intact isoquinoline moiety of (*S*)-scoulerine, indicating that the modification occurred at position 9,10 in ring D. No product formation was detected with either THC or (*S*)-cheilanthifoline. Ctg12 gene expressed in insect cells was confirmed as a methylenedioxy-bridge enzyme and identified as (*S*)-cheilanthifoline synthase (CYP719A14). The sequence of this enzyme has been deposited in the GenBank (accession number EF451152).

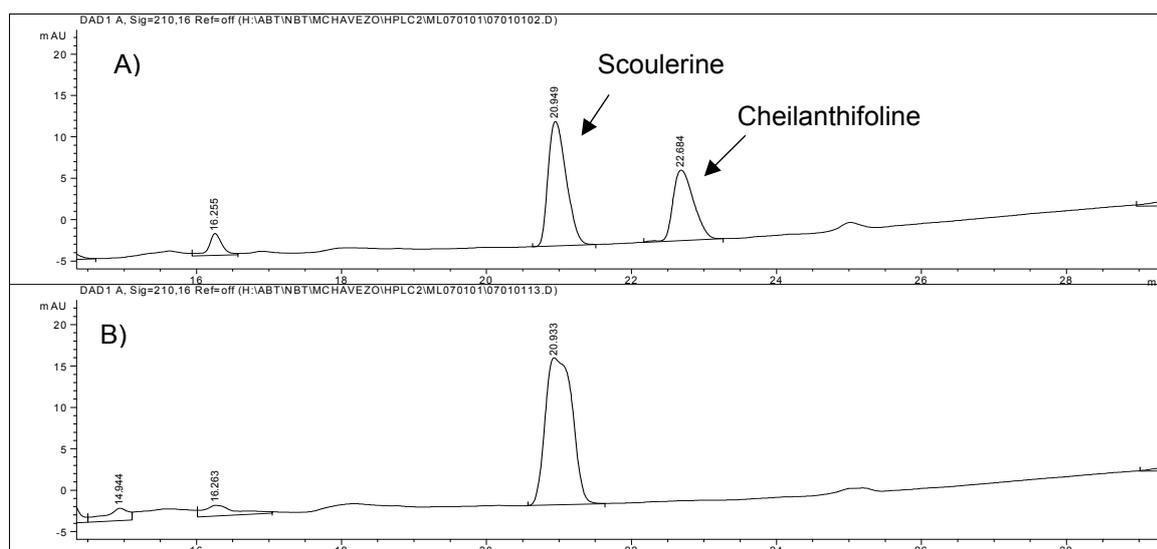


Figure 4.14. HPLC analysis of the reaction products of the recombinant virus AcNPV/ctg12 expressed in insect cells (panel A) and a control reaction with recombinant NADPH-cytochrome P450 reductase (panel B). The solvent system and gradient used were as previously described (3.1.2). The retention times corresponding to (*S*)-scoulerine and (*S*)-cheilanthifoline were approximately 21 and 23 min, respectively.

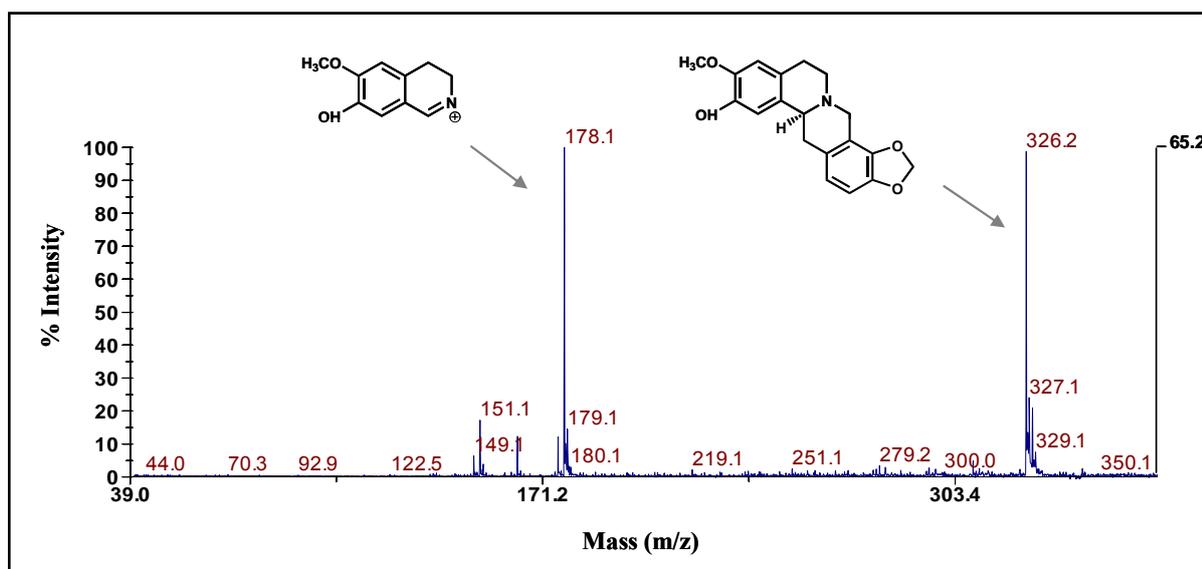


Figure 4.15. LC-MS spectrum of (*S*)-cheilanthifoline obtained from the reaction of cells expressing recombinant virus AcNPV/ctg12 with (*S*)-scoulerine. The molecular ion at m/z 326.2 corresponds to (*S*)-cheilanthifoline.

4.8.2. AcNPV/ctg5 activity

HPLC analysis of the reaction products of cells expressing AcNPV/ctg5 in presence of (*S*)-cheilanthifoline displayed a peak with a retention time of 25 min that corresponds to the substrate and a new peak with a retention time of 29 min identical to (*S*)-stylophine indicating the formation of a methylenedioxy-bridge (Figure 4.16). LC-MS analysis displayed a molecular ion at m/z 324 that correspond to (*S*)-stylophine and a fragment ion at m/z 176 that correspond to the reduction of 2 m/z in the isoquinoline moiety of (*S*)-cheilanthifoline (Figure 4.17), indicating that the modification occurred at position 2,3 in ring A. Neither THC nor (*S*)-scoulerine were accepted as substrate for this enzyme. The recombinant virus AcNPV expressing ctg5 gene was confirmed as a methylenedioxy-bridge enzyme and identified as (*S*)-stylophine synthase (CYP719A13). The sequences of this enzyme have been deposited in the GenBank (accession number EF451151).

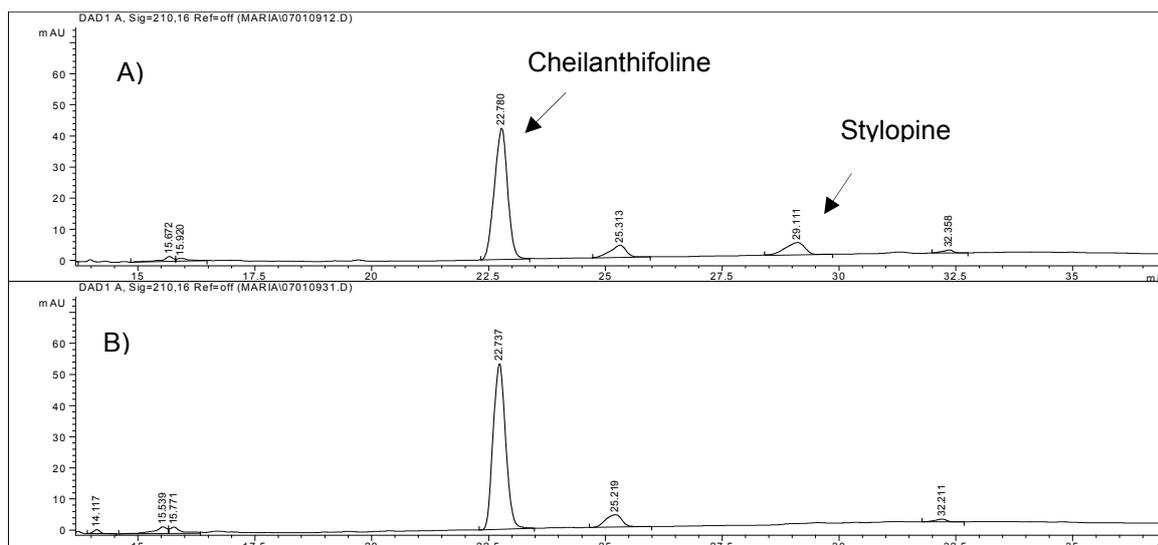


Figure 4.16. HPLC analysis of the reaction products of the recombinant virus AcNPV/ctg5 expressed in insect cells (panel A) and a control reaction with recombinant NADPH-cytochrome P450 reductase (panel B). The solvent system and gradient used were as previously described (3.1.2).

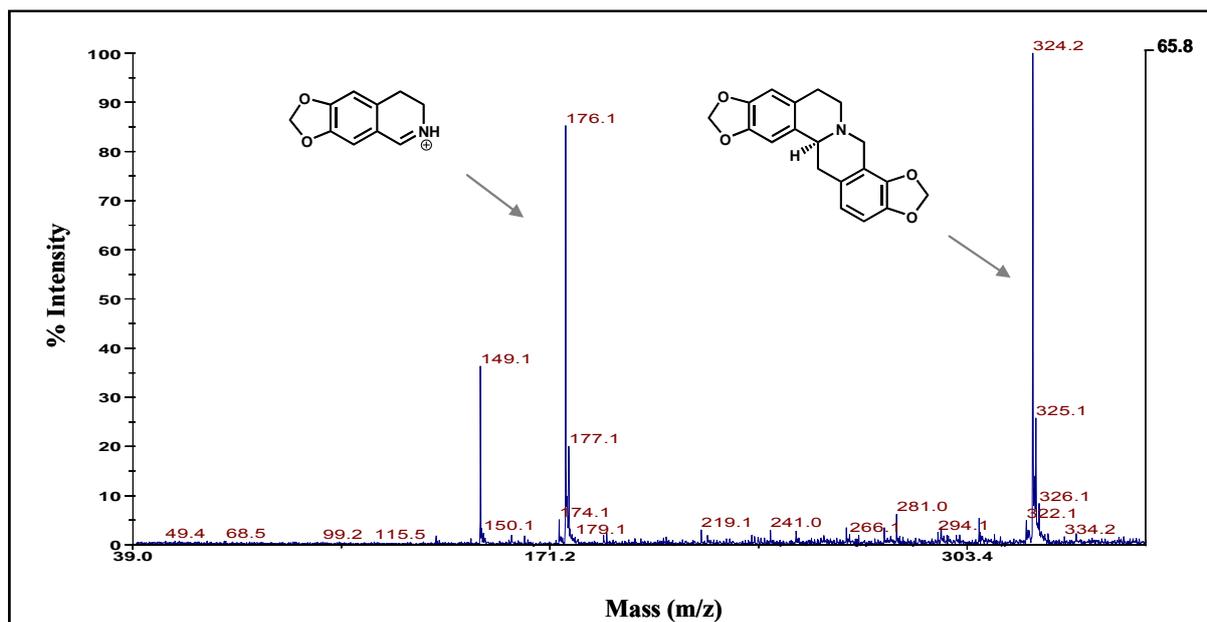


Figure 4.17. Mass spectra of (*S*)-stylopine generated after incubation of recombinant virus CYP719/ctg5 expressed in insect cells in presence of (*S*)-cheilanthifoline.

4.8.3. Substrate specificity

(*S*)-cheilanthifoline synthase and (*S*)-stylophine synthase which recognized (*S*)-scoulerine and (*S*)-cheilanthifoline, respectively, were evaluated for the ability to catalyze a methylenedioxy-bridge group in benzyloquinoline compounds harboring a methoxy group adjacent to a hydroxyl group (Figure 4.18). Reactions were performed under standard conditions in the presence of the following substrates: (*R*)-cheilanthifoline, (*S*)-reticuline, (*R,S*)-norreticuline, (*R,S*)-nororientaline, (*R,S*)-7-*O*-methylnorlaudanoline or (*R,S*)-coclaurine. In all cases activity was not detected, indicating a high affinity for their preferred substrate. In addition, (*S*)-cheilanthifoline synthase did not react with (*R*)-cheilanthifoline, displaying a high stereospecificity for the substrate (*S*)-cheilanthifoline.

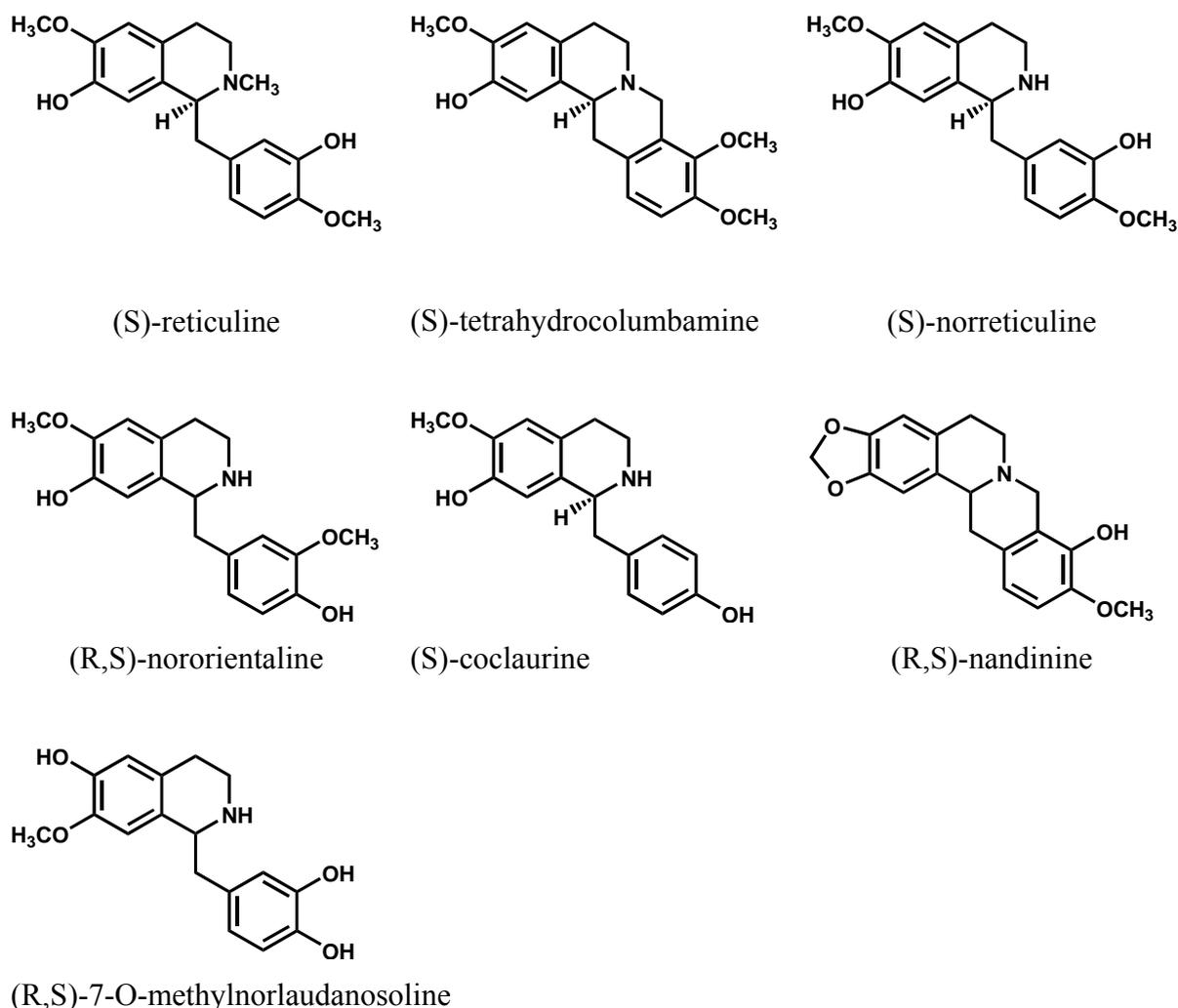


Figure 4.18. Benzyloquinoline alkaloids tested as potential substrates for (*S*)-cheilanthifoline synthase and (*S*)-scoulerine synthase.

4.9. Characterization of (*S*)-cheilanthifoline synthase

The time course of (*S*)-cheilanthifoline formation was analyzed by incubating a reaction mixture containing (*S*)-scoulerine and insect cell expressing (*S*)-cheilanthifoline synthase for 5 to 120 min at 30°C. Results are showed in figure 4.19. After 15 min incubation about 50% product formation was reached and after 60 min there was almost no change.

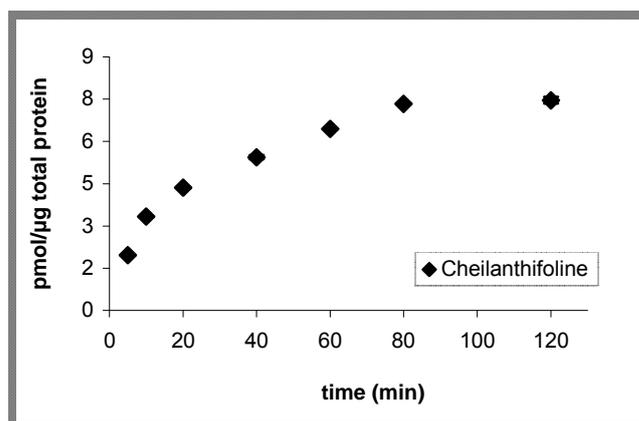


Figure 4.19. Time course of (*S*)-cheilanthifoline formation. Insect cells expressing (*S*)-cheilanthifoline synthase enzyme were incubated at 30°C in 200 mM tricine/NaOH pH 8.0, 0.5 mM NADPH and 60 µM (*S*)-scoulerine. Results are the average of a triplicate.

4.9.1. Determination of pH optimum for (*S*)-cheilanthifoline synthase activity

The optimum pH for cheilanthifoline synthase activity was determined by incubating cells extracts of insect cells expressing cheilanthifoline synthase enzyme with (*S*)-scoulerine, over a range of pH from 6.0 to 10.0. The reaction mixture containing 100 mM sodium phosphate buffer pH 6.0 - 8.5, tricine buffer pH 7.4 - 8.8 or glycine buffer pH 9.0 - 10.0 were incubated at 30°C for 15 min. The results of the quantification of (*S*)-cheilanthifoline are showed in figure 20. The pH optimum for (*S*)-cheilanthifoline synthase activity is about 7.5 with identical enzyme activities in tricine or phosphate buffer. No enzyme activity was detected when the pH changed from 9.0 to 10.0 with glycine buffer.

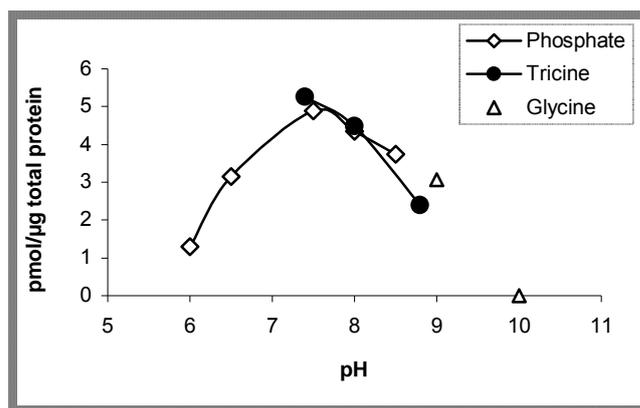


Figure 4.20. pH optimum for (*S*)-cheilanthifoline synthase activity. Assay mixtures contained 100 mM sodium phosphate (pH 6.0, 6.5, 7.5, 8.0 and 8.5), 100 mM of tricine buffer (pH 7.4, 8.0 and 8.8) and 100 mM glycine buffer (pH 9.0 and 10.0). The samples were incubated for 15 min at 30°C. Assays were performed in triplicate.

4.9.2. Determination of optimum temperature

The optimum temperature for (*S*)-cheilanthifoline synthase activity was determined by incubating the reaction mixture for 15 min at different temperatures. As observed in figure 4.21, the enzyme is stable between 30 and 35°C after that the activity diminished.

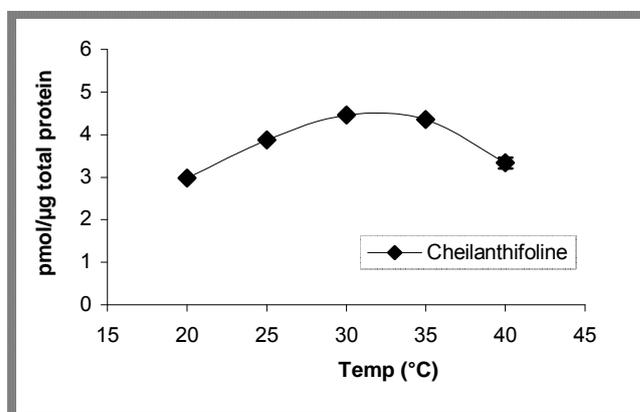


Figure 4.21. Temperature dependence for (*S*)-cheilanthifoline synthase activity. Samples were incubated for 15 min at 20, 25, 30, 35 and 40°C in tricine/NaOH (pH 7.4). Results are the average of a triplicate determinations.

4.10. Characterization of (*S*)-stylophine synthase

(*S*)-stylophine formation was monitored by incubation of (*R,S*)-cheilanthifoline with insect cells expressing (*S*)-stylophine synthase from 5 to 120 min at 30°C. The results displayed in figure 4.22 showed a similar pattern to (*S*)-cheilanthifoline formation. After 15 min

incubation, about 50% of (*S*)-stylophine formation was reached and after 60 min there was almost no change.

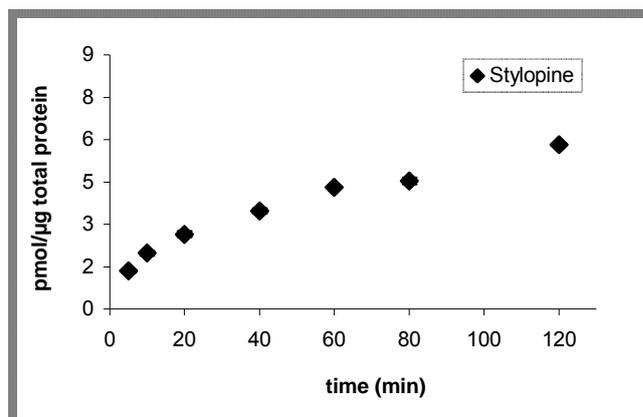


Figure 4.22. Time course of (*S*)-stylophine production with (*S*)-stylophine synthase. The reaction mixture was incubated at 30°C in the presence of 200 mM tricine/NaOH pH 8.0, 0.5 mM NADPH and 60 μM (*R,S*)-cheilanthifoline. Samples were monitored from 5 to 120 min. Results are the average of a triplicate determinations.

4.10.1. Determination of pH optimum

Stylophine synthase activity was measured over a pH range from 6.0 to 9.0 by using 0.1 M sodium phosphate (pH 6.0, 6.5, 7.5, 8.0 and 8.5), tricine buffer (pH 7.4, 8.0 and 8.8) and glycine buffer (pH 9.0 and 10.0). Samples containing (*S*)-cheilanthifoline and cells extracts expressing stylophine synthase were incubated at 30°C for 15 min. The results of the quantification of (*S*)-stylophine are displayed in figure 4.23. Optimal activity was observed in phosphate buffer at pH 7.5 to 8.0. No stylophine formation was detected when the pH changed from 9.0 to 10.0 with glycine buffer.

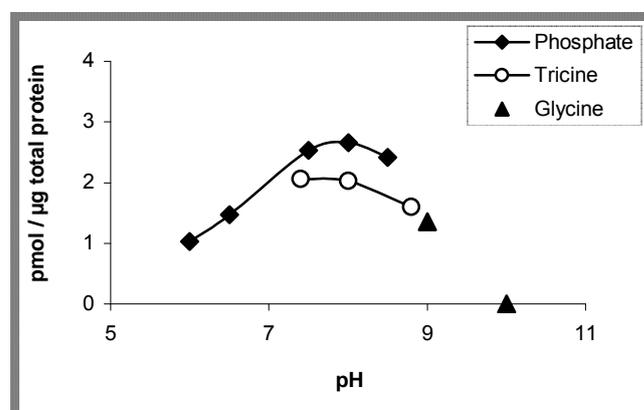


Figure 4.23. Influence of pH on stylophine activity. Assay mixtures contained 100 mM sodium phosphate (pH 6.0, 6.5, 7.5, 8.0 and 8.5), tricine buffer (pH 7.4, 8.0 and 8.8) and glycine buffer (pH 9.0 and 10.0) were incubated at 30°C for 15 min. Assays were in triplicate.

4.10.2. Optimum temperature

The optimum temperature was calculated by incubating the samples at different temperatures ranging from 20 to 40°C. Results of the quantification of (*S*)-stylophine by HPLC are displayed in figure 4.24. The enzyme is catalytically stable at temperatures between 30 to 35°C.

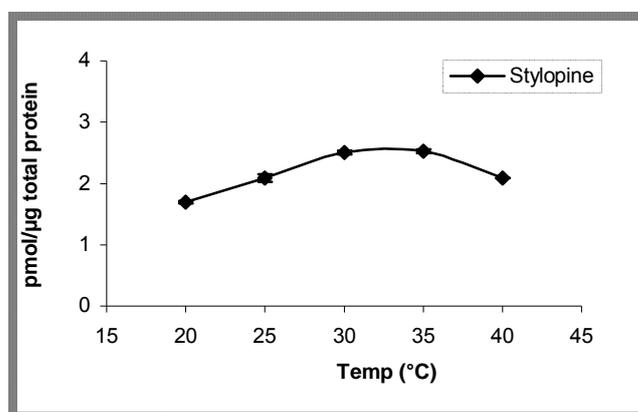
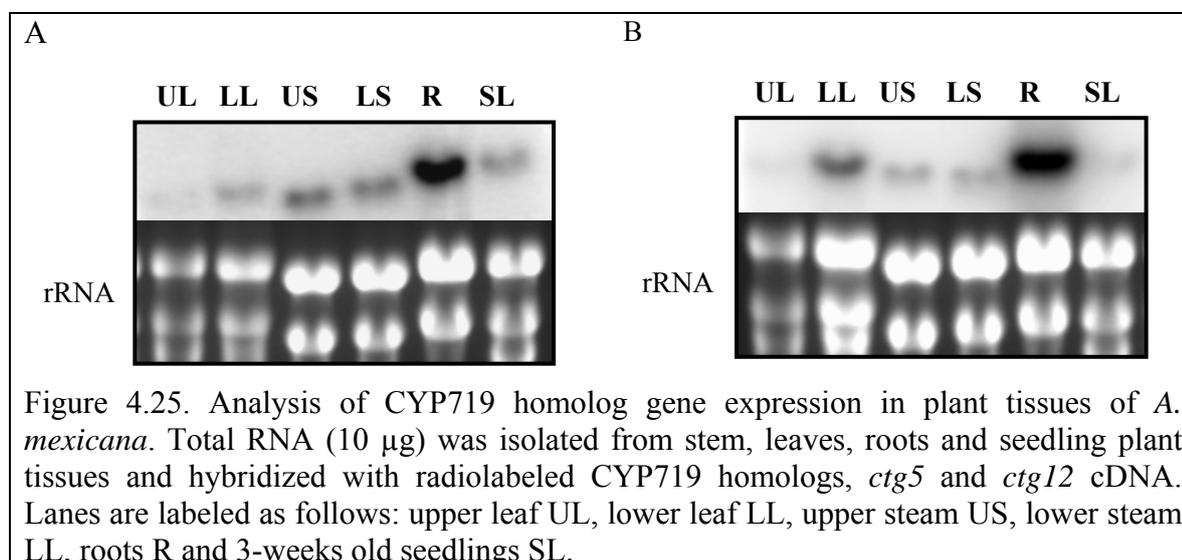


Figure 4.24. Effect of temperature on (*S*)-stylophine synthase activity. Reaction mixtures were incubated for 15 min at 20 to 40°C. Data points represent the mean of triplicate determinations.

4.11. Northern blot analysis

The full-length clones of *ctg5* and *ctg12* were used as a hybridization probe for northern blot analysis using RNA isolated from different tissues of *Argemone mexicana*. Results displayed in figure 4.25 showed that the transcripts of both genes *ctg5* (panel A) and *ctg12* (panel B) were highly expressed in roots and to a lesser extent in stem, leaves and seedlings. This is in accordance with the fact that alkaloids were mainly accumulated in the roots of the plant.

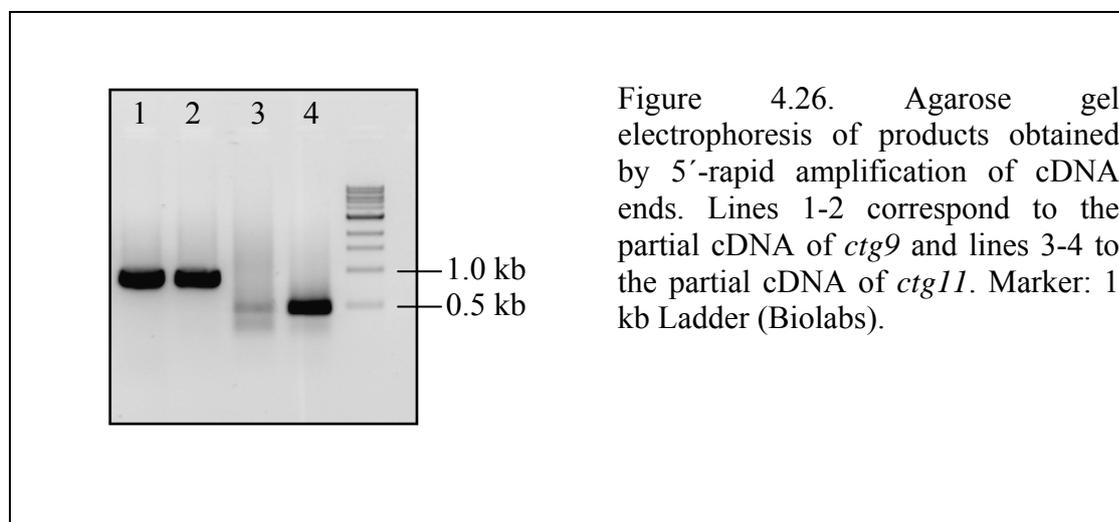


4.12. Generation of full-length cDNAs of BBE homologues

The 5'-terminal region of *ctg9* and *ctg11* cDNA was obtained by 5'-rapid amplification of cDNA ends (5'-RACE) as described in method section (3.13). For this purpose, a set of gene specific primer was designed for *ctg9* and *ctg11* based on its partial sequence.

Name	Primer 5' → 3'
<i>ctg9</i> -GSP1	CTC CGT TTC CGT TGG CAC CGC CG
<i>ctg11</i> -GSP1	TCCTCCTACATATCTTTTCCATATCCCTTCTAACCC
<i>ctg11</i> -GSP2	ACG TGA GAT TCA ACC CAA CTC ATT TCC GTC GTG TC
<i>ctg11</i> flf	ATGATTCCAAATTCATCATCTTC
<i>ctg11</i> flr	TAAATTACAATATTAATGGCTTC
<i>ctg9</i> flf	ATGCTTACTACTGTAACC
<i>ctg9</i> flr	TTCAAAGCTCATCATCAAC

The RACE product of *ctg9* analyzed on agarose gel displayed a discrete band of about 900 bp (Figure 4.26, lines 1-2). This fragment was cloned into the pGEM-T easy vector for sequencing. RACE product of *ctg11* appeared as a smear; in this case a nested PCR was performed substituting *ctg11*-GSP1 for *ctg11*-GSP2 and the primary PCR product instead of 5'-RACE-ready cDNA. The resulted fragment (Figure 4.26, lines 3-4) of about 600 bp was ligated into the pGEM-T easy TA cloning vector for sequencing.



The RACE sequence yielded a 923-bp and 585-bp fragment containing the ATG start codon of *ctg9* and *ctg11*, respectively. The assembly of the 5'-RACE sequence with the partial EST sequence yielded a *ctg9*-cDNA of 1723 bp with an open reading frame of 1611 bp (figure 4.27) and a *ctg11*-cDNA of 1794 bp with an ORF of 1632 bp (figure 4.28). cDNAs were confirmed by comparing them with cDNA clones amplified with native *Pfu* DNA polymerase using 2 μ l of cDNA produced by RT-PCR (3.12) and primers designed from the original sequence. The *ctg9* gene encodes a protein of 537 aa with a predicted molecular mass of 60.2 kDa and an isoelectric point of 5.217. The *ctg11* gene encodes a protein of 543 amino acids with a predicted molecular weight of 61.39 kDa and an isoelectric point of 9.14. A signal peptide of 31 and 26 aa for *ctg9* and *ctg11*, respectively was predicted using the software SignalP V3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) with default settings. This signal peptide directs the protein into specific vesicles of alkaloid biosynthesis in which it is accumulated (Dittrich and Kutchan, 1991).

Ctg9 and *ctg11* share 36% identity at the nucleotide level and 34% identity at the amino acid level. The deduced amino acid sequences of *ctg9* and *ctg11* compared to BBE from *Papaver somniferum* displayed 72% and 34% homology, respectively. The high homology of *ctg9* to BBE indicates a high probability for this enzyme to be involved in the formation of (*S*)-scoulerine from (*S*)-reticuline. The lower homology of *ctg11* to BBE indicates that this enzyme could be involved in another reaction that differs from that leading from (*S*)-scoulerine to (*S*)-reticuline. Thus, after 5'-RACE method, two independent full-length cDNAs were isolated from a cDNA library from *A. mexicana*. Both proteins were analyzed for similarities to known proteins with the BLASTP algorithm at the National Center for Biotechnology Information. As was observed before, Ctg9 protein displayed a higher homology to BBE enzyme from *Papaver somniferum* (figure 4.29) and also to that of *Eschscholzia californica* and *Berberis stolonifera* (Table 4.3). On the other hand, *ctg11* displayed the highest homology to a putative BBE from *Medicago truncatula* (figure 4.30) and *Arabidopsis thaliana* and to a tetrahydrocannabinolic acid synthase (THCA), an oxidoreductase involved in the biosynthesis of tetrahydrocannabinolic acid in *Cannabis sativa* (Sirikantaramas *et al.*, 2004). This last enzyme THCA displays 40.2% identity to BBE from *P. somniferum*.

Table 4.3. BLASTP results of ctg9 and ctg11 compared with known proteins at the NCBI

Protein	BLASTP Homology	Score	E-value
Ctg9	AAC61839.1 Berberine bridge enzyme [<i>Papaver somniferum</i>]	738	0.0
	AAC39358.1 Berberine bridge enzyme [<i>Eschscholzia californica</i>]	729	0.0
	AAD17487.1 Berberine bridge enzyme [<i>Berberis stolonifera</i>]	616	1.0E-174
Ctg11	ABE90048.1 Berberine and berberine like, putative [<i>Medicago truncatula</i>]	489	2.0E-136
	NP_174363.1 Electron carrier [<i>Arabidopsis thaliana</i>]	460	1.0 E-127
	BAE48241.1 Tetrahydrocannabinolic acid synthase [<i>Cannabis sativa</i>]	422	4.0E-116

BBE and THCA are flavinylated oxidases that require molecular oxygen for the oxidation of their substrate. There is another enzyme that was reported to be involved in oxidation reactions in alkaloids biosynthesis (Amann and Zenk, 1988). This enzyme (*S*)-tetrahydroprotoberberine oxidase (STOX) isolated from suspension cultures of *Berberis wilsoniae* catalyzed the oxidation of a series of tetrahydroprotoberberines and benzyloquinoline alkaloids.

Based on the similarities of ctg9 and ctg11 to BBE and THCA synthase, respectively, could suggest these two enzymes to be also flavoproteins involved in oxidation reactions. To elucidate the roll of ctg9 and ctg11 enzymes in the biosynthesis of benzyloquinoline alkaloids, these proteins where further expressed in a baculovirus-insect cells system and its activity tested with diverse benzyloquinoline derivatives.

```

1  ATGCTTACTACTGTAACCATGGAAACAAAAATCACAAAAATTTCTACTCATCTTTGTTTCATCTTTCTTT
   M L T T V T M E T K I T K N F Y S S L F I F F
71  CTGTGCTAACATGCGCACTAAGTGATGATATCTTATCATGTTAACTTCAAATGGAGTTCATAACTATAC
   S V L T C A L S D D I L S C L T S N G V H N Y T
141 TACACCATCATCTGATTCAAATTTCTGATTACCTTAGATTATTTACCTCTCCATACAAAACCCATTATTC
   T P S S D S N S D Y L R L F H L S I Q N P L F
211 AAAAAATCCACAATATCAAAACCGTCCCTAATCGTATTACCCGGTAACAAAGAAGAGTTATCGAATACCG
   K K S T I S K P S L I V L P G N K E E L S N T
281 TTAGATGTTGCACAAGAGGATCTTGGACCATTAGATTAAGAAGTGGTGGTCATAGTTATGAAGGATTATC
   V R C C T R G S W T I R L R S G G H S Y E G L S
351 ATATACCGCAGATACACCATTTGTTCTTATAGATTTAATGAATTTGAATCGGATTTCTATTGATATGGAC
   Y T A D T P F V L I D L M N L N R I S I D M D
421 TCAGAAACAGCTTGGGTTGAATCTGGTGAACAGTTGGAGAACTCTATTACGCAATCAGTGAGTCAACTG
   S E T A W V E S G A T V G E L Y Y A I S E S T
491 ATTCATTCGGATTCACAGCAGGTTGGTGTCTACTGTTGGTACTGGGGTTCATATAAGTGGTGGTGGTTT
   D S F G F T A G W C P T V G T G G H I S G G G F
561 CGGTATGATGTCGAGAAAATACGGTCTCGCAGCCGATAACGTGAAGATGTCATTCTTATAGATAGTAAC
   G M M S R K Y G L A A D N V E D V I L I D S N
631 GGTGCGATTCTAGATCGTAAATTAATGGGTGAAGACGTTTTTTGGGCGTTTCGTGGTGGTGGAGGTGGTG
   G A I L D R K L M G E D V F W A V R G G G G G
701 TTTGGGGTGCAATTTACGCGTGGAAAATCAAATTATTACCCGTTCCAAAAAGGTGACGGTTTTTCGCGT
   V W G A I Y A W K I K L L P V P K K V T V F R V
771 AATGAAAAACGTAAATATCGAAGAAGCTTCAATTTTAATTCACAAATGGCAATATGTTGCAGATGAATTA
   M K N V N I E E A S F L I H K W Q Y V A D E L
841 GATGATGATTTCACTGTAACGATTCTCGGCGGTGCCAACGGAAACGGAGCATGGCTAGTATTCTTAGGTT
   D D D F T V T I L G G A N G N G A W L V F L G
911 TACATTTAGGATGTA AAAACCGTTGCGAAATCTATAATGGATAAAAATGTTCCCGGAATTAGGGTTAATCGA
   L H L G C K T V A K S I M D K M F P E L G L I E
981 AGAAGAATTTTTGGAAAATGAATTTGGGGTGAATCTTTTGCTTACTTATCAGGATTA AAAACAGTTAAGGAA
   E E F L E M N W G E S F A Y L S G L K T V K E
1051 TTGAATAATAGGTTTTTTGAAACTTGATGATAAAGCTTTTAAGACAAAAGTTGATTTTACTAAAGAAACAT
   L N N R F L K L D D K A F K T K V D F T K E T
1121 TACCATTA AAAAGTGATTAATGGTGTATTAGAGATTTTATCGAAAGAGCCACGTGGATTTATCTTGTTAAA
   L P L K V I N G V L E I L S K E P R G F I L L N
1191 TAGTTTCGGAGGAAAAATGAGTAAATAGTAATGATTTTACTCCATTTCTCATCGTAACGGTACCAA
   S F G G K M S K I S N D F T P F P H R N G T K
1261 TTAATGGTGAATATATAGTTTCTTGGAGTAAAGATGAAGAATCAAAGAGCGACGAATTTCTTGATTGGT
   L M V E Y I V S W S K D E E S K S D E F F D W
1331 TACGTAATATTTATGATTATATGGAAGAGTTCGTATCGAAAAACCTAGAGTGGGTTATGTTAATAATAT
   L R N I Y D Y M E E F V S K N P R V G Y V N N I
1401 TGATCTTGATCTCGGAGGAATAGATTGGAGTAACAAAAATAGTTCTAATAATGCAATTGAGATTGCTAGA
   D L D L G G I D W S N K N S S N N A I E I A R
1471 AATTGGGGTGAAAAATATTTTTATCAAATTATGAACGTTAATTAGGGCTAAAACATTGATTGATCCTA
   N W G E K Y F L S N Y E R L I R A K T L I D P
1541 ATAATATTTTTAATCATCCACAAAGTATACCTCCAATGATGAAATTTGATAATGTTGATGATGAGCTTTG
   N N I F N H P Q S I P P M M K F D N V D D E L *
1611 A

```

Figure 4.27. Nucleotide sequence and deduced amino acid sequence of contig 9 (BBE homologue). *Ctg9* cDNA contains an ORF of 1611 bp encoding a putative protein of 537 aa with a predicted molecular mass of 60.2 KDa.

```

1  ATGATTCCAAATTCATCATCTTCATCAATTCCTTTCACCTCCTTGTTTTACTATTATTCTCAACCAGTTCAT
   M I P N S S S S S I L S L L V L L L F S T S S
71  CATGGGCAACTAATTCATGAAGATTTCTCAATTGTCTTTCAATCTATAAATCTTCATTTCCAAT
   S W A T N S I H E D F L N C L S I Y K S S F P I
141 CCCTATTTATACATCCAAGAACTCTTCATTTAATACCCTCTTTAGATCCTCAGCTCGAAACCTTCGATTT
   P I Y T S K N S S F N T L F R S S A R N L R F
211 TTATCACCTAATCAACACAAAAACCTGAATTTATAAATTAATCTCAACACTTGAATCTCATGTTCAAACCA
   L S P N S T Q K P E F I I T P T L E S H V Q T
281 CTGTTGTTTGTCTAAGAAACCGGGTTAGATCTCAAAGTTCGAAGTGGTGGTCACGATGTGCAAGGCTT
   T V V C S K K H G L D L K V R S G G H D V E G L
351 GTCATACGTCTCTGATTACCTTATGTCATGATAGACTTAGTCGATTTTCGAAACATCACTGTTAATGTA
   S Y V S D S P Y V M I D L V D F R N I T V N V
421 AAGAATGCTACTGCTTGGATTCAAGCTGGTCTTCACTTGGAGAAGTTTATTATAAAGTTGGTAATGAAA
   K N A T A W I Q A G S S L G E V Y Y K V G N E
491 GTAAGAATACCCTTGGATTTCCAGCCGGGTTTTGCCGACCGTAGGCGTTGGTGGTCACATTAGCGGAGG
   S K N T L G F P A G F C P T V G V G G H I S G G
561 TGGGTTTGGTTCCTTGGTTAGGAAATATGGACTTGCATCTGATCAAGTCATTGATGCTAGAATTGTGACC
   G F G S L V R K Y G L A S D Q V I D A R I V T
631 GTTAATGGTGAAATACTTAACAAAGAAACCATGGGAAAGATTTATATTGGGCCATTTCGAGGTGGAGGAG
   V N G E I L N K E T M G K D L Y W A I R G G G
701 CTAACAATTTCCGGTGTGCTTCTTTCGTGGAAAGTTAAGTTGGTTCCTGTTACGCCTATCGTGACCGTTGC
   A N N F G V L L S W K V K L V P V T P I V T V A
771 TACAATAGATAGAACGTTAGAACAAGGTGCGACTAACCTAGTTCATAAGTGGCAATTCGTTGCCGATAGG
   T I D R T L E Q G A T N L V H K W Q F V A D R
841 CTTCATGAAGATGTTTACATTGGTCTCACAAATGGTCACTGCTAATACTAGTCGAGCCGGAGAAAAACTG
   L H E D V Y I G L T M V T A N T S R A G E K T
911 TAGTAGCTCAATTCTCGTTCCTTGTCTTAGGAAACACTGATAGACTTCTCCAAATCATGGAAGAGAGTTT
   V V A Q F S F L F L G N T D R L L Q I M E E S F
981 CCCAGAATTAGGTTTAAAACGTAATGACACGACGGAAATGAGTTGGGTTGAATCTCACGTGTACTTTTAT
   P E L G L K R N D T T E M S W V E S H V Y F Y
1051 CGACGAGGACAACCTATAGAATTTTTATGGGATAGAGATCATCTAACGAAGAGTTTTTTGAAAGTGAAT
   R R G Q P I E F L W D R D H L T K S F L K V K
1121 CGGATTACGTAAGAGAACCAGATATCAAAATTAGGGTTAGAAGGGATATGGAAAAAGATATGTAGGAGGAGA
   S D Y V R E P I S K L G L E G I W K R Y V G G D
1191 TTCACCAGCAATGTTATGGACTCCaTTTTGGTGGAAAGAAATGAATCAGATTTCTGAATTTGAAAGTCCTTAT
   S P A M L W T P F G G R M N Q I S E F E S P Y
1261 CCACATAGAGCTGGAAATATTTACAATATTATGTATGTTGGAAACTGGTTAAATGAAATGAGTCTGAAA
   P H R A G N I Y N I M Y V G N W L N E N E S E
1331 AACAGCTGAATTGGATGAGAAGTTTTTATAGTTATATGGGTCGATATGTATCGAAAAATCCGAGATCGGC
   K Q L N W M R S F Y S Y M G R Y V S K N P R S A
1401 GTATTTAAATTACAAGGATCTTGATTTAGGGGTTAATGATAATAATGTTAGTGAATACATAAGGTATTTA
   Y L N Y K D L D L G V N D N N V S E Y I R Y L
1471 AAAGCTAGATCATGGGGTAGAAAAATTTTCAAGAATAATTTTGAAAAATTAGTTAAAGTAAAAAGTATGG
   K A R S W G R K Y F K N N F E K L V K V K S M
1541 TTGATCCTGATAATTTCTTCAAGAACAACAGAGTATTCCTCCTATTAGGTCTTGGGGTAAAGAATTAGA
   V D P D N F F K N K Q S I P P I R S W G K E L E
1611 AGCCATTAATATTGTAATTTAA
      A I N I V I *

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Figure 4.28. Nucleotide sequence and deduced amino acid sequence of *ctg11* (BBE homologue). The cDNA contains an ORF of 1632 bp encoding a putative protein of 543 aa with a predicted molecular mass of 61.39 kDa.

```

                20                40                60
BBE_paso  : ----MCRSLTLRFFLFIVLLQTCVRRGGDVNDNLSSCLNSHGVHNFITLSTDTNSDYF : 55
Ctg9_Amex : MLTTVTMETKIKKNFYSSLFIFFSVLT-CALSDDILS-CLTNSGVHNYITPSSDSNSDYL : 58

                80                100               120
BBE_paso  : KLLHASMQNPLFAKPTVSKPSFIVMPGSKHEELSSTVHCCTRESWTIRLRSGGHSYEGLSY : 115
Ctg9_Amex : RLFHLSIQNPLFKKSTISKPSLIVLPGNKEELSNTVRCCTRGSWTIRLRSGGHSYEGLSY : 118

                140               160               180
BBE_paso  : TADTPFVIVDMNLRNRSIDVLSSETAWVESGATLGELYIAIAQSTDTLGFTAGWCPTVGS : 175
Ctg9_Amex : TADTPFVILIDLMLNLRNRSIDMDSETAWVESGATVGELYIAISESTDSEFTAGWCPTVGT : 178

                200               220               240
BBE_paso  : GGHSGGGFGMMSRKYGLAADNVVDAIILIDSNAGAILDREKMGDDVFWAIVRGGGGGVWGA : 235
Ctg9_Amex : GGHSGGGFGMMSRKYGLAADNVEDVILIDSNAGAILDRKLMGEDVFWAVRGGGGGVWGA : 238

                260               280               300
BBE_paso  : YAWKIKLLPVPKELTVFRVTKNVGIEDASSLLHKWQYVADELDEDFTVSVLGGVNGND : 295
Ctg9_Amex : YAWKIKLLPVPKQVTVFRVMKKNVNIIEEASFLIHKWQYVADELDDDFTVTILGGANG : 298

                320               340               360
BBE_paso  : LMFLGLHLGRKDAAKTIIDEKFPPELGLVDKEFQEMSWGESMAFLSGLDTISELNNRFL : 355
Ctg9_Amex : LVFLGLHLGCKTVAKSITMDKMFPELGLIEEFLMNVGESFAYLSGLKTVKELNNRFL : 358

                380               400               420
BBE_paso  : DERAFKTKVDFTKVSVPINVFRHALEMVSEVQPGGFIALNGFGGKMSIISTDFTPFPHR : 415
Ctg9_Amex : DDKAFKTKVDFTKETLPLKVINWVLEILSKEPRGFILNLSFGGKMSKISNDFTPFPHR : 418

                440               460               480
BBE_paso  : TKLMFEYI IAWNQDEESKIGEFSEWLAKFYDYLEP FVSKEPRVGYVNHIDLDLGGID : 475
Ctg9_Amex : TKLMVEYIIVSWSKDEESKSEFFDWLRNIYDYMEEFVSKNPRVGYVNHIDLDLGGID : 478

                500               520               540
BBE_paso  : KSSSTNAVEIARNWGERYFSNRYERLVKAKTLIDPNNVFNHPQSIPPMMKFEEIYMLKEL : 535
Ctg9_Amex : KNSNNAIEIARNWGEKYFLSNRYERLIRAKTLIDPNNIFNHPQSIPPMMKFDNVVDEL-- : 536

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Figure 4.29. Protein sequence alignment of ctg9 with the berberine bridge enzyme of *Papaver somniferum*. Ctg9 share 72% identity to BBE of *P. somniferum*. Conserved amino acids are highlighted red.

```

                20                40                60
BBE_mtrunc : MIS--SPSSYTAIIILLISISLTKSAS--TEENFVQCLSFYSDKAAPFYASYTTPNNASFNN : 58
Ctg11_Amex : MIPNSSSSSIISLIVLLLFSTSSSWATNSIHEDFLNCLSIY---KSSFPIPIYTSKNSSFNT : 59

                80                100               120
BBE_mtrunc : ILNSSAQNLRYLVP--SAPKPEFIFTELTDSHVQVAVTCSKKLCIHLRVRSGGHDYEGLSYVS : 119
Ctg11_Amex : LFRSSARNLRFLSPNSTQKPEFIFTELTDSHVQTTVVCSKKHCLDLIKVRSGGHDVEGLSYVS : 121

                140               160               180
BBE_mtrunc : EIETPFIIVDLAKLRDVNVDDIGDNSAWIQAGATIGEVYYRIHEKS-DVHGFPAGLCTSLGVG : 180
Ctg11_Amex : --DSPYVMIDLVDFRNITVNVKNATAWIQAGSSSLGEVYYKVGNESKNTLGFPAGFCPTVGVG : 181

                200               220               240
BBE_mtrunc : GHITGCAYGSMMRKYGLGADNVLDAKIVDDANGNILDRKSMCEEFFWAIRGGGGGSFGLLLWW : 242
Ctg11_Amex : GHISGCGFSLVRKYGLASDQVIDARLVTVNGEILNKETMCKDLYWAIRGGGCANNFVLLSW : 243

                260               280               300
BBE_mtrunc : KIKLVPVPKTIVTVFTVTKSLEQDASKIVHKWQEVAPTIDENLFMRVIIQPAASTANKTQRTI : 304
Ctg11_Amex : KVKLVPVTPIVTVATIIDRTLEQGATNLVHKWQFVADRRLHEDVYIGLTMVTAN-ISRAGEKTV : 304

                320               340               360
BBE_mtrunc : TTSYNAQFLCDSEKLLQVMKESFPELGLTKQDCTETSWIKSVMYIAGYPNDTPPEVLEGKS : 366
Ctg11_Amex : VAQFSFLFLCNTDRLLQIMESFPELGLKRNDTTEMSWVESHVYF--YRRGQPIEFTWDRDH : 364

                380               400               420
BBE_mtrunc : TFKNYFKAKSDFVREPIPETGLQCLWRLLEEDSPLMIWNFYCGMMNNFSESDIPPHRNCT : 428
Ctg11_Amex : LTKSFLKVKSDYVREPISKLGLECIWKRYVGGDSPAMLWIFFCGRMNQISEFESPYPHRACN : 426

                440               460               480
BBE_mtrunc : LYKIQYLTLWQDGDKNASKHVDWIRKLNYMTPYVSKFPREAYVNYRDLDLGCMNKNSTSFT : 490
Ctg11_Amex : IYNIMYVGNWLN-ENESEKQLNWMRSFYSYMGRYVSKNPRSAYLNYKDLDLGCVNDNVSEYI : 487

                500               520               540
BBE_mtrunc : ---QATSWGCMNYFKDNFNRLVKIKTKVDPENVFRHEQSIPPLPVSTMQLKDKKCKRWE : 545
Ctg11_Amex : RYLKARSWGCRKYFKNNFEKLVKVKSMVDEDNFFKNKQSIPPIRSWGKELEAINIVI-- : 543

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Figure 4.30. Protein sequence alignment of ctg11 with a putative BBE of *Medicago truncatula*. Ctg11 share 53% identity to BBE of *M. truncatula*. Conserved amino acids are highlighted red.

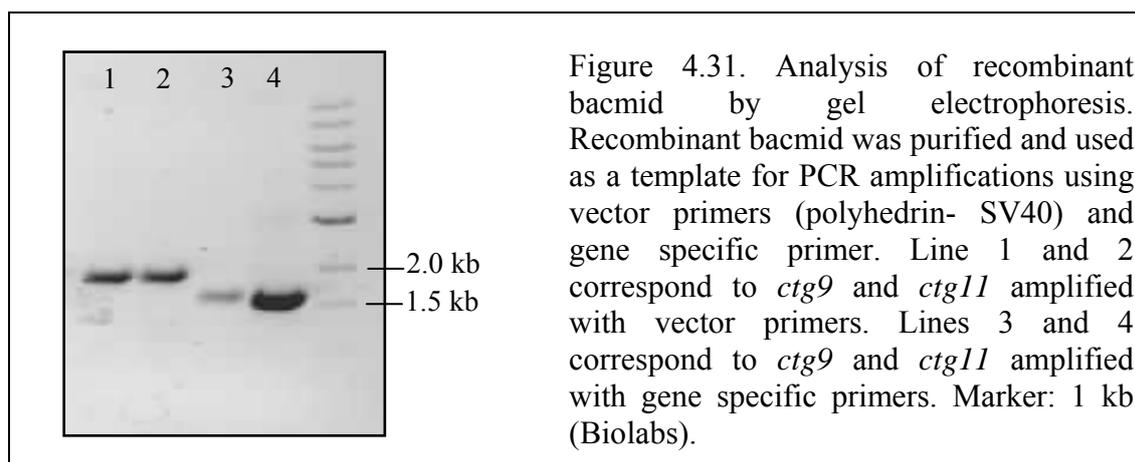
4.13. Heterologous Expression of *ctg9* and *ctg11* cDNAs

4.13.1. Cloning into pFastBac vector

Ctg9 and *ctg11* genes were heterologously expressed in insect cells using the baculovirus expression system Bac-to-Bac from Invitrogen and the transfer vector pFastBac1. This vector contains a multiple cloning site down stream of a polyhedrin promoter derived from a baculovirus, which is then inserted into a bacmid (baculovirus shuttle vector) propagated in *E. coli* cells (DH10Bac™) by site-specific transposition. First, the *ctg9* and *ctg11* cDNAs were excised from the pGEM-Teasy vector with the restriction enzyme NotI, purified by gel electrophoresis, and inserted into the equivalent site of the transfer vector pFastBac1. Approximately 60 ng of insert were required to ligate with 100 ng linearized vector. After plasmid purification, these constructs were sequenced with GSP and vector primers to confirm the integrity of the insert and orientation. The resulting plasmids were named pFastBac1/*ctg9* and pFastBac1/*ctg11*.

4.13.2. Transposition into DH10Bac cells

After purification, approximately 2 ng of the recombinant plasmid were transformed into DH10Bac cells for site-specific transposition into a bacmid (3.18.4). Selection of the recombinant bacmid was done on LB plates containing X-gal, IPTG, kanamycin, gentamicin and tetracycline. White colonies were selected for recombinant bacmid isolation (3.3.1). The insertion of the gene of interest into the bacmid was confirmed by PCR (3.14.1) using vector primers polyhedrin and sv40. The thermal cycling conditions were as follows: 93/3:00 // 30 cycles of 94/0:45 min, 50/0:45, 72/5:00 // 72/7:00, 4/∞. As shows figure 4.31, PCR amplification with vector primers generated a band of about 2 kb that correspond to the 200 bp nucleotides flanking the MCS plus the 1.6 kb from *ctg9* (line 1) or *ctg11* (line 2). This PCR product was used as a template for a second PCR using gene specific primers. Results in figure 4.31 showed a PCR product of about 1.6 kb that confirms the insertion of *ctg9* cDNA (line 3) and *ctg11* cDNA (line 4) into the bacmid. Recombinant bacmid obtained was designated AcNPV/*ctg9* and AcNPV/*ctg11*.

Primer designed for *ctg9* and *ctg11* and pFastBac1 vector primers

Name	Primer 5'→3'
Ctg11flf	ATGATTCCAAATTCATCATCTTC
Ctg11flr	TTAAATTACAATATTAATGGCTTC
Ctg9flf	ATGCTTACTACTGTAACC
Ctg9flr	TTCAAAGTCATCATCAAC
Polyhedrin	AAATGATAACCATCTCGC
sv40	CCTCTAGTACTTCTCGACAA

Recombinant bacmid DNA was used for transfection in *Sf9* insect cells using the method described in methods section 3.18.5. Recombinant virus was used to prepare a high recombinant baculovirus stock by serial infection of insect cells (3.18.6).

4.13.3. Protein expression

For expression of recombinant *ctg9* and *ctg11* in insect cells the protocol described in method section 3.18.8 was followed. Basically, 50 ml of suspension culture (2×10^6 cells/ml) were infected with the virus stock. Cells were harvested 72 h after infection and recovered by centrifugation. The supernatant was used direct in activity assays and the pellet was resuspended in 3 ml of resuspension buffer:

- 50 mM Tricine/NaOH pH 7.5, 5 mM TGA, 10% v/v glycerol, 2 mM EDTA, 10 mM 2-mercaptoethanol.
- 50 mM phosphate buffer pH 7.5 or 9.0
- 50 mM phosphate buffer pH 7.5 or 9.0, 1.0 % emulgen 913 and 2 mM EDTA.

4.14. Enzyme assay

Figure 4.32 shows the compounds tested as substrates for these possible flavoproteins *ctg9* and *ctg11*. The activity assays were performed in 100 mM phosphate buffer (pH 7.5 and pH 9.0) or Tricine/NaOH (pH 7.5) with 50 μ M substrate and 140 μ l of cell suspension or supernatant in a total volume of 150 μ l. The reaction mixtures were incubated for 2 h at 30 and 37°C, and then terminated by the addition of 1 volume methanol. After centrifugation, the supernatant was concentrated and resuspended in 20 μ l EtOH 80%. Five microliters were injected in the HPLC (3.1.2). Control studies were performed with uninfected *Sf9* cells.

The reaction mixture containing (*S*)-reticuline with the cell suspension of *ctg9* (BBE-like protein) at pH 7.5 or 9.0 produced a colored sample (brown) in comparison with the control which displayed a light brown coloration. But when the sample was analyzed by HPLC, no product responsible for the coloration could be detected. In case of *ctg11* (STOX-like protein) no product formation was observed with the substrates tested. These results suggested a low expression level of protein or degradation of the same due to proteolytic activity. In some other works, it has been reported that the yield of heterologously expressed proteins in insect cells decreased as a result of degradation due to the activity of proteases and that the addition of protease inhibitors resulted in the successful production of the recombinant protein (Gotoh *et al.*, 2001). Considering the possibility of proteolytic degradation, an *in vivo* assay (Gesell, 2008) was followed using protease inhibitors.

4.14.1. In vivo assays

After 24 hrs of recombinant virus infection, the pH of the medium was adjusted between 7.5-8.0 and substrate and protease inhibitor solutions were added. After three days incubation, only cells infected with *ctg11* (STOX-like protein) in presence of (*S*)-tetrahydropalmatine, or (*S*)-coreximine produced a colorful yellow solution which later was analyzed by HPLC, LC-MS and fluorescence spectrum (345 nm excitation wave length). There is also the possibility that the enzyme reacts with (*R,S*)-canadine to produce berberine, but this result could not be confirmed due to the low amounts produced and since traces of the same product were found also in the control cells (Figure 4.33). Cells expressing *ctg11* showed clear oxidase activity *in vivo* with (*S*)-tetrahydropalmatine as a substrate. HPLC revealed a single product peak with a retention time of 31.42 minutes (Figure 4.34). The mass spectra of the product displayed a molecular mass of 352 (Figure 4.36, A), consistent with that of palmatine. The MS fragmentation pattern (Figure 4.36, B) and its fluorescence spectrum (Figure 4.35) identical to

a standard of palmatine confirmed the identity of this product. In case of (*S*)-coreximine as substrate, HPLC analysis showed that cells expressing *ctg11* transformed this substrate completely to a new product with a retention time of 17.086 minutes (Figure 4.37) and a UV spectrum with absorption peaks at 250, 310 and 370 nm (Figure 4.38). The molecular mass of the product formed contained two hydrogen atoms less than the substrate coreximine (m/z 330 – 2, since labelled coreximine was used). Besides this molecular ion, the fragmentation for the isoquinoline ion displayed an m/z of 180 as was determined by LC-MC analysis (Figure 4.39). That means that the formation of the double bond occurred in the ring C which did not contained any isotopic label.

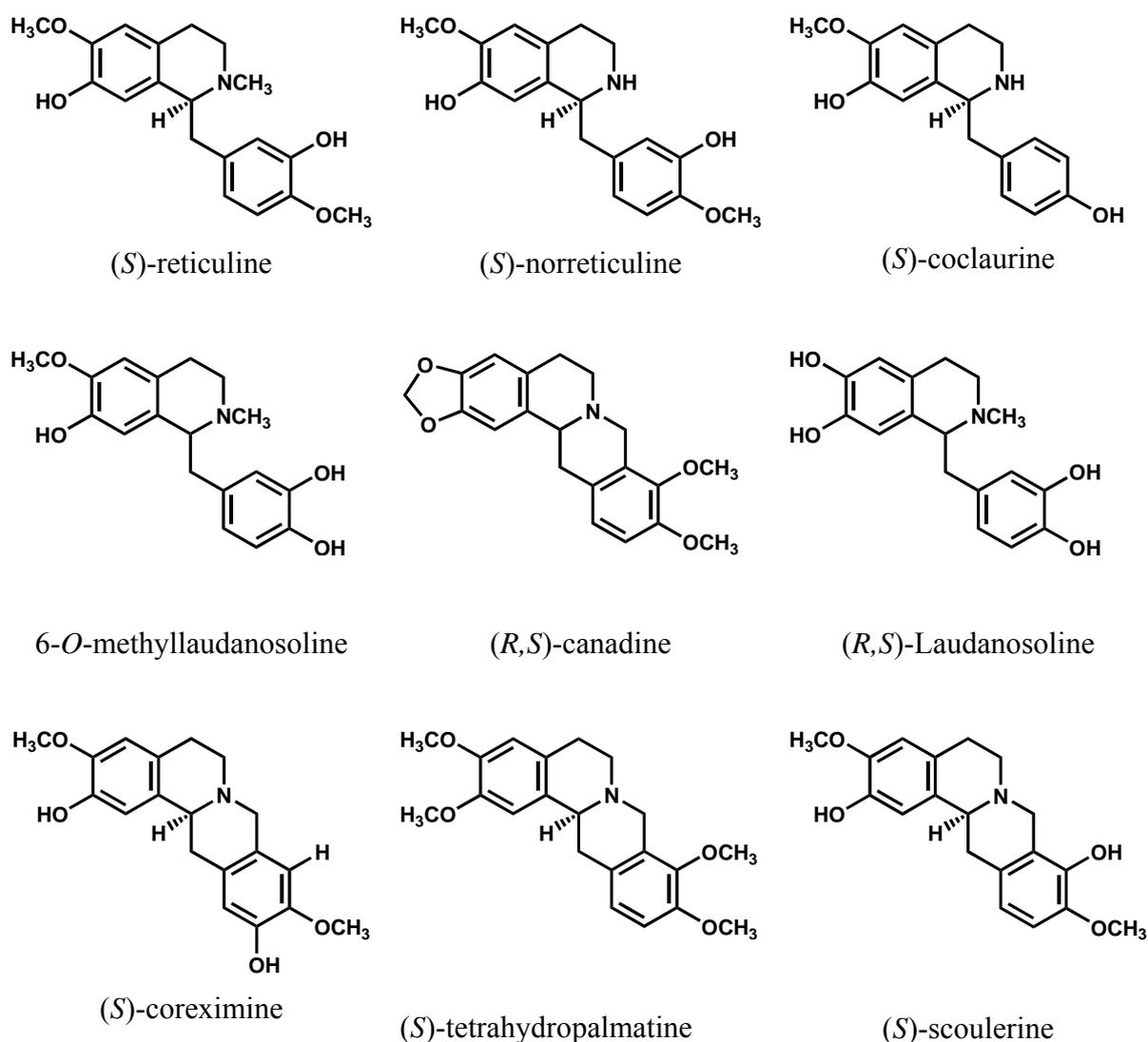


Figure 4.32. Compounds tested as possible substrates for *ctg9* and *ctg11*.

More works like HNMR are needed to know the exact position of the double bond in this dehydrogenation product and to confirm this activity, but whatever the role of this protein in *Argemone mexicana*, it appears to be involved mainly in the biosynthetic pathway derived from coreximine. The complete conversion of this substrate suggested also the occurrence of coreximine in the plant even though no reports about this compound in *Argemone mexicana* have been published so far. After LC-MS analysis of plant's extracts it was possible to confirm the presence of coreximine in the same. These results might indicate the possible roll of the enzyme in the biosynthesis of protoberberines derived from coreximine.

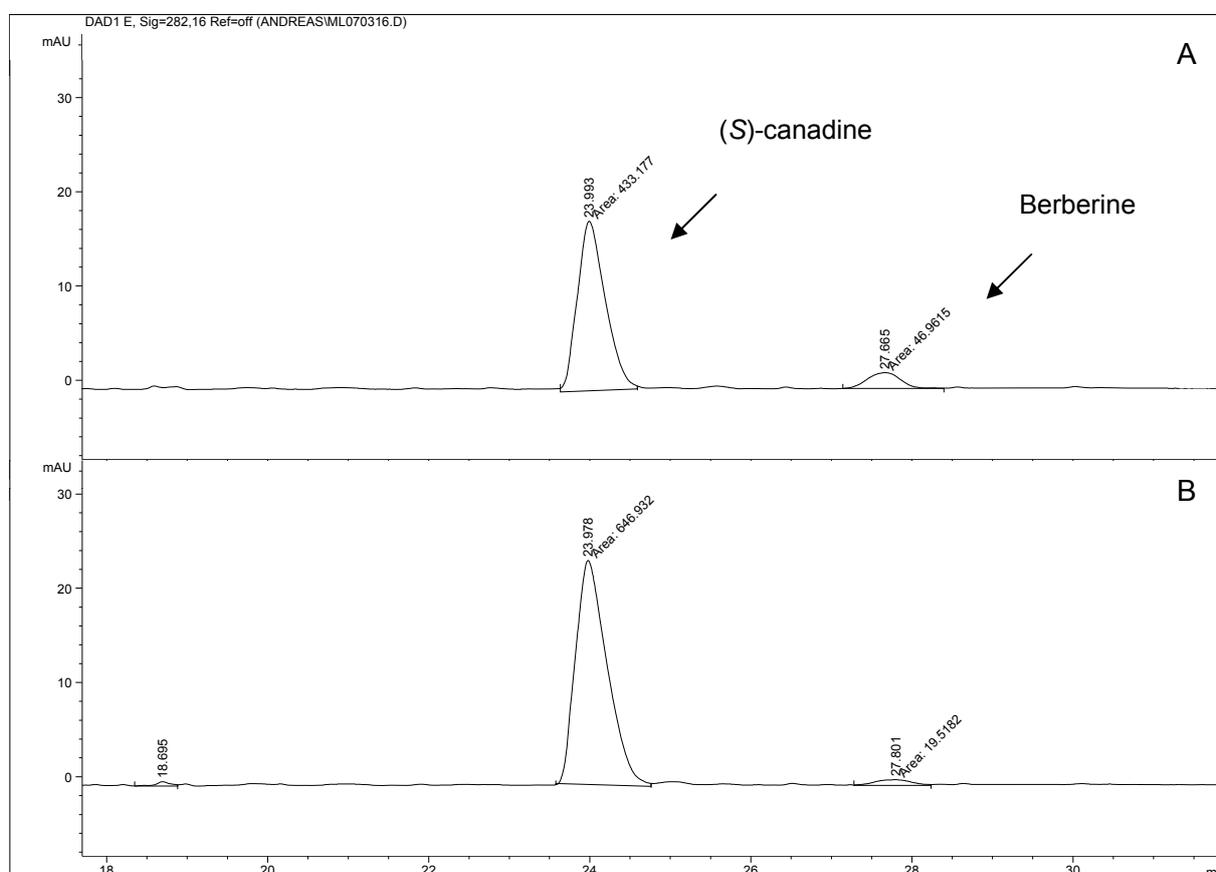


Figure 4.33. High performance liquid chromatography (HPLC) profiles derived from the reaction of the recombinant ctg11-protein expressed in *Sf9* insect cells (panel A) and a control reaction (panel B) in the presence of (R,S)-canadine. The solvent system and gradient used was as previously described (3.1.2).

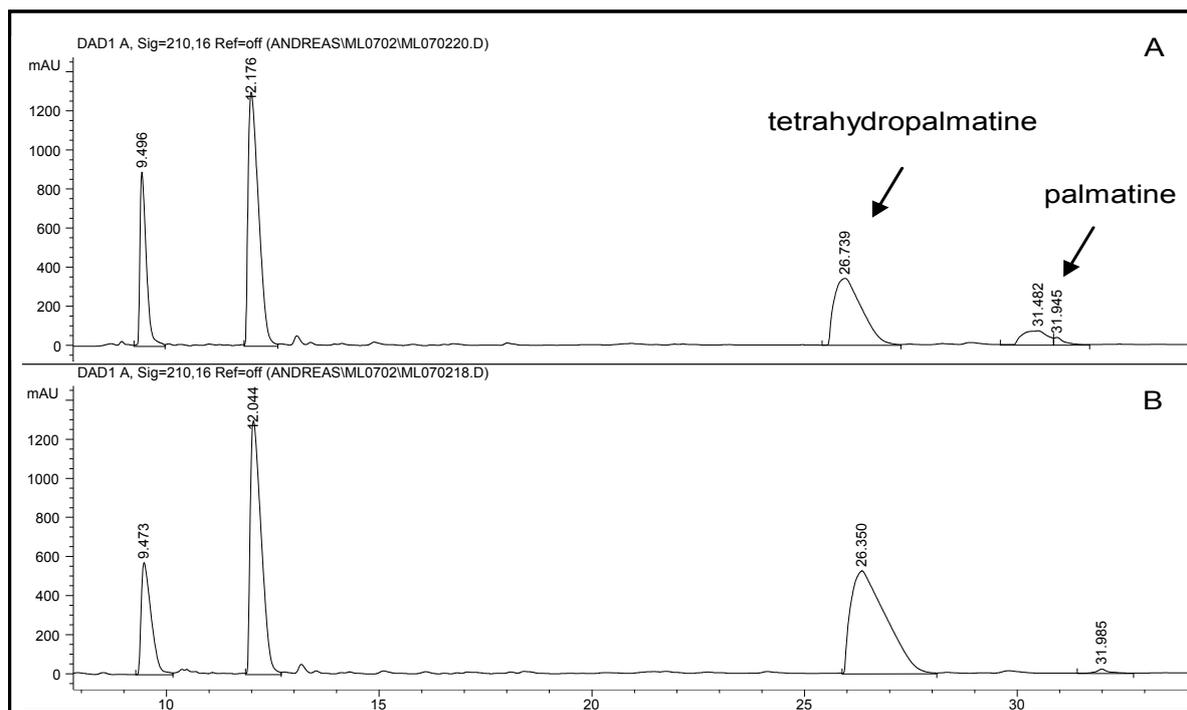


Figure 4.34. High performance liquid chromatography (HPLC) profiles derived from the reaction of the recombinant ctg11-protein expressed in *Sf9* insect cells (panel A) and a control reaction (panel B). The solvent system and gradient used was as previously described (3.1.2).

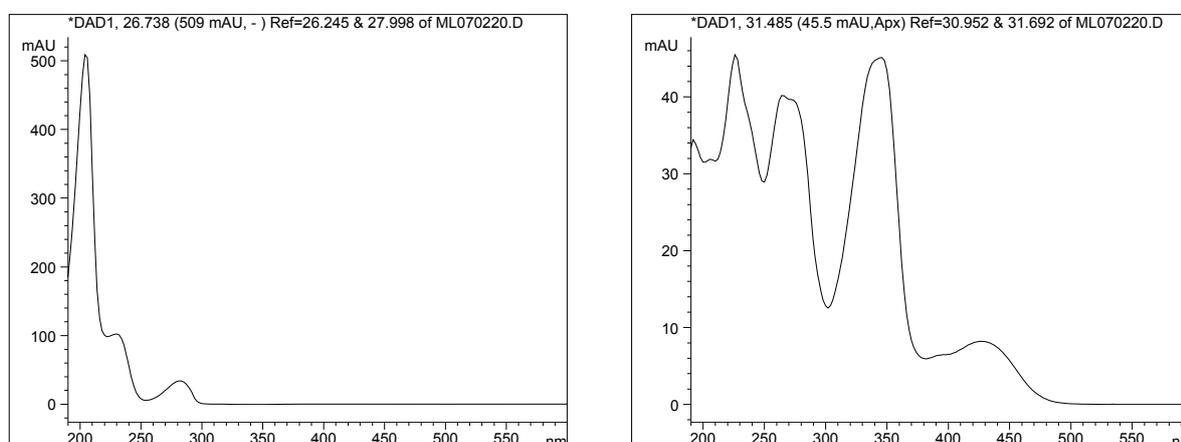
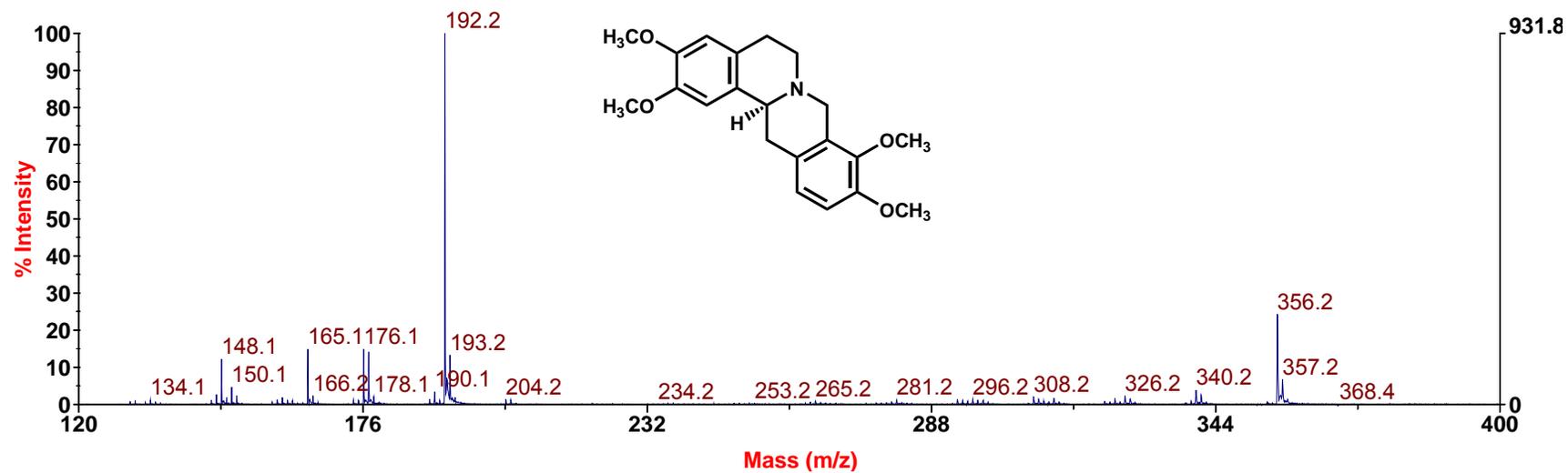
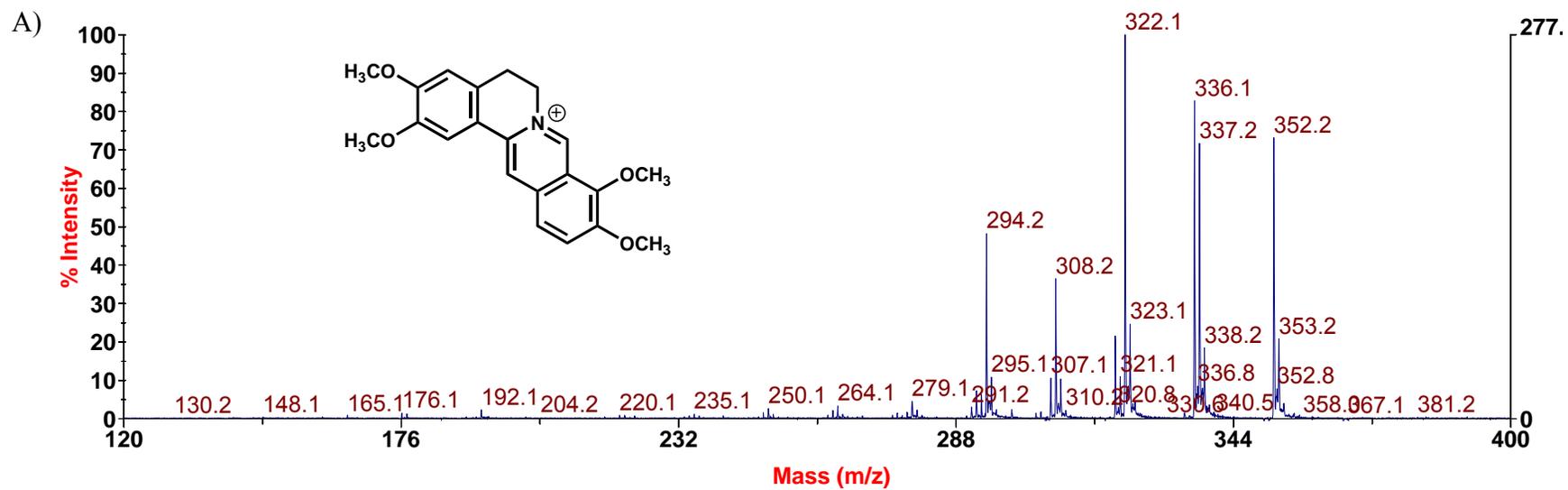


Figure 4.35 Ultraviolet absorption spectra profiles derived from the reaction of ctg11-protein in presence of (S)-tetrahydropalmitate. Panel A, the substrate (S)-tetrahydropalmitate and panel B, the reaction product palmitate.



B)

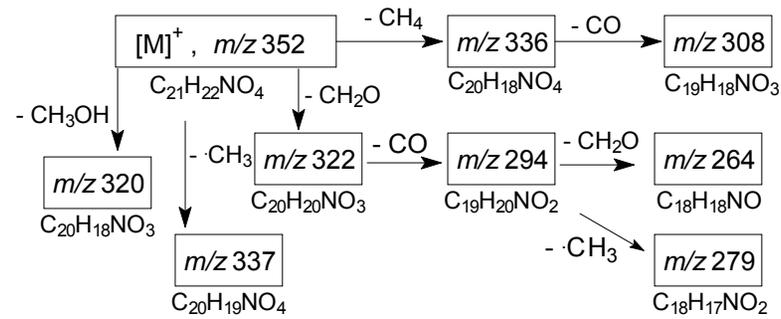


Figure 4.36. A) MS/MS data of palmatine (upper panel) produced after incubation of recombinant *ctg11* expressed in insect cells in presence of (*S*)-tetrahydropalmatine (lower panel). B) Fragmentation pattern of palmatine ion.

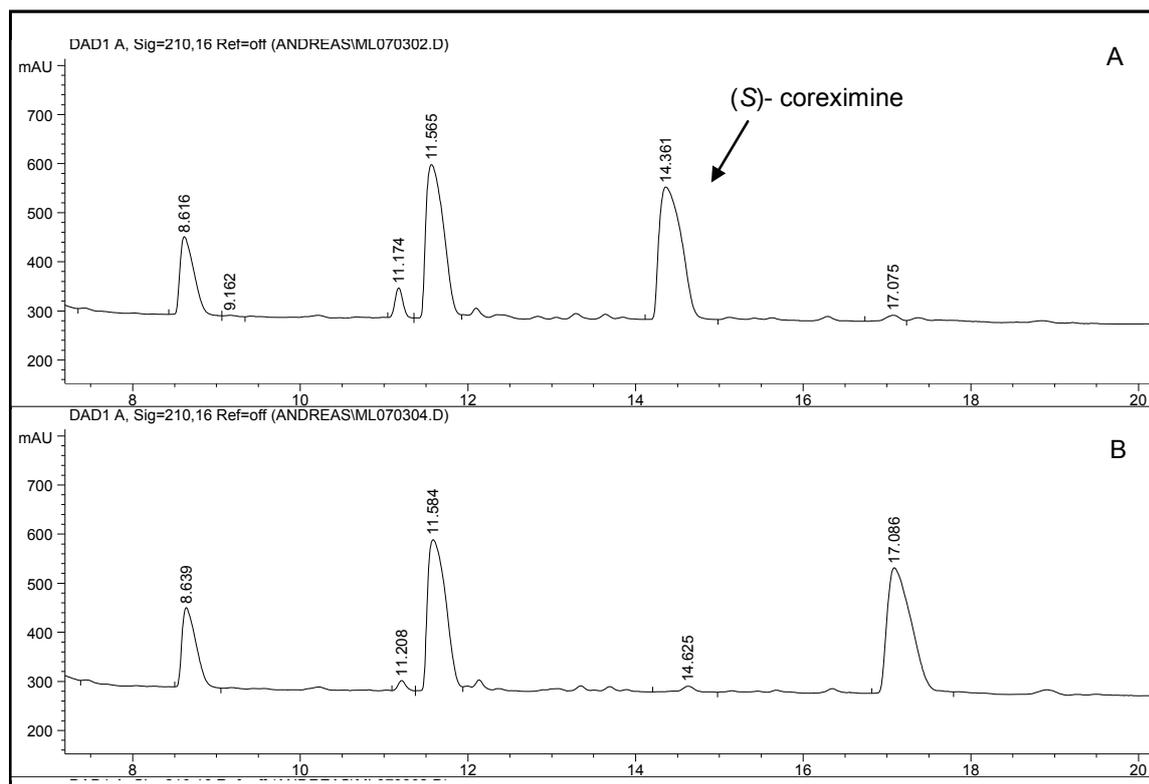


Figure 4.37. High performance liquid chromatography (HPLC) profiles derived from the reaction of the recombinant ctg11 expressed in insect cells (panel B) and a control reaction (panel A) in the presence of (S)-coreximine. The solvent system and gradient used was as previously described (3.1.2).

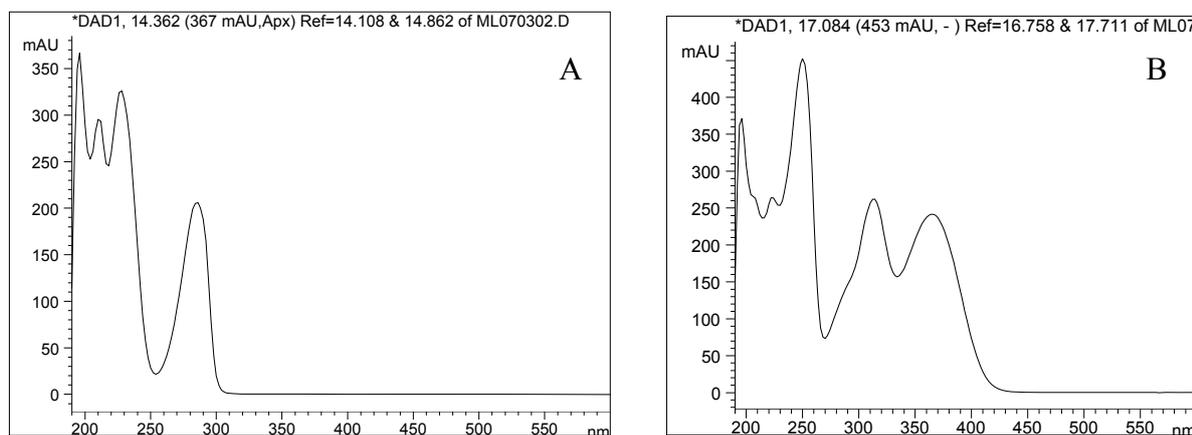


Figure 4.38. Ultraviolet absorption spectra profiles derived from the reaction of ctg11 in presence of (S)-coreximine. Panel A, the substrate (S)-coreximine and panel B, the reaction product.

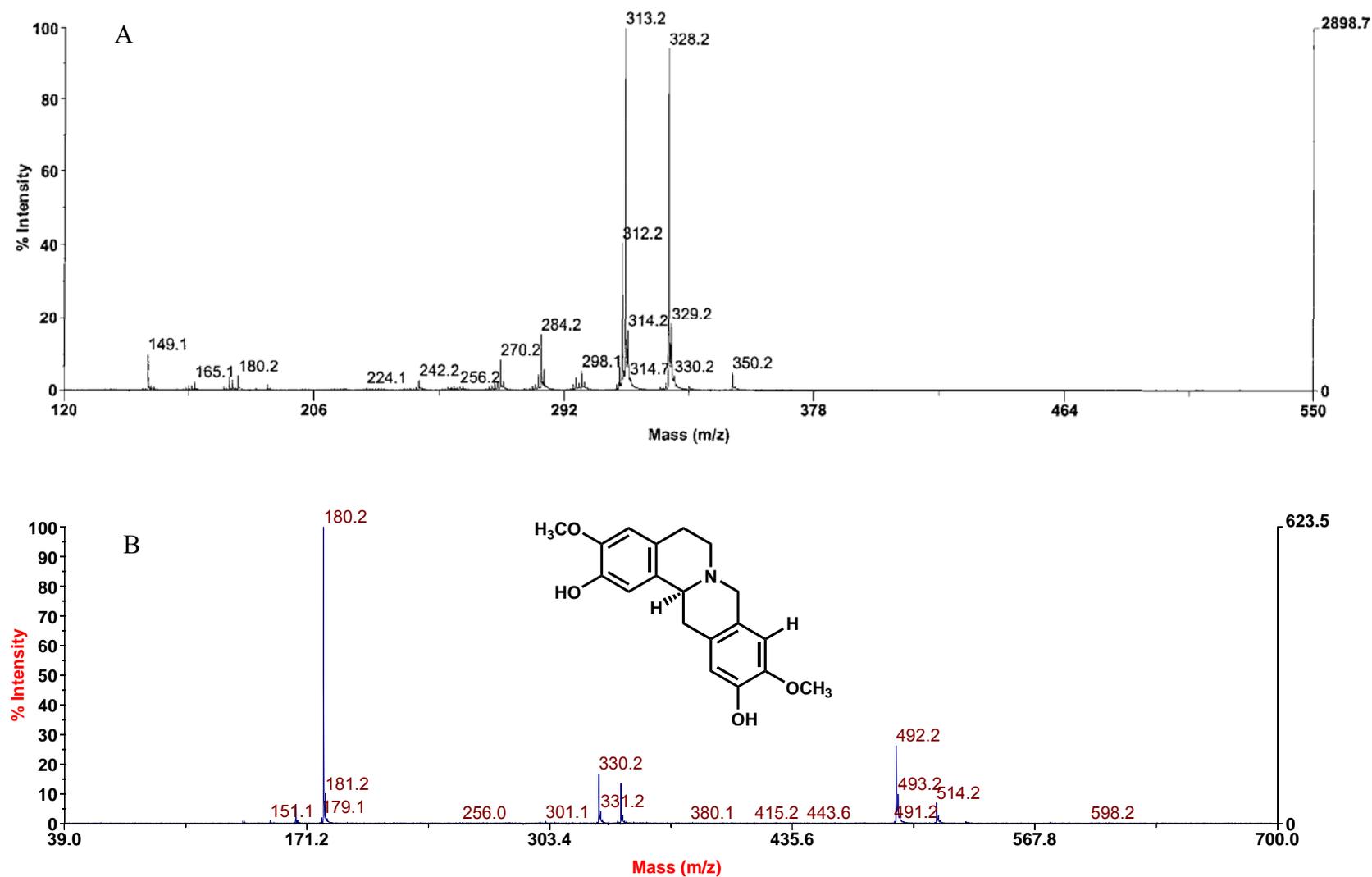


Figure 4.39. MS/MS data of the product produced (A) after incubation of recombinant *ctg11* expressed in insect cells in presence of (*S*)-coreximine (B).

5. Discussion

5.1. *Argemone mexicana*

Previous studies on *Argemone mexicana*, a medicinal plant used for the treatment of pain, diarrhea and cancer, have shown the presence of alkaloids belonging to various classes such as protopines, protoberberines and benzophenanthridine type alkaloids; some of them with antimicrobial and antiallergenic activity. In this work, alkaloid analysis in *A. mexicana* quantified protopine and allocryptopine as the main compounds in the plant being protopine accumulation three times higher in roots than in leaves. Berberine, reticuline, cryptopine, dihydrosanguinarine and chelerythrine were also found in roots, leaves and stem and sanguinarine was mainly found in roots otherwise only traces were found.

The construction of a cDNA library from *Argemone mexicana* roots generated approximately 160,000 cDNAs. About 1600 randomly selected cDNAs were partially sequenced from the 5'-end and the thus generated expressed sequence tags (5'-ESTs) were clustered into 1255 unique sequences. Similarity search of the non-redundant ESTs against the public non-redundant database at the NCBI indicated that 43% of these sequences displayed similarity to genes of known function, 36% to hypothetical genes, and the remaining 21% were novel sequences. ESTs were classified into 7 different categories according to putative protein function. To our knowledge, this is the first report of a cDNA library from *Argemone mexicana* and therefore it may represent a valuable source for gene discovery. In addition, benzyloquinoline alkaloid biosynthesis in this plant can now potentially be modified, since a transformation protocol using *Agrobacterium tumefaciens* is established (Godoy-Hernandez *et al.*, 2008).

Analysis of the ESTs lead to the identification of several clones homologous to methyltransferases, P450 monooxygenases and FAD dependent oxidoreductases from the biosynthesis of benzyloquinoline alkaloids. Two CYP450 clones were selected for their homology to a previously described cDNA identified as canadine synthase (CYP719A1). This enzyme isolated from *C. japonica* cells (Ikezawa *et al.*, 2003) is involved in the biosynthesis of canadine leading to berberine. Two other FAD oxidoreductase cDNA clones selected presented homology to the berberine bridge enzyme isolated from *Eschscholzia californica* (Dittrich and Kutchan, 1991). These four cDNAs were generated as full-length clones and heterologously expressed in insect cells for further characterization. Reaction products were identified by HPLC, UV spectra and Liquid Chromatography-Mass Spectrometry (LC-MS).

5.2. Cytochrome CYP719 family

The cytochrome CYP719 family has been until now identified as part of the biosynthesis of isoquinoline alkaloids. (*S*)-canadine synthase (CYP719A1), catalyzes methylenedioxy bridge formation in (*S*)-tetrahydrocolumbamine to form (*S*)-tetrahydroberberine. This enzyme was first studied by using microsomal fractions of *Thalictrum tuberosum* cells (Rueffer and Zenk, 1994) and later isolated from a cDNA library from *Coptis japonica* cells (Ikezawa *et al.*, 2003). This plant cytochrome was demonstrated to be highly substrate specific. Studies using *Eschscholzia californica* cells led to the partial characterization of two other methylenedioxy bridge-forming enzymes leading to the biosynthesis of sanguinarine. The first one, (*S*)-cheilanthifoline synthase, converts (*S*)-scoulerine to (*S*)-cheilanthifoline and the second one, (*S*)-stylophine synthase catalyzes the biosynthesis of (*S*)-stylophine from (*S*)-cheilanthifoline (Bauer and Zenk, 1989, 1991). Ikezawa (2007) reported the isolation of two cytochrome P450 enzymes (CYP719A2 and CYP719A3) from *E. californica* cells. Both enzymes catalyze the formation of a methylenedioxy bridge in (*S*)-cheilanthifoline to form (*S*)-stylophine. They also recognized (*S*)-scoulerine as substrate to form (*S*)-nandinine, a secondary metabolite with a methylenedioxy bridge in ring A. Neither CYP719A2 nor CYP719A3 displayed (*S*)-cheilanthifoline synthase activity to form (*S*)-cheilanthifoline (methylenedioxy bridge in ring D) from (*S*)-scoulerine. From all the substrates tested, both enzymes catalyzed the formation of a methylenedioxy bridge formation exclusively in ring A displaying the possibility of the existence of another enzyme with preference for ring D. Both CYP719A2 and CYP719A3 transcripts were induced in seedling after methyl jasmonate treatment. This induction was previously demonstrated in *E. californica* cell suspension culture by Blecher *et al.* (1995). In that work, four cytochromes of the sanguinarine pathway including (*S*)-cheilanthifoline synthase and (*S*)-stylophine synthase together with the BBE enzyme were induced after treatment with a yeast cell wall elicitor, methyl jasmonate or 12-oxo-PDA. Also the accumulation of benzophenanthridine alkaloids was observed due to an increase of the activity of these enzymes (Gundlach *et al.*, 1992).

5.3. CYP719A13 and CYP719A14

In the present work, two full-length cDNA cytochromes isolated from *A. mexicana* designated as CYP719A13 and CYP719A14 by the P450 nomenclature committee (Dr. Nelson, University of Tennessee, Memphis, TN), presented methylenedioxy bridge-formation activity. Both sequences have been deposited in the GenBank database (accession number EF451151 and EF451152, respectively). CYP719A13 named (*S*)-stylophine synthase catalyzes the transformation of (*S*)-cheilanthifoline into (*S*)-stylophine and CYP719A14 identified as (*S*)-cheilanthifoline synthase, recognized (*S*)-scoulerine as substrate to form (*S*)-cheilanthifoline. Both CYP719 enzymes presented a pH optimum between 7.5-8.0 and an optimum temperature of 30-35°C. No reaction was observed in both cases in the presence of substrates with an (*R*)-configuration or any other analogues substrates such as (*S*)-nandinine. Northern blot analysis reveals that CYP719A13 and CYP719A14 transcripts are much higher in roots than stems and leaves. So these enzymes might influence the production of benzophenanthridine alkaloids in this organ which was found to be the main organ for alkaloid accumulation.

5.3.1. Amino acid sequence analysis

Amino acid sequence analysis of CYP719A13 displayed 58% identity to CYP719A14. This enzyme presented 78%, 74% and 65% identity to CYP719A2, CYP719A3 and CYP719A1, respectively; whereas CYP719A14 displayed 60%, 59% and 62% sequence identity, compared to CYP719A2, CYP719A3 and CYP719A1, respectively. Even though only the activities from CYP719A1-A3 enzymes have been tested, some other CYP719A sequences isolated from several plants (Ranunculales) have been deposited in the cytochrome P450 home page (<http://drnelson.utmem.edu/CytochromeP450.html>). The relative homology among this CYP719A subfamily is shown in table Table 5.1.

Table 5.1. Percentage identity of the amino acid sequences of the cytochromes P450 isolated from *Argemone mexicana* with members of the same family.

		1	2	3	4	5	6	7	8
1	CYP719A1 Cj								
2	CYP719A2 Eca	64.8							
3	CYP719A3 Eca	64.3	84.6						
4	CYP719A4 Tf	85.9	63.8	62.6					
5	CYP719A6 Af	80.4	60.2	59.4	85				
6	CYP719A7 Af	86.2	63.6	62.4	93.9	85			
7	CYP719A9 Eca	60.7	77.8	78.6	60.4	57.1	60.9		
8	CYP719A13 Amex	64.8	78.4	74.7	64.8	61.7	65.6	71.1	
9	CYP719A14 Amex	62.1	59.2	57.8	61.8	61.1	61.5	54.5	57.4

Pair Distances CYP719 family, ClustalW (Slow/Accurate, Gonnet). Shortenings: Cj, *Coptis japonica*; Eca, *Eschscholzia californica*; Tf, *Thalictrum flavum*; Af, *Aquilegia formosa*; Amex, *Argemone mexicana*.

5.3.2. P450 primary sequence motifs

The deduced amino acid sequences of CYP719A13 and CYP719A14 displayed characteristic structural motifs that have been associated with eukaryotic P450s. Eukaryotic cytochromes are membrane-bound proteins with a hydrophobic signal peptide at the N-terminus incorporated into the endoplasmic reticulum, whereas the remaining part of the protein is exposed on the cytoplasmic side of the membrane (Szczesna *et al.*, 1988; Sakaguchi *et al.*, 1992). In CYP719A13 and CYP719A14, this signal peptide corresponds to the first 35 and 24 amino acids, respectively. Also, microsomal P450s have a conserved proline region immediately after the signal peptide sequence. This consensus sequence, (P/I)PGPx(G/P)xP is thought to act as a “hinge” that is required for optimal orientation of the enzyme with regard to the membrane. Deletion of this motif resulted in high protease susceptibility and complete loss of enzyme activity. In other cases, mutation of the proline residues to alanine disrupted protein structure sufficiently to eliminate heme incorporation. In mitochondrial and bacterial P450s, this proline region is not present (Yamazaki *et al.*, 1993; Szczesna *et al.*, 1993; Chapple C., 1998). This motif is highly conserved in CYB80A and B subfamily from the biosynthesis of isoquinoline alkaloids and in the family CYP81Q with methylenedioxy bridge-formation activity like the CYP719A subfamily. But in case of CYP719A13 and

CYP719A14 and all the CYP719 enzymes described so far (Figure 5.1), this region is not highly conserved; the consensus sequence here is represented by PxGPxxLP.

In the I helix, the consensus sequence (A/G)Gx(D/E)I(T/S) contains a threonine (Thr-252 in P450cam) that has been implicated in oxygen activation (Imai *et al.*, 1989; Durst and Nelson, 1995). In the CYP719A subfamily, this sequence is represented by LGVDST in which threonine is replaced by serine. Also in the CYP81Q family this threonine is replaced by alanine indicating that this amino acid is not essential to methylenedioxy bridge forming activity.

Other conserved motifs found in cytochrome P450s correspond to the tetrapeptide ExxR found in the K-helix and the heme-binding region FxxGxRxCxG where cysteine serves as the fifth ligand to the heme iron (Chapple, 1998; Yoshioka *et al.*, 2001). In most plant P450s, there is a proline residue immediately after the invariant heme-binding cysteine (Schalk *et al.*, 1999); however, in CYP719A13 and CYP719A14, this proline is replaced by alanine.

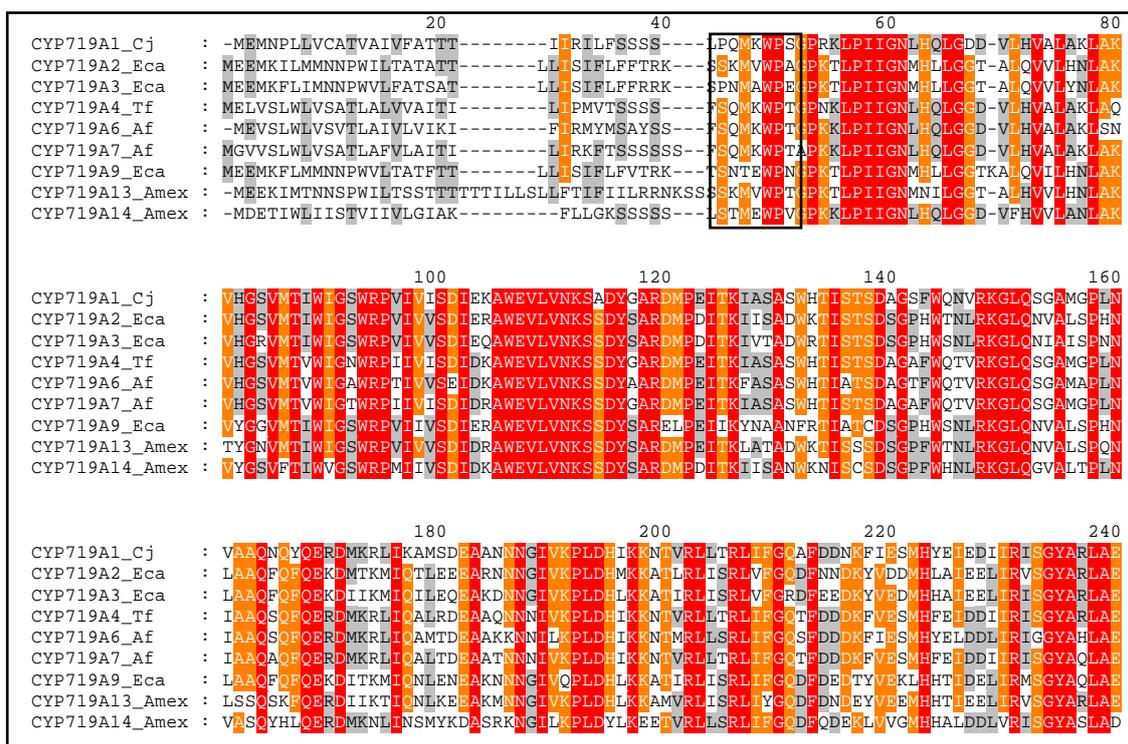
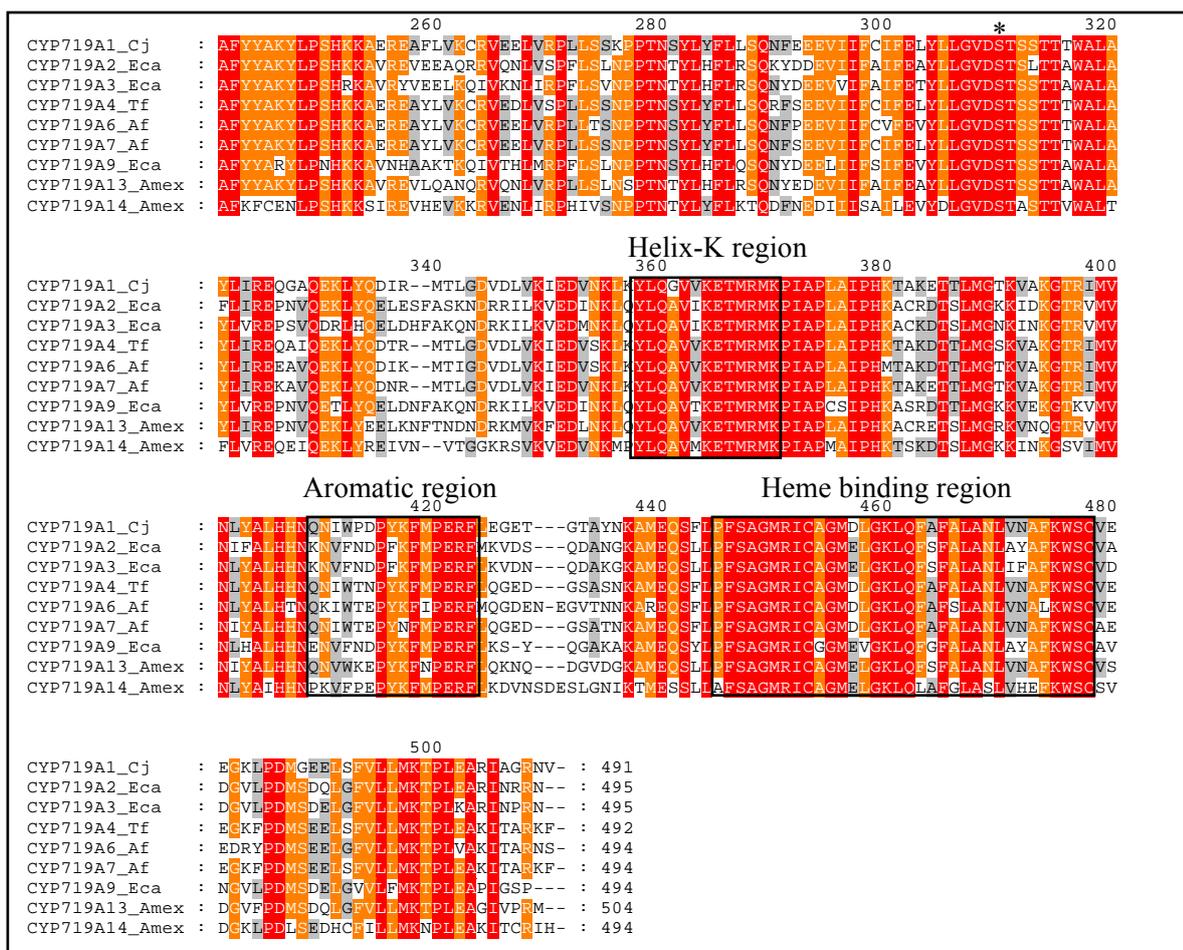


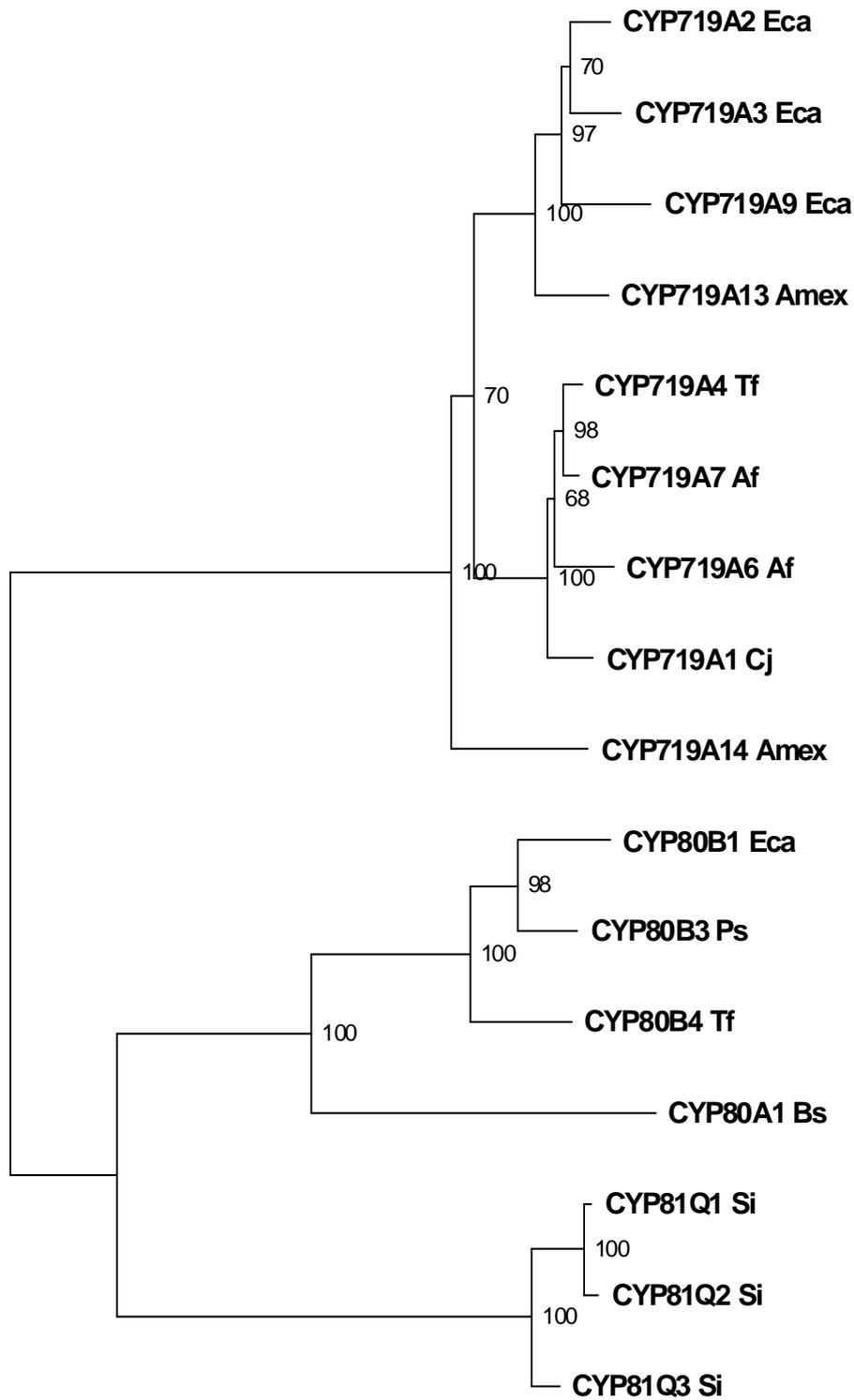
Figure 5.1. Multiple sequence alignment of cytochrome CYP719A subfamily. The sequence were compared using Clustal W. Red background represents 100% identities, orange background represents 80% identities and gray background represents 60% identities. The proline region is boxed. Cj, *Coptis japonica*; Eca, *Eschscholzia californica*; Tf, *Thalictrum flavum*; Af, *Aquilegia formosa*; Amex, *Argemone mexicana*.



Continue from figure 5.1. Multiple sequence alignment of cytochrome CYP719 family. The sequence were compared using Clustal W. Red background represents 100% identities, orange background represents 80% identities and gray background represents 60% identities. Conserved motifs in cytochromes P450s are boxed; Position equivalent to Thr-252 in P450cam is shown with asterisk. Cj, *Coptis japonica*; Eca, *Eschscholzia californica*; Tf, *Thalictrum flavum*; Af, *Aquilegia formosa*; Amex, *Argemone mexicana*.

5.3.3. Phylogenetic analysis

A phylogenetic tree for the CYP719A13 and CYP719A14 proteins from *A. mexicana* and several other CYP719A proteins was constructed using full-length amino acid sequences. These sequences were analysed together with the CYP80A and B subfamilies from isoquinoline alkaloid biosynthesis and CYP81Q subfamily from lignan biosynthesis. In the results displayed on Figure 5.2, the CYP81Q subfamily forms an independent cluster separated from the other methylenedioxy bridge-forming enzymes of the CYP719A subfamily. The CYP719A subfamily forms three different clades that have relation with the activity that they developed with CYP719A14 representing the root of the others.



0.1

Figure 5.2. Neighbor-joining tree of CYP450s. Bootstrap values are from 500 replications. Shortenings: Af *Aquilegia fromosa*, Amex *Argemone mexicana*, Bs *Berberis stolonifolium*, Cj *Coptis japonica*, Eca *Eschscholzia californica*, Ps *Papaver somniferum*, Tf *Thalictrum flavum*, Si *Sesamum indicum*

5.3.4. Substrate recognition sites

Due to the low quantities and membrane localization of plant P450 enzymes, no structures have yet been solved. The first crystal structure from cytochromes P450 that could be elucidated correspond to the soluble cytochrome P450 101A (P450cam) from *Pseudomonas putida* (Poulos *et al.*, 1987). Later modification at the N-terminus of membrane-bound proteins from mammals facilitated expression in bacteria and subsequent crystallization (Barnes *et al.*, 1991; Scott *et al.*, 2001; Rupasinghe and Schuler, 2006). In the last years, homology modeling and substrate-docking techniques have been used as alternative methods to analyze structure-function relationships of several P450s. It has been observed that even with low sequence identities among cytochromes from bacteria and eukaryotic origin, the overall fold that includes α -helices and β -sheets is still conserved (Nelson and Strobel, 1989).

Based on a group-to-group comparison between mammalian CYP2 sequences and the bacterial cytochrome P450 101A as template, Gotoh (1992) identified six putative substrate recognition sites (SRS1-SRS6) along the primary structure of the CYP2 family. The SRS1 region located between helices B' and C was found the most variable region and to be involved in controlling substrate specificity. Other regions are located at the C-terminal end of helix F (SRS2), the N-terminal end of helix G (SRS3), at the middle of helix I (SRS4), the beta-3 sheet (SRS5) and at a central region of beta-4 sheet (SRS6). The same pattern of substrate recognition sites was also observed among divergent P450s. For example, homology modeling of CYP73A5, CYP75B1, CYP84A1 and CYP98A3 proteins from the phenylpropanoid pathway in *Arabidopsis thaliana*, showed that these enzymes bind their substrate in a similar orientation over the heme plane, being the C-terminal end of the SRS4, SRS5 and SRS6 regions important in contacting the aromatic ring of their cinnamate-derived substrate and the variable regions SRS1, SRS2 and the N-terminal end of SRS4 important in contacting the aliphatic regions of these substrates (Rupasinghe *et al.*, 2003).

Sequence alignment of representative sequences from the CYP719A subfamily together with CYP101A lead us to identify the location of putative SRS regions in the methylenedioxy bridge enzymes by the location of several residues conserved in most cytochrome P450s. In this alignment, the SRS5 region was found to be conserved among all CYP719A enzymes that might be related by the kind of reaction they catalyze; regions SRS1, SRS4 and SRS6 stand out as having the most variability in residues inside this subfamily. Within these regions, differences in amino acid substitutions were observed indicating a possible role in substrate

recognition or catalytic activity. For example, in the SRS1 region (Figure 5.3), the conserved threonine (filled in green) in the CYP719A subfamily is replaced by asparagine (Asn120) in CYP719A14. Homology modeling of the human CYP2B6 enzyme with CYP2B4 located isoleucine 114 (Asn120 in CYP719A14) in close contact with the substrate cyclophosphamide; mutation of this residue to valine (I114V) improved the affinity of this enzyme for its substrate (Nguyen *et al.*, 2008).

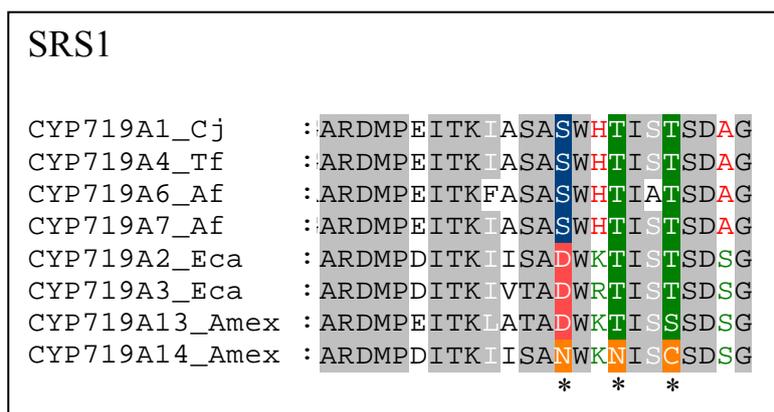


Figure 5.3. Substrate recognition site SRS1. In orange Asn117, Asn120 and Cys123 from CYP719A14. Amino acids conserved among all sequences except CYP719A14 are filled in green; positions in which mutations have been reported for other enzymes are indicated with asterisks. Differences between stylophine synthase enzymes and canadine synthase and homologous enzymes are filled in red and blue, respectively. Differences in amino acid substitution between canadine synthase and homologous enzymes and those from the biosynthesis of protopine are marked in red and green, respectively.

Another change in amino residue corresponds to cysteine 123 in CYP719A14, the position at which members of this subfamily have threonine/serine (filled in green). Mutation at this position in CYP51H10 (A124V) increased the amount of β -amyrin formed in comparison to the wild type (Qi *et al.*, 2006). Also in the SRS1 region, the charged residue aspartate (filled in red) in CYP719A2, A3 and A13 (stylophine synthase enzymes) is replaced by serine (Ser117) (filled in blue) in CYP719A1 (canadine synthase) and homologous enzymes and by asparagine (Asn117) in CYP719A14 (cheilanthifoline synthase). Since cheilanthifoline synthase and stylophine synthase enzymes catalyze subsequent steps in the biosynthesis of protopine, they could contain some similarities in amino residues for substrate recognition that could differ from that of CYP719A1 from the berberine pathway. As the figure shows, both stylophine synthase and cheilanthifoline synthase present a lysine/arginine (Lys119 in CYP719A14) that is substituted by histidine in CYP719A1 and homologous sequences, however in all cases, a positively charged residue is presented. Also in this region, the small

residue alanine in CYP719A1 (Ala126) and homologous sequences is replaced by another small residue in the stylophine and cheilanthifoline synthase enzymes, in this case serine.

In the SRS2 region (Figure 5.4), the amino acid glycine (Gly207) in CYP719A14 is replaced by serine (filled in blue) in CYP719A1 or aspartate/glutamate (filled in red) in stylophine synthase enzymes. Further amino acid substitutions are located at position 210 and 211 in CYP719A1. Whereas this enzyme has tyrosine and glutamate at those positions respectively, histidine and alanine are the major residues in the cheilanthifoline synthase/stylophine synthase enzymes. In SRS3 (Figure 5.4), the conserved residue alanine (filled in green) is substituted by serine (Ser240) in CYP719A14. Also the hydrophilic residue glutamate (E241) (filled in blue) in CYP719A1 and homologues is replaced by a hydrophobic residue (V/I) in the rest of this subfamily.

SRS2		SRS3	
CYP719A1_Cj	: SMH YE TIEDIIRI	CYP719A1_Cj	: AER EAF L V
CYP719A4_Tf	: SMH FE IDDIIIRI	CYP719A4_Tf	: AER EAY L V
CYP719A6_Af	: SMH YE LDDLIRI	CYP719A6_Af	: AER EAY L V
CYP719A7_Af	: SMH FE IDDIIIRI	CYP719A7_Af	: AER EAY L V
CYP719A2_Eca	: DMH LA IEELIRV	CYP719A2_Eca	: AVR E VEE A
CYP719A3_Eca	: DMH HA IEELIRI	CYP719A3_Eca	: AVR Y VEE L
CYP719A13_Amex	: EMH HT IEELIRV	CYP719A13_Amex	: AVR E VLQ A
CYP719A14_Amex	: GMH HA LDDL V RI	CYP719A14_Amex	: SIR E VHE V
	*		**

Figure 5.4. Substrate recognition sites SRS2 and SRS3. In orange Gly207, Ser240 and Ile241 from CYP719A14. Amino acids conserved among all sequences except CYP719A14 are filled in green; positions in which mutations have been reported for other P450s are indicated with asterisks. Differences between stylophine synthase enzymes and canadine synthase and homologous enzymes are filled in red and blue, respectively. Differences in amino acid substitution between canadine synthase and homologous enzymes and those from the biosynthesis of protopine are marked in red and green, respectively.

In SRS4 region (Figure 5.5), CYP719A14 is the only member of this subfamily that differs from the other enzymes with amino acids serine (Ser284), leucine (Leu287) and aspartate (D291); positions in which members of this subfamily present phenylalanine, phenylalanine, and leucine, respectively (all filled in green). The last position (aspartate 291), was identified to line the active site cavity in CYP51H11 (Qi *et al.*, 2006). Other differences observed correspond to the amino acid alanine in the stylophine synthase/cheilanthifoline synthase enzymes that is replaced by cysteine in CYP719A1.

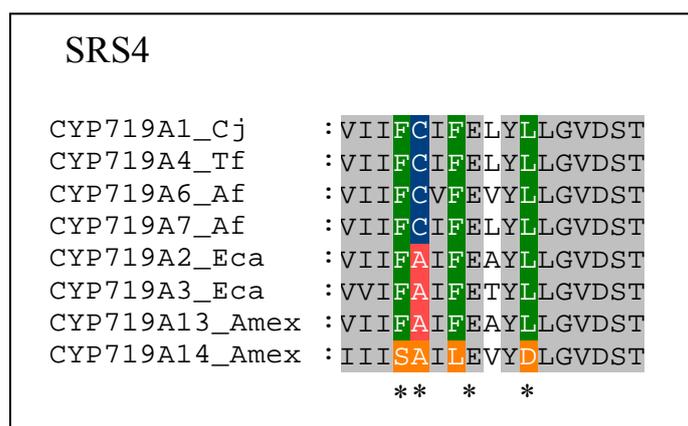


Figure 5.5. Substrate recognition site SRS4. In orange Ser283, Ala284, Leu286 and Asp290 from CYP719A14. Amino acids conserved among all sequences except CYP719A14 are filled in green; position in which mutation affected substrate specificity in P450s is indicated with an asterisk. Differences between stylopine synthase enzymes and canadine synthase and homologous enzymes are filled in red and blue, respectively.

In SRS6 (Figure 5.6), the conserved leucine (filled in green) in the CYP719A subfamily is replaced by the hydrophilic residue histidine (His475) in CYP719A14. This enzyme has also a cysteine (Cys476) that is replaced by serine or glycine in the other members of this subfamily. Those positions correspond to I396 and V397 in CYP101A, identified as substrate-binding residues. In CYP73A1, lysine 484 (Asp474 in CYP719A14) was predicted to exist within the catalytic site. Mutation of this residue to methionine decreased the catalytic activity probably because it affects substrate positioning or product release (Schoch *et al.*, 2003). Also in CYP94A2, a hydroxylase enzyme of *Vicia sativa*, mutagenesis of F494 (His475 in CYP719A14) to smaller residues, including L, V and A, increased the amount of product hydroxylated at internal positions in short chain fatty acids (Kahn *et al.*, 2001). Another change observed is isoleucine 478 in CYP719A14, which differs from the conserved residue valine (filled in green) in the members of this subfamily. The mutation of the analogous residue in CYP2B6 (Val477) to isoleucine, phenylalanine or tryptophan improved the affinity of this enzyme for its substrate (Nguyen *et al.*, 2008).

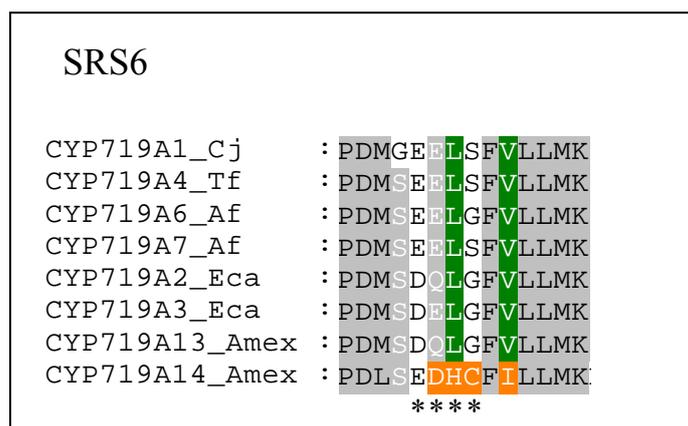


Figure 5.6. Substrate recognition site SRS6. In orange Asp474, His475, Cys476 and Ile478 from CYP719A14. Amino acids conserved among all sequences except CYP719A14 are filled in green; positions in which mutations have been reported for other P450s are indicated with asterisks.

5.4. (S)-tetrahydroprotoberberine oxidase and berberine bridge enzyme

In this work also two FAD-dependent oxidoreductases, (S)-tetrahydroprotoberberine oxidase (STOX-like protein) and a berberine bridge enzyme (BBE-like protein) were isolated. BBE displayed 34% identity at the amino acid level to STOX and 72% to the BBE from *Papaver somniferum*. The protein sequence of STOX displayed 53% identity to a putative BBE from *Medicago truncatula* and 40% identity to an oxidoreductase involved in the biosynthesis of tetrahydrocannabinolic acid in *Cannabis sativa* (Sirikantaramas *et al.*, 2004). STOX and BBE contain a predicted signal peptide of 26 and 31 aa, respectively that redirects the protein into specific vesicles (Dittrich and Kutchan, 1991). Both enzymes contain a conserved covalently bound FAD moiety that is required for activity (Winkler *et al.*, 2006). These residues correspond to histidine 104 and cysteine 173 in BBE and histidine 112 and cysteine 175 in STOX (Figure 5.7).

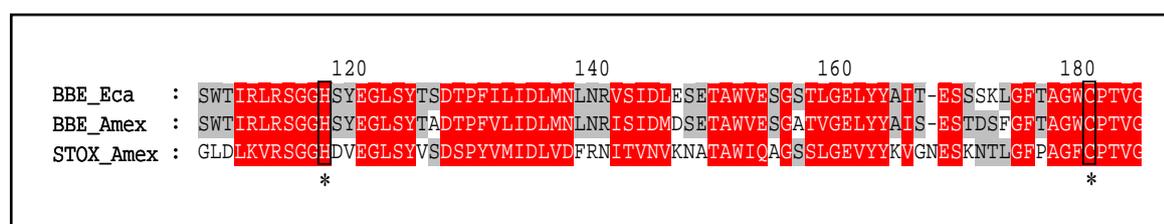


Figure 5.7. Amino acid sequence alignment of BBE from *Eschscholzia californica* (BBE_Eca) and BBE and STOX from *Argemone mexicana*. A (*) indicates the sites of covalent attachment (H104 and C166 for EscBBE). Conserved residues are marked in red and semiconservative amino acids in gray.

5.4.1. Activity assays

It was not possible to identify product formation using cells extracts of insect cells (*Sf9*) containing the corresponding recombinant protein due possibly to a low protein expression or protein degradation. *In vivo* assays were developed in which the pH of the medium was adjusted and a protease inhibitors solution and the substrate were added. In case of BBE-like protein, no product could be identified by HPLC or LC-MS even though the reaction mixture in presence of scoulerine displayed a dark brown coloration that was not presented in the control reaction. The reaction mixtures of STOX in presence of different tetrahydroprotoberberines (Table 5.2) produced a colorful yellow solution. The enzyme catalyzed the removal of four hydrogen atoms in the protoberberine (*S*)-tetrahydropalmatine and possibly also in (*S*)-canadine which lead to the aromatization of the ring C producing palmatine and berberine, respectively. It also catalyzed the oxidation of coreximine (*m/z* 330, since a radiolabelled coreximine in the isoquinoline part was used) into a yellow product with a molecular mass of *m/z* 328. In this case there was a reduction of two protons in the ring C instead of four which did not generate a conjugated ring. The exact position of this double bond was not identified but Amman and coworkers (1988) discard the possibility of an intermediate with a double bond at position 13,14 since a synthetic 13,14-dehydroberberine was not taken as substrate by the (*S*)-tetrahydroprotoberberine oxidase purified from *Berberis wilsoniae*. Interestingly, this protein did not yield a product in the presence of scoulerine even though coreximine and scoulerine are structural isomers.

Table 5.2. (*S*)-tetrahydroprotoberberines used as substrates by STOX

Substrate	R ₁	R ₂	R ₃	R ₄
(<i>S</i>)-coreximine	OCH ₃	OH	H	OH
(<i>S</i>)-tetrahydropalmatine	OCH ₃	OCH ₃	OCH ₃	H
(<i>S</i>)-canadine	O	CH ₂	O	OCH ₃

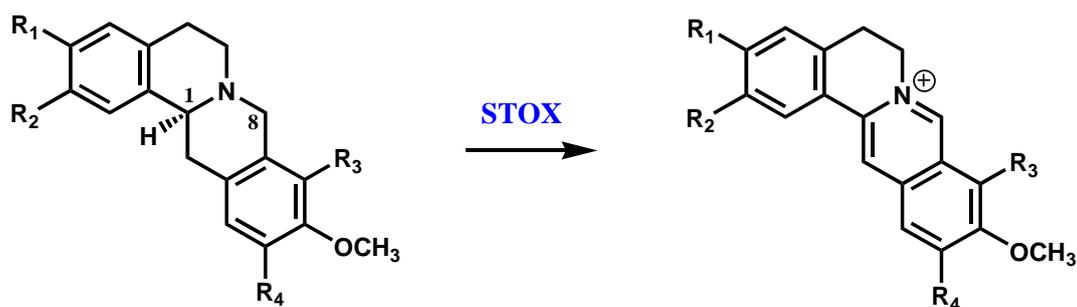


Figure 5.8. Reaction catalyzed by (*S*)-tetrahydroprotoberberine oxidase (STOX).

STOX was previously purified from *Berberis wilsoniae* cell cultures (Amman *et al.*, 1988). The enzyme presented broad substrate specificity oxidizing a series of (*S*)-tetrahydroprotoberberines and benzyloquinoline alkaloids into their corresponding protoberberines (Figure 5.8); nevertheless, STOX from *Argemone mexicana* isolated in the present work could be involved in the biosynthesis of protoberberines derived from coreximine, since this compound was preferred for the enzyme and was also found to be present in the plant. Probably there is more than one “STOX” enzyme involved in the biosynthesis of benzyloquinoline alkaloids since different substrate specificities were found between STOX isolated from *A. mexicana* and STOX isolated from *Berberis wilsoniae* (Gesell, 2008). In the first one, no product formation was observed in presence of scoulerine and in the last one, the enzyme did not react with coreximine but recognized scoulerine, canadine and tetrahydropalmatine as substrates. Both enzymes presented 50% identity with each other at amino acid level.

In summary, the presence of ESTs homologous to known alkaloid biosynthetic genes starting from (*S*)-norcoclaurine to berberine or stylophine showed that the created EST library was an excellent cDNA source. An FAD dependent oxygenase, “STOX” with affinity for coreximine could be obtained as recombinant protein. It was also possible to functionally express the missing methylenedioxy bridge forming enzyme from the biosynthesis of stylophine. So far it is not studied if additional methylenedioxy bridge forming enzymes are part of the biosynthesis of the *Argemone*-specific alkaloids argemexicaine A and B, both structural isomers of allocryptopine (Chang *et al.*, 2003). Unfortunately, mass spectrometric analysis failed to detect these compounds in our plant extracts, probably because these compounds occur in very low concentrations or are present just under certain conditions. On the other hand, it was possible to identify coreximine in the plant, which contains an unusual ring D substitution pattern and the cyclization of which from reticuline has not yet been clarified.

6. References

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