Gene 510 (2012) 193–201

Contents lists available at SciVerse ScienceDirect

Gene

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

Short Communication

Characterization of cytochrome P450 monooxygenases isolated from trichome enriched fraction of *Artemisia annua* L. leaf

Amita Misra [a], Chandan S. Chanotiya [b], Madan M. Gupta [b], Upendra N. Dwivedi [c], Ajit K. Shasany [a,*]

[a] *Biotechnology Division, Central Institute of Medicinal and Aromatic Plants (CSIR), P.O. CIMAP, Lucknow-226015, U.P., India*
[b] *Analytical Chemistry Division, Central Institute of Medicinal and Aromatic Plants (CSIR), P.O. CIMAP, Lucknow-226015, U.P., India*
[c] *Department of Biochemistry, University of Lucknow, Lucknow-226007, U.P., India*

ARTICLE INFO

Article history:
Accepted 5 September 2012
Available online 14 September 2012

Keywords:
Biosynthesis
Lauric acid
RACE
trans-cinnamic acid
CPR

ABSTRACT

*CYP*s have major role in the biosynthesis and modification of secondary metabolites. Predicting the possible involvement of *CYP*s in secondary metabolism, 20 partial sequences were amplified from the cDNA of trichome enriched tissue of *Artemisia annua*. Seven *CYP*s were converted to full length and assigned to different families based on sequence homology. These were co-expressed with CPR in *Saccharomyces cerevisiae* and microsome fractions were assayed for conversion of sesquiterpenes, phenols and fatty acid substrates. *CIM_CYP02(c73)* and *CIM_CYP05(c81)* converted *trans*-cinnamic acid to *p*-coumaric acid; and capric acid, lauric acid to their hydroxylated products, respectively. Higher expression of *CIM_CYP71AV1*, *CIM_CYP03(c72a)*, *CIM_CYP06(c72b)*, *CIM_CYP02(c73)* and *CIM_CYP04(c83)* was observed in the mature leaf, whereas expression of *CIM_CYP05(c81)* was more in the seedling. *CIM_CYP71AV1*, *CIM_CYP02(c73)* and *CIM_CYP04(c83)* expressed more in the flower bud compared to the leaf, with minor expression in stem. All *CYP*s' expression increased progressively with time after wounding except for *CIM_CYP07(c92)*. These results relate involvement of *CIM_CYP02(c73)* to phenyl-propanoid metabolism in the leaf and *CIM_CYP05(c81)* to fatty acid metabolism in the seedling. Expression of *CIM_CYP71AV1* and *CIM_CYP02(c73)* significantly increased when sprayed with *trans*-cinnamic acid indicating a relationship between phenylpropanoid and artemisinic acid pathways.

1. Introduction

Cytochrome P450 monooxygenases (CYPs) are multifunctional, heme-iron containing enzymes, catalyzing NADPH and oxygen dependent hydroxylation reactions. CYPs in different organisms are involved in biosynthetic and detoxicating pathways (Nelson, 2011). In plants, these membrane bound enzymes are responsible for biosynthesis of a variety of compounds like phenylpropanoids, terpenoids, fatty acids, hormones, pigments and defense related molecules (Schuler and Werck-Reichhart, 2003). Many CYPs, alone or in combinations are involved in multistep catalysis of substrates. Example of such CYPs are CYP71C1–C4 involved in DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) biosynthesis and CYP71AV1 in the biosynthesis of artemisinic aldehyde from its precursor amorpha 4, 11-diene (Bailey and Larson, 1991; Frey et al., 1997; Ro et al., 2006; Liu et al., 2011). *Artemisia annua* produces an anti-malarial sesquiterpene endoperoxide lactone, "artemisinin" which is extensively used in artemisinin-based combined therapy (ACT) to cure chloroquine resistant malaria. Though, the biosynthetic pathway of artemisinin is yet to be deciphered completely, tremendous progress has been taking place in recent years providing a clear picture of the intermediate steps (Teoh et al., 2009; Zhang et al., 2008). It is believed that arteannuin B and artemisinin are the end products of the bifurcated pathway, competing with each other for precursors (Liu et al., 2011). Weathers et al. (1999) showed better artemisinin production in a highly oxygenated environment, compared to a hypoxic one. Wallaart et al. (1999, 2001) suggested DHAA as a reactive oxygen species (ROS) scavenger which is probably converted to artemisinin by more than one non-enzymatic, spontaneous photo-oxidation reactions (Brown and Sy, 2007; Wallaart et al., 2001). In plants, an enormous diversity of *CYP*s is observed and correlated to the secondary metabolite diversity (Bolwell et al., 1994; Nelson et al., 1996). Though *Arabidopsis*is not very rich in secondary metabolites, still, 272 *CYP*s belonging to 45 families have been described (Werck-Reichhart et al., 2002). As very few *CYP*s are reported and analyzed from *A. annua*, in this study we analyzed and reported new *CYP*s from *A. annua* trichome enriched tissue. These were characterized for their possible role in secondary metabolism under various conditions.

Abbreviations: CPR, Cytochrome P450 reductase; CYP, Cytochrome P450 monooxygenase; DHAA, Di-hydro Artemisinic acid; RACE, Rapid amplification of cDNA ends.

* Corresponding author. Tel.: +91 522 2718548; fax: +91 522 2342666.
E-mail addresses: akshasany@yahoo.com, ak.shasany@cimap.res.in (A.K. Shasany).

0378-1119/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2012.09.015

2. Materials and methods

2.1. Plant material and treatments

Seeds of *Artemisia annua* var. 'CIM-Arogya' (Khanuja et al., 2008, United States Patent No. US 7, 375,260), obtained from National Gene Bank for Medicinal and Aromatic Plants (NGBMAP) maintained at the Central Institute of Medicinal and Aromatic Plants in India, were grown in the fields during February to August. The plants were also grown in pots and maintained in the glass house. Leaf material was collected from 5 month old plants for RNA isolation. For wound induction plants were pricked at various parts with the help of syringe and forceps. Immediately after the wounding leaf samples were collected at 0 h, 1 h, 2 h and 4 h for RNA extraction. For exogenous treatment of different compounds, plant leaves were sprayed with either 1 mM *trans*-cinnamic, *p*-coumaric, caffeic acid or artemisinin in methanol according to Perradin et al. (1983), Park et al. (2006), Melgar et al. (2009), and Vimala and Suriachandraselvan (2009). Control plants were sprayed with methanol only. After 6 h, leaf tissue was harvested for RNA isolation and RT-PCR.

2.2. Isolation and cloning of partial and full length CYPs

Glandular trichomes were isolated from young leaves of *A. annua* following the protocol described by Teoh et al. (2006) and100 mg trichome was used for total RNA isolation (Chomczynski and Sacchi, 1987). From this 5 μg RNA was taken for cDNA synthesis using Thermoscript RT-PCR System (Invitrogen, USA). The partial *CYP*s from trichome enriched tissue were isolated by two step PCR amplifications. The first RT-PCR was performed with a set of 4 primers directed against the conserved EEF(R)PER-motif in combination with oligo dT primer. After amplification (Fischer et al., 2001), PCR-products were diluted by 100 fold and used as template for the second nested RT-PCR. A set of eight primers directed against the PFG-motif was used in combination with oligo dT primer. All primers to isolate the partial *CYP* sequences and conditions for amplification were the same as described by Fischer et al. (2001). The resulting amplified fragments were cloned in pGEM-T Easy vector (Promega, USA) and sequence characterized. SMART™ RACE cDNA Amplification System (Clontech) was used to clone full length *CYP*s in pGEM-T Easy vector and identities were confirmed through sequence homology using BLAST N, BLAST X and BLAST P analyses (Altschul et al., 1997). Multiple sequence alignment (MSA) of the amino acid sequences using the ClustalW (http://www.ebi.ac.uk/Tools/clustalw/) with the default parameters was performed.

2.3. Co-expression of CPR, CYPs, microsome isolation and substrate conversion

Full length isolated *CYP*s were cloned at *Eco*RI and *Not*I restriction sites in MCS1 of pESC-URA containing a CPR (JN594507) sequence amplified from *A. annua* at *Bam*HI and *Sal*I restriction enzyme sites of MCS2 (Ro et al., 2006). List of primers used for full length gene amplification and cloning in yeast expression vector is provided in Table 1. *S. cerevisiae*, YPH501 competent cells were transformed with 1–3 μg of the pESC::CPR and pESC::CPR/*CYP* plasmids. These were plated separately on URA dropout plates with D-glucose (SD-URA) as carbon source and incubated at 30 °C for 2–3 days. For CYP-protein induction in the YPH501 strain, transformed yeast colonies were streaked on SD-URA dropout plates and incubated at 30 °C overnight. Single colony was sub-cultured in 3 ml of SD-URA dropout medium overnight and 250 ml of the same medium was subsequently inoculated by a 1:100 (v/v) dilution of the culture. This was incubated at 30 °C with shaking at 200 rpm for 12 to 15 h until cultures reached mid-log phase (approximately $3–5\times10^7$ cells/ml). Cells were harvested by centrifuging at 3000×*g* for 5 min at 20 °C. Pellet was resuspended in 10 ml sterile distilled water, centrifuged under the same conditions and resuspended in fresh synthetic galactose-URA dropout medium to initiate CYP-protein induction. Further, the yeast cells were cultured for an additional 20 h at 30 °C. Microsome fraction from yeast was isolated following the protocol described by Pompon et al. (1996). The concentration of protein was determined according to Bradford (1976) with bovine serum albumin (BSA) as the standard. Approximately 5 mg of total microsomal protein was added in 1 ml reaction containing 100 mM Tris–HCl buffer pH-7.5, 25 μM substrate (sesquiterpenes, sesquiterpnoids, and fatty acids), 100 μM NADPH and NADPH regeneration system (5 mM glucose-6-phosphate and two units of glucose-6-phosphate dehydrogenase). Reaction was started by adding microsomal protein and incubated for 2 h at 30 °C with gentle agitation. After incubation the reaction was acidified to pH 2 by 6 M HCl, and the reaction mixture was extracted twice with equal volume of ethyl acetate, followed by evaporation of organic phase in vacuum. Conversion of sesquiterpenes by CYP-CPR combination was checked by GC and HPLC following the method described earlier (Ro et al., 2006; Teoh et al., 2006). Fatty acids were resolved by TLC as described by Cabello-Hurtado et al. (1998). Conversion of *trans*-cinnamic acid to *p*-coumaric acid was monitored by TLC (Ro et al., 2001).

Table 1
List of primer sequences used for full length gene amplification.

S no	Primer name	Sequence (5′ to 3′)
1	CIM_CPRF	ATG*GATCC*TATGCAATCAACAACTTCCGTTAAGTTAT
2	CIM_CPRR	TAT*GTCGAC*CCATACATCACGGAGATATCTTCCT
3	CIM_CYP02F	TCG*GAATTC*ATGGATCTTCTCCTTTTGGA
4	CIM_CYP02R	ATA*GCGGCCGC*AAAGGATCTTGGTTTA
5	CIM_CYP03F	GCG*GAATTC*ATGACAGCAACATTTAC
6	CIM_CYP03R	ATC*GCGGCCGC*TATGTTATGTAAAATTAAG
7	CIM_CYP04F	TAC*GAATTC*ATGGACTCTCTTCTTCAATTTCTAC
8	CIM_CYP04R	TAC*GCGGCCGC*ATTTGGAGAATTGGAGTTAAA
9	CIM_CYP05F	GCA*GAATTC*ATGGAAGTTCCTTATCTATACATC
10	CIM_CYP05R	TAA*GCGGCCGC*CAACTCGGAAAGTAAAGTTT
11	CIM_CYP06F	TGC*GAATTC*ATGGAGTCACCTACATC
12	CIM_CYP06R	ATA*GCGGCCGC*AACTTTGTGTAAAATTAAG
13	CIM_CYP07F	TCG*GAATTC*ATGGAACCACTTTCTAATTCTTAC
14	CIM_CYP07R	TTA*GCGGCCGC*GAGATGGTATATTTCAACT
15	CIM_CYP71AV1F	ATGGCACTCTCACTGACCACTTC
16	CIM_CYP71AV1R	CTAGAAACTTGGAACGAGTAACAAC

2.4. Quantitative and semi-quantitative RT-PCR analysis of full length CYPs

The expression levels of full length *CYP*s at various conditions were measured by real time PCR with SYBR green I chemistry (Applied Biosystems, USA). Primers were designed with the Primer Express software (Applied Biosystems, USA) and tested to ensure amplification of single discrete fragment of size ~150 bp with no primer–dimers. Two micrograms of DNase (Promega, USA) treated total RNA was used for first-strand cDNA synthesis with random hexamer primer using ThermoScript™ RT-PCR System (Invitrogen, USA). The reaction was set up with 50 ng cDNA, 5 pmol of each forward and reverse primers, 15 μl of SYBR Green PCR master mix (2×) (Applied Biosystems, USA) and the final volume was adjusted to 30 μl with milliQ water. The reactions were carried out (5 replicates) in 7900HT Fast Real Time PCR System (Applied Biosystems) and the specificity of the reactions was verified by melting curve analysis. The level of gene expression was analyzed with Sequence Detection System (SDS) software version 2.2.1 and normalized with β-actin expression (endogenous control). Threshold cycle (Ct) values obtained after real time PCR were used for calculation of ΔCt value (target-endogenous control). The relative quantification was carried out by calculation of ΔΔCt to determine the fold difference in gene expression [ΔCt target − ΔCt calibrator]. Relative quantity (RQ) was determined by computing $2^{-\Delta\Delta CT}$. Semi-quantitative RT-PCR was carried out using total RNA (Invitrogen, USA ThermoScript RT-PCR kit), *CYP*

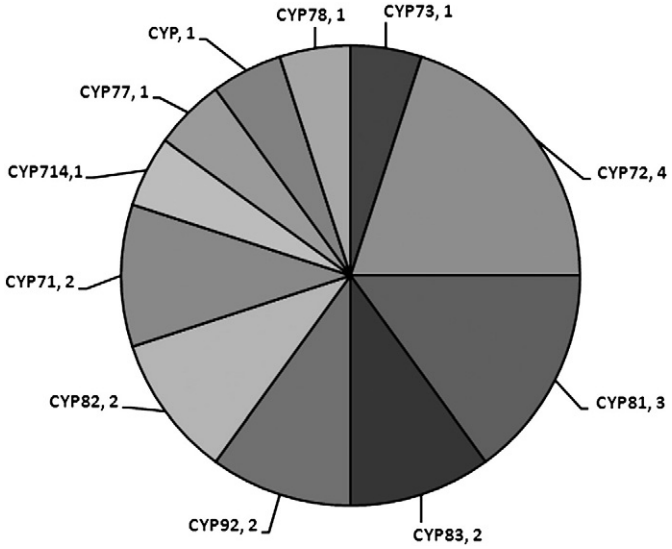


Fig. 1. Partial *CYP*s isolated from of *A. annua*. These were tentatively named as per their homology to the CYP family genes. Frequency of similar family genes is also indicated.

specific primers and compared with β-actin amplification. Further, to ascertain the expression of CYP-transcripts in yeast after transformation and induction, semi-quantitative RT-PCR was also carried out.

2.5. Artemisinin extraction and analysis

Artemisinin content of plant was estimated by thin layer chromatography. Dry powdered plant material (0.1 g) was extracted in 10 ml of hexane by heating at 60 °C till half the volume and left overnight at room temperature. The next day the volume of the extract was made up to 10 ml with hexane, filtered and evaporated on water bath at 50 °C. The extract was dissolved in 1 ml hexane and spotted on silica gel 60 F_{254} TLC plates (Merck) at 1 cm apart along with standard artemisinin (1 mg/ml). Spotted TLC plate was dipped in solvent (mobile phase) hexane:diethyl ether (1:1). The plate was dried in air and dipped in developing reagent, anisaldehyde:sulphuric acid (1:2) and heated at 120 °C for 10–15 min. The spots were scanned (540 nm, visible) (Densitometer CAMAG: Switzerland) to quantify the artemisinin content of the individual plant.

3. Results and discussion

As less number of full length *CYP*s have been reported from *A. annua* it is required to isolate and identify more *CYP*s to explore their role in secondary metabolism. Since trichomes are described to be highly active in the synthesis and storage of secondary metabolites including the highly desired artemisinin, this tissue was selected for isolating novel *CYP*s for characterization.

3.1. CYP family and isolation from A. annua trichome enriched tissue

NCBI database was searched for available *CYP* sequences in *A. annua* and *CYP71* (DQ667171), *CYP71AV1* (DQ872632, EU684540), *taxadiene-5-hydroxylase* (DQ363134), *taxadiene-13-hydroxylase* (DQ363133), *sterol-23-hydroxylase* (DQ363132), *flavonoid-3-hydroxylase* (DQ363131), *amorpha-4,11-diene oxidase* (EF197889) and *CYP* (DQ370065) sequences were retrieved. These were used for multiple sequence alignment analysis which showed the presence of conserved regions specific for *CYP*s. Further, when *CYP*s from Asteraceae family were compared, a total of five regions were found to be almost conserved (Supplementary Table 1). These regions include the heme binding domain, the ERR triad (KETLR motif and PERF motif), T-containing oxygen binding domain and a proline rich region at the 5′ end of the sequence.

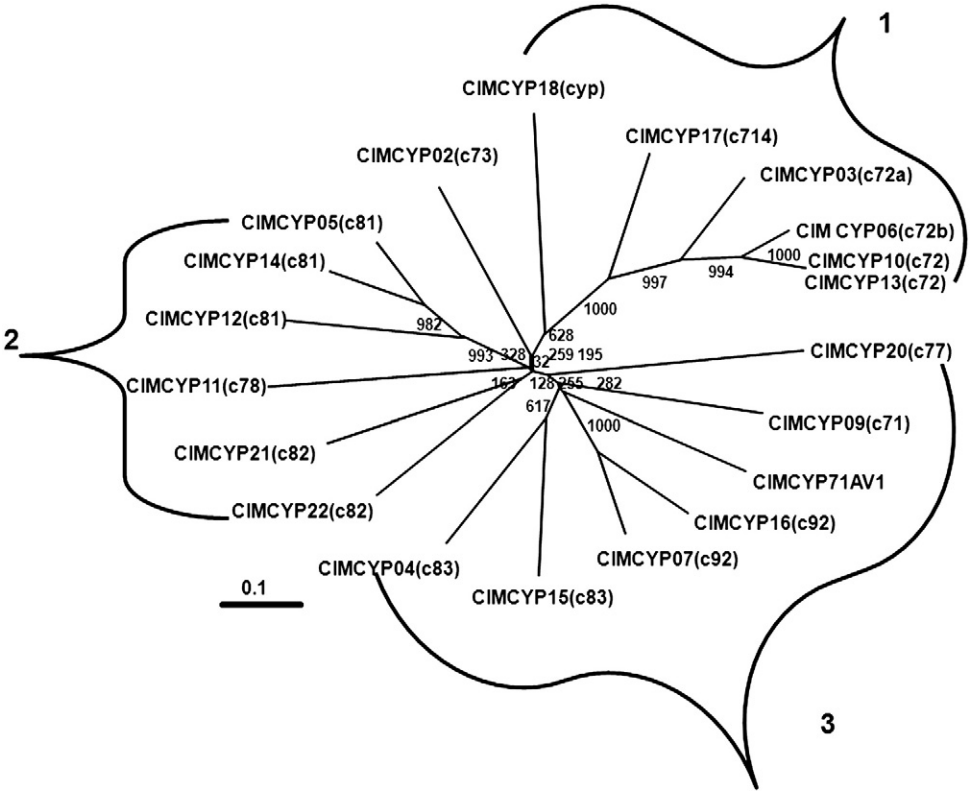
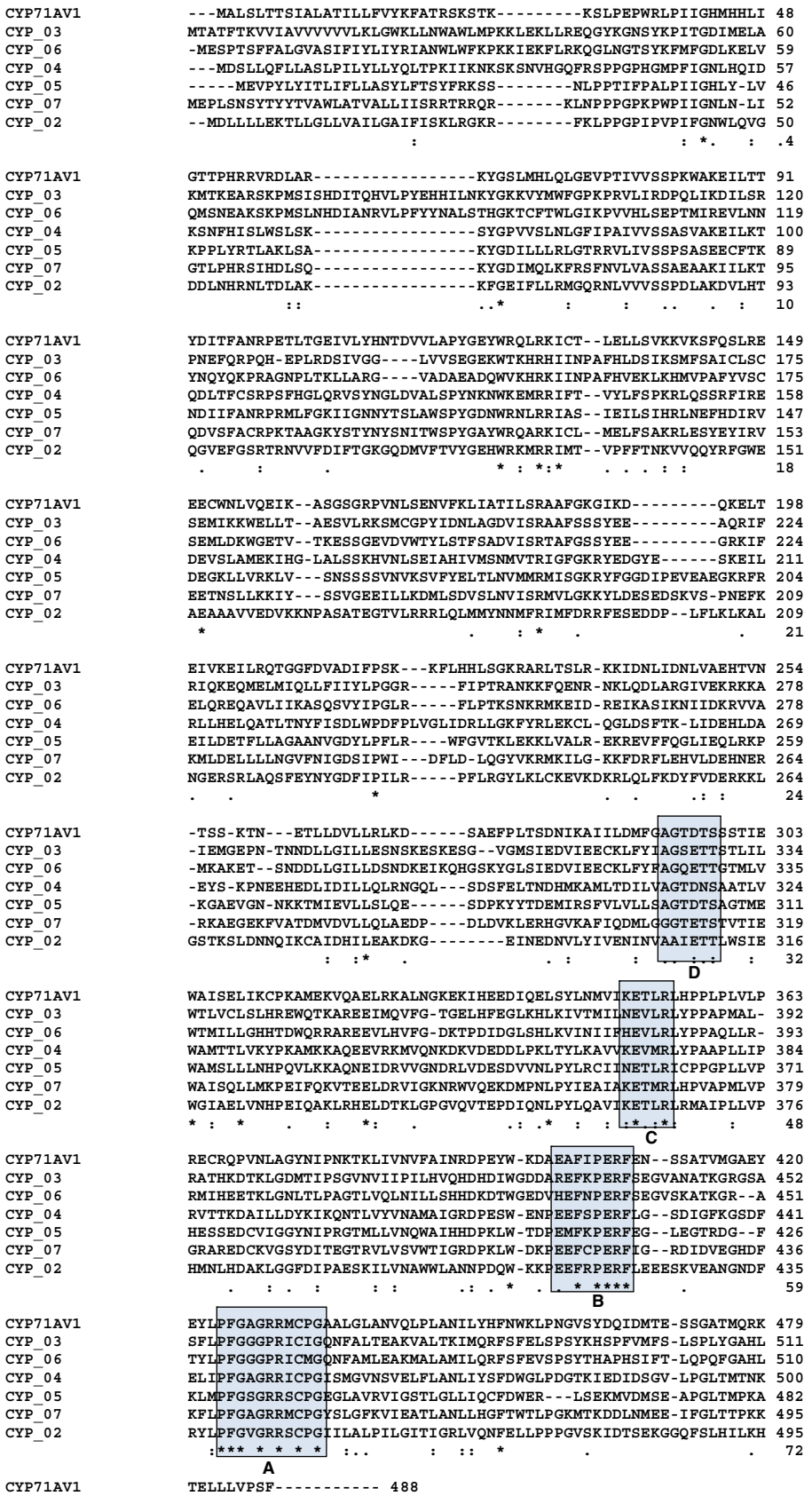


Fig. 2. Unrooted neighbor-joining tree comparing the amino acid sequences of partial *CYP*s isolated from *A. annua* (homology based family name is within the brackets).


CYP71AV1 ---MALSLTTSIALATILLFVYKFATRSKSTK---------KSLPEPWRLPIIGHMHHLI 48
CYP_03 MTATFTKVVIAVVVVVVLKLGWKLLNWAWLMPKKLEKLLREQGYKGNSYKPITGDIMELA 60
CYP_06 -MESPTSFFALGVASIFIYLIYRIANWLWFKPKKIEKFLRKQGLNGTSYKFMFGDLKELV 59
CYP_04 ---MDSLLQFLLASLPILYLLYQLTPKIIKNKSKSNVHGQFRSPPGPHGMPFIGNLHQID 57
CYP_05 -----MEVPYLYITLIFLLASYLFTSYFRKSS--------NLPPTIFPALPIIGHLY-LV 46
CYP_07 MEPLSNSYTYYTVAWLATVALLIISRRTRRQR-------KLNPPPGPKPWPIIGNLN-LI 52
CYP_02 --MDLLLLEKTLLGLLVAILGAIFISKLRGKR--------FKLPPGPIPVPIFGNWLQVG 50
CYP71AV1 GTTPHRRVRDLAR----------------KYGSLMHLQLGEVPTIVVSSPKWAKEILTT 91
CYP_03 KMTKEARSKPMSISHDITQHVLPYEHHILNKYGKKVYMWFGPKPRVLIRDPQLIKDILSR 120
CYP_06 QMSNEAKSKPMSLNHDIANRVLPFYYNALSTHGKTCFTWLGIKPVVHLSEPTMIREVLNN 119
CYP_04 KSNFHISLWSLSK----------------SYGPVVSLNLGFIPAIVVSSASVAKEILKT 100
CYP_05 KPPLYRTLAKLSA----------------KYGDILLLRLGTRRVLIVSSPSASEECFTK 89
CYP_07 GTLPHRSIHDLSQ----------------KYGDIMQLKFRSFNVLVASSAEAAKIILKT 95
CYP_02 DDLNHRNLTDLAK----------------KFGEIFLLRMGQRNLVVVSSPDLAKDVLHT 93
CYP71AV1 YDITFANRPETLTGEIVLYHNTDVVLAPYGEYWRQLRKICT--LELLSVKKVKSFQSLRE 149
CYP_03 PNEFQRPQH-EPLRDSIVGG----LVVSEGEKWTKHRHIINPAFHLDSIKSMFSAICLSC 175
CYP_06 YNQYQKPRAGNPLTKLLARG----VADAEADQWVKHRKIINPAFHVEKLKHMVPAFYVSC 175
CYP_04 QDLTFCSRPSFHGLQRVSYNGLDVALSPYNKNWKEMRRIFT--VYLFSPKRLQSSRFIRE 158
CYP_05 NDIIFANRPRMLFGKIIGNNYTSLAWSPYGDNWRNLRRIAS--IEILSIHRLNEFHDIRV 147
CYP_07 QDVSFACRPKTAAGKYSTYNYSNITWSPYGAYWRQARKICL--MELFSAKRLESYEYIRV 153
CYP_02 QGVEFGSRTRNVVFDIFTGKGQDMVFTVYGEHWRKMRRIMT--VPFFTNKVVQQYRFGWE 151
CYP71AV1 EECWNLVQEIK--ASGSGRPVNLSENVFKLIATILSRAAFGKGIKD---------QKELT 198
CYP_03 SEMIKKWELLT--AESVLRKSMCGPYIDNLAGDVISRAAFSSSYEE---------AQRIF 224
CYP_06 SEMLDKWGETV--TKESSGEVDVWTYLSTFSADVISRTAFGSSYEE---------GRKIF 224
CYP_04 DEVSLAMEKIHG-LALSSKHVNLSEIAHIVMSNMVTRIGFGKRYEDGYE------SKEIL 211
CYP_05 DEGKLLVRKLV---SNSSSSVNVKSVFYELTLNVMMRMISGKRYFGGDIPEVEAEGKRFR 204
CYP_07 EETNSLLKKIY---SSVGEEILLKDMLSDVSLNVISRMVLGKKYLDESEDSKVS-PNEFK 209
CYP_02 AEAAAVVEDVKKNPASATEGTVLRRRLQLMMYNNMFRIMFDRRFESEDDP--LFLKLKAL 209
CYP71AV1 EIVKEILRQTGGFDVADIFPSK---KFLHHLSGKRARLTSLR-KKIDNLIDNLVAEHTVN 254
CYP_03 RIQKEQMELMIQLLFIIYLPGGR-----FIPTRANKKFQENR-NKLQDLARGIVEKRKKA 278
CYP_06 ELQREQAVLIIKASQSVYIPGLR-----FLPTKSNKRMKEID-REIKASIKNIIDKRVVA 278
CYP_04 RLLHELQATLTNYFISDLWPDFPLVGLIDRLLGKFYRLEKCL-QGLDSFTK-LIDEHLDA 269
CYP_05 EILDETFLLAGAANVGDYLPFLR----WFGVTKLEKKLVALR-EKREVFFQGLIEQLRKP 259
CYP_07 KMLDELLLLNGVFNIGDSIPWI---DFLD-LQGYVKRMKILG-KKFDRFLEHVLDEHNER 264
CYP_02 NGERSRLAQSFEYNYGDFIPILR-----PFLRGYLKLCKEVKDKRLQLFKDYFVDERKKL 264
CYP71AV1 -TSS-KTN---ETLLDVLLRLKD------SAEFPLTSDNIKAIILDMFGAGTDTSSSTIE 303
CYP_03 -IEMGEPN-TNNDLLGILLESNSKESKESG--VGMSIEDVIEECKLFYIAGSETTSTLIL 334
CYP_06 -MKAKET--SNDDLLGILLDSNDKEIKQHGSKYGLSIEDVIEECKLFYFAGQETTGTMLV 335
CYP_04 -EYS-KPNEEHEDLIDILLQLRNGQL---SDSFELTNDHMKAMLTDILVAGTDNSAATLV 324
CYP_05 -KGAEVGN-NKKTMIEVLLSLQE------SDPKYYTDEMIRSFVLVLLSAGTDTSAGTME 311
CYP_07 -RKAEGEKFVATDMVDVLLQLAEDP----DLDVKLERHGVKAFIQDMLGGGTETSTVTIE 319
CYP_02 GSTKSLDNNQIKCAIDHILEAKDKG--------EINEDNVLYIVENINVAAIETTLWSIE 316
CYP71AV1 WAISELIKCPKAMEKVQAELRKALNGKEKIHEEDIQELSYLNMVIKETLRLHPPLPLVLP 363
CYP_03 WTLVCLSLHREWQTKAREEIMQVFG-TGELHFEGLKHLKIVTMIINEVLRLYPPAPMAL- 392
CYP_06 WTMILLGHHTDWQRRAREEVLHVFG-DKTPDIDGLSHLKVINIIFHEVLRLYPPAQLLR- 393
CYP_04 WAMTTLVKYPKAMKKAQEEVRKMVQNKDKVDEDDLPKLTYLKAVVKEVMRLYPAAPLLIP 384
CYP_05 WAMSLLLNHPQVLKKAQNEIDRVVGNDRLVDESDVVNLPYLRCIINETLRICPPGPLLVP 371
CYP_07 WAISQLLMKPEIFQKVTEELDRVIGKNRWVQEKDMPNLPYIEAIAKETMRLHPVAPMLVP 379
CYP_02 WGIAELVNHPEIQAKLRHELDTKLGPGVQVTEPDIQNLPYLQAVIKETLRLRMAIPLLVP 376
CYP71AV1 RECRQPVNLAGYNIPNKTKLIVNVFAINRDPEYW-KDAEAFIPERFEN--SSATVMGAEY 420
CYP_03 RATHKDTKLGDMTIPSGVNVIIPILHVQHDHDIWGDDAREFKPERFSEGVANATKGRGSA 452
CYP_06 RMIHEETKLGNLTLPAGTLVQLNILLSHHDKDTWGEDVHEFNPERFSEGVSKATKGR--A 451
CYP_04 RVTTKDAILLDYKIKQNTLVYVNAMAIGRDPESW-ENPEEFSPERFLG--SDIGFKGSDF 441
CYP_05 HESSEDCVIGGYNIPRGTMLLVNQWAIHHDPKLW-TDPEMFKPERFEG--LEGTRDG--F 426
CYP_07 GRAREDCKVGSYDITEGTRVLVSVWTIGRDPKLW-DKPEEFCPERFIG--RDIDVEGHDF 436
CYP_02 HMNLHDAKLGGFDIPAESKILVNAWWLANNPDQW-KKPEEFRPERFLEEESKVEANGNDF 435
CYP71AV1 EYLPFGAGRRMCPGAALGLANVQLPLANILYHFNWKLPNGVSYDQIDMTE-SSGATMQRK 479
CYP_03 SFLPFGGGPRICIGQNFALTEAKVALTKIMQRFSFELSPSYKHSPFVMFS-LSPLYGAHL 511
CYP_06 TYLPFGGGPRICMGQNFAMLEAKMALAMILQRFSFEVSPSYTHAPHSIFT-LQPQFGAHL 510
CYP_04 ELIPFGAGRRICPGISMGVNSVELFLANLIYSFDWGLPDGTKIEDIDSGV-LPGLTMTNK 500
CYP_05 KLMPFGSGRRSCPGEGLAVRVIGSTLGLLIQCFDWER---LSEKMVDMSE-APGLTMPKA 482
CYP_07 KFLPFGAGRRMCPGYSLGFKVIEATLANLLHGFTWTLPGKMTKDDLNMEE-IFGLTTPKK 495
CYP_02 RYLPFGVGRRSCPGIILALPILGITIGRLVQNFELLPPPGVSKIDTSEKGGQFSLHILKH 495
CYP71AV1 TELLLVPSF---------- 488
CYP_03 ILHNI---------- 516
CYP_06 ILHKV---------- 515
CYP_04 KGLCLLAHFNSNSPN----- 515
CYP_05 EPLVAKCKPRLEIQTLLSEL 502
CYP_07 FPLVTVAQPRLPVEIYHL-- 513
CYP_02 STIVAKPRSF---------- 505
A
B
C
D
72

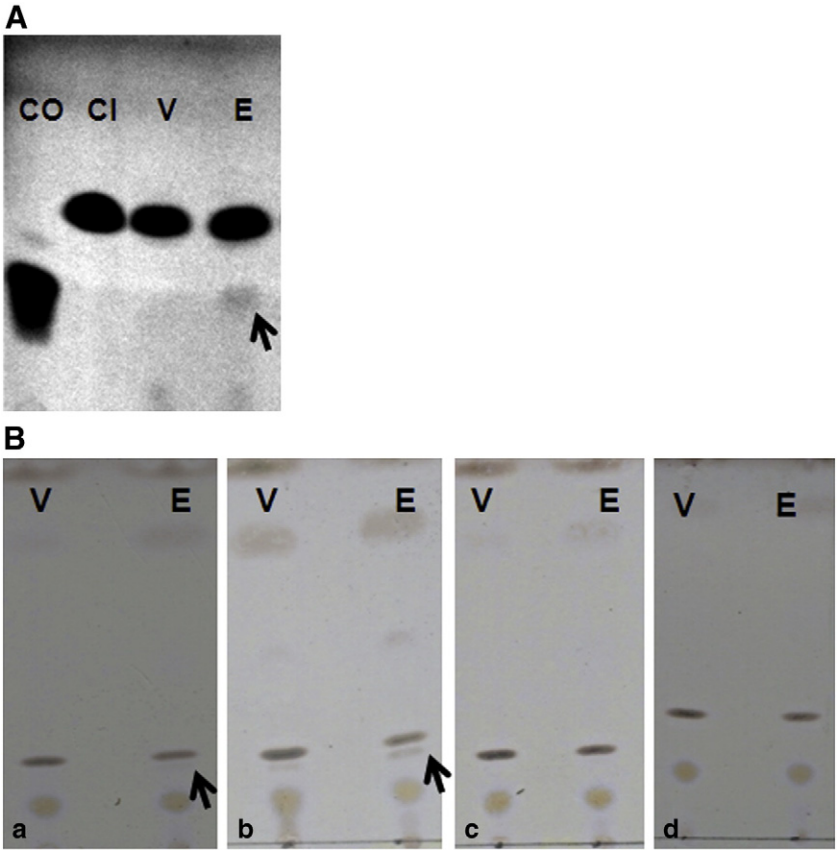


Fig. 4. Substrate conversion by CIM_CYP02(c73) and CIM_CYP05(c81). A. TLC showing conversion of *trans*-cinnamic acid to *p*-coumaric acid by microsomal protein of *S. cerevisiae* expressing CPR and CIM_CYP02(c73). CO: *p*-coumaric acid, CI: *trans*-cinnamic acid, V: control reaction of *trans*-cinnamic acid with microsomal fraction from *S. cerevisiae* transformed with vector pESC::CPR only, E: enzyme reaction of *trans*-cinnamic acid with microsomal fraction from *S. cerevisiae* transformed with the construct and pESC::CPR/CIM_CYP02(c73). Arrow indicates the formation of *p*-coumaric acid. B. TLC showing conversion of fatty acids to their hydroxylated products by microsomal protein of *S. cerevisiae* expressing CPR and CIM_CYP05 (c81). a, b, c and d represents reaction with capric acid, lauric acid, myristic acid and palmitic acid respectively. V: control reaction of fatty acid with microsomal fraction from *S. cerevisiae* transformed with vector pESC::CPR only, E: enzyme reaction of fatty acid with microsomal fraction from *S. cerevisiae* transformed with the construct and pESC::CPR/CIM_CYP05 (c81). Arrow indicates the formation of hydroxylated product of fatty acid.

A total of 20 partial *CYP*s were isolated and sequence characterized from *A. annua*. Based on their homology (Nelson et al., 1993, 1996) these partial sequences ranging between 200 and 700 bp were assigned to different families (Fig. 1) and submitted to the NCBI database with accession numbers GU318231 to GU318243. In all the partial sequences heme binding domain (PF(G/S)XG(R/P)(R/F)XC) was observed. Cysteine residue which acts as 5th ligand for heme iron along with a phenylalanine (F) at +30 after heme binding domain was also detected. The tree generated after MSA and neighbor-joining analysis grouped these partial *CYP*s in three major clusters (Fig 2). In the first cluster *CIMCYP13*, *CIMCYP10*, *CIMCYP06*, *CIMCYP03*, *CIMCYP17* and *CIMCYP18* grouped together. *CIMCYP02* also originated from this cluster as an out-group. The second cluster included *CIMCYP14*, *CIMCYP05*, *CIMCYP12*, *CIMCYP11*, *CIMCYP21* and *CIMCYP22*. In the third cluster, *CIMCYP71AV1* grouped with *CIMCYP16*, *CIMCYP07*, *CIMCYP15*, *CIMCYP04*, *CIMCYP09* and *CIMCYP20.* Of 20, seven full length genes *CIM_CYP02*, *CIM_CYP03*, *CIM_CYP04*, *CIM_CYP05*, *CIM_CYP06*, *CIM_CYP07* and *CIM_CYP71AV1* were amplified through RACE and the sequence characterized. The Genbank accession number assigned to these genes are GU318229, GU318227, JN594506, JN594505, GU318230, GU318226 and JN594504 respectively. All the conserved regions for *CYP*s were observed in the multiple sequence alignment carried out for the isolated full length *CYP*s (Fig. 3). The size of these genes ranged between 488 and 516 amino acids and predicted substrate recognition sites (SRS) were identified according to Gotoh (1992) (Supplementary Fig. 1).

3.2. Substrate utilization

Predotar v. 1.03 software (Small et al., 2004) predicted expression of all *CYP*s in endoplasmic reticulum except *CIM_CYP07*. As *CIM_CYP07* was predicted to be a member of *CYP92* family and some members of this family are reported to be expressing in the microsome (Petkova-andonova et al., 2002), the microsome fraction along with the cytosolic fraction was taken for the assay. For all other CYPs only the microsome fraction was taken for analysis to determine their role in secondary metabolism. CYP71AV1 of *A. annua* catalyzes three step oxidation of the amorpha 4, 11-diene to artemisinic acid (Ro et al., 2006). Other isolated *CYP*s [*CIM*_CYP02, CIM_*CYP03*, *CIM_CYP04*, *CIM_CYP05*, *CIM_CYP06*, and *CIM_CYP07*] of this investigation were found to be novel and hence assayed for enzyme activity. *CIM_CYP02* showed more than 90% similarity with *CYP73A* subfamily, which are predicted

Fig. 3. Multiple sequence alignment for the deduced amino acid sequences of isolated CYPs by ClustalW. Conserved domains for CYPs are shown as boxed (A: Heme binding domain, B: PERF motif, C: KETLR motif, D: Oxygen binding pocket).

Table 2
Substrate utilization by microsome fraction from *Saccharomyces cerevisiae* expressed CPR and CYPs.

		CIM_CYP02 (c73)	CIM_CYP 03 (c72a)	CIM_CYP04 (c83)	CIM_CYP05 (c81)	CIM_CYP06 (c72b)	CIM_CYP07(c92)
1	Valencene	No	No	No	No	No	No
2	β-caryophyllene	No	No	No	No	No	No
3	Ledene	No	No	No	No	No	No
4	Gurjunene	No	No	No	No	No	No
5	Farnesene	No	No	No	No	No	No
6	Epicedrol	No	No	No	No	No	No
7	Artemisinic acid	No	No	No	No	No	No
8	Arteannuin B	No	No	No	No	No	No
9	Artemisinin	No	No	No	No	No	No
10	Dihydroartemisinic acid	No	No	No	No	No	No
11	Norannuic acid	No	No	No	No	No	No
12	Cinnamic acid	**Yes**	No	No	No	No	No
13	Capric acid	No	No	No	**Yes**	No	No
14	Lauric acid	No	No	No	**Yes**	No	No
15	Myristic acid	No	No	No	No	No	No
16	Palmitic acid	No	No	No	No	No	No

to be catalyzing 4-hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid (Russell, 1971; Teutsch et al., 1993). CIM_CYP02 was also observed to be converting *trans*-cinnamic acid to *p*-coumaric acid in this analysis (Fig 4a). *CIM_CYP03* and *CIM_CYP06* showed ~50% and ~60% similarity, respectively with *CYP72A* subfamily members involved in terpene indole alkaloid biosynthesis and other stress related responses. The *CYP72* family is however quite diverse, with 155 named sequences in grape and majority of these have unknown functions. But, the first member of the family, *CYP72A1* is characterized to be secologanin synthase, active in *Catharanthus roseus* indole alkaloid biosynthesis (Irmler et al., 2000). *CIM_CYP04* showing ~50% homology with CYP83 family members is predicted to be involved in the biosynthesis of the glucosinolates and most members of these families are involved in glucosinolate biosynthesis in plants (Bak and Feyereisen, 2001). CYP83A1, CYP83B1, CYP71E1 have also been reported to be involved in oxime metabolism (Jorgensen et al., 2011; Naur et al., 2003). *CIM_CYP05* showed ~80% homology with the *CYP81B* subfamily and the function of the members of this family is yet to be worked out in detail. *Helianthus tuberosus CYP81B1* is involved in in-chain hydroxylation of C:10, C:12, and C:14 fatty acids in higher plants (Cabello-Hurtado et al., 1998). As shown in Table 2 and Fig. 4b, the conversion of capric acid and lauric acid to their hydroxylated products was observed for CIM_CYP05. Some conversion of lauric acid in microsome fraction of *S. cerevisiae* transformed with pESC::CPR may be due to the endogenous enzyme of yeast but, the conversion was higher in the case of pESC:: CPR/ CIM_CYP05(c81) transformed microsome fraction. The hydroxylation is better for lauric acid compared to capric acid. *CIM_CYP07* showed more than 70% similarity with other *CYP92* family members. CYP92 of *Actinidia chinensis* is described to be a putative flavonoid 3′ hydroxylase (Montefiori et al., 2011) and that of *Petunia* is involved in in-chain hydroxylation of fatty acids (Petkova-andonova et al., 2002). Thus, all the *CYP*s of *A. annua* were tentatively named as *CIM_CYP02(c73)*, *CIM_CYP03(c72a)*, *CIM_CYP04(c83)*, *CIM_CYP05(c81)*, *CIM_CYP06(c72b)* and *CIM_CYP07(c92)*. These were also assayed for their involvement in secondary metabolism with different sesquiterpenes, phenolics, fatty acids and sesquiterpenoids. No conversion was detected in Gas chromatography (GC) analysis with sesquiterpenes valencene, β-caryophyllene, ledene, gurjunene, farnesene and epicedrol having similar type of structure as that of amorpha 4, 11-diene. Sesquiterpenoids like artemisinic acid, dihydroartemisinic acid, norannuic acid, arteannuin B and artemisinin were also not utilized as substrate by these CYPs as analyzed through HPLC (Table 2). The cytosolic protein from yeast transformed with pESC::CPR/CIM_CYP07 also did not show any activity with the substrates used in this investigation. Though, these CYPs did not react with the sesquiterpenes used in this investigation on one hand, their involvement in other pathways involving new sesquiterpenes cannot be ruled out.

3.3. Expression analysis of the CYPs

The quantitative expression analysis of *CYP*s along with artemisinin was carried out at the seedling (20 days after sowing) and mature leaf (150 days after sowing) stages of the plant. The formation of artemisinin in the seedling was not detected by HPTLC analysis while in the mature leaf it was approximately 1%. Increased expression of *CIM_CYP71AV1* was observed in the mature leaf. Similar trend was observed for *CIM_CYP02(c73)*, *CIM_CYP03(c72a)*, *CIM_CYP04(c83)* and *CIM_CYP06(c72b)* with no significant change in *CIM_CYP07(c92)* expression. Expression of *CIM_CYP05(c81)* was more in the seedling compared to the mature leaf (Fig. 5a). When the tissue specific expression was compared in mature plants, none of the *CYP*s showed expression in the root (Fig. 5b). Higher expression of *CIM_CYP71AV1* was detected in the flower bud compared to the leaf and minor expression in the stem. Similar trend was observed for *CIM_CYP02(c73)* and *CIM_CYP04(c83)*. Higher leaf expression compared to flower bud was detected for *CIM_CYP03(c72a)* and *CIM_CYP07(c92)* with very low expression in the stem. *CIM_CYP05(c81)* expressed in low amount in the leaf and flower bud with no expression in the stem and root. High expression was obtained for *CIM_CYP07(c92)* in the flower bud, leaf and stem. The genes responsible for the synthesis of artemisinin are supposed to be upregulated as the artemisinin content of the leaf is increased. The transcript abundance studies for the artemisinin biosynthetic pathway genes was carried out by Teoh et al. (2006) in different tissues of the plant and maximum abundance was observed in the trichomes (present in the leaf, flower bud and stem) where the synthesis and storage of the genes are described (Duke et al., 1994). Since negligible amount of artemisinin is detected in the root compared to the leaf, the gene expression in the leaf with the artemisinin content was correlated by Teoh et al. (2006). Though some of the *CYP*s showed similar correlation, their involvement in artemisinin biosynthetic pathway is not conclusive because activity against the available substrates was not observed. Hence, *CYP*s showing similar expression pattern with *CIM_CYP71AV1* may be predicted to be taking part in the biosynthesis of similar kind of metabolites/or leaf/trichome development by metabolizing other compounds. Expression patterns of *CIM_CYP02(c73)* and *CIM_CYP04(c83)* are very much similar to that of *CIM_CYP71AV1* as upregulated in the floral bud and mature leaf. *CIM_CYP02(c73)* showed more than 90% similarity with *CYP73A* subfamily, members of which are described to be *trans*-cinnamic 4 hydroxylase (C4H), catalyzing 4-hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid (Russell,

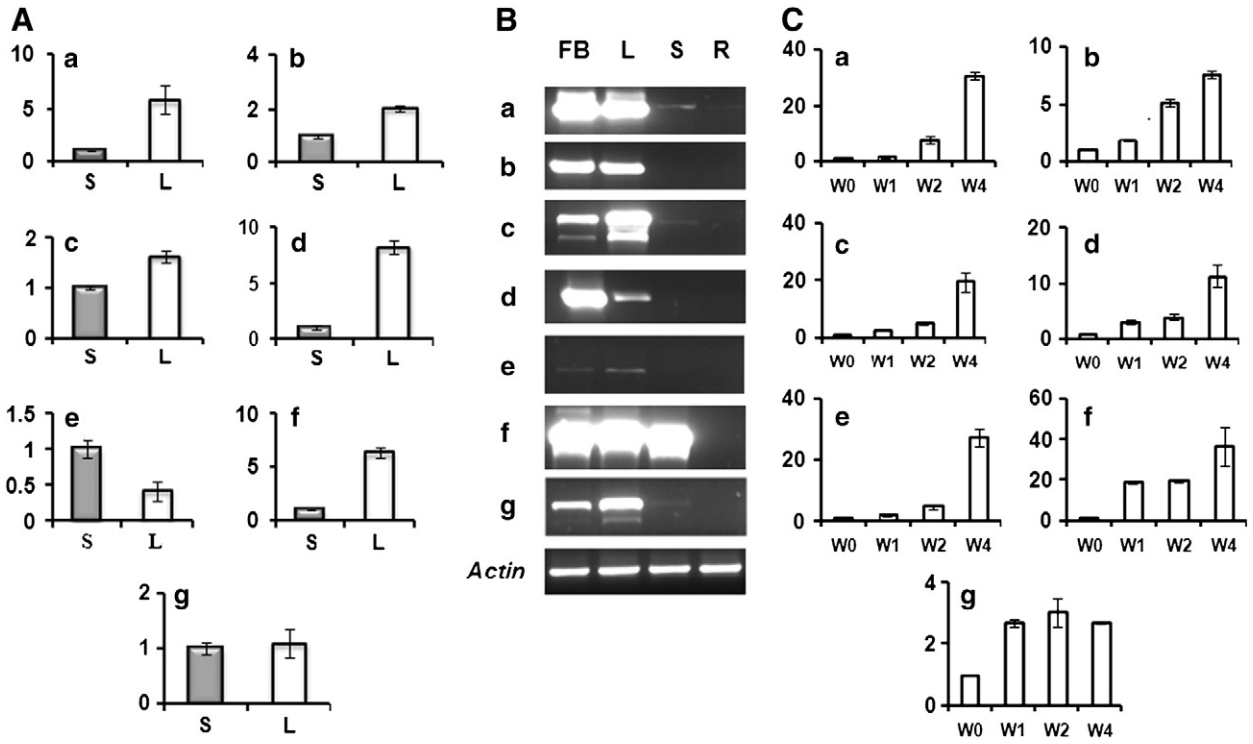


Fig. 5. Expression pattern of full length *CYP*s [a: *CIM_CYP71AV1*, b: CIM_CYP02(c73), c: CIM_CYP03(c72a), d: CIM_CYP04(c83), e: CIM_CYP05(c81), f: CIM_CYP06(c72b) and g: CIM_CYP07(c92)]. A. Comparison of quantitative expression levels at seedling and mature leaf stage of *A. annua* (S: 20 days old seedling and L: mature leaf from 150 days old plant). Y-axis represents relative quantity (RQ) equilibrating the expression in seedling as 1RQ value. Data represent mean ± standard error of 3–5 biological replicates. B. Comparison of semi-quantitative expression levels in different tissues of *A. annua* (FB: flower bud, L: leaf, S: stem, r: Root). C. Comparison of quantitative expression levels after wound induction in mature leaf of *A. annua* (W1, W2, W3, and W4 indicate 0, 1, 2 and 4 h after wound induction). Y-axis represents relative quantity (RQ) equilibrating the expression in seedling as 1RQ value. Data represent mean ± standard error of 3–5 biological replicates.

1971; Teutsch et al., 1993). Higher expression of this gene in mature leaf and flower bud, instead of seedling, stem and root indicates biosynthesis of phenyl-propanoids and benzoid compounds in the leaves. Similarly, *CIM_CYP04(c83)* may be implicated for hydroxylation of unknown compounds in the leaf trichome.

All *CYP*s expressed more after wounding and the expression was increased with the time except for *CIM_CYP07(c92)* showing no significant change after initial increase (Fig. 5c). Most of the *CYP*s involved in secondary metabolite biosynthesis are reported to be upregulated after wounding (Frank et al., 1996; Howe et al., 2000; Major and Constabel, 2006). The expression level of *CIM_CYP07(c92)* was increased slightly after wounding and remained almost constant till 4 h. Members of CYP72A subfamily were induced by wounding in plants (Imaishi and Matumoto, 2007; Mujer and Smigocki, 2001). They also catalyze the metabolism of herbicides and predicted to be having possible role in plant defense (Mujer and Smigocki, 2001). *CIM_CYP06(c72b)* and *CIM_CYP03(c72a)* belonging to *CYP72A* subfamily are also induced by wound treatment. *CYP81B1* of *Helianthus tuberosus* catalyzing the in-chain hydroxylation of fatty acids has been reported to be induced by wound and other metal ions (Cabello-Hurtado et al., 1998).

The observation of similar expression patterns for *CIM_CYP02(c73)* and *CIM_CYP71AV1* and the fact that both were isolated from trichome enriched tissue, prompted analysis of the effect of phenolics and artemisinin on the expression of both the genes. Both *CIM_CYP71AV1* and *CIM_CYP02(c73)*, were found to be overexpressed when treated with cinnamic acid (Figs. 6a, b). Higher expression of *CIM_CYP02(c73)* may be due to higher availability of substrate. Treatment with

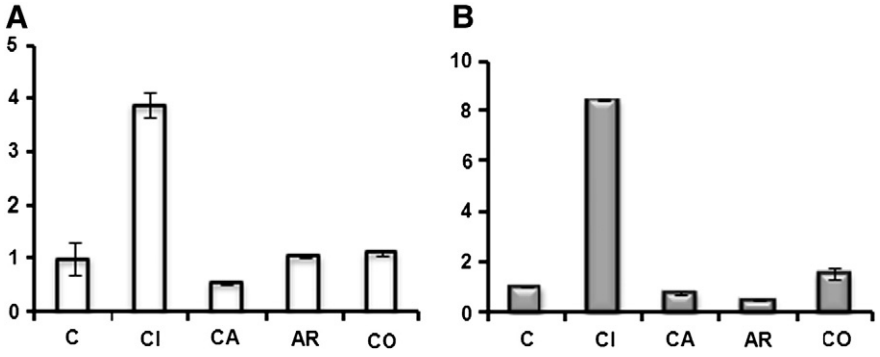


Fig. 6. Quantitative expression pattern of a. *CIM_CYP02(c73)* and b. *CIM_CYP71AV1* when twigs were sprayed with C: methanol (control), CI: *trans*-cinnamic acid, CA: caffeic acid, AR: artemisinin, CO: *p*-coumaric acid.

artemisinin led to the decrease in the expression of *CIM_CYP71AV1* and is in agreement with the finding reported by Arsenault et al. (2010). Expression of *CIM_CYP71AV1* and *CIM_CYP02(c73)* decreased in the plants treated with caffeic acid. Though *p*-coumaric acid induced the expression of *CIM_CYP71AV1* to a lesser extent compared to cinnamic acid, it had no significant effect on *CIM_CYP02(c73)* expression at the same concentration (1 mM). This is in agreement with the finding that coumarate does not inhibit the cinnamate 4-hydroxylation reaction at a concentration as high as 2 mM (Urban et al., 1994). This indicates the modulation of artemisinic acid biosynthesis by the intermediates of phenyl-propanoids and a probable relationship between these two pathways.

4. Conclusion

This study characterizes the expression pattern and function of 7 full length *CYP*s isolated from the trichome enriched tissue fraction of *A. annua* leaf. None of the CYPs utilized sesquiterpene as substrates used in this study except *CIM_CYP71AV1* which was earlier reported to be utilizing amorpha 4, 11-diene. *CIM_CYP02(c73)* was confirmed to be a cinnamate 4-hydroxylase converting *trans*-cinnamic acid to *p*-coumaric acid with flower and leaf tissue specific expression. The conversion of capric acid and lauric acid to their hydroxylated products was observed for *CIM_CYP05(c81)*. Also this hydroxylase expressed more in the seedling compared to the leaf tissue. *Trans*-cinnamic acid was found to be inducing the expression of both *CIM_CYP71AV1* and *CIM_CYP02(c73)* whereas artemisinin inhibited the expression of *CIM_CYP71AV1* indicating a relationship between the two pathways.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2012.09.015.

Acknowledgment

The authors express their sincere gratitude to the Director, CIMAP for keen interest and for providing facilities for the experiments. The work was carried out in CSIR funded EFY (NWP08) project. AM was supported by UGC-CSIR (JRF-SRF). Help of Dr. Anil K. Gupta, Curator, National Gene Bank for Medicinal and Aromatic Plants is also acknowledged. Dr. R. S. Bhakuni provided the arteannuin B, dihydroartemisinic acid and norannuic acid to analyze the conversion.

References

Altschul, S.F., et al., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 389–402.
Arsenault, P.R., Vail, D., Wobbe, K.K., Erickson, K., Weathers, P.J., 2010. Reproductive development modulates gene expression and metabolite levels with possible feedback inhibition of artemisinin in *Artemisia annua*. Plant Physiol. 154, 958–968.
Bailey, B.A., Larson, R.L., 1991. Maize microsomal benzoxazinone N-monooxygenase. Plant Physiol. 95, 792–796.
Bak, S., Feyereisen, R., 2001. The involvement of two P450 enzymes, CYP83B1 and CYP83A1, in auxin homeostasis and glucosinolate biosynthesis. Plant Physiol. 127, 108–118.
Bolwell, G.P., Bozak, K., Zimmerlin, A., 1994. Plant cytochrome P450. Phytochemistry 37, 1491–1506.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Brown, G.D., Sy, L.K., 2007. In vivo transformations of dihydro-epi-deoxyarteannuin B in *Artemisia annua* plants. Tetrahedron 63, 9536–9547.
Cabello-Hurtado, F., Batard, Y., Salaun, J.P., Durst, F., Pinot, F., Werck-Reichhart, D., 1998. Cloning, expression in yeast, and functional characterization of CYP81B1, a plant cytochrome P450 that catalyzes in-chain hydroxylation of fatty acids. J. Biol. Chem. 273, 7260–7267.
Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.
Duke, M.V., Paul, R.N., Elsohly, H.N., Sturtz, G., Duke, S.O., 1994. Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua* L. Int. J. Plant Sci. 155, 365–372.
Fischer, T.C., Klattig, J.T., Gierl, A., 2001. A general cloning strategy for divergent plant cytochrome P450 genes and its application in *Lolium rigidum* and *Ocimum basilicum*. Theor. Appl. Genet. 103, 1014–1021.
Frank, M., Deyneka, J.M., Schuler, M.A., 1996. Cloning of wound-induced cytochrome P450 monooxygenases expressed in pea. Plant Physiol. 110, 1035–1046.
Frey, M., Chomet, P., Glawischnig, E., Stettner, C., Grun, S., Winklmair, A., 1997. Analysis of a chemical plant defense mechanism in grasses. Science 277, 696–699.
Gotoh, O., 1992. Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. J. Biol. Chem. 267, 83–90.
Howe, G.A., Lee, G.I., Itoh, A., Li, L., De-Rocher, A.E., 2000. Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. Plant Physiol. 123, 711–724.
Imaishi, H., Matumoto, S., 2007. Isolation and functional characterization in yeast of CYP72A18, a rice cytochrome P450 that catalyzes (−1)-hydroxylation of the herbicide pelargonic acid. Pestic. Biochem. Physiol. 88, 71–77.
Irmler, S., et al., 2000. Indole alkaloid biosynthesis in *Catharanthus roseus*: new enzyme activities and identification of cytochrome P450 CYP72A1 as secologanin synthase. Plant J. 24, 797–804.
Jorgensen, K., et al., 2011. Biosynthesis of the cyanogenic glucosides linamarin and lotaustralin in cassava: isolation, biochemical characterization, and expression pattern of CYP71E7, the oxime-metabolizing cytochrome P450 enzyme. Plant Physiol. 155, 282–292.
Khanuja, S.P.S., Paul, S., Shasany, A.K., Gupta, A.K., Darokar, M.P., Gupta, M.M., Verma, R.K., Ram, G., Kumar, A., Lal, R.K., Bansal, R.P., Singh, A.K., Bhakuni, R.S., Tandon, S., 2008. High artemisinin yielding artemisia plant named 'CIM-arogya'. United States Patent No. US 7375260.
Liu, B., Wang, H., Du, Z., Li, G., Ye, H., 2011. Metabolic engineering of artemisinin biosynthesis in *Artemisia annua* L. Plant Cell Rep. 30, 689–694.
Major, I.T., Constabel, C.P., 2006. Molecular analysis of poplar defense against herbivory: comparison of wound- and insect elicitor-induced gene expression. New Phytol. 172, 617–635.
Melgar, J.C., Dunlop, J., Syvertsen, J.P., 2009. Tree water status and exogenous abscisic acid affect freeze tolerance in 'Valencia' trees. Proc. Fla. State Hort. Soc. 122, 78–80.
Montefiori, M., et al., 2011. Identification and characterisation of F3GT1 and F3GGT1, two glycosyltransferases responsible for anthocyanin biosynthesis in red-fleshed kiwifruit (*Actinidia chinensis*). Plant J. 65, 106–118.
Mujer, C.V., Smigocki, A.C., 2001. Cytokinin- and wound-inducible cytochrome P450 from *Nicotiana plumbaginifolia*. Physiol. Plant. 111, 172–181.
Naur, P., et al., 2003. CYP83A1 and CYP83B1, two nonredundant cytochrome P450 enzymes metabolizing oximes in the biosynthesis of glucosinolates in *Arabidopsis*. Plant Physiol. 133, 63–72.
Nelson, D.R., 2011. Progress in tracing the evolutionary paths of cytochrome P450. Biochim. Biophys. Acta 1814, 14–18.
Nelson, D.R., et al., 1993. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. DNA Cell Biol. 12, 1–51.
Nelson, D.R., et al., 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers, and nomenclature. Pharmacogenetics 6, 1–41.
Park, E., Jeknic, Z., Chen, T.H.H., 2006. Exogenous application of glycinebetaine increases chilling tolerance in tomato plants. Plant Cell Physiol. 47, 706–714.
Perradin, Y., Mottet, M.J., Lalonde, M., 1983. Influence of phenolics on in vitro growth of *Frankia* strains. Can. J. Bot. 61, 2807–2814.
Petkova-andonova, M., Imaishi, H., Ohkawa, H., 2002. CYP92B1, a cytochrome P 450, expressed in petunia flower buds, that catalyzes mono-oxidation of long chain fatty acids. Biosci. Biotechnol. Biochem. 66, 1819–1828.
Pompon, D., Louerat, B., Bronine, A., Urban, P., 1996. Yeast expression of animal and plant P450s in optimized redox environments. Methods Enzymol. 272, 51–64.
Ro, D.K., Mah, N., Ellis, B.E., Douglas, C.J., 2001. Functional characterization and subcellular localization of poplar (*Populus trichocarpa*×*Populus deltoids*) cinnamate 4-hydroxylase. Plant Physiol. 126, 317–329.
Ro, D.K., et al., 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature 440, 940–943.
Russell, D.W., 1971. The metabolism of aromatic compounds in higher plants. X. Properties of the cinnamic acid 4-hydroxylase of pea seedlings and some aspects of its metabolic and developmental control. J. Biol. Chem. 246, 3870–3878.
Schuler, M.A., Werck-Reichhart, D., 2003. Functional genomics of P450s. Annu. Rev. Plant Biol. 54, 629–667.
Small, I., Peeters, N., Legeai, F., Lurin, C., 2004. Predotar: a tool for rapidly screening proteomes for *N*-terminal targeting sequences. Proteomics 4, 1581–1590.
Teoh, K.H., Polichuk, D.R., Reed, D.W., Nowak, G., Covello, P.S., 2006. *Artemisia annua* L. (Asteraceae) trichome-specific cDNAs reveal CYP71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. FEBS Lett. 580, 1411–1416.
Teoh, K.H., Polichuk, D.R., Reed, D.W., Covello, P.S., 2009. Molecular cloning of an aldehyde dehydrogenase implicated in artemisinin biosynthesis in *Artemsia annua*. Botany 87, 635–642.
Teutsch, G.H., et al., 1993. Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. Proc. Natl. Acad. Sci. U. S. A. 90, 4102–4106.
Urban, P., et al., 1994. Characterization of recombinant plant cinnamate 4-hydroxylase produced in yeast. Eur. J. Biochem. 222, 843–850.
Vimala, R., Suriachandraselvan, M., 2009. Induced resistance in bhendi against powdery mildew by foliar application of salicylic acid. J. Biopest. 2, 111–114.
Wallaart, T.E., van Uden, W., Lubberink, H.G.M., Woerdenbag, H.J., Pras, N., Quax, W.J., 1999. Isolation and identification of dihydroartemisinic acid from *Artemisia annua* and its role in the biosynthesis of artemisinin. J. Nat. Prod. 62, 430–433.

Wallaart, T.E., Bouwmeester, H.J., Hille, J., Poppinga, L., Maijers, N.C., 2001. Amorpha-4,11-diene synthase: cloning and functional expression of a key enzyme in the biosynthetic pathway of the novel antimalarial drug artemisinin. Planta 212, 460–465.

Weathers, P.J., Wyslouzil, B.E., Wobbe, K.K., Kim, Y.J., Yigit, E., 1999. The biological response of hairy roots to O2 levels in bioreactors. In Vitro Cell. Dev. Biol. Plant 35, 286–289.

Werck-Reichhart, D., Bak, S., Paquette, S., 2002. Cytochrome P450. In: Somerville, C.R., Meyerowitz, E.M. (Eds.), The Arabidopsis Book. American Society of Plant Biologists, Rockville, MD.

Zhang, Y., et al., 2008. The molecular cloning of artemisinic aldehyde D11(13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in *Artemisia annua*. J. Biol. Chem. 283, 21501–21508.