Rana et al. BMC Biotechnology 2014, 14:89
http://www.biomedcentral.com/1472-6750/14/89

BMC Biotechnology

RESEARCH ARTICLE Open Access

Molecular characterization of two A-type P450s, *WsCYP98A* and *WsCYP76A* from *Withania somnifera* (L.) Dunal: expression analysis and withanolide accumulation in response to exogenous elicitations

Satiander Rana[1], Wajid Waheed Bhat[1], Niha Dhar[1], Shahzad A Pandith[1], Sumeer Razdan[1], Ram Vishwakarma[2] and Surrinder K Lattoo[1*]

Abstract

Background: Pharmacological investigations position withanolides as important bioactive molecules demanding their enhanced production. Therefore, one of the pivotal aims has been to gain knowledge about complete biosynthesis of withanolides in terms of enzymatic and regulatory genes of the pathway. However, the pathway remains elusive at the molecular level. P450s monooxygenases play a crucial role in secondary metabolism and predominantly help in functionalizing molecule core structures including withanolides.

Results: In an endeavor towards identification and characterization of different P450s, we here describe molecular cloning, characterization and expression analysis of two A-type P450s, *WsCYP98A* and *WsCYP76A* from *Withania somnifera*. Full length cDNAs of *WsCYP98A* and *WsCYP76A* have open reading frames of 1536 and 1545 bp encoding 511 (58.0 kDa) and 515 (58.7 kDa) amino acid residues, respectively. Entire coding sequences of *WsCYP98A* and *WsCYP76A* cDNAs were expressed in *Escherichia coli* BL21 (DE3) using pGEX4T-2 expression vector. Quantitative real-time PCR analysis indicated that both genes express widely in leaves, stalks, roots, flowers and berries with higher expression levels of *WsCYP98A* in stalks while *WsCYP76A* transcript levels were more obvious in roots. Further, transcript profiling after methyl jasmonate, salicylic acid, and gibberellic acid elicitations displayed differential transcriptional regulation of *WsCYP98A* and *WsCYP76A*. Copious transcript levels of both P450s correlated positively with the higher production of withanolides.

Conclusions: Two A-types P450 *WsCYP98A* and *WsCYP76A* were isolated, sequenced and heterologously expressed in *E. coli*. Both P450s are spatially regulated at transcript level showing differential tissue specificity. Exogenous elicitors acted as both positive and negative regulators of mRNA transcripts. Methyl jasmonate and salicylic acid resulted in copious expression of *WsCYP98A* and *WsCYP76A*. Enhanced mRNA levels also corroborated well with the increased accumulation of withanolides in response to elicitations. The empirical findings suggest that elicitors possibly incite defence or stress responses of the plant by triggering higher accumulation of withanolides.

Keywords: *Withania somnifera*, Withanolides, Cytochrome P450 monooxygenase, mRNA, Phytohormones, *E.coli*

* Correspondence: sklattoo@iiim.ac.in
[1]Plant Biotechnology Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu Tawi-180001, India
Full list of author information is available at the end of the article

Background

Cytochrome P450s form a huge superfamily of heme-containing monooxygenases present in all domains of life. These are pivotal in detoxification of xenobiotics, drug metabolism, assimilation of carbon sources and formation of secondary metabolites. Presently, there are more than 18500 P450 genes that have been identified across all the kingdoms of life [1,2]. The preponderance of P450s is more in plants per species than in animals and their content in angiosperm genomes can reach up to 2% [3,4]. Plant cellular and metabolic processes are dependent on P450s to drive the reactions such as hydroxylation, oxidative demethylation, desaturation, epoxidation, oxidative rearrangement of the carbon skeleton and oxidative C–C bond cleavage [5]. P450s are endoplasmic reticulum (ER) localised, requiring auxiliary reductases for the activation of molecular oxygen for different reactions. These reductases transfer two electrons in a single step from cofactor NAD(P)H to heme centre of P450s [6]. P450 monooxygenases constitute a highly regio and stereo-specific class of definite substrate-specific enzymes. By gene annotations approximately, 1% of the total genes in *Arabidopsis thaliana* and other model plants are found to be cytochrome P450s. *Arabidopsis* genome contains 244 genes and 28 pseudo-genes representing cytochrome P450 [3]. Involvement of P450s in diverse physiological processes *in vivo,* leads to biosynthesis and catabolism of huge array of complex metabolites like lignin, pigments, defence compounds, fatty acids, hormones and signaling molecules [7-9].

Owing to their regio and stereo-specific catalysing versatility, these are potential targets for industrial biocatalysis. P450s have been applied in industry for the investigation of new drugs, medicine or xenobiotics [10-12]. Remarkable variety of chemical reactions catalysed and enormous number of substrates attacked, have earned P450s the reputation of "the most versatile biological catalysts in nature" [13-15]. Thus, emphasizing identification and characterization of P450s for elucidation of various biosynthetic pathways. Plant P450s are presumed to be polyphyletic in their origin and generally classified into two main clades, A-type and non-A-type. A-type P450s are involved in 'plant specific metabolism' and are responsible for the biosynthesis of diverse natural products [7].

Withania somnifera is a medicinal plant of immense repute known to synthesize a suite of low molecular weight natural products such as flavonoids, alkaloids, terpenoids, tannins, resins and sterols through secondary metabolism [16-18]. These metabolites are derived from distinct metabolic pathways. The main constituents reported for significant pharmacological activities are withanolides, a group of naturally occurring triterpenoids. Withanolides are synthesized *via* both mevalonate (MVA) and non-mevalonate (MEP/DOXP) pathways [19]. Recently, we have elucidated the regulatory role of three branch point oxidosqualene cyclases (β-amyrin synthase, lupeol synthase and cycloartenol synthase) which showed that repression of β-amyrin synthase and lupeol synthase led to diversion of common precursor flux towards cycloartenol synthase resulting in enhanced accumulation of withanolides [20]. We have also characterized two paralogs of cytochrome P450 reductase (*WsCPR1* and *WsCPR2*) wherein *WsCPR2* expression was inducible in response to exogenous elicitors [21]. Two more genes namely squalene synthase and squalene epoxidase were also characterized from same plant species [22]. The regulation pattern of squalene synthase, squalene epoxidase, cycloartenol synthase and *WsCPR1* and *WsCPR2* in leaves and roots at various developmental stages in relation to dynamics of withanolides accumulation, indicated towards their tissue-specific biosynthesis [22]. In our earlier findings, we have reported that exogenous application of methyl jasmonate (MeJA) and salicylic acid (SA) increases withanolide content due to the presence of stress regulatory elements within the upstream promoter regions of their genes [20,23].

We, in present study report isolation, heterologous expression and transcript profiling of two A-type P450 monooxygenases viz. *WsCYP98A* (GenBank: HM585369) and *WsCYP76A* (GenBank: KC008573). These are supposed to be involved mainly in the shikmate and phenylpropanoid pathway biosynthesizing the various defence molecules during biotic and abiotic stress. CYP98A (p-coumarate 3-hydroxylase) catalyzes the meta-hydroxylation of *p*-coumarate derivatives for the biosynthesis of lignins and chlorogenic acid, which constitutes a crucial bottleneck in phenylpropanoid pathway [24]. The members of CYP76 gene family characterized from many plant species are known to catalyse various oxidative reactions. For instance, *Catharanthus roseus* CYP76A26 converts both iridodial and iridotrial into 7-deoxyloganetic acid. Whereas, *C. roseus* CYP76B6 and CYP76C1 from *A. thaliana* execute hydroxylation of geraniol [25,26].

In present investigation, full length genes of *WsCYP98A* and *WsCYP76A* were cloned and heterologously expressed in *Escherichia coli. WsCYP98A* shares maximum homology with reported *CYP98A* of *Capsicum annum* (95%) and *Solanum tuberosum* (94%) whereas, *WsCYP76A* was similar to *CYP76A2* of *S. tuberosum* (83%) and *S. melongena* (83%). The phylogenetic analysis demonstrated that both genes belong to their respective families and are close homologs to other *CYP98A* and *CYP76A* genes of Solanaceae family. The prediction of three dimensional structures, ligand binding site and conserved amino acid residues of both proteins were done using homology modelling analysis. The transcript profiling of *WsCYP98A* and *WsCYP76A* in different tissues of *W. somnifera* was performed using quantitative real time PCR (qRT-PCR). Expression profiles in response to exogenous elicitors

namely MeJA, SA and gibberellic acid (GA_3) were also studied for both the P450s. Empirical findings reveal their possible role in defence mechanism *via* enhanced accumulation of withanolides. Purview of literature suggests that this is the only report of type-A *WsCYP98A* and *WsCYP76A* from *W. Somnifera*.

Methods

Plant material

A WS-3 rich genetic stock of *W. somnifera* designated as WS-Y-08 grown at IIIM experimental farm (CSIR-Indian Institute of Integrative Medicine, Jammu, India, 32°44′N longitude, 74°55′E latitude; 305 m in altitude) was used as a source material. *In vitro* plants were raised through induction of axillary buds. These cultures were used for MeJA, SA and GA_3 treatments. Samples were collected, frozen immediately in liquid nitrogen, and stored at −80°C.

RNA isolation and cDNA synthesis

Total RNA was extracted using Trizol reagent (Sigma, St. Louis, USA) as per manufacturer's protocol. Concentration of isolated RNAs was estimated by measuring the absorbance at 260 nm in a spectrophotometer (AstraAuriga, Cambridge, UK). Further, quality of RNA was assessed by determining the ratio of absorbance at 260 and 280 nm ($A_{260/280}$) and formaldehyde-denatured agarose gel electrophoresis. Total RNA (5 μg) was incubated with RNase free DNase (Fermentas, Burlington, Canada) at 37°C for 30 min. First strand cDNA synthesized using the RevertAid cDNA synthesis kit (Fermentas, Burlington, Canada) in a total volume of 20 μl containing 3 μg total RNA, 10 mM dNTPs, 10 μM oligo (dT) primer, 1 μl M-MuLV reverse transcriptase (200 U/μl) and 1X first strand buffer (250 mM Tris–HCl, pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT). The reaction was incubated for 60 min at 42°C followed by 5 min at 70°C to inactivate the reverse transcriptase.

Amplification of *WsCYP98A* and *WsCYP76A*

Degenerate primers (Table 1) based on the conserved regions of P450s were designed by multiple sequence alignment of different P450 monooxygenases sequences retrieved from the GenBank database at National Center for Biotechnology Information (NCBI). Optimization of polymerase chain reaction conditions allowed amplification of cDNA fragments of *WsCYP98A* and *WsCYP76A* under following cycling conditions: one cycle of 94°C for 3 min, 35 cycles of 94°C for 30 s, 55–60°C for 30 sec and 72°C for 1 min followed by a final extension of 72°C for 10 min in a thermal cycler (Bio-Rad Laboratories, Hercules, USA). Amplicons examined by agarose gel electrophoresis were cloned into pTZ57R/T vector (Fermentas, Burlington, Canada), and then transformed into *E. coli* DH5α host strain. Cloned amplicons were sequenced using an automated DNA sequencer (ABI Prism 3130XL; Applied Biosystems, Foster City, USA). The nucleotide sequences obtained were analysed using the similarity search BLAST program and subsequently used for designing gene specific primers (GSPs).

5′ and 3′ RACE PCR

Remaining 5 & 3 cDNA ends of putative *WsCYP98A* and *WsCYP76A* were obtained using RLM-RACE kit according to the product manual (Ambion, Austin, TX, USA). cDNAs obtained were subjected to nested PCR using GSPs as listed in Table 1. Two rounds of PCR were performed, first round with 5′ RACE-OUT corresponding to the 5′ RACE adapter sequence and 5′ CYP98-OUT and 5′ CYP76-OUT as specific outer primers, followed by a second round of PCR with 5′ RACE-IN as inner adapter specific primer and 5′ CYP98-IN and 5′ CYP76-IN as inner GSPs. In both rounds, PCR reactions of 50 μl containing 1.0 μl cDNA as template (except for second round where amplified products of 1st round were used as template), 2 μl of 10 μM 5′ CYP98-OUT and 5′ CYPP76-OUT , 2 μl of 5′ RACE-OUT, 45.0 μl master Mix (34.5 μl PCR-grade water, 10 mM Tris HCl; pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM dNTPs, 2.5 U Taq DNA polymerase) were subjected to following cycling conditions: One cycle of 94°C for 3 min and 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min with a final step at 72°C of 10 min. Similarly for 3′ RACE, first strand cDNA was synthesized from total RNA using supplied 3′ RACE adapter primer. cDNA was subjected to nested PCR using outer and inner primers specific to 3′ RACE adapter along with 3′ GSPs with same reaction volume and cycling conditions as described for 5′ RACE. The nested amplification products of both 5′ RACE PCR and 3′ RACE PCR were purified and cloned into pTZ57R/T cloning vector (Fermentas, Burlington, Canada). The ligation mixtures were transformed into *E. coli* cells (New England Biolabs, Herts, UK). The clones were picked individually and amplified in 10 mL of Luria-Bertani (LB) medium at 37°C overnight. The plasmid DNA from each clone was extracted using a DNA plasmid Miniprep Kit (Promega, Madison, USA) and sequenced using M13 primers.

Full-length cloning of *WsCYP98A* and *WsCYP76A*

By comparing and aligning the sequences of core fragments, 5′ RACE and 3′ RACE products, the full-length cDNAs of *WsCYP98A* and *WsCYP76A* were generated and subsequently amplified with primers FullCYP98F, FullCYP98R and FullCYP76F, FullCYP76R (Table 1). A high fidelity proof-reading DNA polymerase (New England Biolabs, Herts, UK) was employed for amplification under PCR conditions; One cycle of 94°C for 3 min , 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min. The final extension was at 72°C for 10 min. PCR products were

Table 1 List of primers used in the study

Primers	Sequence (5′ – 3′)	Direction
Degenerate		
CYP450-1 F.	CCN(A/C/G/T)TAY(C/T)GGH(A/C/T)GD(G/A/T)TACTGGAGR(A/G)CAG	Forward
CYP450-1R.	AGTCD(G/A/T)GD(G/A/T)CAW(A/T)D(G/A/T)GCCCATTCD(G/A/T)AG	Reverse
CYP450-2 F.	ATATGGGCY(C/T)GATTATGGV(G/A/C)CCTCA	Forward
CYP450-2R.	AGCATH(A/C/T)AGH(A/C/T)GGAGTTGGAGGY(C/T)TG	Reverse
5′ and 3′ RACE		
5′ Adapter*	GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA	
5′ RACE-OUT*	GCTGATGGCGATGAATGAACACTG	Forward
5′ RACE-IN*	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG	Forward
5′ CYP98-OUT	TGTTATCAGAATTATTGCAATCTCTGT	Reverse
5′ CYP98-IN	AGCCGTAACCTCATCTTCACGAATGG	Reverse
5′ CYP76-OUT	CGTCTCTGTACCAGCTAAAAACATTTCCAC	Reverse
5′ CYP76-IN	GATCTCATGTTCTGATAACTTGGCTGGTT	Reverse
3′ Adapter*	GCGAGCACAGAATTAATACGACTCACTATAGGT$_{12}$V(G/A/C)N(A/C/T/G)	
3′ RACE-OUT*	GCGAGCACAGAATTAATACGACT	Reverse
3′ RACE- IN*	CGCGGATCCGAATTAATACGACTCACTATAGG	Reverse
3′ CYP98-OUT	TGATCGGCTATGAGCGCGTGATGAA	Forward
3′ CYP98-IN	AAGCGCTAAGGTTGCAGCCTCCAA	Forward
3′ CYP76-OUT	AGAGATCCTGAATGTTGGGATGACCCTAT	Forward
3′ CYP76-IN	AGAGATCCTGAATGTTGGGATGACCCTAT	Forward
Full length		
FullCYP98F	ATGGCAATTCCCTTAGCTGCTGCAATCCCTC	Forward
FullCYP98R	TTATATGTCCACTGCAATTCGTTTATACAACTCAGC	Reverse
FullCYP76F	ATGGAATGGGAATGGAGCTATTTGT	Forward
FullCYP76R	CTAGATAAGATTGATGAGTGTCTCA	Reverse
Expression (*E. coli*)		
CYP98*BamHI*F	CG**GGATCC**ATGGCAATTCCCTTAGCTGCTGCAA	Forward
CYP98*SalI*R	GCTC**GTCGAC**TTATATGTCCACTGCAATTCGT	Reverse
CYP76*BamHI*F	CG**GGATCC**ATGGAATGGGAATGGAGCTATTTGT	Forward
CYP76*SalI*R	GCTC**GTCGAC**CTAGATAAGATTGATGAGTGTCTCA	Reverse
Real-Time analysis		
RTCYP98F	GTGATGGATGAACAAGGAAACGA	Forward
RTCYP98R	CCGTGCTTAGAAAATGCATCCTC	Reverse
RTCYP76 F.P	TAGAATGGGCACTAGCAGAGCTTTTGCG	Forward
RTCYP76 R.P	CTTGCATATAAGGGAGATTGTCGATGTCGTT	Reverse
ActinF	GAGAGTTTTGATGTCCCTGCCATG	Forward
ActinR	CAACGTCGCATTTCATGATGGAGT	Reverse

*Primers marked with star were provided with the kits. Added restriction sites for cloning into pGEX4T-2 vector are bold.

analysed on 1.2% agarose gels and visualized under UV light. The resulted amplified products were ligated in pJET vector and transformed into *E.coli* DH5α.

In silico analysis

BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to find similarity of amplified P450s in GenBank database. Translate tool (http://www.expasy.ch/tools/dna.html) was used to predict the open reading frame (ORF). The properties of deduced amino acid sequences of *WsCYP98A* and *WsCYP76A* were estimated by using ProtParam (http://www.expasy.ch/tools/protparam.html), SPLIT v.4.0 (http://split.pmfst.hr/split/4/) and TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) programs.

Protein sequences were retrieved from the GenBank through BLASTp algorithm at the National Center for Biotechnology Information (NCBI) using *WsCYP98A* and *WsCYP76A* sequences as query and several homologous sequences of different plant species were selected. Multiple sequence alignment was performed using ClustalX program with default parameters [27]. For phylogenetic analysis, sequences were aligned employing ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and phylogenetic tree was constructed by Neighbor-joining method using MEGA 6 software [28]. Bootstrap analysis with 100 replicates was also conducted in order to obtain confidence levels for the branches. Protein secondary structures were determined by SOPMA program [29].

Prediction of three-dimensional structures of *WsCYP98A* and *WsCYP76A*

Three-dimensional structures of *WsCYP98A* and *WsCYP76A* were predicted using Phyre^2 server (Protein Homology/analogY Recognition Engine V 2.0) [30] using crystal structure of cytochrome P450 1a2 (PDB: 2hi4) as a template. Ligand binding sites were predicted using 3DLigandSite. Ramachandran plot analysis of both *WsCYP98A* and *WsCYP76A* was performed online using RAMPAGE web server [31]. Structurally, evolutionary and functionally important regions were identified in deduced protein sequences by ConSurf [32].

Recombinant protein expression in *E. coli*

Full length coding sequences of *WsCYP98A* and *WsCYP76A* genes were modified by adding restriction sites (Table 1). Full length primers were modified by adding *BamH*I/*Sal*I restriction sites for both genes for directional cloning in pGEX4T-2 vector and heterologous expression in *E. coli*. ORFs containing modified restriction sites were excised from pJET cloning vector and ligated into pre-digested, purified pGEX4T-2. Further, ligated mixtures containing pGEX-*WsCYP98A* and pGEX-*WsCYP76A* expression cassettes were transformed into *E. coli* BL21 (DE3). Individual positive colony harbouring transformed expression cassettes were inoculated separately into 100 ml of LB containing 100 μg/ml of ampicillin and incubated overnight at 37°C. 1% culture was transferred into 100 ml of LB media containing the corresponding antibiotic and incubated at 37°C, until optical density ($A_{600\ nm}$) reached 0.4-0.5. Protein expression was induced by adding Iso-propyl β-D-1-thiogalactopyranoside (IPTG; Fermentas, Berligton, Canada) into the cultures at the concentration of 0.2 mM to 1 mM. The cultures were constantly incubated at 25°C for 8 h. The induced bacterial cells were harvested at an interval of 2 h by centrifugation and resuspended in 6X sodium dodecyl sulphate–polyacrylamide gel electrophoresis sample buffer (SDS-PAGE; 0.375 M Tris pH 6.8, 12% SDS, 60% glycerol, 0.6 M DTT, 0.06% bromophenol blue). The expression of target proteins was analysed on 10% SDS-PAGE.

Tissue-specific gene expression analysis using qRT-PCR

To study tissue-specific expression, total RNA was extracted from leaves, stalks, roots, flowers and berries respectively. Total RNA (5 μg) was incubated with RNase free DNase (Fermentas, Burlington, Canada) at 37°C for 30 min. cDNA was synthesized from 3 μg of RNA using RevertAid cDNA synthesis kit according to manufacturer's instruction. qRT-PCR amplification was performed using the Step One Real-time PCR System (Applied Biosystems, Foster City, USA). Briefly, the standard 20 μl of reaction included 0.2 μL cDNA template, 200 nM of each primers, and 10 μL SYBR Premix Ex (Takara, Otsu, Japan) under following cycling conditions: one cycle of 94°C for 1 min, 40 cycle of 94°C for 10 s, 60°C for 15 s and 72°C for 15 s. Two primers, Actin F and Actin R were used to amplify housekeeping actin gene as control. All samples were analysed in triplicate and the specificity of each primer pair was validated by a dissociation curve (a single peak was observed for each primer pair). The real-time PCR amplification data were exported into Microsoft Excel and gene expression levels were calculated based on the comparative C_t method [33].

Effect of elicitors on the expression of *WSCYP98A* and *WsCYP76A*

For elicitor treatment the micro-propagated plantlets were pre-cultured in Murashige and Skoog (MS) liquid medium supplemented with 3% sucrose, inositol (100 mg l^{-1}) and incubated at 25 ± 1°C under 16 h photoperiod with light intensity of 30 μmol m^{-2} s^{-1} provided by cool, white fluorescent tubes of 40 W (Philips, Calcutta, India) for 2 weeks. Relative humidity was maintained at 50-60%. The plantlets growing in liquid MS medium were supplemented with MeJA (0.1 mM), SA (0.1 mM), and GA_3 (0.1 mM) dissolved in ethanol and untreated were kept as control with same amount of ethanol. The tissue from each treated sample was harvested after 6, 12, 24 and 48 h for RNA isolation. cDNA was obtained from each treated sample including control, using the same RNA isolation and cDNA synthesis protocols as described above. Effect of elicitor treatment on expression profile was studied using qRT- PCR as described above.

Withanolide analysis using HPLC

Withanolides were extracted and quantified as described by Dhar *et al.* [34]. Concisely, samples of *W. somnifera* were powdered and extracted with ethanol-water (50:50; *v/v*) with magnetic stirring at room temperature (25 ± 2°C). Extracts were filtered and the solvent was removed under vacuum. The extracts (20 mg/ml) obtained from each sample of the plant material were prepared in HPLC-

grade methanol–water (50:50; *v/v*) for quantitative analysis. 1.2 mg per 2 ml of the standards of withanolide A (WS-1), withanone (WS-2) and withaferine A (WS-3) were prepared in HPLC-grade methanol. HPLC analysis was performed with Shimadzu HPLC system (Shimadzu, Tokyo, Japan) equipped with 515 quaternary gradient pump, 717 Rheodyne injector, 2996 PDA detector and CLASS-VP software v 6.14. All samples were filtered through 0.45 μM filters (Millipore, Bedford, USA). Extracts of *W. somnifera* samples were separated on a RP-18e (4.6 × 100 mm, 5 μm) (Merck, Bangalore, India) column. The mobile phase consisted of methanol–water (60:40; *v/v*) delivered at a flow rate of 0.5 ml/min. The samples were analyzed at 30°C to provide efficiency to the peaks. The UV chromatograms were recorded at 237 nm.

Results and discussion

Molecular cloning of *WsCYP98A* and *WsCYP76A*

P450 superfamily contain genes of diverse functions involved both in primary as well as secondary metabolism. These are heme-thiolate proteins containing iron atom coordinated to a proximal cysteine and receive electrons from NAD(P)H *via* a FAD-domain of auxiliary reductases. In the present investigation, two monooxygenases from *W. somnifera, WsCYP98A* and *WsCYP76A* have been isolated and expressed in *E. coli* BL21 (DE3). Quantitative assessment of gene expression pattern in different plant organs and relative transcript abundance in response to exogenous elicitors was also evaluated and corroborated with withanolide production to extrapolate and infer their possible role during biotic and abiotic stresses.

Full length cDNAs of *WsCYP98A* and *WsCYP76A* genes were obtained from the leaf tissue of WS-3 rich chemo-variant by degenerate PCR and RACE methods (*WsCYP98A*: HM585369 and *WsCYP76A*: KC008573). Degenerate primers based on conserved regions were designed by employing multiple sequence alignment strategy using ClustalW. A 550 bp and 520 bp core fragments were obtained from initial RT-PCR reactions. The amplicons were confirmed as members of CYP98 and CYP76 families by sequencing and similarity searches using BLASTn. Further, extension of both amplified core fragments toward 5′ and 3′ ends by using RACE-PCR succeeded in amplification of remaining cDNA portions. Using GSPs designed from start codons and stop codons, 1536 bp and 1548 bp open reading frames (ORFs) were amplified from the pool of leaf cDNA designated as *WsCYP98A* and *WsCYP76A* and theses encoded 511 and 515 amino acid residues respectively (Figure 1A and B). The first methionine as per First-AUG rule was considered as initiator codon. *WsCYP98A* contains downstream untranslated region (UTR; 230 bp) at 3′ ends whereas *WsCYP76A* comprises of 3′-UTR of 180 bp. Similarity search showed *Ws*CYP98A shares 65-95% homology with protein sequences of many other species while *Ws*CYP76A shares 39-89% homology.

In silico characterization of deduced *Ws*CYP98A and *Ws*CYP76A

*Ws*CYP98A and *Ws*CYP76A have predicted isoelectric points 8.13 and 7.99 and molecular masses of 58.0 kDa and 58.7 kDa respectively. Multiple sequence alignment with P450s of taxonomically diverse species showed presence of cysteine heme ligand signature which is characteristic signature of P450 sequences This motif was detected at positions 434–443 aa (FGAGRRVCPG) and 444–453 aa (FGAGRRMCVG) for *Ws*CYP98A and *Ws*CYP76A respectively (Figure 2A and B). Like all eukaryotic monooxygenases, a string of amino acid residues which helps in anchoring to ER membrane was detected at the N-terminal end of each monooxygenase. This membrane anchor region is also essential for normal interaction between P450s and their redox partners. Their presence was also predicted by TMHMM and SPLIT 4.0 bioinformatics programs (Additional file 1: Figure S1). To ascertain the degree of evolutionary relatedness, Neighbor-joining phylogenetic tree was constructed with MEGA 6.0 software from the ClustalW alignment of *Ws*CYP98A and *Ws*CYP76A with a number of homologous P450 sequences of different plants retrieved from the NCBI GenBank database. *Ws*CYP98A and *Ws*CYP76A corresponded to two separate phylogenetic clans in accordance with the amino acid similarity among their proteins (Figure 3). Prediction of secondary structure of *Ws*CYP98A and *Ws*CYP76A proteins by SOPMA program revealed that they consist of α-helixes (52.84%; 50.68%), β-turns (6.07%; 4.47%) joined by extended strands (8.61%; 10.49%), and random coils (32.49%; 34.37%). Three dimensional structural models for *Ws*CYP98A and *Ws*CYP76A were generated using Phyre^2 with >90% accuracy on the basis of homology based modelling using cytochrome P450 1a2 (PDB: 2HI4) as template (Figure 4A and C). The amino acid residues involved in ligand binding were also predicted using the 3DLigandSite tool as depicted in Figure 4B and D. Analysis of the evolutionary conservation of *Ws*CYP98A and *Ws*CYP76A amino acids were performed using ConSurf program. Several residues with high scores were found to be functional and structural residues of the proteins by ConSeq servers (Additional file 2: Figure S2). Ramachandran plot analysis of *Ws*CYP98A showed 461 aa (90.6%) residues in the most favourable region, 32 aa (6.3%) residues in the additional allowed region and 16 (3.1%) in the outlier region. Similarly, in case of *Ws*CYP76A, 480 aa (93.6%) residues were in the most favourable region, 20 aa (3.9%) residues in the additional allowed region and 13 (2.5%) in the outlier region (Additional file 3: Figure S3).

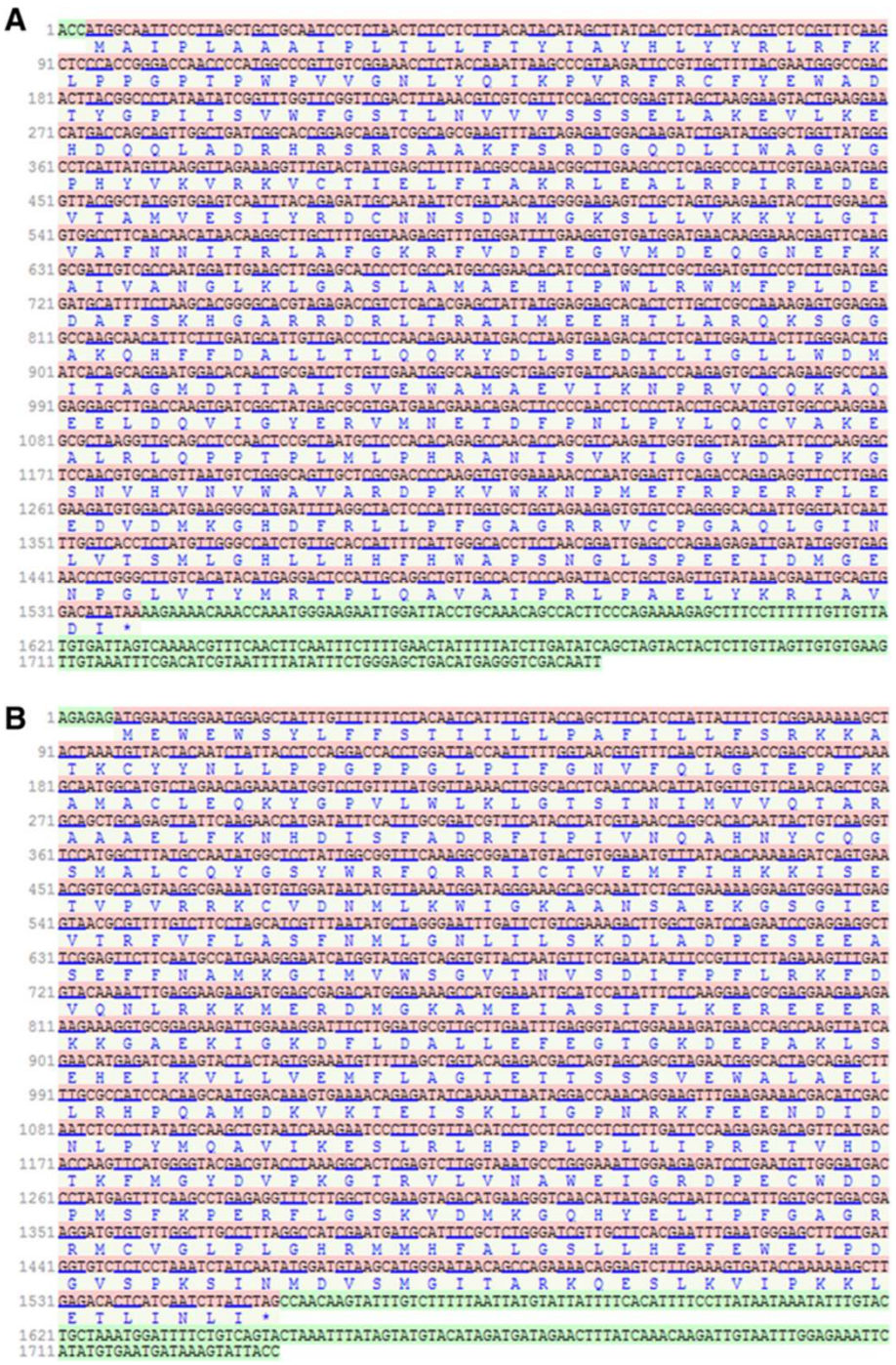

Figure 1 Nucleotide and the deduced amino acid sequence of *WsCYP98A* (A) and *WsCYP76A* (B) from *Withania somnifera*. The start codon (ATG) present at 4th and 7th positions whereas stop codons at 1552 and 1537 bp, respectively.

Heterologous expression in *E. coli*

For heterologous expression, entire coding sequences of *WsCYP98A* and *WsCYP76A* cDNAs were expressed in *E. coli* using pGEX 4 T-2 expression vector system. The ORFs were released from pJET-*WsCYP98A* and pJET-*WsCYP76A* using *BamH*I/*Sal*I restriction enzymes, and inserted into vector pGEX4T-2. The recombinant expression vectors with the inserted *WsCYP98A* and *WsCYP76A* constructs were identified by PCR analysis and restriction digestion using *BamH*I/*Sal*I. Heterologous expression of

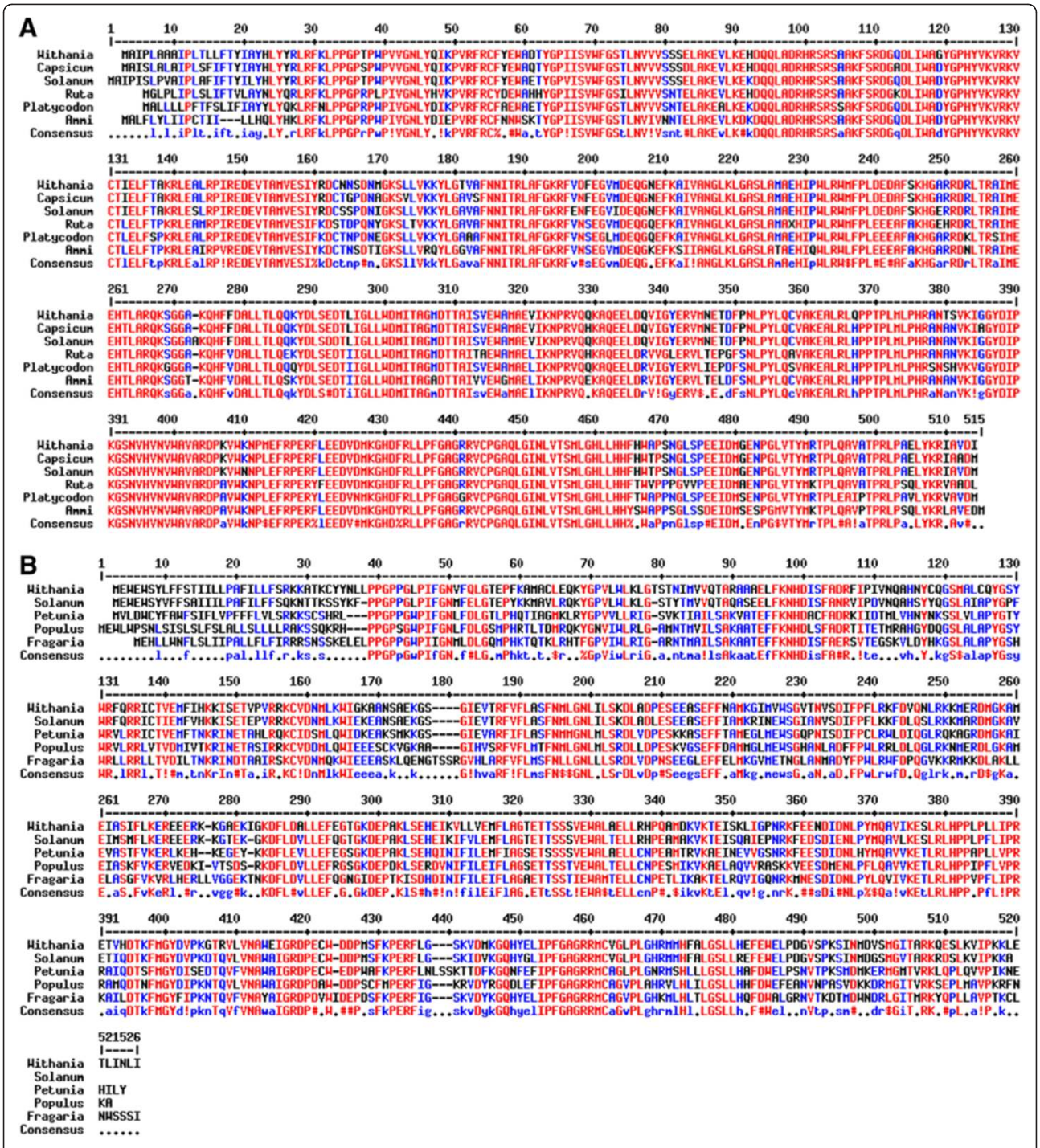

Figure 2 Multiple sequence alignment of deduced amino acid sequences of *WsCYP98A* and *Ws*CYP76A with their respective homologs using Multalign tool. For *Ws*CYP98A **(A)**, sequences used for alignment were from *Withania somnifera* (*WsCYP98A*: HM036710), *Solanum lycopersicum* (ACF17644.1) *Capsicum annuum* (ACF17644.1), *Ruta graveolens (AEG19446.1), Platycodon grandiflorus* (AEM63674.1), *Ammi majus* (AAT06912.1). Consensus residues present were shown below. For *Ws*CYP76A **(B)**, sequences used for alignment were from *Withania somnifera* (*WsCYP98A*: HM036710), *Solanum lycopersicum* (XP_004249463), *Populus trichocarpa* (XP_002309107.1), *Fragaria vesca* (XP_004305369.1), *Petunia x hybrida* (BAC53892.1). Consensus residues present were shown below.

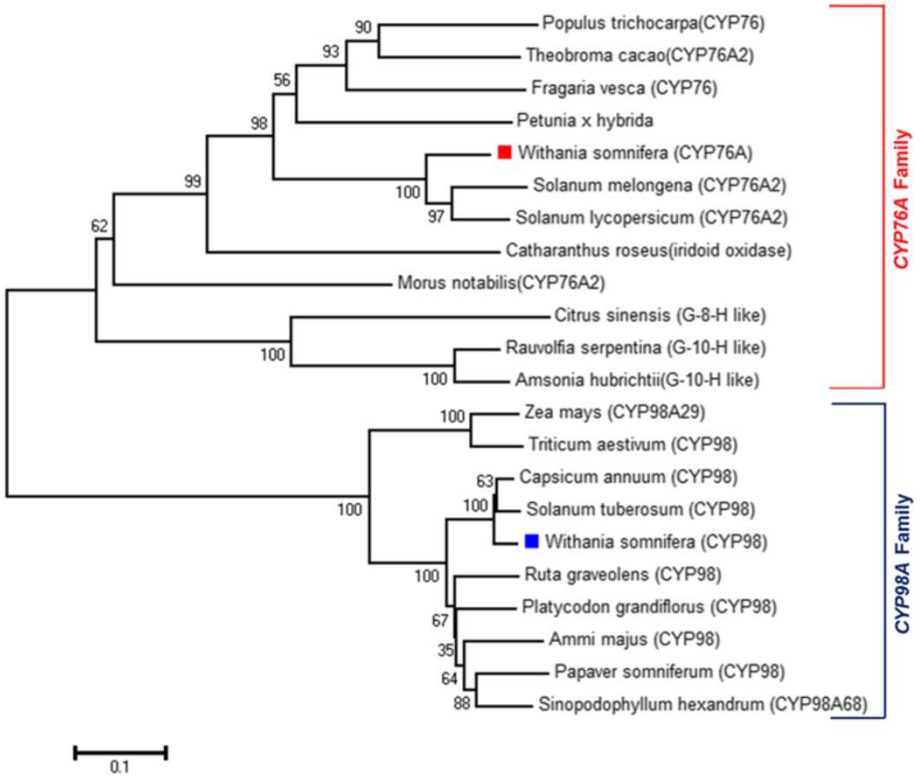

Figure 3 Phylogenetic analysis of deduced amino acid sequences of *WsCYP98A* and *WsCYP76A* was inferred using the Neighbour-joining method employing MEGA 6.0 software. For *WsCYP98A* total of 10 sequences and for *WsCYP76A*, 12 sequences including *Withania somnifera* were used for analysis. The sequences used were from following plant specieis: *Withania somnifera* (*WsCYP98A*: HM036710; *WsCYP76A*: GU808569), *Solanum lycopersicum* (ACF17644.1) *Capsicum annuum* (ACF17644.1) *Solanum tuberosum (XP_006347680.1), Ruta graveolens (AEG19446.1), Platycodon grandiflorus* (AEM63674.1), *Ammi majus* (AAT06912.1), *Zea mays* (ACG25686.1), *Triticum aestivum* (CAE47490.1), *Papaver somniferum* (AFK73720.1), *Sinopodophyllum hexandrum* (AGC29945.1), *Solanum melongena* (CAA50648.1), *Solanumlycopersicum* (XP_004249463), *Populus trichocarpa* (XP_002309107.1), *Fragaria vesca* (XP_004305369.1), *Petunia x hybrida* (BAC53892.1), *Theobroma cacao* (XP_007027589.1), *Catharanthus roseus (iridoid oxidase;* AHK60833.1*), Morus notabilis* (EXC33897.1), *Rauvolfia serpentina* (G-10-H like; AGX93053.1), *Amsonia hubrichtii* (G-10-H like; AGX93052.1, *Citrus sinensis* (G-8-H like; XP_006477331.1). Both P450 confined to separated clan and exhibited maximum similarity with their respective members of CYP98 and CYP76 families.

proteins was induced with different concentrations of IPTG. SDS-PAGE analysis demonstrated that optimum expression of proteins was observed at 25°C using 0.8 mM IPTG after 6–8 h of induction. The fusion protein having molecular weight of ~84.06 kDa and ~84.7 kDa appeared in the lysate of recombinant *E. coli* transformed with the expression cassettes pGEX-*WsCYP98A* and pGEX-*WsCYP76A*, respectively (Figure 5). The apparent molecular masses of these recombinant proteins were in good agreement with molecular mass of GST (26 kDa) and those calculated from the deduced primary structures of *Ws*CYP98A and *Ws*CYP76A proteins. Previously, we were able to optimize the functional expression of *WsCPRs* in soluble fraction with its membrane anchor [21]. Nevertheless, *Ws*CYP98A and *Ws*CYP76A were not expressed in soluble fraction in ample amount. Maximum amount of recombinant protein of two P450s were localized to inclusion bodies which hindered their purification.

Tissue-specific gene expression analysis

To study *WsCYP98A* and *WsCYP76A* gene expression pattern in different tissues of *W. somnifera*, cDNA libraries were prepared separately from RNA samples extracted from leaves, stalks, roots, flowers and berries (unripen) of four month old plant. Tissue-specific cDNAs were used as templates for qRT-PCR. The results showed highest expression of *WsCYP98A* in stalk (Figure 6A). Similar findings have been reported for coumarate 3-hydroxylase in *A. thaliana* where the distribution of transcripts was more preponderant in stem. High frequencies of *CYP98A* transcripts have also been observed in poplar and pine xylem, soybean hypocotyl and stem, as well as cotton fibres indicating towards its higher expression in lignin synthesizing tissues [35-37]. However, two closely related homologs *CYP98A8* and *CYP98A9* expressed predominantly in floral tissues which is very distinct from other CYP98 members. The higher expression in floral tissue probably helps in

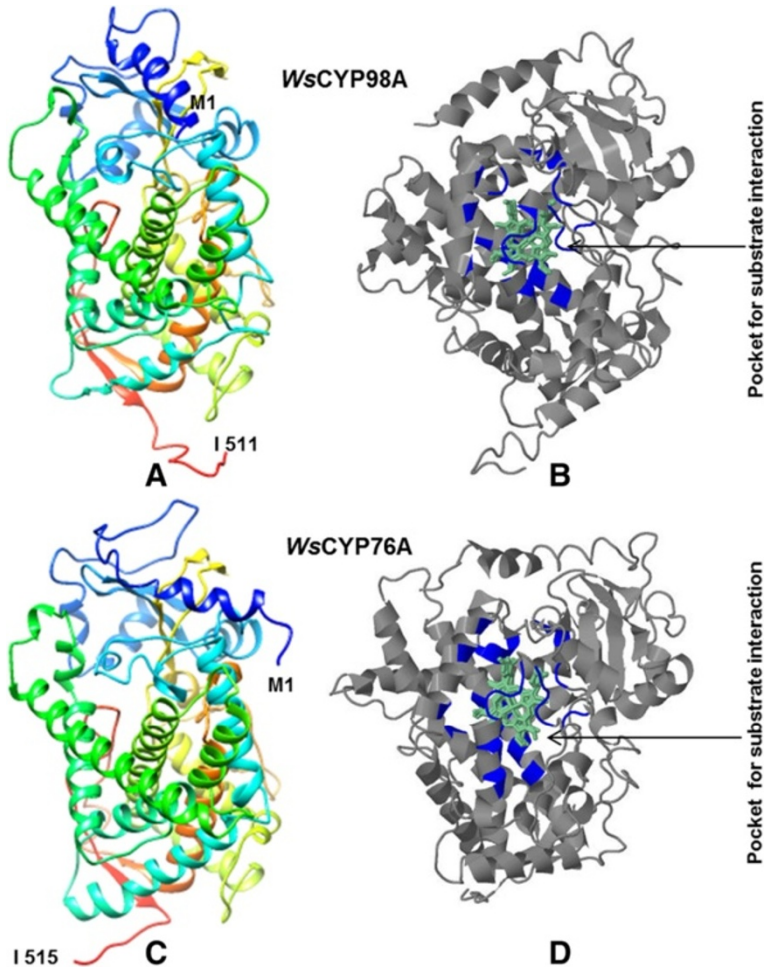

Figure 4 Prediction of three dimensional structures of *Ws*CYP98A and *Ws*CYP76A using Phyre[2] based on homology modelling approach. **A** & **C**: Cartoon display of the 3-D structures of *Ws*CYP98A and *Ws*CYP76A as predicted by Phyre[2] using crystal structure of cytochrome P450 1a2 (PDB: 2HI4) as template. **B** & **D**: Predicted ligand (shown in green) binding sites as predicted by 3DLigandSite Web Server. The residues involved in substrate binding and active site are shown in the center core of structure.

pollen coat development [38]. *WsCYP76A* transcripts were found predominantly expressing in roots of *W. somnifera* (Figure 6B). Likewise, expression of *CYP76A3* was reported higher in petunia roots. Higher expression of *WsCYP76A* in roots possibly protects plant against attack by plant pathogens [39]. The comparative analysis of transcriptomes in *Salvia miltiorrhiza* showed abundant expression of *CYP76AH1* in roots rather than other plant tissues and found to be involved in tanshinone biosynthetic pathway [40]. The least expression of both monooxygenases was observed in berries.

Effect of elicitors on *WsCYP98A* and *WsCYP76A*

In vitro cultures established *via* micro-propagation were used for MeJA, SA and GA_3 treatments. Micro-shoots were grown for 2 weeks in MS liquid medium. Subsequently, micro-shoots were supplemented with MeJA (0.1 mM), SA (0. 1 mM) and GA_3 (0.1 mM) for 48 h. The untreated were kept as control. Samples were harvested after 6, 12, 24, and 48 h of interval. Equal amounts of DNase-treated RNA (3 μg) of control and treated samples were converted into cDNA. The effect of MeJA, SA and GA_3 on expression profile of *WsCYP98A* and *WsCYP76A* was studied using qRT-PCR.

One of the important endogenous plant hormones MeJA is known to modulate biosynthesis of many of the secondary metabolites that play crucial role in the adaptation of plants to various biotic and biotic stresses [41]. MeJA has been reported to stimulate the biosynthesis of alkaloids by inducing the expression of several genes from the monoterpenoid branch of the monoterpene indole alkaloid biosynthetic pathway [42]. In present study, MeJA induced expression of both P450s post 48 h of treatment. *WsCYP98A* showed 2-fold increase in expression after

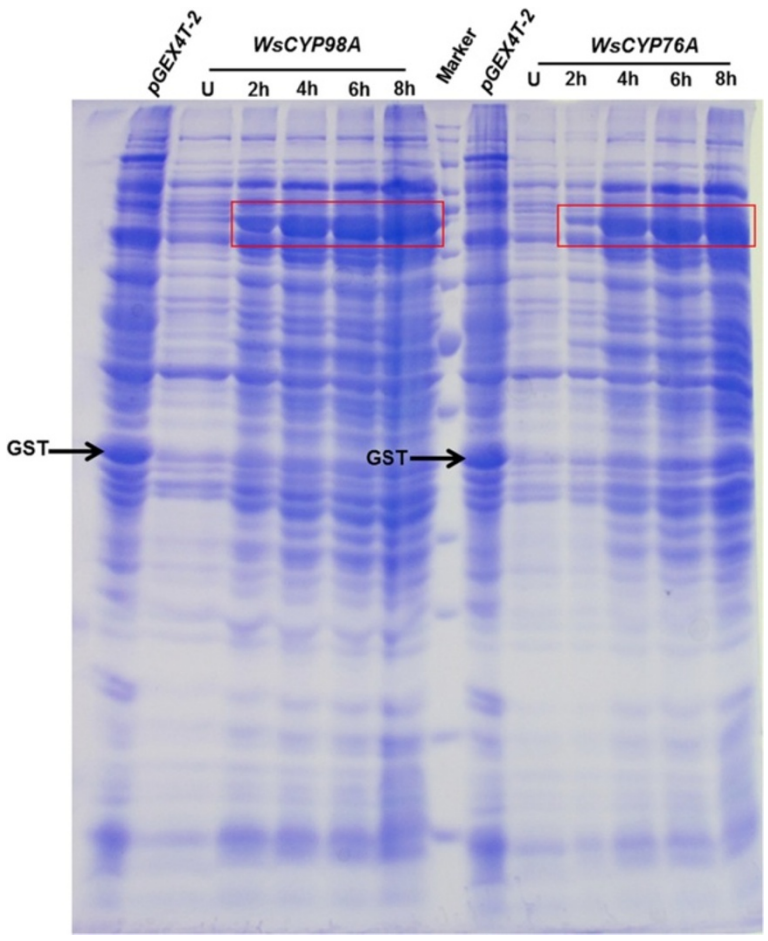

Figure 5 Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE: 10%) pattern of proteins obtained from *E.coli* BL21 (DE3) transformed with pGEX-*WsCYP98A* and pGEX-*WsCYP76A*. Lane 1 & 8; Cell lysate of *E. coli* BL21 (DE3) cells containing the empty vector pGEX-4 T2 obtained at 4 h post-induction with 0.8 mM IPTG. Lane 2 & 9; Whole cell lysate of uninduced *Ws*CYP98A and *Ws*CYP76A, Lane 7; Standard protein marker Lane 3–6 & 9–12; Cell lysate of *Ws*CYP98A and *Ws*CYP76A induced with 0.8 mM IPTG harvested after 2 h, 4 h, 6 h and 8 h respectively.

48 h of application of MeJA while *WsCYP76A* showed 6-fold increase in the expression after 48 h of MeJA application (Figure 7A).

SA has been studied in detail as stress signaling molecule. It is implicated in signal transduction of numerous processes including the biosynthesis of some important secondary metabolites in plants [43-45]. SA has been demonstrated to induce the accumulation of triterpenoids, ginsenosides in *Panx ginseng* and glycyrrhizin in *Glycyrrhizaglabra*, respectively [44,46]. Production of sesquiterpenoids, such as bilobalide in *Ginkgo biloba* and artemisinin in *Artemisia annua* are also stimulated by exogenous application of SA [47,48]. SA treated plantlets showed upto 2.5-3 fold increase in accumulation of *WsCYP98A* and *WsCYP76A* after time points of 24 h and 48 h, respectively (Figure 7B). The results obtained are fully in agreement with the previous studies wherein microarray analysis revealed up-regulation of several P450s from *A. thaliana* in response to SA treatment [49]. Likewise, enhanced expression of *CYP98A5* gene has been noticed in *Physilus vulgaris* when leaves were treated with derivatives of salicylic acids [50]. The induction of *LjC3H* in *Lonicera japonium* has been induced by the treatment of MeJA and UV radiation [51,52]. Up-regulation of transcript levels of *WsCYP98A* and *WsCYP76A* in response to elicitors may be due to their possible implication in defence mechanism.

CYP98A is key gene of phenylpropanoid pathway which leads to formation of flavonoids, phenolic and lignin. During pathogens attack and wound healing, deposition of lignin and suberin synthesis occurs in the cell wall [53]. Thus, the abundance of transcripts of *WsCYP98A* in treated plantlets appears to be a defence response due to elicitor triggered stimulus. The expression of *WsCYP76A* have also enhanced in response to MeJA and SA treatment. Members of same family like *CYP76B6* is strongly induced

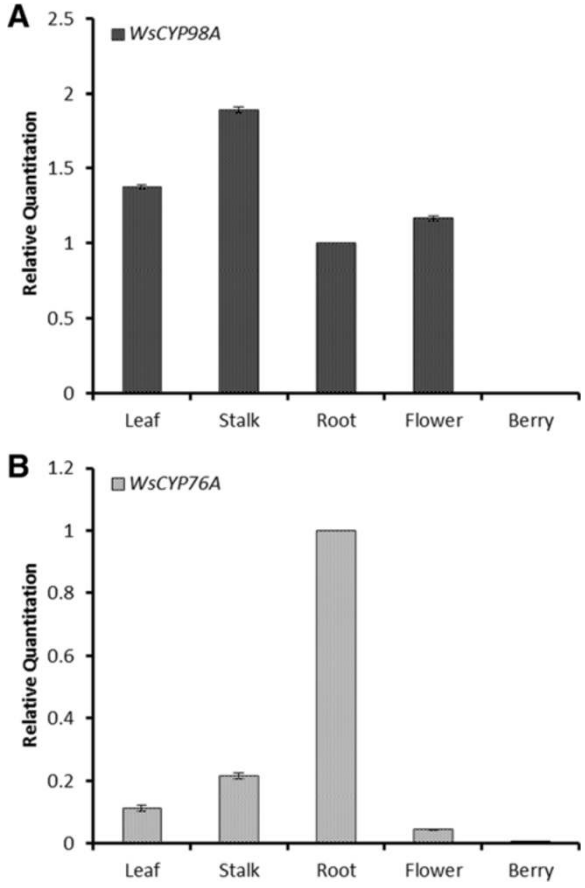

Figure 6 Quantitative assessment of the expression of (A) *WsCYP98A* and (B) *WsCYP76A* in different tissues of *Withania somnifera*. Data were compared and analysed with analysis of variance (*ANOVA*). Values are means, with standard errors indicated by bars, representing three independent biological samples, each with three technical replicates.

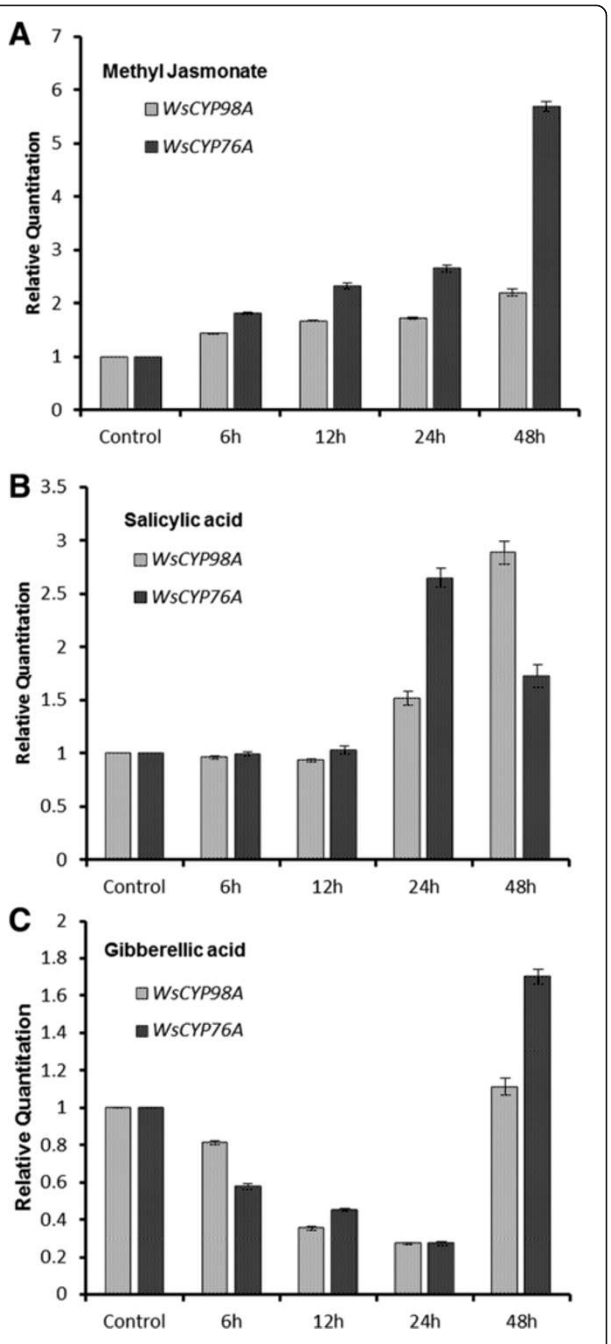

Figure 7 Quantitative real-time analysis of *WsCYP98A* and *WsCYP76A* expression in micro-propagated *Withania somnifera* induced by (A) methyl jasmonate (MeJA; 0.1 mM), (B) salicylic acid (SA; 0.1 mM) and (C) gibberellic acid (GA_3; 0.1 mM) treatments. MeJA, SA and GA_3 were added to the micro-propagated plantlets pre-cultured for 2 weeks on MS liquid medium. The samples were harvested at time points of 6, 12, 24, and 48 h. Actin was kept as internal control. Data were compared and analysed with analysis of variance (*ANOVA*). Experiments were performed in triplicate with similar results with standard errors indicated by bars, representing three independent biological samples, each with three technical replicates.

by MeJA treatment along with other terpenoid indole alkaloid biosynthesis genes in *C. roseus* cell culture. After 48 h of MeJA treatment the expression of *WsCYP76A* was about 6-fold higher than control. Closely related homologs of CYP76 family are mainly involved in biosynthesis of phytoalexin and are mostly multifunctional oxidases such as CYP76M7, which seem to be involved in the production of antifungal phytocassanes [54]. *CYP76B6* has been shown to be a highly specialized multifunctional enzyme catalyzing two sequential oxidation steps leading to the formation of 8-oxogeraniol from geraniol [55].

GA_3 is a diterpene plant growth regulator, controlling various growth and developmental process from seed germination to flower and fruit development. Broadly, GAs have been implicated in various cross talk mechanisms among different signaling pathways triggered by other plant growth regulators [56]. It impinges on a common transcription module of other phytohormones thus promoting many similar developmental responses

in plants [57]. It has also been contended that GA_3 orchestrates the metabolite fluxes between the primary and secondary metabolism influencing the production of isoprenoids and carotenoids [58,59]. Exogenous application of GA_3 resulted in decrease in the expression of *WsCYP98A* as well as *WsCYP76A* (Figure 7C). Lowest transcript levels were observed after 24 h of treatment which indicated that GA_3 acts as negative regulator of these two genes. After 48 h of treatment the transcript accumulation of *WsCYP98A* was comparable to control while as *WsCYP76A* showed 0.5-fold higher expression in comparison to control.

Effect of elicitors on accumulation of withanolides

Elicitor treatment is an efficient strategy to induce *de novo* synthesis of secondary metabolites. Endogenous signal molecules like MeJA and SA act as key signaling compounds in various stress responses and plants subjected to various vagaries often result in higher accumulation of secondary metabolites such as alkaloids, terpenoids, flavonoids, phenolic compounds and phytoalexins [60,61]. To establish a correlation between expression profiles and metabolite flux, withanolides extracted from the treated samples were subjected to HPLC analysis (Additional file 4: Figure S4). There was appreciable increase in WS-1 and WS-3 over a period of time in response to elicitor treatments. In MeJA treated samples, there was a significant increase in WS-1 ($53.583 \pm 1.02 - 137.530 \pm 1.76$ μg g^{-1} of dry weight) and WS-3 ($584.012 \pm 2.67 - 2504.937 \pm 3.52$ μg g^{-1} of dry weight) (Figure 8A). SA treated samples showed marked increase in both WS-1 ($129.934 \pm 1.04 - 230.864 \pm 1.54$ μg g^{-1} of dry weight) and WS-3($448.257 \pm 2.59 - 1467.235 \pm 2.58$ μg g^{-1} of dry weight) with no traces of WS-2 (Figure 8B). Estimation of withanolide accumulation in GA_3 treated samples demonstrated a gradual decline in WS-3 ($397.326 \pm 3.54 - 295.1673 \pm 3.18$ μg g^{-1} of dry weight) while as WS-1 increased moderately up to 24 h ($58.475 \pm 2.2 - 112.667 \pm 2$ μg g^{-1} of dry weight) but after 48 h there was a slight decrease in WS-1 (84.214 ± 2.01 μg g^{-1} of dry weight). WS-2 level also peaked at 6 h (44.323 ± 2.49 μg g^{-1} of dry weight) in GA_3 treated plantlets followed by a decrease at 24 h (10.867 ± 0.73 μg g^{-1} of dry weight) and un-detectable concentration after 48 h (Figure 8C). Absence or low production of WS-2 is possibly linked to inherently lower levels of WS-2 accumulation even in cultivated accessions of *W. somnifera* [37]. Application of exogenously applied phytohormones has been shown to impinge on physiological and metabolic processes in many plant organs and cell cultures. In some of the reports accumulation of anthocyanins, glutathione and flavonoids have shown varied response to GA_3 treatments [62].

In our previous reports, MeJA and SA up-regulated the expression of squalene synthase, squalene epoxidase and *WsCPR2* genes which play an important regulatory role in the phytosterol biosynthesis [63]. These elicitors are important components of signal transduction cascades activating plant's defence response against pathogen attack and often result in increased metabolite accumulation.

Conclusion

The increased demand of secondary metabolites for medicinal purposes coupled with low yield and supply has spurred the interest in enhanced production of bioactive metabolites. Recent developments and upsurge in synthetic biology approach in combination with rapid advances in systems biology and metabolic engineering have enabled the manipulation of genome of microorganisms to produce heterologous molecules in a manner that was previously much more demanding in terms of integrating the metabolic circuitries in heterologous host [64]. P450s play a critical role in heterologous and homologous systems as they are able to catalyse regio- and stereospecific hydroxylation reactions that are extremely difficult to carry out using chemical methods [65]. These P450-catalysed reactions are pivotal steps in the biosynthesis of variety of compounds and optimization of P450 enzyme activities are key targets in yield improvement efforts to render such approaches economically feasible [66]. The characterization of P450s and their role in biosynthesis of plant secondary metabolites is a fascinating area of investigation. Present endeavour was aimed towards identification and characterization of different P450 monooxygenases from *W. somnifera*. We were able to clone two important P450 monoxygenases from *Withania* which seem to play an active role in defence mechanism by coping up with biotic or abiotic stresses. It also gets reflected in higher accumulation of withanolides. The full length genes were successfully transformed in *E. coli* for heterologous expression. Time-course study revealed that IPTG had positive influence on protein expression in *E. coli*. Expression pattern for *WsCYP98A* and *WsCYP76A* in different plant tissues was studied using qRT-PCR which showed highest expression of *WsCYP98A* in stalk but the expression of *WsCYP76A* was more obvious in roots. In present study, MeJA and SA acted as positive regulators whereas GA_3 behaved as a negative regulator for *WsCYP98A* and *WsCYP76A*. To establish a correlation between enhanced transcript levels in response to activation of defence mechanism we also analysed withanolide accumulation. As the elicitors play key role as signaling molecules during stress conditions and enhance production of secondary metabolites, we observed discernible changes in withanolide concentrations. MeJA elicitation significantly increased the WS-3 accumulation over a period of 48 h. These results were in conformity with our earlier studies where MeJA and SA mediated induction of squalene synthase, squalene

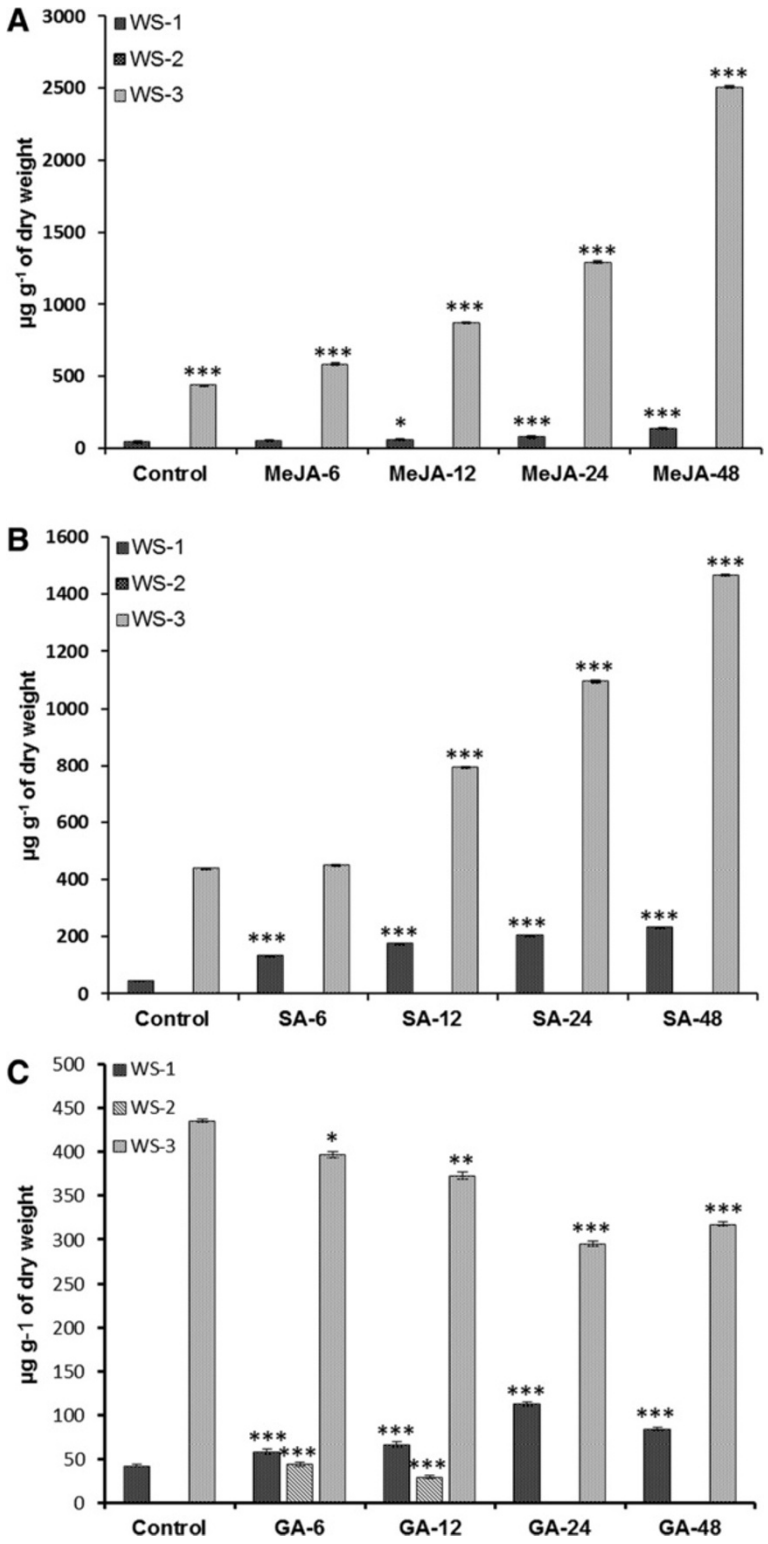

Figure 8 Time-course effect of elicitor treatments on withanolides accumulation in response to (A) methyl jasmonate (0.1 mM), (B) salicylic acid (0.1 mM) and (C) gibberellic acid (0.1 mM) at different time points. Variation in three key withanolides viz. withanolide A (WS-1), withanone (WS-2) and withaferine A (WS-3) was confirmed by HPLC analysis at 6, 12, 24 and 48 h. All values obtained were means of triplicate with standard errors. Time course accumulation of WS-1, WS-2 and WS-3 was statistically significant at $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ levels.

epoxidase and *Ws*CPR2 also led to enhanced withanolide content [63,67]. GA_3 was found to be a negative regulator and resulted in low levels of mRNA transcripts. The accumulation of withanolides in GA_3 treated samples showed remarkable decrease in WS-3 and WS-1 accumulation. These results are positively corroborating with some of our earlier findings where the exogenous application of GA_3 resulted in decrease in the cycloartenol synthase at translational level resulting in the decline of withanolide content [20]. Possibly, GA_3 application diverts the flux towards the formation of primary metabolites for cell elongation and development because it has been reported to be associated with the promotion of germination, growth, and flowering [62]. For homologous modulation of withanolide biosynthetic pathway, we have successfully incited the induction of transformed hairy roots using *Agrobacterium rhizogenes*. Hairy roots provide an excellent experimental system to understand the regulatory role of different P450s with their redox partners for enhanced production of withanolides (Unpublished).

Additional files

Additional file 1: Figure S1. Transmembrane domain prediction of **(A)** *WsCYP98A* and **(B)** *WsCYP76A* using TMHMM web server.

Additional file 2: Figure S2. Evolutionary conserved key residue analysis of **(A)** *Ws*CYP98A and **(B)** *Ws*CYP76A identified using Consurf, an empirical Bayesian inference based web server. Residue conservation from variable to conserve is shown in blue (1) to violet (9). The residues involved in binding of the donor moieties are shown in the centre of the structures.

Additional file 3: Figure S3. Ramachandran plot of *Ws*CYP98A *and Ws*CYP76A 3D models of *Withania somnifera* using RAMPAGE server. Most favoured regions are coloured red, additional allowed, generously allowed, and disallowed regions are indicated as yellow, light yellow and white fields, respectively.

Additional file 4: Figure S4. HPLC analysis of withanolide A (WS-1), withanone (WS-2) and withaferin A (WS-3) at 6 h and 48 h from methyl jasmonate (0.1 mM), salicylic acid (0.1 mM) and gibberellic acid (0.1 mM) treated micro-shoots of *Withania somnifera*.

Abbreviations

ER: Endoplasmic reticulum; GSPs: Gene specific primers; RACE: Rapid amplification of cDNA ends; ORF: Open reading frame; UTR: Untranslated region; *E. coli*: *Escherichia coli*; IPTG: Isopropyl- β-D-thiogalactopyranoside; SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis; qRT-PCR: Quantitative real-time polymerase chain reaction; MeJA: Methyl jasmonate; SA: Salicylic acid; GA_3: Gibberellic acid; HPLC: High performance liquid chromatography.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Conceived and designed the experiments: SKL, RV, SR. Performed the experiments: SR, WWB, ND, SAP, SR. Analyzed the data: SR, SKL. Contributed reagents/materials/analysis tools: RV, SKL. Wrote the paper: SR, SKL.
All authors read and approved the final manuscript.

Acknowledgements

This work was supported by a grant from the Council of Scientific and Industrial Research (CSIR), Government of India, New Delhi under Network Project BSC-0108. S. Rana, W.W.B, N.D. and S. Razdan are thankful to CSIR, Government of India, New Delhi for Senior Research Fellowship (CSIR-SRF). S A. Pandith is grateful to University Grants Commission, Government of India, New Delhi for Senior Research Fellowship (UGC-SRF).

Author details

[1]Plant Biotechnology Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu Tawi-180001, India. [2]Medicinal Chemistry Division, CSIR-Indian Institute of Integrative Medicine, Canal Road, Jammu Tawi-180001, India.

Received: 25 May 2014 Accepted: 6 October 2014
Published online: 23 November 2014

References

1. Cheng Q, Guengerich FP: **Identification of Endogenous Substrates of Orphan Cytochrome P450 Enzymes Through the Use of Untargeted Metabolomics Approaches.** In *Cytochrome P450 Protocols*, Volume 987. Edited by Phillips IR, Shephard EA, de Montellano PR O. New York: Humana Press; 2013:71–77.
2. Nelson DR, Nebert DW: **Cytochrome P450 (CYP) Gene Superfamily.** In *eLS*. West Sussex: John Wiley & Sons, Ltd; 2011.
3. Nelson D, Ming R, Alam M, Schuler M: **Comparison of cytochrome P450 genes from six plant genomes.** *Trop Plant Biol* 2008, **1**(3–4):216–235.
4. Nelson D, Werck-Reichhart D: **A P450-centric view of plant evolution.** *Plant J* 2011, **66**(1):194–211.
5. Schuler MA, Werck-Reichhart D: **Functional genomics of P450s.** *Annu Rev Plant Biol* 2003, **54:**629–667.
6. de Montellano PRO: *Cytochrome P450: Structure, Mechanism, and Biochemistry*. New York: Springer; 2005.
7. Bak S, Beisson F, Bishop G, Hamberger B, Hofer R, Paquette S, Werck-Reichhart D: **Cytochromes P450.** *Arabidopsis Book* 2011, **9**(10):6.
8. Werck-Reichhart D, Feyereisen R: **Cytochromes P450: a success story.** *Genome Biol* 2000, **1**(6):8.
9. Schuler MA: **The role of cytochrome P450 monooxygenases in plant-insect interactions.** *Plant Physiol* 1996, **112**(4):1411–1419.
10. Miners JO: **Evolution of drug metabolism: hitchhiking the technology bandwagon.** *Clin Exp Pharmacol Physiol* 2002, **29**(11):1040–1044.
11. Guengerich FP: **Cytochrome P450 enzymes in the generation of commercial products.** *Nat Rev Drug Discov* 2002, **1**(5):359–366.
12. Guengerich FP: **Special issue: P450 catalysis mechanisms introduction.** *Arch Biochem Biophys* 2011, **507**(1):1–2.
13. Sono M, Roach MP, Coulter ED, Dawson JH: **Heme-containing oxygenases.** *Chem Rev* 1996, **96**(7):2841–2888.
14. Guengerich FP: **Uncommon P450-catalyzed reactions.** *Curr Drug Metab* 2001, **2**(2):93–115.
15. Coon M: **Cytochrome P450: Nature's most versatile biological catalyst.** *Annu Rev Pharmacol Toxicol* 2005, **45:**1–25.
16. Kirson I, Glotter E, Lavie D, Abraham A: **Constitutents of *Withania somnifera* Dun. Part XII The withanolides of an indian chemotype.** *J Chem Soc C: Organic* 1971, 2032–2044.
17. Lattoo SK, Dhar RS, Khan S, Bamotra S, Dhar AK: **Temporal sexual maturation and incremental staminal movement encourages mixed mating in Withania somnifera - An insurance for reproductive success.** *Curr Sci* 2007, **92**(10):1390–1399.
18. Rana S, Dhar N, Bhat WW, Razdan S, Khan S, Dhar RS, Dutt P, Lattoo SK: **A 12-deoxywithastramonolide-rich somaclonal variant in *Withania somnifera* (L.) Dunal-molecular cytogenetic analysis and significance as a chemotypic resource.** *In Vitro Cell Dev Biol-Plant* 2012, **48**(5):546–554.
19. Chaurasiya ND, Sangwan NS, Sabir F, Misra L, Sangwan RS: **Withanolide biosynthesis recruits both mevalonate and DOXP pathways of isoprenogenesis in Ashwagandha *Withania somnifera* L. (Dunal).** *Plant Cell Rep* 2012, **31**(10):1889–1897.
20. Dhar N, Rana S, Razdan S, Bhat WW, Hussain A, Dhar RS, Vaishnavi S, Hamid A, Vishwakarma R, Lattoo SK: **Cloning and functional characterization of three branch point oxidosqualene cyclases from *Withania somnifera* (L.) dunal.** *J Biol Chem* 2014, **289**(24):17249–17267.

21. Rana S, Lattoo SK, Dhar N, Razdan S, Bhat WW, Dhar RS, Vishwakarma R: **NADPH-cytochrome P450 reductase: molecular cloning and functional characterization of Two paralogs from *Withania somnifera* (L.) dunal.** *PLoS One* 2013, **8**(2):e57068.
22. Dhar N, Rana S, Bhat WW, Razdan S, Pandith SA, Khan S, Dutt P, Dhar RS, Vaishnavi S, Vishwakarma R, Lattoo SK: **Dynamics of withanolide biosynthesis in relation to temporal expression pattern of metabolic genes in *Withania somnifera* (L.) Dunal: a comparative study in two morpho-chemovariants.** *Mol Biol Rep* 2013, **40**(12):7007–7016.
23. Bhat WW, Dhar N, Razdan S, Rana S, Mehra R, Nargotra A, Dhar RS, Ashraf N, Vishwakarma R, Lattoo SK: **Molecular characterization of UGT94F2 and UGT86C4, Two glycosyltransferases from *picrorhiza kurrooa*: comparative structural insight and evaluation of substrate recognition.** *PLoS One* 2013, **8**(9):e73804.
24. Karamat F, Olry A, Doerper S, Vialart G, Ullmann P, Werck-Reichhart D, Bourgaud F, Hehn A: **CYP98A22, a phenolic ester 3′-hydroxylase specialized in the synthesis of chlorogenic acid, as a new tool for enhancing the furanocoumarin concentration in *Ruta graveolens*.** *BMC Plant Biol* 2012, **12**(1):152.
25. Miettinen K, Dong L, Navrot N, Schneider T, Burlat V, Pollier J, Woittiez L, van der Krol S, Lugan R, Ilc T, Verpoorte R, Oksman-Caldentey KM, Martinoia E, Bouwmeester H, Goossens A, Memelink J, Werck-Reichhart D: **The seco-iridoid pathway from *Catharanthus roseus*.** *Nat Commun* 2014, **5**. doi:10.1038/ncomms4606.
26. Hofer R, Dong L, Andre F, Ginglinger JF, Lugan R, Gavira C, Grec S, Lang G, Memelink J, Van der Krol S, Bouwmeester H, Werck-Reichhart D: **Geraniol hydroxylase and hydroxygeraniol oxidase activities of the CYP76 family of cytochrome P450 enzymes and potential for engineering the early steps of the (seco)iridoid pathway.** *Metab Eng* 2013, **20**:221–232.
27. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG: **Clustal W and ClustalX version 2.0.** *Bioinformatics* 2007, **23**(21):2947–2948.
28. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S: **MEGA6: molecular evolutionary genetics analysis version 6.0.** *Mol Biol Evol* 2013, **30**(12):2725–2729.
29. Geourjon C, Deleage G: **SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments.** *Comput Appl Biosci* 1995, **11**(6):681–684.
30. Kelley LA, Sternberg MJE: **Protein structure prediction on the Web: a case study using the Phyre server.** *Nat Protoc* 2009, **4**(3):363–371.
31. Lovell SC, Davis IW, Arendall WB 3rd, de Bakker PI, Word JM, Prisant MG, Richardson JS, Richardson DC: **Structure validation by Calpha geometry: phi, psi and Cbeta deviation.** *Proteins* 2003, **50**(3):437–450.
32. Celniker G, Nimrod G, Ashkenazy H, Glaser F, Martz E, Mayrose I, Pupko T, Ben-Tal N: **ConSurf: using evolutionary data to raise testable hypotheses about protein function.** *Isr J Chem* 2013, **53**(3–4):199–206.
33. Livak KJ, Schmittgen TD: **Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method.** *Methods* 2001, **25**(4):402–408.
34. Dhar RS, Verma V, Suri KA, Sangwan RS, Satti NK, Kumar A, Tuli R, Qazi GN: **Phytochemical and genetic analysis in selected chemotypes of *Withania somnifera*.** *Phytochemistry* 2006, **67**(20):2269–2276.
35. Sterky F, Regan S, Karlsson J, Hertzberg M, Rohde A, Holmberg A, Amini B, Bhalerao R, Larsson M, Villarroel R, Van Montagu M, Sandberg G, Olsson O, Teeri TT, Boerjan W, Gustafsson P, Uhlen M, Sundberg B, Lundeberg J: **Gene discovery in the wood-forming tissues of poplar: analysis of 5, 692 expressed sequence tags.** *Proc Natl Acad Sci U S A* 1998, **95**(22):13330–13335.
36. Allona I, Quinn M, Shoop E, Swope K, St Cyr S, Carlis J, Riedl J, Retzel E, Campbell MM, Sederoff R, Whetten RW: **Analysis of xylem formation in pine by cDNA sequencing.** *Proc Natl Acad Sci U S A* 1998, **95**(16):9693–9698.
37. Schoch G, Goepfert S, Morant M, Hehn A, Meyer D, Ullmann P, Werck-Reichhart D: **CYP98A3 from *Arabidopsis thaliana* is a 3′-hydroxylase of phenolic esters, a missing link in the phenylpropanoid pathway.** *J Biol Chem* 2001, **276**(39):36566–36574.
38. Matsuno M, Compagnon V, Schoch GA, Schmitt M, Debayle D, Bassard JE, Pollet B, Hehn A, Heintz D, Ullmann P, Lapierre C, Bernier F, Ehlting J, Werck-Reichhart D: **Evolution of a novel phenolic pathway for pollen development.** *Science* 2009, **325**(5948):1688–1692.
39. Imaishi H, Ishitobi U: **Molecular cloning of CYP76A3, a novel cytochrome P450 from Petunia hybrida catalyzing the omega-hydroxylation of myristic acid.** *Biologia Plantarum* 2008, **52**(2):242–250.
40. Yang L, Ding G, Lin H, Cheng H, Kong Y, Wei Y, Fang X, Liu R, Wang L, Chen X, Chen X, Yang C: **Transcriptome analysis of medicinal plant *Salvia miltiorrhiza* and identification of genes related to tanshinone biosynthesis.** *PLoS One* 2013, **8**(11):e80464.
41. Martin D, Tholl D, Gershenzon J, Bohlmann J: **Methyl jasmonate induces traumatic resin ducts, terpenoid resin biosynthesis, and terpenoid accumulation in developing xylem of Norway spruce stems.** *Plant Physiol* 2002, **129**(3):1003–1018.
42. Oudin A, Mahroug S, Courdavault V, Hervouet N, Zelwer C, Rodriguez-Concepcion M, St-Pierre B, Burlat V: **Spatial distribution and hormonal regulation of gene products from methyl erythritol phosphate and monoterpene-secoiridoid pathways in *Catharanthus roseus*.** *Plant Mol Biol* 2007, **65**(1–2):13–30.
43. Draper J: **Salicylate, superoxide synthesis and cell suicide in plant defence.** *Trends Plant Sci* 1997, **2**(5):162–165.
44. Ali MB, Yu KW, Hahn EJ, Paek KY: **Methyl jasmonate and salicylic acid elicitation induces ginsenosides accumulation, enzymatic and non-enzymatic antioxidant in suspension culture *Panax ginseng* roots in bioreactors.** *Plant Cell Rep* 2006, **25**(6):613–620.
45. Hayat Q, Hayat S, Irfan M, Ahmad A: **Effect of exogenous salicylic acid under changing environment: a review.** *Environ Exp Bot* 2010, **68**(1):14–25.
46. Shabani L, Ehsanpour AA, Asghari G, Emami J: **Glycyrrhizin production by *in vitro* cultured *Glycyrrhiza glabra* elicited by methyl jasmonate and salicylic acid.** *Russ J Plant Physiol* 2009, **56**(5):621–626.
47. Kang SM, Min JY, Kim YD, Kang YM, Park DJ, Jung HN, Kim SW, Choi MS: **Effects of methyl jasmonate and salicylic acid on the production of bilobalide and ginkgolides in cell cultures of *Ginkgo biloba*.** *In Vitro Cell Dev Biol-Plant* 2006, **42**(1):44–49.
48. Pu GB, Ma DM, Chen JL, Ma LQ, Wang H, Li GF, Ye HC, Liu BY: **Salicylic acid activates artemisinin biosynthesis in *Artemisia annua* L.** *Plant Cell Rep* 2009, **28**(7):1127–1135.
49. Narusaka Y, Narusaka M, Seki M, Umezawa T, Ishida J, Nakajima M, Enju A, Shinozaki K: **Crosstalk in the responses to abiotic and biotic stresses in *Arabidopsis*: analysis of gene expression in cytochrome P450 gene superfamily by cDNA microarray.** *Plant Mol Biol* 2004, **55**(3):327–342.
50. Basson AE, Dubery IA: **Identification of a cytochrome P450 cDNA (CYP98A5) from *Phaseolus vulgaris*, inducible by 3,5-dichlorosalicylic acid and 2,6-dichloro isonicotinic acid.** *J Plant Physiol* 2007, **164**(4):421–428.
51. Pu G, Wang P, Zhou B, Liu Z, Xiang F: **Cloning and characterization of *Lonicera japonica* p-coumaroyl ester 3-hydroxylase which is involved in the biosynthesis of chlorogenic acid.** *Biosci Biotechnol Biochem* 2013, **77**(7):1403–1409.
52. Peng X, Li W, Wang W, Bai G: **Cloning and characterization of a cDNA coding a hydroxycinnamoyl-CoA quinate hydroxycinnamoyl transferase involved in chlorogenic acid biosynthesis in *Lonicera japonica*.** *Planta Med* 2010, **76**(16):1921–1926.
53. Nawrath C: **The biopolymers cutin and suberin.** *The Arabidopsis book/Am Soc Plant Biol* 2002, **1**:e0021.
54. Swaminathan S, Morrone D, Wang Q, Fulton DB, Peters RJ: **CYP76M7 is an ent-cassadiene C11 alpha-hydroxylase defining a second multifunctional diterpenoid biosynthetic gene gluster in rice.** *Plant Cell* 2009, **21**(10):3315–3325.
55. Ehlting J, Sauveplane V, Olry A, Ginglinger JF, Provart NJ, Werck-Reichhart D: **An extensive (co-)expression analysis tool for the cytochrome P450 superfamily in *Arabidopsis thaliana*.** *BMC Plant Biol* 2008, **8**:47.
56. Olszewski N, Sun TP, Gubler F: **Gibberellin signaling: biosynthesis, catabolism, and response pathways.** *Plant Cell* 2002, **14**(Suppl):S61–S80.
57. Bai MY, Shang JX, Oh E, Fan M, Bai Y, Zentella R, Sun TP, Wang ZY: **Brassinosteroid, gibberellin and phytochrome impinge on a common transcription module in *Arabidopsis*.** *Nat Cell Biol* 2012, **14**(8):810–817.
58. Mansouri H, Asrar Z, Mehrabani M: **Effects of gibberellic acid on primary terpenoids and delta-tetrahydrocannabinol in *Cannabis sativa* at flowering stage.** *J Integr Plant Biol* 2009, **51**(6):553–561.
59. Jones AMP, Saxena PK, Murch SJ: **Elicitation of secondary metabolism in *Echinacea purpurea* L. by gibberellic acid and triazoles.** *Eng Life Sci* 2009, **9**(3):205–210.
60. Ebel J, Mithofer A: **Early events in the elicitation of plant defence.** *Planta* 1998, **206**(3):335–348.

61. van der Fits L, Memelink J: **ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism.** *Science* 2000, **289**(5477):295–297.
62. Weiss D, Ori N: **Mechanisms of cross talk between gibberellin and other hormones.** *Plant Physiol* 2007, **144**(3):1240–1246.
63. Bhat WW, Lattoo SK, Razda S, Dhar N, Rana S, Dhar RS, Khan S, Vishwakarma RA: **Molecular cloning, bacterial expression and promoter analysis of squalene synthase from *Withania somnifera* (L.) Dunal.** *Gene* 2012, **499**(1):25–36.
64. Paddon CJ, Keasling JD: **Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development.** *Nat Rev Microbiol* 2014, **12**(5):355–367.
65. Morant M, Bak S, Moller BL, Werck-Reichhart D: **Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation.** *Curr Opin Biotechnol* 2003, **14**(2):151–162.
66. Keasling JD: **Synthetic biology and the development of tools for metabolic engineering.** *Metab Eng* 2012, **14**(3):189–195.
67. Razdan S, Bhat WW, Rana S, Dhar N, Lattoo SK, Dhar RS, Vishwakarma RA: **Molecular characterization and promoter analysis of squalene epoxidase gene from *Withania somnifera* (L.) Dunal.** *Mol Biol Rep* 2013, **40**(2):905–916.

doi:10.1186/s12896-014-0089-5
Cite this article as: Rana *et al.*: Molecular characterization of two A-type P450s, *WsCYP98A* and *WsCYP76A* from *Withania somnifera* (L.) Dunal: expression analysis and withanolide accumulation in response to exogenous elicitations. *BMC Biotechnology* 2014 **14**:89.