

An inducible NADPH–cytochrome P450 reductase from *Picrorhiza kurrooa* — an imperative redox partner of cytochrome P450 enzymes

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Abstract *Picrorhiza kurrooa* synthesizes a large array of pharmacologically important monoterpenoid iridoid glycosides called picrosides. Although chemical profile and pharmacological activities of *P. kurrooa* have been extensively studied, limited attempts have been made to decipher the biosynthetic route and to identify the key regulatory genes involved in picroside biosynthesis. In the present study, NADPH–cytochrome P450 reductase, a key enzyme involved in electron transfer to cytochrome P450s was identified from *P. kurrooa*. The full length cDNA (2679 bp) contained an open reading frame of 2133 bp, corresponding to 710 amino acids. *PkCPR* was heterologously expressed in *Escherichia coli* and the kinetic parameters of the recombinant enzyme were determined. Specific activity, $V_{\max}$ and K_m of *PkCPR* were found to be $5.8 \pm 0.05 \mu\text{mol min}^{-1} \text{mg}^{-1}$, $8.1 \pm 0.12 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and $7.8 \mu\text{M}$, respectively. *PkCPR* was found to be spatially regulated at transcript level, being maximally expressed in leaf tissues. Altitude was found to have a positive effect on the picroside concentration and the picroside content positively correlated with the *PkCPR* transcript levels in samples collected at varied altitudes. Further, transcript profiling under methyl jasmonate, salicylic acid, 2,4-dichlorophenoxy acetic acid and UV-B elicitations displayed differential transcriptional regulation of *PkCPR* that fully corroborated with the identified

cis-elements within the *PkCPR* promoter. Expression of *PkCPR* was inducible by UV-B and phytohormone elicitation, indicating that the *PkCPR* is possibly related to defence reactions, including biosynthesis of secondary metabolites. Present study is so far the only report of identification and functional characterization of CPR ortholog from *P. kurrooa*.

Keywords Elicitors · Functional characterization · Homology modelling · *Picrorhiza kurrooa* · Picrosides · NADPH–cytochrome P450 reductase

Abbreviations

IPTG	Isopropyl- β -D-thiogalactopyranoside
ORF	Open reading frame
CPR	Cytochrome P450 reductase
RACE	Rapid amplification of cDNA ends
RT-PCR	Reverse transcription-polymerase chain reaction
MeJA	Methyl jasmonate
SA	Salicylic acid
2,4-D	2,4-Dichlorophenoxy acetic acid
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TSS	Transcriptional start site
UTR	Untranslated region

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Introduction

Cytochrome P450s (CYP450s) are heme thiolate-proteins that catalyse extremely diverse variety of reactions like hydroxylations, dealkylations, sulphoxidations, epoxidations, reductive dehalogenations, peroxidations and various types of isomerization for the synthesis of a suite of primary and

secondary metabolites essential for plant growth and development (Schuler and Werck-Reichhart 2003). However, the catalytic activities of most eukaryotic P450 monooxygenases, including plant cytochrome P450s rely on a key redox partner, NADPH–cytochrome P450 reductase (CPR; E.C.1.6.2.4). NADPH–CPR are membrane-bound proteins localized to endoplasmic reticulum (ER) and contain N-terminal FMN binding domain linked to NADPH binding domain via FAD domain. CPRs serve as the electron donors to almost all eukaryotic cytochromes P450s and together with CYP450, constitute the multicomponent monooxygenase system (Wang et al. 1997). CPR belongs to a small family of diflavin proteins and is built of cofactor binding domains with high structural homology to those of bacterial flavodoxins and ferredoxin-NADP + oxidoreductases. CPR shuttles electrons from NADPH through the FAD and FMN-cofactors into the central heme-group of the P450s (Vermilion et al. 1981). A large number of CPRs have been isolated and characterized from various plant species, such as *Withania somnifera* (Rana et al. 2013), *Vigna radiata* (Shet et al. 1993), *Catharanthus roseus* (Meijer et al. 1993), *Arabidopsis thaliana* (Mizutani and Ohta 1998), *Petroselinum crispum* (Koopmann and Hahlbrock 1997) and *Helianthus tuberosus* (Benveniste et al. 1986). Plant CPRs share relatively low sequence identities with those from animals and fungi (30–40 %), but are highly identical within flowering plants (65–80 %). It has been found that most organisms, including animals and yeasts, have only one CPR gene in each genome, whose expression product interacts with different P450s (Porter et al. 1990). However, in vascular plants, the number of CPR paralogs varies from one to three depending on the species. A single form of CPR has been characterized from some plants like *Coleus blumei* and *V. radiata* (Shet et al. 1993; Eberle et al. 2009). *H. tuberosus*, *P. crispum*, *W. somnifera*, *A. thaliana* and *Gossypium hirsutum* have two CPR paralogs each (Koopmann and Hahlbrock 1997; Mizutani and Ohta 1998; Yang et al. 2010; Benveniste et al. 1986; Rana et al. 2013) while *Nothapodytes foetida* and Hybrid poplar contain three paralogs (Ro et al. 2002; Jennewein et al. 2005; Huang et al. 2012).

Picrorhiza kurroa Royle ex Benth. (Plantaginaceae) is a highly valued, high-altitude medicinal herb, endemic to the North Western Himalayan regions of India (3000–4300 m) (Bhat et al. 2012b). It has been reported to possess a variety of pharmacological activities that include anticarcinogenic (Joy et al. 2000), antioxidant (Rajkumar et al. 2011), immunomodulatory (Gupta et al. 2006), antiallergic, antiasthmatic (Dorsch et al. 1991), superoxide scavenging (Chander et al. 1992) and antidiabetic (Joy and Kuttan 1999) properties. It is also widely used as a hepatoprotective (Saraswat et al. 1997) in various formulations such as Picroliv (Ansari et al. 1991), Katuki, Arogya, Livomap and Kutaki (Bhandari et al. 2009). Most of these pharmacological properties are attributed to a novel group of iridoid monoterpenes (C10) known as picrosides.

P. kurroa contains two major phytoconstituents, picroside I (PK-I) and picroside II (PK-II), along with minor iridoid glycosides including kutkoside, picroside III (PK-III), picroside IV (PK-IV), verminoside, specioside, etc. (Mondal et al. 2012). *P. kurroa* figures prominently among ‘endangered’ list of plant species as it is being indiscriminately collected from its wild habitats because of its immense therapeutic importance (Sood and Chauhan 2010).

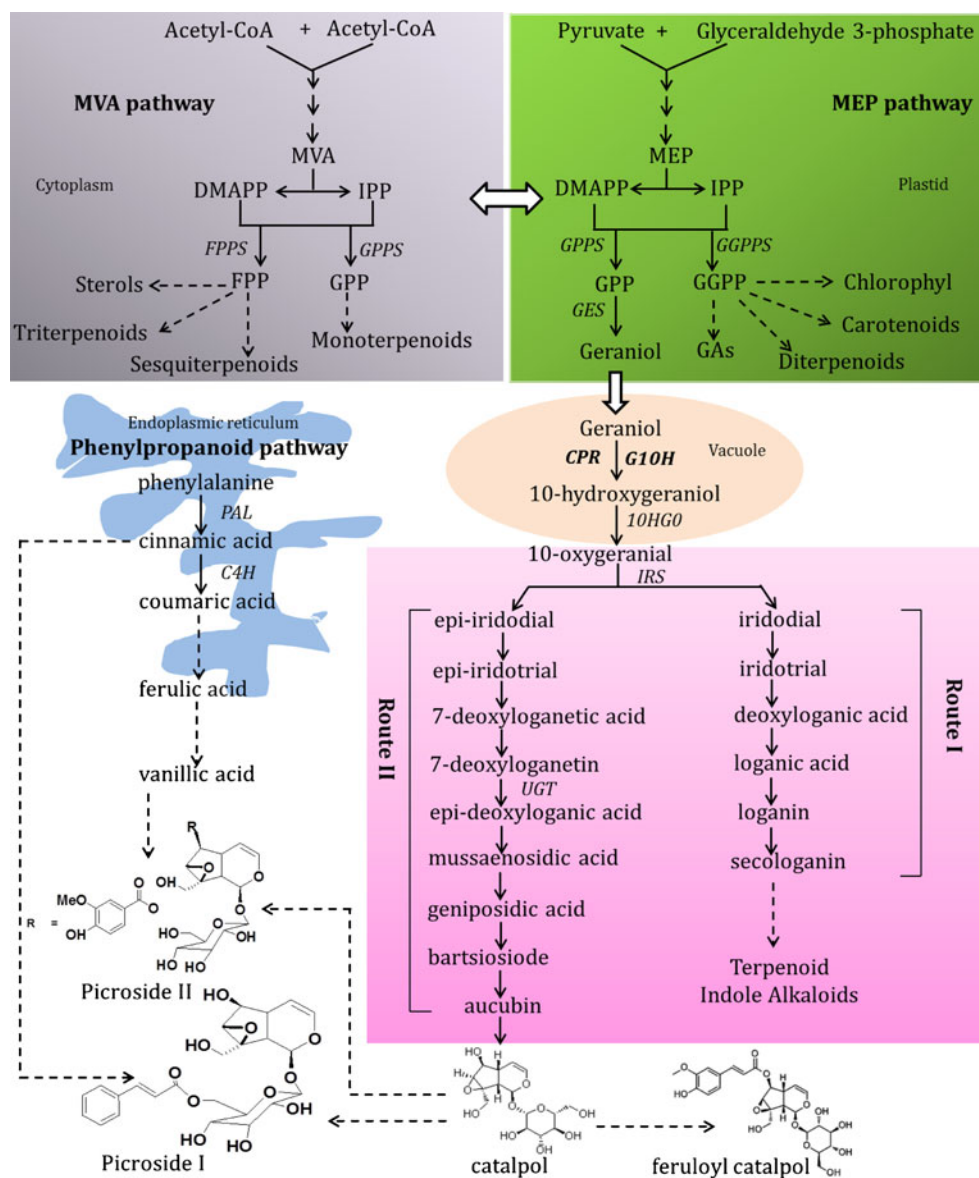
Metabolic engineering provides a highly efficient alternative for production of picrosides in heterologous hosts. However, an important prerequisite for any attempt at metabolic engineering is the detailed knowledge of the underlying biosynthetic and regulatory pathways in plants. Despite the pharmacological significance of picrosides, their biosynthetic pathway is yet to be fully elucidated. In recent times there has been surge in the molecular pathway elucidation studies related to picroside biosynthesis. It has led to the characterization of some of the key pathway genes (Kawoosa et al. 2010; Bhat et al. 2012a; Pandit et al. 2012b; Gahlan et al. 2012; Bhat et al. 2013; Singh et al. 2013; Kumar et al. 2013). Picrosides are essentially derived from geranyl diphosphate, synthesized both from cytoplasmic mevalonate and plastidic non-mevalonate pathways through a series of hydroxylation, epoxidation, oxidation, glucosylation, acylation and cyclization steps (Fig. 1). Most of these reactions are catalysed by various P450 monooxygenases along with their imperative redox partner, NADPH–CPR. One of the limiting factors associated with the introduction of plant P450s into a heterologous host such as bacteria, yeasts or other plant species is to guarantee an adequate supply of electrons from CPR to optimize P450 activity. The heterologous expression of P450s is often constrained because of inadequate interface of endogenous cytochrome P450 oxidoreductase partners resulting in no or low activity (Duan and Schuler 2006). Thus, in an endeavour towards the metabolic engineering prospect, we herein report the molecular characterization and functional validation of NADPH-CPR gene from *P. kurroa* — an essential partner of P450 monooxygenases. Upstream promoter region of *PkCPR* was also isolated and scanned for the identification of different *cis*-regulatory elements. Further, the influence of varying altitudes on picroside accumulation was also investigated. Present study also includes comparative tissue-specific expression analysis of *PkCPR* in response to elicitor stimuli including methyl jasmonate (MeJA), salicylic acid (SA), 2,4-dichlorophenoxy acetic acid (2,4-D) and UV-B.

Materials and methods

Plant materials and treatments

Plants were collected during the months of June–July (2009, 2010) when *P. kurroa* exhibits luxuriant growth with the

Fig. 1 Overview of the proposed picroside pathway in *Picrorhiza kurrooa* adapted from (Bhat et al. 2013). *CPR* cytochrome P450 reductase, *DMAPP* dimethylallyl diphosphate, *FPP* farnesyl diphosphate, *FPPS* farnesyl diphosphate synthase, *GPP* geranyl diphosphate, *GPPS* geranyl diphosphate synthase, *GES* geraniol synthase, *GGPP* geranylgeranyl diphosphate, *GGPPS* geranylgeranyl diphosphate synthase, *G10H* geraniol 10-hydroxylase, *10HGO* 10-hydroxygeraniol oxidoreductase, *IRS* iridoid synthase, *IPP* isopentenyl diphosphate, *IDI* IPP isomerase, *MEP* 2-C-methyl-D-erythritol 4-phosphate, *PAL* phenylalanine ammonia lyase, *C4H* cinnamoyl 4-hydroxylase, *MVA* mevalonic acid; *Route I* the route leading to the biosynthesis of secologanin, *Route II* the route leading to the biosynthesis of catalpol to picrosides



initiation of flowering under wild conditions, from three different altitudinal locations of Kashmir valley. These included Sonamarg (34°14'34"N 75°18'7"E; 3,500 m in altitude), Dhanwas (33°59'39.6"N 74°26'55.9"E; 3,500 m in altitude), and Yarikah (34°04'06.6"N 74°25'38.7"E; 2,400 m in altitude) of Jammu and Kashmir state, India. Plants collected from Sonamarg were cultivated and habituated at Indian Institute of Integrative Medicine (IIIM), Srinagar (34°5'24" N, 74°47'24" E; 1,730 m in altitude). For elicitor treatments, the plants were mist-sprayed with SA (1 mM), 2,4-D (50 µM) and MeJA (0.1 mM) on both sides of leaves until liquid dripped from the leaves. The plants were covered with transparent polythene. Shoot tips including four to five true leaves, from three to four plants were excised after 6, 12, 24 and 48 h after treatment and combined as one biological replicate. Fresh tissue samples were immediately frozen in liquid

nitrogen and kept at −80 °C for further analysis. For UV-B treatment, the in vitro raised plantlets were exposed in a dark closed chamber to 1,500 µJ/m² UV-B irradiation and the control plantlets were placed in a dark closed chamber. The source of UV-B radiation was a band of four UV-B lamps (Daavlin, UVA/UVB Research Irradiation Unit, Bryan, OH, USA) equipped with digital controller to regulate UV dosage at a fixed distance of 24 cm from the UV lamps. The resulting wavelengths were in the UV-B range of 290–320 nm (above 90 %) and the peak emission was recorded at 314 nm.

RNA isolation and cDNA synthesis

In vitro cultures established from field grown plants at IIIM, Srinagar (Bhat et al. 2012b) were used for the identification of CPR gene from *P. kurrooa*. Total RNA was extracted from the

young leaves of *P. kurroa* plantlets using TRI Reagent (Sigma, St. Louis, MO, USA) as per manufacturer's protocol. First-strand cDNA was synthesized using the RevertAid cDNA synthesis kit (Fermentas, Burlington, Canada) in a total volume of 20 µl containing 3 µg total RNA, 2 µl of 10 mM dNTPs, 1 µl of 10 mM oligo(dT) primer, 1 µl M-MuLV reverse transcriptase (200 U/ml) and 4 µl of 5× first strand buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 20 mM MgCl₂, 50 mM DTT). The reaction was incubated for 60 min at 42 °C followed by 5 min at 70 °C to terminate the reaction.

by a final extension of 72 °C for 10 min in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR product was gel-purified, ligated into the pTZ57R/T vector (Fermentas, Burlington, Canada), transformed into *Escherichia coli* DH5α competent cells and sequenced in both directions using an automated DNA sequencer (ABI Prism 3130XL; Applied Biosystems, Foster City, CA, USA). The nucleotide sequence obtained was analysed using the similarity search BLAST program and subsequently used for designing gene-specific primers (GSPs).

Degenerate primer design and PCR amplification of *PkCPR* core amplicon

Degenerate primers (Table 1) based on the conserved regions of CPRs were designed by multiple sequence alignment of different CPR sequences retrieved from the GenBank database at National Center for Biotechnology Information (NCBI). Optimization of reverse transcriptase-polymerase chain reaction (RT-PCR) conditions allowed amplification of a cDNA fragment corresponding to *PkCPR* under following cycling conditions: one cycle of 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 48 °C for 1 min and 72 °C for 2 min followed

5' and 3' RACE

To obtain the complete open reading frame (ORF) of *PkCPR*, the rapid amplification of cDNA ends (RACE) method was performed by using the SMART™ RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA), in accordance with the manufacturer's instructions. For amplification of 5' end of *PkCPR*, *PkCPR*-5R1 and UPM (Universal Primer A Mix; Table 1) were used as primers and 5' RACE-Ready cDNA as template. For nested PCR amplification, *PkCPR*-5R2 and NUP (Nested Universal Primer A; Table 1) were used as nested PCR primers and product of the primary PCR

Table 1 List of primers used in the study

S. no	Primer code	Primer sequence (5'-3')	Direction
1.	Degenerate		
2.	DEGCPR-1 F	GAGCCACIGATAATGCTGCN(A/C/T/G)A(C)G	Forward
3.	DEGCPR-1R	TTGCY(C/T)GAW(A/T)GGR(A/G)AAM(C/A)CAGCCA	Reverse
	RACE		
4.	<i>PkCPR</i> -5R1	TCCCTCGGTAAACCATTTGTAAAATCTC	Reverse
5.	<i>PkCPR</i> -5R2	GCCAAGACCAAATATACCATACTGAAGA	Reverse
6.	<i>PkCPR</i> -3R1	ATGTTGCAGTAAGAAAGAGCTCACAGTC	Forward
7.	<i>PkCPR</i> -3R2	GAATTCGACATATCCGGCAGTGGAAAT	Forward
8.	UPM ^a	Long: 5'-CTAAT ACGAC TCACT ATAGG GCAAG CAGTG GTATC AACGC AGAGT-3'	Forward/Reverse
9.	NUP ^a	Short: 5'-CTAAT ACGAC TCACT ATAGG GC-3'	Forward/Reverse
	Expression		
10.	CPR-F	CGGGATCCATGCAATCCACGCCGAGAAACT	Forward
11.	CPR-R	TTGCGGCCGCTCACCATACATCACGTAAATACC	Reverse
	Real-time PCR		
12.	RT-CPR-F	TATTTGGTCTTGGCAACAGA	Forward
13.	RT-CPR-R	CCCACTGGGACAAGCCGTTT	Reverse
14.	β-ACTIN-F	GAGAGTTTGTATGTCCCTGCCATG	Forward
15.	β-ACTIN-R	CAACGTCGCATTTTCATGATGGAGT	Reverse
	Genome walking		
16.	GWCP-1	AGTTTCTCCGGCGTGGATTGCAT	Reverse
17.	GWCP-2	TGAATTGGGTGAGACAGACTGA	Reverse
18.	WALKER-AP1 ^a	GTAAT ACGAC TCACT ATAGG GC	Forward
19.	WALKER-AP2 ^a	ACTAT AGGGC ACGCG TGGT	Forward

^a These primers were provided with the kit. Primers used in the study were synthesised by Integrated DNA Technologies (<http://www.idtdna.com/site>)

amplification was used as template. Both primary and the nested PCR amplification procedures were carried out under following conditions: 3 min at 95 °C, 39 cycles (30 s at 95 °C, 35 s at 67 °C, 3 min at 72 °C) and 8 min at 72 °C.

Similarly, for isolation of the 3' end of *PkCPR* gene, 3' RACE-Ready cDNA was used as template, PkCPR-3R1 and UPM (Universal Primer A Mix; Table 1) were used as primers. For nested PCR amplification, PkCPR-3R2 and NUP (Nested Universal Primer A; Table 1) were used as nested PCR primers, while product of the primary PCR amplification was used as template. Both primary and the nested PCR amplification procedures were carried out under following conditions: 2 min at 95 °C, 35 cycles (30 s at 95 °C, 45 s at 65 °C, 2 min at 72 °C) and 8 min at 72 °C.

The amplification products of both 5' RACE PCR and 3' RACE PCR were purified and cloned into pTZ57R/T TA cloning vector (Fermentas, Burlington, Canada). The ligation mixtures were transformed into *E. coli* cells (New England Biolabs, Ipswich, MA, USA). The clones (20 colonies each) were picked individually and grown 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, WI, USA) and sequenced using M₁₃ primers. DNA sequencing was performed with ABI PRISM 3130XL Genetic Analyser and Big Dye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). The full-length cDNA of *PkCPR* was generated by comparing and aligning the sequences of the core fragments, 5' RACE and 3' RACE products.

Computational analysis of *PkCPR*

The full length nucleotide sequence of *PkCPR* was translated using Translate Tool (<http://www.expasy.ch/tools/dna.html>) and the properties of deduced amino acid sequence was estimated using ProtParam (<http://www.expasy.ch/tools/protparam.html>), SPLIT v.4.0 (<http://split.pmfst.hr/split/4/>), TMHMM (<http://www.cbs.dtu.dk/services/>) and Phobius (<http://www.ebi.ac.uk/Tools/pfa/phobius/>) programs. BLAST (<http://www.ncbi.nlm.nih.gov>) was used to find similarity of amplified CPRs in Genbank database. Translate tool (<http://www.expasy.ch/tools/dna.html>) was used to predict the ORF. Predictions of *N*-glycosylation and chloroplast targeting sites were computed using NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc/>) and ChloroP (<http://www.cbs.dtu.dk/services/ChloroP/>). ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) was used for multiple sequence alignment. To assess the evolutionary relationships between the *PkCPR* and CPR homologs from different plants species, sequences were retrieved using BLASTp searches using *PkCPR* as query. Neighbour-joining tree was constructed with MEGA 5.10 software (Tamura et al. 2011). Bootstrap analysis with 1,000 replicates was also conducted in order to obtain confidence levels for the branches.

Heterologous expression of *PkCPR* in *E. coli*

For the heterologous expression and functional validation of *PkCPR*, full-length cDNA was amplified by PCR using a High-Fidelity proofreading DNA polymerase system (New England Biolabs, Herts, UK). Both forward (PkCPR-F) and reverse primers (PkCPR-R; Table 1) were engineered to introduce *Bam*HI and *Not*I restriction sites at the beginning and end of the coding sequence, respectively. The PCR conditions used were: One cycle of 98 °C for 1 min, 39 cycles of 98 °C for 15 s, 60 °C for 30 s, 68 °C for 3 min. The final extension was at 68 °C for 10 min. The resulting 2,133-bp fragment was purified and ligated into the pJET blunt vector (Fermentas, St. Leon-Rot, Germany), subcloned in *E. coli* (DH5 α) and then transferred into *Bam*HI and *Not*I restriction sites of the bacterial expression vector pGEX-4T-2. *E. coli* BL21 (DE3) cells were transformed with pGEX-*PkCPR* expression cassette. A single colony of each recombinant culture was inoculated separately into 10 ml of LB broth containing 100 μ g/ml of ampicillin and incubated overnight at 37 °C. Then, 1 % of the overnight culture was transferred into 100 ml of LB medium containing the corresponding antibiotic and incubated at 37 °C, until optical density (OD) (A_{600} nm) reached 0.4–0.5. Protein expression was induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG; Fermentas, Burlington, Canada) into the cultures at the concentration of 0.2–1 mM. The cultures were constantly incubated at 20–37 °C for 8–12 h. The induced bacterial cells were harvested at an interval of 2 h by centrifugation and resuspended in 6 $\times$ 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 protein was analysed on 10 % SDS-PAGE.

Purification of *PkCPR*

Protein expression was induced by addition of 0.8 mM IPTG when OD₆₀₀ of the secondary culture was around 0.4. Cells were grown for further 8–12 h at 20–37 °C and then harvested by centrifugation (6,000 $\times$ g at 4 °C for 10 min; Eppendorf, Hamburg, Germany). Pelleted cells were resuspended in the 1 $\times$ PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 10 mM KH₂PO₄, pH 7.3) and lysed by adding 100 mM DTT followed by lysozyme (100 mg/ml) for 45 min on ice. The lysates were sonicated four times for 30 s each time (amplitude 1, 20 % duty cycle) using a probe sonicator (Sartorius, Gottingen, Germany). Triton X-100 (10 %) was added to the lysates and incubated for 30 min on ice. Furthermore, soluble and insoluble fractions were separated by centrifugation (14,500 $\times$ g at 4 °C for 15 min). The supernatant was incubated overnight with glutathione-sepharose beads (1 ml l⁻¹ of culture) (GE Healthcare, Little Chalfont, UK) at 4 °C. The beads were washed five times with 10 bead volumes of 1 $\times$ PBS and

the fusion protein was eluted with a buffer containing 10 mM reduced glutathione/50 mM Tris–HCL pH 8.0/5 % glycerol. To remove the glutathione *S*-transferase (GST) moiety, thrombin (4 U/ml of beads) was added to the beads, and cleavage was allowed to proceed for 10–12 h at 4 °C. The beads were pelleted (600×g at 4 °C for 5 min), supernatant containing *PkCPR* protein was incubated overnight further with benzamidine beads (Sigma Aldrich, St. Louis, MO, USA) to remove the thrombin. The purified protein samples were analysed on 10 % SDS-PAGE and their concentration was directly measured on spectrophotometer.

NADPH-reductase assay

Activities of purified NADPH-reductases were determined by the reduction of cytochrome *c* (20 mM) in presence of NADPH (100 mM) as described earlier (Rana et al. 2013). All assays and incubations were carried out in 300 mM potassium phosphate buffer, containing 0.1 mM EDTA, pH 7.8 at 25 °C. The rate of reduction was monitored by increase of absorbance at 550 nm. For calculating the reduction rate of cytochrome *c*, a specific molar absorption coefficient ($21.1 \text{ mM}^{-1} \text{ cm}^{-1}$) of equine heart cytochrome *c* was used. Furthermore, for measurement of kinetic parameters, NADPH (100 mM) with varying concentrations of cytochrome *c* (10–250 mM) was used in the reaction mixture. The kinetic constants K_m and V_{max} , were calculated with nonlinear regression analysis using GraphPad Prism 5 software.

Gene expression analysis

About 2 µg of total RNA was used to synthesize first strand cDNA primed with Oligo(dT) in a 20 µl reaction mix using Revert-aid Premium M-MuLV reverse transcriptase (Fermentas, Burlington, Canada) following manufacturer's instructions. Real-time PCR reactions were performed in triplicates using SYBR *Premix Ex Taq* (Takara, Dalian, China) in 48-well optical plates using ABI StepOne Real-time qPCR system (Applied Biosystems). The PCR reaction (20 µl) included 0.2 µl cDNA template, 200 nM each of the primers (Table 1), and 10 µl 2× SYBR *Premix Ex Taq*. The cycling parameters were 95 °C for 20 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of each primer pair was validated by a dissociation curve (a single peak was observed for each primer pair). The primers used for real-time PCR analysis were designed with the help of Primer Express Version 3.0. (Applied Biosystems). A constitutive active gene, β -actin was used as endogenous control (β -ACTIN-F and β -ACTIN-R). To study the modulation of expression of *PkCPR* by MeJA, SA, 2,4-D and UV-B, leaves were excised at different time intervals and analysed for mRNA transcript abundance. The experiments were repeated thrice and the data were analysed statistically ($\pm$ SD and two-

tailed Student's *t*-test). The real-time PCR amplification data were exported into Microsoft Excel and gene expression levels were calculated based on the comparative CT method.

Chemoprofiling of *P. kurrooa* using HPLC

HPLC analysis of *P. kurrooa* was performed as previously mentioned (Bhat et al. 2013). In brief, plant samples were completely dried under gentle air stream (temperature 25 ± 2 °C and relative humidity 65 ± 5 %). The samples were ground into fine powder with a mortar and pestle. The powdered samples (10 g of each sample) were serially extracted with ethanol: water (1:1) for 3 h at 25 ± 2 °C. The aqueous ethanolic extracts were filtered and dried by evaporation at reduced pressure using rotary evaporator at 40 °C. The crude extracts of all the samples were dissolved in the methanol (20 mg/ml) and were filter sterilized with 0.22 µm membrane filters (Millipore, Bedford, MA, USA). Stock solutions of the pure reference compounds (1 mg/ml) were prepared in HPLC grade water and stored in a refrigerator at 4 °C. From the stock solutions, working solutions for each reference compound were prepared by dilution with HPLC-grade water. These working solutions of both the reference compounds (5 µl of each) were mixed together in equal volumes for further analysis. A 10-µl aliquot of this solution was taken for the calibration curve. The HPLC (Shimadzu CLASS-VP V 6.14 SPI model) equipped with RP-18e column (E-Merck; 5 µm, 4.6×250 nm), a photo-diode array detector (SPD-M10AVP model) and a pump (LC-10AT VP model) was used for analysis of marker compounds. The analysis was carried out using a mobile phase of methanol: water (2:3) which was delivered at a flow rate of 0.7 ml/min. The detection wavelength was set at 270 nm for picroside I and picroside II, while as the detection wavelength for feruloylcatalpol, androsin and apocynin was 283 nm. Injection volume of the sample was 10 µl and the column temperature was 30 °C.

Promoter cloning and analysis

GenomeWalker DNA libraries were constructed following the user manual provided by the manufacturer (Universal GenomeWalker™ Kit; Clontech). Genomic DNA of *P. kurrooa* was isolated from fresh leaves using DNeasy Plant mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol with minor modifications (Bhat et al. 2012c). Purified genomic DNA (3 µg) was digested with *Dra*I, *Eco*RV, *Pvu*I and *Stu*I, respectively, and recovered using a PCR purification Kit (Qiagen). PCR-purified digested DNA samples were then ligated separately to the AP adaptor (provided with the kit) to construct the genome walking libraries. For isolation of the *PkCPR* gene promoter, the primary PCR was performed using AP1 (provided with the kit) and GWCPR-1 as primers, and constructed libraries as template

via the following protocol: 5 cycles at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 3 min; 30 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 3 min; and a final extension at 72 °C for 7 min. The product was diluted 10-fold and used in nested PCR, which was performed using AP2 (provided with the kit) and GWCP2 as nested primers under the following conditions: 10 cycles at 94 °C for 30 s, 67 °C for 30 s and 72 °C for 3 min; 30 cycles at 94 °C for 30 s, 62 °C for 30 s and 72 °C for 3 min; and a final extension at 72 °C for 8 min. Putative *cis*-acting elements upstream to start codon of the *PkCPR* were identified by searching PlantCare (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>), PLACE (<http://www.dna.affrc.go.jp/PLACE/>) and PLANTPAN (<http://plantpan.mbc.nctu.edu.tw/>) databases.

Homology modelling and prediction of three-dimensional structure of *PkCPR*

The three-dimensional structure of *PkCPR* was constructed using PHYRE2 server (Protein Homology/analogy Recognition Engine V 2.0) (<http://www.sbg.bio.ic.ac.uk/phyre2/html/>) using the crystal structure of *Rattus norvegicus* (PDB ID: 1J9Z) as template. Protein model refinement was performed using KoBa^{MIN}Server2012 (Knowledge-based Potential Refinement for Proteins; (<http://csb.stanford.edu/kobamin/>)). The stereo-chemical analysis of the modelled protein was carried out using Ramachandran plot obtained from Procheck module of the SAVES server (<http://services.mbi.ucla.edu/SAVES/>). Structurally, evolutionary and functionally important regions were identified in deduced protein sequence by ConSurf (<http://consurf.tau.ac.il/>). Topology of the modelled *PkCPR* protein was analysed using PDBSum (<http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html>).

Results

Cloning of full length cDNA encoding *PkCPR*

A homology based strategy was employed to identify CPR gene from *P. kurrooa*, taking advantage of the highly conserved amino acid domains present in plant CPRs. A 600-bp fragment of the *PkCPR* gene was amplified by PCR using degenerate primers derived from the FAD and NADPH binding domains, respectively. The fragment exhibited a high degree of homology with other plant CPR genes. Subsequently, 3' and 5' RACE PCR generated a 1,500- and a 778-bp fragment, respectively. From overlapping sequences of three fragments, the full-length *PkCPR* gene was obtained, which was subsequently confirmed by sequencing. It was 2,679 bp long and contained a 2,133-bp ORF encoding for a 710-amino-acid protein. It was flanked by 144-bp 5' untranslated region (UTR) and 402-bp 3' UTR (Fig. S1), including a poly (A) tail. The nucleotide sequence

of full-length cDNA of *PkCPR* identified from *P. kurrooa* was submitted to NCBI GenBank under accession number JN968968.

Computational analysis of *PkCPR*

Using the ExPASy online tool, the theoretical isoelectric point (pI) and molecular weight of the deduced *PkCPR* protein was predicted to be 5.45 and 78.6 kDa, respectively. Furthermore, the Self-Optimized Prediction Method with Alignment (SOPMA) online tool was used to predict the secondary structure of *PkCPR*. SOPMA indicated that *PkCPR* is predominantly an α -helical protein, consisting of α -helices (42.25 %) and random coils (39.15 %), while extended strands (13.8 %) and β -turns (4.79 %) are also present. Protein sequences when subjected to analysis for glycosylation sites were found to have two glycosylation sites for *PkCPR* (at positions 27 and 286). For the prediction of chloroplast localization, *PkCPR* amino acid sequence was analysed in silico with ChloroP program. Scores for potential chloroplast targeting of CPR was 0.493 for *PkCPR*. There was high serine content at N-terminal in *PkCPR*, which is predicted to be required for chloroplast targeting. Sub-cellular localization study of CPR proteins in hybrid poplar has been experimentally demonstrated that they are confined to ER only. However, in silico prediction does not rule out the possibility of their localization to chloroplast membrane also. Similarity search showed that *PkCPR* shares 68–83 % identities with CPRs from other plant species. On the basis of N-terminal anchoring sequences, *PkCPR* contained extended amino acid sequence at N-terminal, thus conforms to Ro et al. (2002) classification of Class II CPRs. By aligning deduced sequences of *PkCPR* with other CPRs from taxonomically diverse species, all functional domains involved in the binding of the P450 monooxygenase, and the cofactors of FMN, FAD and NADPH, were identified (Fig. 2). At the N-terminal end of each CPR, there is a membrane anchor region which is essential for normal interaction between CPR and P450 monooxygenases. It was also predicted by Phobius and TMHMM programs (Fig. S2). FMN domain is connected to FAD domain via a flexible linker region and NADPH-binding domain is present near C-terminal end (Fig. 2). *PkCPR* contains a conserved acidic amino acid motif (LGDDDQCIEDD) which is proposed to interact with cytochrome *c* and P450s, existing nearby FMN domain. The linker region which joins the FAD and FMN somehow allows the conformational changes in the position of FMN domain to interact with various P450s. Principally, the N-terminal membrane anchoring region shows the highest divergence, while as the rest of the protein is conserved among plant CPRs (Fig. 2).

To investigate the degree of evolutionary relatedness, neighbour-joining phylogenetic tree was constructed with MEGA 5.10 software from the ClustalW2 alignment of *PkCPR* with number of CPR orthologs from different plants,

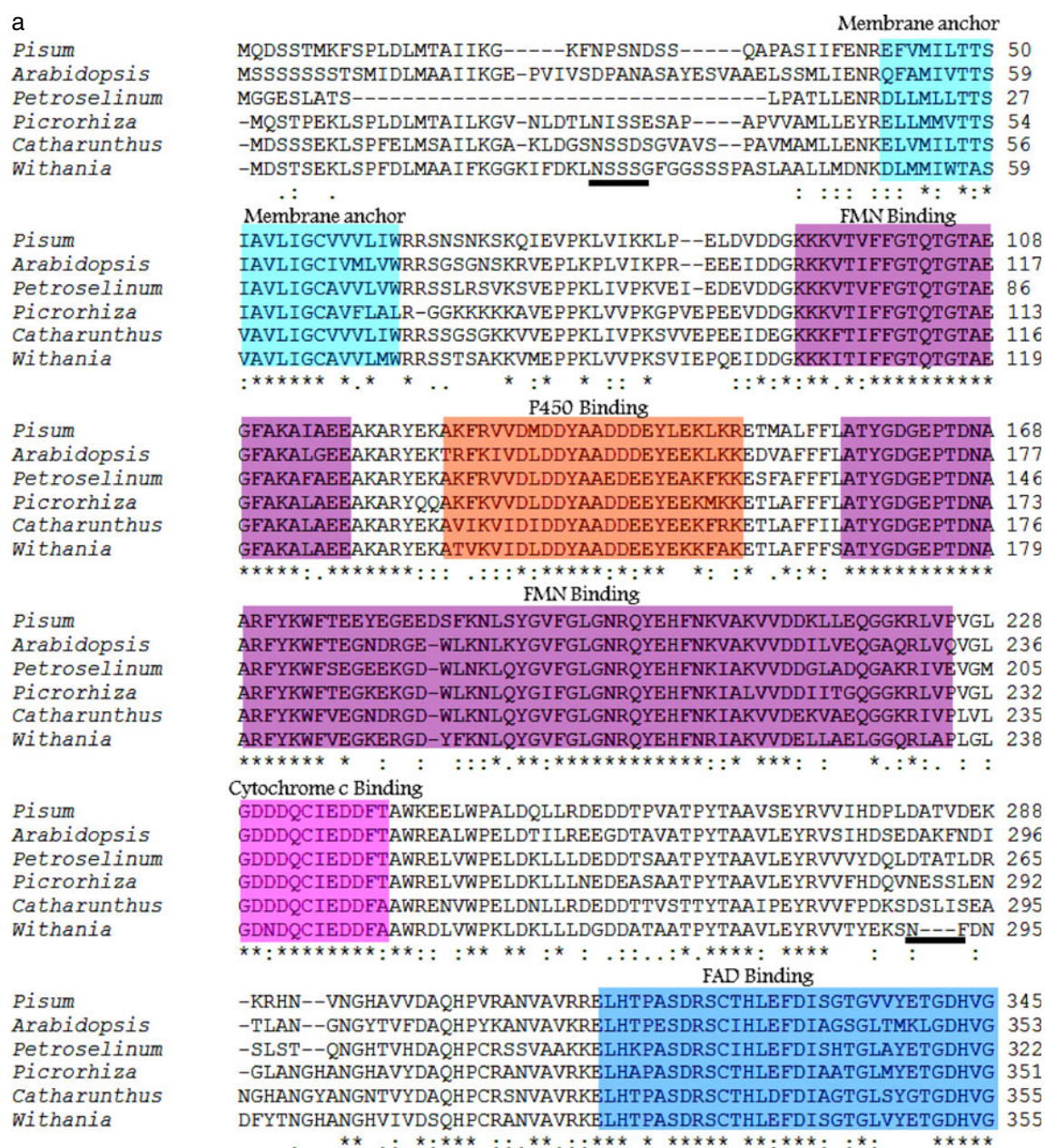


Fig. 2 Multiple sequence alignment of deduced amino acid sequences of *PkCPR* with other plant orthologs using ClustalW2 multiple alignment tool. The sequences were from *Picrorhiza kurroa* (AGA84059.1), *Petroselinum crispum* (AAB97736), *Arabidopsis thaliana* (NP_194750), *Catharunthus roseus* (Q05001), *Withania somnifera* (ADG29353) and *Pisum sativum* (AAC09468). Conserved regions and binding sites are shaded. Underlined residues are possible *N*-

glycosylation sites at positions 27 and 286 (**a**). Schematic representation of the key domains of *PkCPR*. *ANC* membrane anchor sequence, *FMN* flavin mononucleotide binding domain, *P450* cytochrome P450 binding domain, *CYT C* cytochrome *c* interacting domain, *FAD* flavin adenine dinucleotide binding domain, *LINKER* FAD linking region, *NADPH* nicotinate amide dinucleotide phosphate binding domain (**b**)

retrieved from the NCBI GenBank database. As depicted in phylogenetic tree (Fig. S3), *PkCPR* showed high amino acid sequence identity to *Perilla frutescens* (ADC94831), *Salvia miltiorrhiza* (AGL46979), *C. roseus* (Q05001), *P. crispum* (AAB97737; AAB97736), *W. somnifera* (ADI49691; ADG29353), *Pisum sativum* (AAC09468), *A. thaliana* (CAA46814; NP_194750), *Gossypium hirsutum* (ACN54323; ACN54324) and *Petunia × hybrida* (AAZ39648; AAZ39649).

The topology of the phylogenetic tree is generally in good agreement with the traditional taxonomy classification. *PkCPR* was closely related to CPRs from *P. frutescens* and *S. miltiorrhiza*. Both these species along with *P. kurroa* belong to the order Lamiales. Furthermore, phylogenetic analysis clustered *PkCPR* in Class II of CPRs in accordance with the amino acid similarity among their proteins (Fig. S3).

<i>Pisum</i>	VYCNLS	SDTVEEAERILGLSPDTYLSIHTDDEEGKPLGGSSLPFPFPCTLRALTALTRYAD	405
<i>Arabidopsis</i>	VLCDNL	SETVDEALRLLDSPDTYFSLHAEKEDGTPIS-SSLPPFPFPCTLRALTALTRYAC	412
<i>Petroselinum</i>	VYCNLI	VEIVEEAERLLGMQNTYFSVHIDDEDGTPLTGGSLPFPFPCTVRSLALAKYAD	382
<i>Picrorhiza</i>	VYCNLI	ENVEEAERLLNMPQTYFSIHTDKEDGTPLSGSSLPFPFPCTLRALTALTRYAD	411
<i>Catharunthus</i>	VYCDNL	SETVEEAERLLNLPETYFSLHADKEDGTPLAGSSLPFPFPCTLRALTALTRYAD	415
<i>Withania</i>	VYCNLI	VETVEEAERLLNISPDTFFSIHTDKEDGTPGGSLPFPFPCTLRALTALTRYAD	415
		* : *	
<i>Pisum</i>	LLSSPKKS	SALVALAAHASDPSEADRLRLHASPAGKDEYAEWISSQSRSLLEVMAEFSSAK	465
<i>Arabidopsis</i>	LLSSPKKS	SALVALAAHASDPTEAERLKLHASPAGKDEYSKWVVSQSRSLLEVMAEFSSAK	472
<i>Petroselinum</i>	LLSSPKKS	SALLALAAHASDPTEADRLRLHASPAGKDEYAQWVVASHRSLLEVLAEFSSAK	442
<i>Picrorhiza</i>	LLSAPKK	STLVALAAYASDLNEADRLRLHASPVGKEEYTYIILTNMKSLLLEVMAEFSSAK	471
<i>Catharunthus</i>	LLNTPKK	SALLALAAAYASDPNEADRLKYLASPAGKDEYAQSLVANQSRSLLEVMAEFSSAK	475
<i>Withania</i>	LLSSPKKS	SALLALAAACASDPNEADRLRYLVSPAGKEEYAQWIVASQSRSLLEVMEGFH--Q	473
		** : *	
		FAD Binding	NADPH Binding
<i>Pisum</i>	PPIGVFFA	AVAPRLQPRYYSISSSPRMAPSRIH-VTCALVHDKMPTGRIHKGVCSTWMKN	524
<i>Arabidopsis</i>	PPLGVFFA	GAVAPRLQPRFYYSISSSPKIAETRIH-VTCALVYEKMPGRIHKGVCSTWMKN	531
<i>Petroselinum</i>	PPLGVFFA	SVAPRLQPRYYSISSSPRMVPSRIH-VTCALVYEKTPGRIHKGVCSTWMKN	501
<i>Picrorhiza</i>	PPLGVFFA	SVAPRLQPRFCSISSSPKIAPNRIH-VTCALVYEKTPGRIHKGVCSTWMKN	530
<i>Catharunthus</i>	PPLGVFFA	AIAPRLQPRFYYSISSSPRMAPSRIH-VTCALVYEKTPGGRIHKGVCSTWMKN	534
<i>Withania</i>	PSLQLVFS	LLLLLALYNRDSTLSHLLGWRHLESMFVHWFTTRCQLDVHKGVCSTWMKN	533
		* : *	
		NADPH Binding	
<i>Pisum</i>	SVPLEKNQ	DCSWA-PIFVRQSNFRLPADNKVPVIMIGPGTGLAPFRGFLQERLALKEDGA	583
<i>Arabidopsis</i>	AVPYEKSE	KLFLGRPIFVRQSNFKLPDSKVPIIMIGPGTGLAPFRGFLQERLALVESGV	591
<i>Petroselinum</i>	AVSLEESH	DCSWA-PIFVRQSNFKLPSTDKVPIIMIGPGTGLAPFRGFLQERQALKDAGA	560
<i>Picrorhiza</i>	AIPSEESL	DCSSA-PIFVRTSNFRLPADPKVPIIMIGPGTGLAPFRGFLQERLALKESGA	589
<i>Catharunthus</i>	AIPLEESR	DCSWA-PIFVRQSNFKLPADPKVPVIMIGPGTGLAPFRGFLQERLALKKEGA	593
<i>Withania</i>	AIPLEESL	SCSAA-PIFVRQSNFKLPADNKVPIIMIGPGTGLAPFRGFLQERLALKKEGA	592
		:: : *	
<i>Pisum</i>	ELGPSVL	FFGCRNRQVDYIYEDELNFVNGGALSELIVAFSRDGPTEKEYVQHKMMEKASD	643
<i>Arabidopsis</i>	ELGPSVL	FFGCRNRMDYIYEELQRFVESGALAELSVAFSREGPTKEYVQHKMMDKASD	651
<i>Petroselinum</i>	ELGTAVLY	FGCRNRNLDYIYEDELNFVSGSISELIVAFSREGPTKEYVQHKMLQKASE	620
<i>Picrorhiza</i>	ELGPAVL	FFGCRNRQMDYIYEDELNFVKGAVISELVAFSRQGPTEKEYVQHKMAQKASD	649
<i>Catharunthus</i>	ELGTAVFF	FGCRNRKMDYIYEDELNFLEIGALSELLVAFSREGPTKQYVQHKMAEKASD	653
<i>Withania</i>	GLGPAVL	FFGCRNRQMDYIYQDELNTFLEAGALSELVAFSREGPNKQYVQHKMTQKASD	652
		** : *	
		NADPH Binding	
<i>Pisum</i>	IWNMISQ	GAYVYVCGDAKGMDVHRTLHTILQEQQSLDSSKTESMVKNLQMTGRYLDRV	703
<i>Arabidopsis</i>	IWNMISQ	GAYLYVCGDAKGMDVHRTLHTIAQEQQSMDSTKAEGFVKNLQTSGRYLDRV	711
<i>Petroselinum</i>	IWNLISE	GAYIYVCGDAKGMDVHRTLHTIAQEQQGALDSSKAESWVKNLQMTGRYLDRV	680
<i>Picrorhiza</i>	IWNMISE	GAYVYVCGDAKGMDVHRTLHTIVQEQQSLDSSKTEALVKNLQMTGRYLDRV	709
<i>Catharunthus</i>	IWRMISD	GAYVYVCGDAKGMDVHRTLHTIAQEQQSMDSTQAEQFVKNLQMTGRYLDRV	713
<i>Withania</i>	IWNMISQ	GGHVYVCGDAKGMDVHRTLHTIAQDQGSMDSSNAESLVKNLQMTGRYLDRV	712
		** : *	
<i>Pisum</i>	W	704	
<i>Arabidopsis</i>	W	712	
<i>Petroselinum</i>	W	681	
<i>Picrorhiza</i>	W	710	
<i>Catharunthus</i>	W	714	
<i>Withania</i>	W	713	
		*	



Fig. 2 (continued)

Heterologous expression in BL21 cells and protein purification

To confirm whether *PkCPR* encodes for a functional protein, the full-length ORF of *PkCPR* was cloned in pGEX-4T-2 vector and expressed in *E. coli* BL21 (DE3) as a fusion protein with GST. The full length ORF of *PkCPR* was cleaved from

the pJET-*PkCPR* using *Bam*HI and *Not*I and the digested product was inserted into vector pGEX-4T-2. The expression of protein was observed at 20 °C using 0.8 mM IPTG after 8–16 h of induction. The fusion protein having molecular weight of 104.5 kDa was expressed by the pGEX-*PkCPR* and detected by SDS-PAGE analysis. The molecular weight was identical to the predicted molecular mass of the recombinant protein

composed of GST (26 kDa) and *PkCPR* (~77 kDa). Time-course study revealed that the optimum expression of *PkCPR*, as examined from SDS-PAGE profile was obtained after 6 h of induction at 20 °C (Fig. 3). The cloning of *PkCPR* in pGEX-4T-2 having N-terminal GST-tag allowed its expression in soluble fraction along with the membrane anchor region. The recombinant fusion protein having a GST-tag was purified using glutathione sepharose beads. The purified fusion protein band of *PkCPR* at 104.5 kDa in molecular mass was observed on SDS-PAGE. The GST-*PkCPR* fusion protein was cleaved by thrombin and purified using benzamidine beads (Fig. 3). The amount of purified *PkCPR* was 0.20 mg/ml.

NADPH-reductase assay

It has been earlier demonstrated that CPRs from different plant species have different specific activities and most of them have been assayed using microsomal fraction or truncated polypeptide (without membrane anchor). In this investigation, we were able to purify the protein along with its membrane anchor domain. Purified *PkCPR* used NADPH as electron donor for reducing its substrate cytochrome *c*, wherein

PkCPR was observed to possess a specific activity of $5.8 \pm 0.05 \mu\text{mol min}^{-1} \text{mg}^{-1}$ with 20 μM NADPH and 20 μM cytochrome *c*. For kinetic studies of *PkCPR*, the enzyme and NADPH were kept constant whereas the concentration of cytochrome *c* was taken in increasing order. As the substrate concentration was increased, the amount of products produced also increased. V_{max} and K_m of the purified protein was found to be $8.1 \pm 0.12 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and 7.8 μM , respectively. This is explained by the Michaelis–Menten plot (Fig. 4).

PkCPR is highly expressed in leaves of *P. kurrooa*

To investigate the *PkCPR* expression pattern in different tissues of *P. kurrooa*, total RNA extracted from leaf, inflorescence and rhizome tissues were used as the template in quantitative RT-PCR analysis. Results indicated that *PkCPR* expressed in a constitutive manner in the tissues of leaf, inflorescence and rhizome, with the maximum expression in leaves (Fig. 5). There was more than a 7-fold increase in the transcript abundance in leaves as compared to the rhizomes. Also the mRNA expression in the young inflorescence of *P. kurrooa* was around 4-fold higher in comparison to rhizomes.

Effect of altitude on chemical profile of *P. kurrooa*

Altitude has a pronounced effect on the accumulation of secondary metabolites in higher plants. In addition to incurring many climatic differences, altitude influences the quality of radiation, especially UV-B radiation is high in alpine niches compared with lower habitats (Barnes et al. 1987). The higher solar radiation at higher altitudes has often been implicated to influence secondary metabolite profiles. For example, an increase in phenolic compounds with altitude as a response to increasing UV radiation has been demonstrated (Turunen and

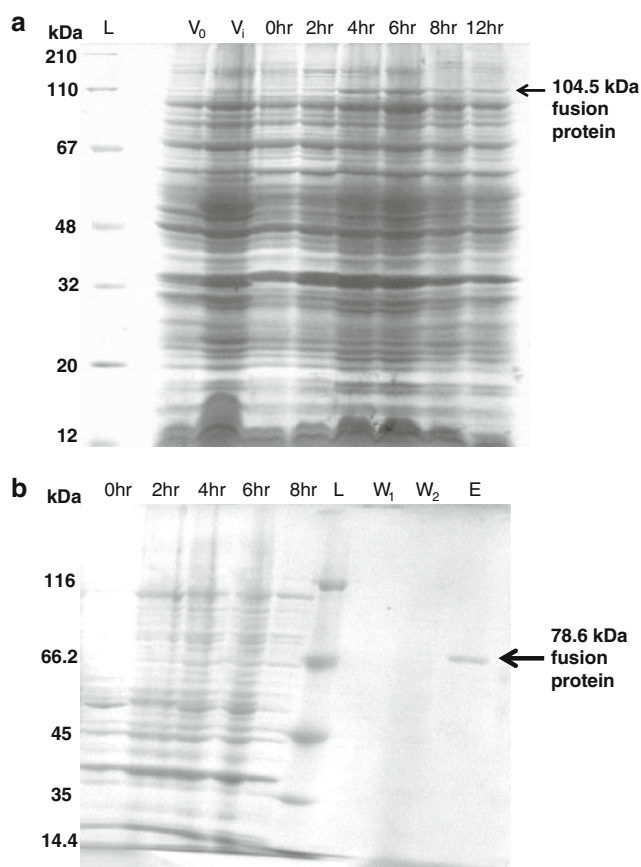


Fig. 3 SDS-PAGE profile of purified recombinant protein. SDS-PAGE (10 %) of purified recombinant protein from *E. coli* BL21 transformed with pGEX-*PkCPR*

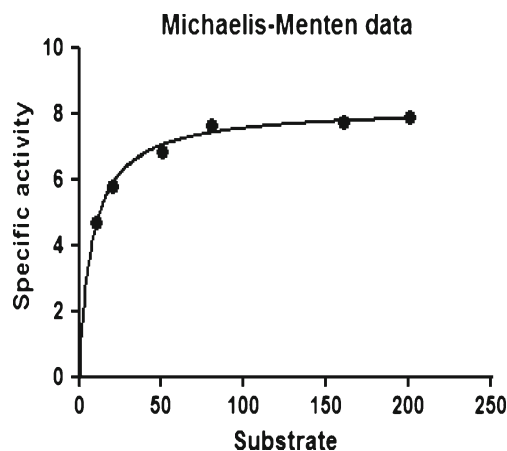


Fig. 4 Steady-state kinetic constants of recombinant *PkCPR* at 25 °C, pH 7.4. Determination of kinetic parameters for cytochrome *c* was performed in a reaction mixture containing 100 μM NADPH and various amounts of cytochrome *c*

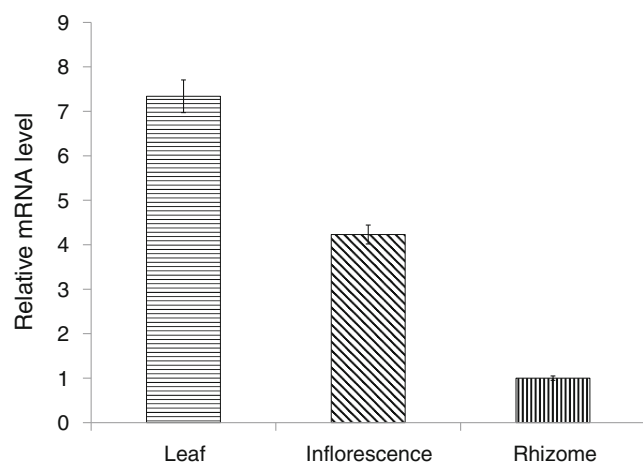


Fig. 5 Expression characteristics of *PkCPR* in different tissues, including leaves, rhizome and inflorescence. Data were normalised to abundance of endogenous control actin and are shown as the mean of three replicate reactions with standard deviations illustrated as vertical bars

Latola 2005). In the present study, five marker compounds including picroside I, picroside II, feruloylcatalpol, androsin and apocynin were quantified in samples collected from three different altitudes in the North-Western Himalayas. HPLC results revealed variations in the distribution and relative content of all the marker compounds extracted from three different altitudinal populations. This variation was found associated with both organ/plant parts and altitude. The highest content of picroside I (7.07 % D.W.B.) and picroside II (5.83 % D.W.B.) was found in leaf and rhizome tissues of Dhanwas population, respectively (3,500 m) (Fig. 6a and Fig. S4). Furthermore, feruloylcatalpol, which is also produced via the iridoid pathway, also showed highest content in the leaf sample of *P. kurroa* collected from Dhanwas area (0.1 % D.W.B.). Apocynin content was found to vary between 0.03 % D.W.B. (Leaf-Dhanwas) to 0.065 % D.W.B. (rhizome-Dhanwas), while as maximum content of androsin was found in the leaf tissues of the samples collected from Yarikah 0.08 % D.W.B. (Fig. 6b). Metabolite content was also found to vary spatially. Rhizome from plants of all altitudes had maximum content of picroside II than leaves while as Picroside I content in the leaves were maximum (Fig. 6a).

PkCPR shows differential expression pattern at different altitudes

Picroside accumulation was found to be higher at high altitude, owing to the low temperature in the upper reaches of the Himalayas and/or high UV-B as shown in Fig. 6. Therefore, it was tempting to find out the transcript variation in the expression of *PkCPR* at different altitudes. Plant samples were collected from three different altitudinal gradients of Kashmir Himalayan range. RNA was extracted from leaf and rhizome tissues and analysed for transcript abundance. The expression level of

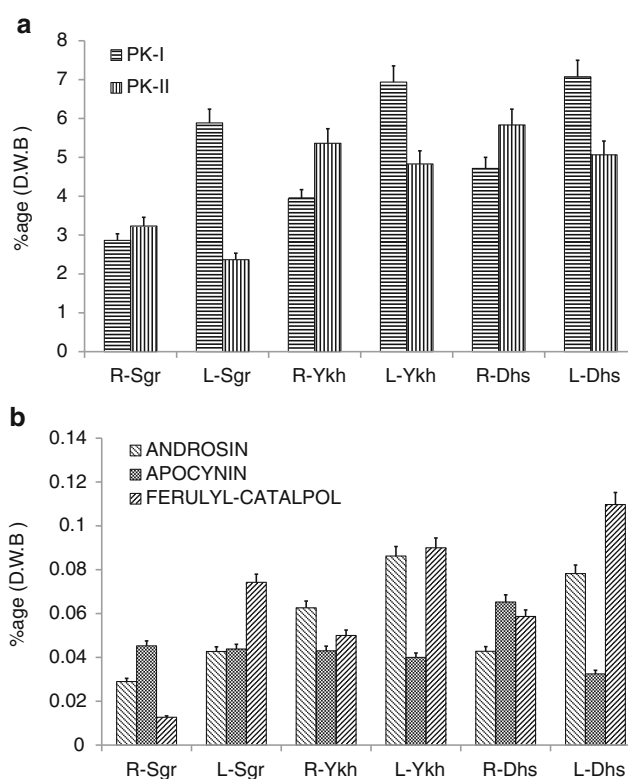


Fig. 6 Differential accumulation pattern of picroside I (PK-I) picroside II (PK-II), apocynin, androsin and feruloylcatalpol in response to varying altitudinal gradients. *R-Sgr* Rhizome-Srinagar (1,730 m asl), *L-Sgr* Leaf-Srinagar (1,730 m asl), *R-Ykh* Rhizome-Yarikah (2,400 m asl), *L-Ykh* Leaf-Yarikah (2,400 m asl), *R-Dhs* Rhizome-Dhanwas (3,500 m asl), *L-Dhs* Leaf-Dhanwas (3,500 m asl). Samples were analysed using HPLC and all the values obtained were means of triplicate with standard errors. Time course accumulation was statistically significant at $P < 0.001$ level

PkCPR was highest in plant samples collected from Dhanwas (3500 m asl) followed by Yarikah (2400 m asl) and the expression of *PkCPR* was lowest in the Sonamarg samples that were habituated and cultivated at a lower altitude of Srinagar (1730 m asl). Our results show a positive correlation between the altitude and the expression level of *PkCPR* (Fig. 7).

Promoter isolation and identification of *cis*-regulatory elements

To elucidate the transcriptional regulation of the *PkCPR* via its 5' flanking promoter element, the DNA sequence of the 5' flanking region of *PkCPR* was identified using genome walking approach and scanned for various putative *cis*-regulatory elements using in silico tools. Promoter analysis revealed *PkCPR* to be a highly regulated gene at the transcriptional level, responsive to various abiotic and biotic elicitors including MeJA, SA, light, temperature, oxygen, auxins, and fungal elicitors. In a 1,058-bp promoter region of *PkCPR*, the predicted transcription start site (TSS; +1) was identified 144 bp upstream of the ATG translation initiation codon, by

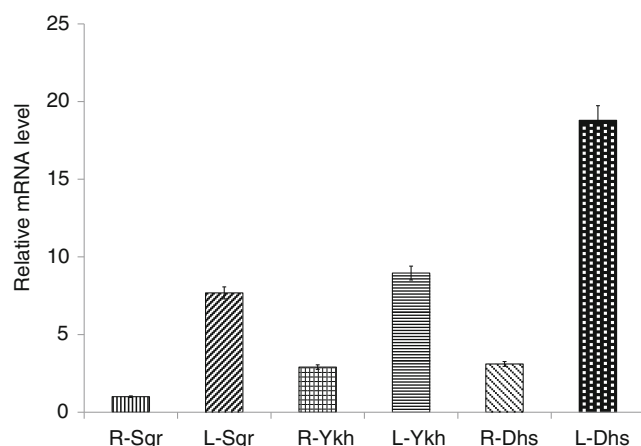


Fig. 7 Differential expression pattern of *PkCPR* in response to varying altitudinal gradients. *R-Sgr* Rhizome-Srinagar (1,730 m asl), *L-Sgr* Leaf-Srinagar (1,730 m asl), *R-Ykh* Rhizome-Yarikhah (2,400 m asl), *L-Ykh* Leaf-Yarikhah (2,400 m asl), *R-Dhs* Rhizome-Dhanwas (3,500 m asl), *L-Dhs* Leaf-Dhanwas (3,500 m asl). 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. Differences were scored as statistical significance at the * $P < 0.05$ and ** $P < 0.01$ levels

sequencing and analysing a number of colonies of full length 5' RACE product. The promoter region contained high A+T content (65.1 %), typical of other plant promoters. Further, sequence analysis of the putative *cis*-acting elements was performed using PLACE and PlantCare promoter databases and the results are depicted in Fig. S5. Functions of in silico identified putative *cis*-acting elements are summarized in Table 2. Putative TATA box and putative CCAAT box were found 19 and 147 bp upstream of the transcriptional start site, respectively. The promoter of the rat P450 reductase gene has been shown to possess neither a TATA nor a CCAAT box, but contains GC-rich consensus sequence for the transcription factor Sp1 and is similar to those of housekeeping genes. In contrast to the rat CPR promoter, both the TATA and CCAAT boxes, along with Sp1 consensus sequences, were found in the *PkCPR* promoter. It was noteworthy that two key *cis*-regulators including LTR elements and GATA boxes were identified in the promoter region of *PkCPR*. These key sites have been earlier identified in some other genes of picroside biosynthesis (Bhat et al. 2013; Kawoosa et al. 2010, 2013) and have been linked to the picroside biosynthesis in a manner that light and low temperature positively regulate the picroside biosynthesis (Kawoosa et al. 2010, 2013). Two types of *cis*-acting elements related to light responsiveness were found in *PkCPR* promoter region, including three ACE-motif/G-boxes and two ATCT-motif/GAP-boxes. Search for promoter boxes also resulted in the detection of several *cis*-elements including light, sugar, hormone, stress and a meristem-specific responsive elements (Table 2; Fig. S5). Bioinformatic analysis also revealed the presence of several motifs essential for tissue-specific expression and other putative *cis*-acting motifs

involved in various biological processes such as MYB-binding site and W-box recognizing WRKY-type transcription factors.

PkCPR expression is inducible by MeJA, SA, 2,4-D and UV-B

Information regarding the regulation of picroside biosynthesis encompassing the various signal transduction pathways and transcription factors involved is unavailable. The recent advances in the understanding of plant signalling pathways has paved the way for using elicitor-induced plant metabolite variation as a prognostic tool for prospective pathway engineering and regulation based studies. Thus, to understand the inducible or constitutively active transcriptional nature of *PkCPR*, and to corroborate these results with the identified important *cis*-regulatory elements, *PkCPR* transcripts were assayed in response to MeJA, SA, UV-B and 2,4-D elicitors. MeJA is known to induce the biosynthesis of many secondary metabolites that play important role in the adaptation of plants to biotic stresses (Martin et al. 2002; Tripathy et al. 2012). In the present study, MeJA up-regulated the expression of *PkCPR* significantly and the mRNA level peaked at 6 h of treatment, with about 6-fold increase in the mRNA transcripts. A similar trend has been observed in *Artemisia annua* (Zhang et al. 2012). Afterwards, a decrease in *PkCPR* transcript level was seen at 12 h which continued up to 48 h. However, even after 48 h the transcript abundance was 4-fold higher than the untreated control. The time-course of *PkCPR* expression is shown in Fig. 8a.

SA belongs to a group of phenolics with wide and ubiquitous distribution among plants. It has been shown to regulate a large variety of physiological processes. In recent years, SA has received particular attention, because it is essential for expression of multiple modes of plant stress resistance (Clarke et al. 2004). In the present study, SA elicited differential increase in *PkCPR* transcript levels. It showed a maximum of 3.4-fold increase in the expression profile, 12 h post induction (Fig. 8b). Transcript abundance was less obvious with exogenous application of 2,4-D in comparison to the effect of MeJA or SA (Fig. 8c). *PkCPR* showed a delayed 3-fold increase in the transcript level after 48 h of induction.

To understand the effect of UV on the expression of *PkCPR*, we treated in vitro raised plantlets with UV-B radiations. The results showed a gradual increase up to a maximum of 5-fold increase in the transcript levels of *PkCPR* 12 h post induction, followed by a steady decrease up to 48 h (Fig. 8d). CPR gene has been found to be induced by UV light in some of the earlier studies as well (Brosché et al. 1999). Solar UV-B radiation flux increases with high elevation, because there is less air mass and greater transparency of the atmosphere to shorter wavelength radiation (Madronich et al. 1998).

Table 2 Putative *cis*-acting regulatory elements identified in the promoter of *PkCPR* using PLACE (<http://www.dna.affrc.go.jp/PLACE>) and PLANTCARE databases (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>)

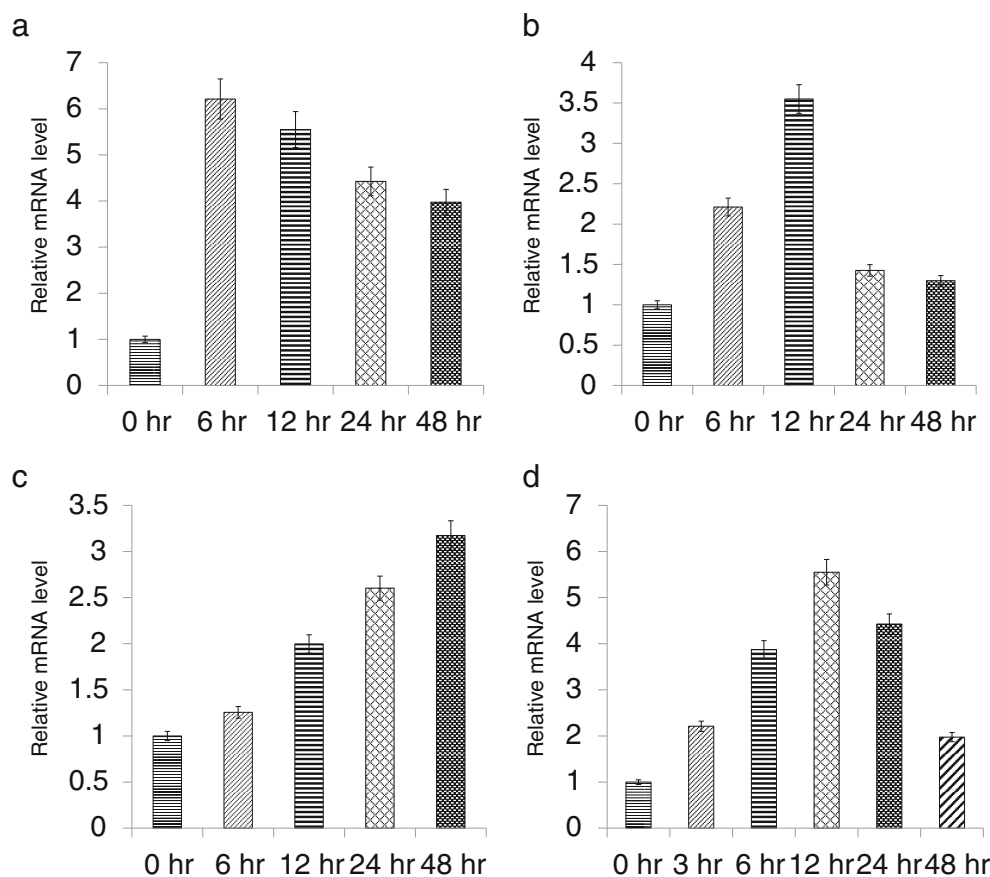
<i>cis</i> -Element	Position	Signal sequence	Putative function
TATA-box	19 (−), 78 (+), 123 (+), 124 (+), 126 (−), 138 (−), 171 (+), 194 (−), 231 (+), 406 (−), 411 (−), 627 (+), 633 (+), 760 (+), 797 (+), 809 (−), 890 (+), 968 (+), 1019 (+)	TAATA, TATA, ATTATA, TATAA	Core promoter element around −30 transcription start
CGTCA motif	435 (+)	CGTCA	<i>cis</i> -Acting regulatory element involved in the MeJa responsiveness
5UTR Py-rich stretch	784 (+)	TTTCTTCTCT	<i>cis</i> -Acting element conferring high transcription levels
TGA element	825 (+)	AACGAC	Auxin-responsive element
CAAT-BOX	147 (−), 403 (+), 309 (−), 830 (+), 183 (−), 556 (+), 360 (−), 922 (+), 175 (−), 504 (+), 351 (−), 839 (+), 308 (−), 574 (+), 361 (−)	CAAT	Common <i>cis</i> -acting element in promoter and enhancer regions
ACE motif/G-box	392 (+), 337 (−), 652 (+)	CTAACGTATT	<i>cis</i> -Acting element involved in light responsiveness
ARE motif	128 (−), 158 (−)	TGGTTT	<i>cis</i> -Acting regulatory element essential for the anaerobic induction
ATCT motif/GAP-box	950 (+), 346 (−)	AATCTAATCT	Part of a conserved DNA module involved in light responsiveness
Box-W1	464 (−)	TTGACC	Fungal elicitor responsive element
CCAAT-box	857 (−)	CAACGG	MYBHv1 binding site
GARE motif	13 (−)	TCTGTTG	Gibberellin-responsive element
HSE	142 (+), 164 (+)	AAAAAATTC	<i>cis</i> -Acting element involved in heat stress responsiveness
LTR	303 (+), 529 (+)	CCGAAA	<i>cis</i> -Acting element involved in low-temperature responsiveness
MBS	463 (+), 747 (+)	CGGTCA	MYB Binding Site
W-BOX	464 (−)	TTGACC	“W box” found in the promoter region of a transcriptional repressor ERF3 gene in tobacco; may be involved in activation of ERF3 gene by wounding

Prediction of three-dimensional structure of *PkCPR*

The three-dimensional structure of the *PkCPR* was built using the crystal structure of *R. norvegicus* (PDB ID: 1J9Z) as template (Fig. 9a). This deduced structure confirmed the presence of four individual domains, a FMN-binding domain, a linker domain and the FAD- and NADPH-binding domains. The amino acid residues involved in ligand binding were also predicted using the 3DLigandSite tool as depicted in Fig. 9b. Analyses of the evolutionary conservation of *PkCPR* surface 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 (Fig. 9c). Superimposition of *PkCPR* with template CPR showed all major domains aligned at same coordinates (Fig. 9d). The predicted structure of *PkCPR* was further refined by employing KoBa^{MIN} web

server. The stereo-chemical qualities of the energy refined predicted model of *PkCPR* proteins was validated by PROCHECK server. Ramachandran plot analysis of *PkCPR* showed 85.8 % residues in the most favourable region, 10.2 % residues in the additional allowed region, 2.4 % in the generously allowed region and 1.6 % in the disallowed region (Fig. S6). The results of the PROCHECK analysis indicate that a relatively low percentage of residues have phi/psi angles in the disallowed regions suggesting the acceptability of Ramachandran plot for *PkCPR* protein. The percentage of residues in the allowed region of *PkCPR* predicted structure were found to be 98.4 %, while residues in disallowed regions were found to be merely 1.6 %, further validating the efficacy of the predicted structure. The energy refined and validated model of *PkCPR* was submitted to the public domain PMDB database and assigned the PMDB ID PM0078444.

Fig. 8 Time-course expression characteristics of *PkCPR* in response to methyl jasmonate (a), salicylic acid (b), 2,4-dichlorophenoxyacetic acid (c) and UV-B (d) at different time points. Data were normalized relative to abundance of endogenous control actin gene. 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. Differences were scored as statistical significance at the * $P < 0.05$ and ** $P < 0.01$ levels

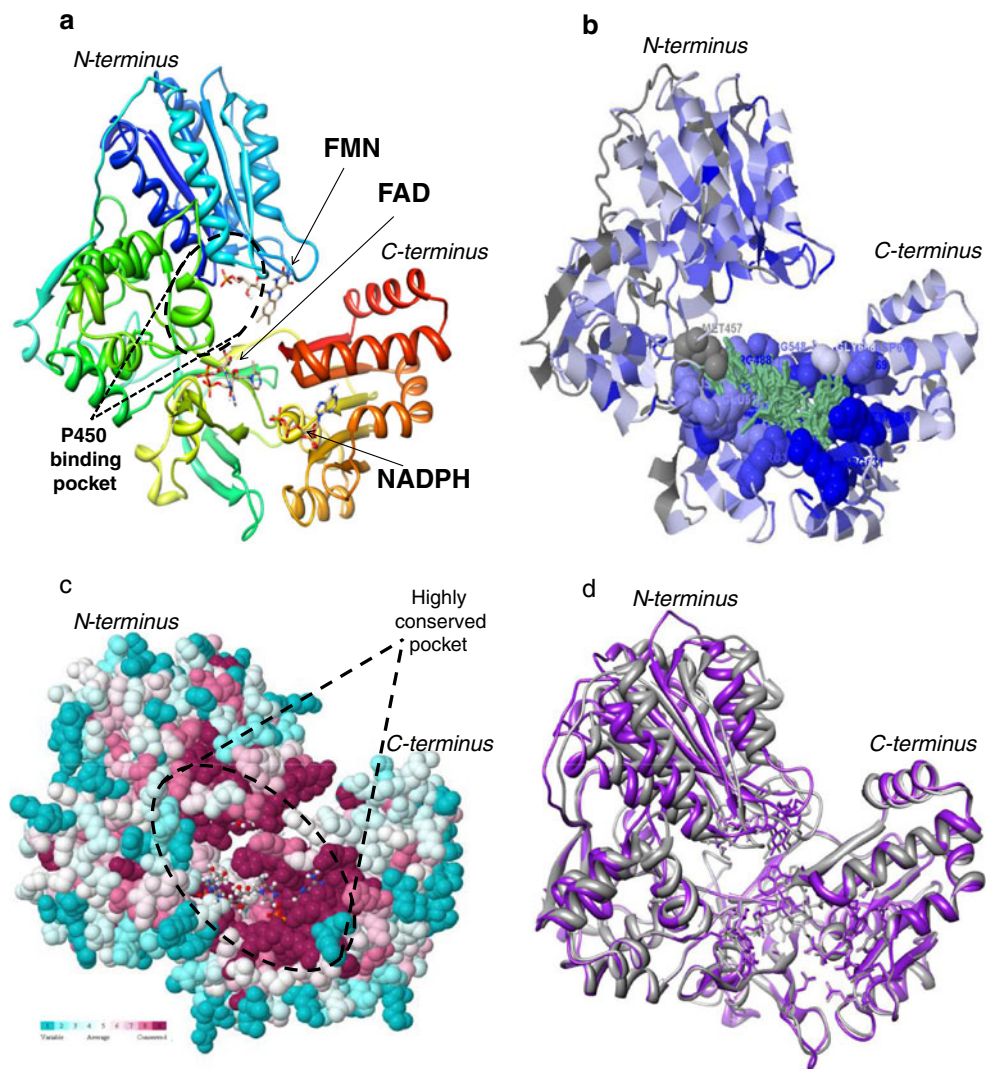


Discussion

There has been a remarkable success vis-à-vis engineered production of number of commercially important secondary metabolites such as flavours, fragrances, costly and complex pharmaceuticals like artemisinin, taxol, and thapsigargin from microorganisms and mosses. It has triggered parallel interest to manipulate secondary pathways in plants through genetic engineering. 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 (Morant et al. 2007). These P450-catalysed reactions are pivotal steps in the biosynthesis of a variety of compounds and optimization of P450 enzyme activities are a key target in yield improvement efforts to render such approaches economically feasible (Keasling 2012). Plant P450s catalyse diverse array of reactions during secondary metabolite synthesis which require involvement of their redox partner, NADPH-cytochrome P450 reductase. One of the limiting factors associated with the introduction of plant P450s into a heterologous host such as bacteria, yeasts or other plant species is to guarantee an adequate supply of electrons from CPR to optimize P450 activity. Owing to the endemic and endangered nature of *P. kurrooa*, overexploitation for herbal drug preparations and

dwindling populations, it becomes all the more necessary to embark on a metabolic engineering program for enhanced production of picrosides. Therefore, as a prerequisite for heterologous and/or homologous production of picrosides, an ortholog of CPR gene was isolated from *P. kurrooa* and functionally characterized in *E. coli*. Full-length cDNAs of *PkCPR* gene was obtained from the in vitro raised *P. kurrooa* plantlets by degenerate PCR and RACE methods. A full length cDNA (2679 bp) of *PkCPR* was isolated from *P. kurrooa* with an ORF of 2,133 bp, corresponding to 710 amino acids. The full length coding sequence of *PkCPR* was overexpressed in *E. coli* as a fusion protein with GST, using an inducible tac promoter based vector system, pGEX-4T-2. The cloning of *PkCPR* in pGEX-4T-2 having N-terminal GST-tag allowed its expression in soluble fraction along with the membrane anchor region. Initially, its expression was localized to inclusion bodies at 37 °C but lowering the temperature to 20 °C allowed protein expression in solubilised form. Similar strategy has been employed by Rana et al. (2013). It has been shown that addition of FMN to the reaction mixture increases the specific activities of other CPRs which are mainly due to depletion of FMN domain during the isolation of microsomal fraction. Contrary to these observations, addition of FMN to *PkCPR* did not affect the activity as the purified protein contained all the domains intact including membrane anchor.

Fig. 9 Three-dimensional protein model and conserved residue prediction for *PkCPR*. Ribbon display of the 3-D structures of *PkCPR* as predicted by PHYRE2 web server, using crystal structure of *Rattus norvegicus* (PDB ID: 1J9Z) as template. N- and C-terminal domains are shown (a). Predicted ligand (shown in green) binding site as predicted by 3DLigandSite Web Server (b). Conserved residue analysis of *PkCPR* using Consurf and Conseq Web Servers (c). Residue conservation from variable to conserved is shown in blue (1) to violet (9). The highly conserved pocket is shown. The residues involved in substrate binding and active site are shown in the center core of the structure. Superimposition of 3-D ribbon model of *PkCPR* (purple) with Rat CPR (grey) using 3d-SS superimposition web server (d)



In several different species, the expression pattern of CPRs has been examined using the distribution of the corresponding mRNAs as an indicator of gene expression. Predominantly, the higher expression has been observed in flowers and leaves of different plant species (Meijer et al. 1993). In our experiments, *PkCPR* showed highest expression in leaves. This may be attributed to the high metabolic rate in the young dividing cells since CPRs are redox partners to wide range of P450s that are involved in an array of metabolic circuitries. The expression pattern of *PkCPR* is in agreement with the higher content of picrosides in leaves of *P. kurrooa* as reported earlier (Pandit et al. 2012a; Bhat et al. 2012a, 2013) and probably indicates its involvement to meet the high reductive demand of different P450 monooxygenases for driving the biosynthesis of picrosides. mRNA levels of multiple orthologs of different plants like *P. crispum*, *A. thaliana* and hybrid poplar have been shown to vary in relation to tissues analysed depending on the species (Koopmann and Hahlbrock 1997; Ro et al. 2002; Mizutani and Ohta 1998). CPR expression in *A. thaliana* was examined in

roots, stems, flowers and leaves and the expression of *AtATR1* was found to be highest in roots and stems whereas *AtATR2* expression was highest in leaves, stems and flowers (Mizutani and Ohta 1998). Moreover, two paralogs of *W. somnifera*, *WsCPR1* and *WsCPR2* expressed with varying expression levels where in *WsCPR2* was found to be constitutively active while as *WsCPR1* was observed to be highly expressed in roots among all the tissues tested (Rana et al. 2013). The tissue-specific expression of a key gene of picroside biosynthesis, Geraniol 10-hydroxylase (*PkG10H*) was found to corroborate positively with the *PkCPR* expression (unpublished data). It possibly indicates that higher mRNA expression levels of CPRs are matched by the higher expression of P450s in the same tissues (Reichhart et al. 1980; Ohta and Mizutani 2004). Similar results have been found in *A. thaliana* wherein seven of the P450s had the highest expression in either flowers or leaves where *AtCPR* expression was also copious (Mizutani and Ohta 1998).

Altitude encompasses an array of environmental factors. As the altitude changes, ecological niche also changes,

including various factors like temperature, UV radiation, abiotic and biotic factors. In different ecological niches, plants behave differently in terms of biochemical aspects in order to better adapt to their environment. Altitude has pronounced effect on the accumulation of secondary metabolites in higher plants. In addition to incurring many climatic differences, altitude influences the quality of radiation, especially UV-B radiation is high in alpine niches compared with lower habitats (Barnes et al. 1987). The higher solar radiation at higher altitudes has often been implicated to influence secondary metabolite profiles. For example, an increase in phenolic compounds with altitude as a response to increasing UV radiation has been demonstrated (Turunen and Latola 2005). In our study, HPLC results revealed variations in the distribution and relative content of all the marker compounds extracted from three different altitudinal populations. This variation was found associated with both organ/plant parts and altitude. The results of the present study noted that as the height above mean sea level increases, the content of picrosides and other associated metabolites also increases (Fig. 6a). Similar results were demonstrated in some previous reports (Singh et al. 2011; Sood and Chauhan 2010) that suggested that leaves are good source of picrosides and can be used as a resource for picrosides, hence, not essential to uproot the whole plant and our results are also in accordance to earlier reports that leaves contain good amount of picrosides. Sood and Chauhan (2010) reported that accumulation of picroside I is developmentally regulated in different morphogenetic stages of *P. kurrooa*, while Singh et al. (2011) reported that picroside I content was higher in leaf tissue as compared to root and rhizomes and also the total picrosides content in roots was less as compared to leaves. Our data revealed that with the decline in altitude, there is concomitant decrease in the concentration of picroside I, picroside II and feruloylcatalpol. The higher concentration of these constituents at higher altitudes may probably have an adaptational role in response to stressful conditions. Other earlier studies reported that the quantity and quality of secondary metabolites depends to a great extent on altitude (Bahuguna et al. 2000; Spitaler et al. 2006, 2008; Camas et al. 2013). Metabolite content was also found to vary spatially. Two of the constituents viz. picroside I and picroside II showed differential tissue-specific accumulation in rhizome and above ground leaves of *P. kurrooa*. Picroside I was predominant in leaves while as picroside II showed maximum accumulation in rhizomes. (Fig. 6a). There is direct a correlation between picroside I and II content in different plant parts with altitude, whereas cultivation and acclimatization of high altitude plants at lower altitude reduces picroside I and II content. These results are positively corroborating with earlier findings (Katoch et al. 2011). It was also tempting to find out the transcript variation in the expression of *PkCPR* at different altitudes so as to understand the underlying mechanism of picroside accumulation and the regulatory role of *PkCPR* in

picroside biosynthesis. Our results show a positive correlation between the altitude and the expression level of *PkCPR* (Fig. 7). These observations regarding increased transcript levels of *PkCPR* suggest that higher accumulation of picrosides at high altitudes may be in response to low temperature and high UV radiation stresses, and picrosides may have an important role in enabling the plant to survive under extreme stresses. It has been amply demonstrated that flavanoids, anthocyanins, phenolics and other secondary metabolites of phenylpropanoid pathway have a well-established role in protecting plants against high altitude stresses (Matsuura et al. 2013; Bartwal et al. 2013; El-Beltagi et al. 2011; Schreiner et al. 2012; Vickers et al. 2009).

In a 1,058-bp promoter region of *PkCPR*, in silico analysis revealed *PkCPR* to be a highly regulated gene at the transcriptional level, responsive to various abiotic and biotic elicitors including MeJA, SA, light, temperature, oxygen, auxins, and fungal elicitors. To understand the inducible or constitutively active transcriptional nature of *PkCPR*, and to corroborate these results with the identified important *cis*-regulatory elements, *PkCPR* transcripts were assayed in response to MeJA, SA, UV-B and 2,4-D elicitors. Transcript profiling under various elicitors displayed differential transcriptional regulation of *PkCPR* which fully corroborated with the identified *cis*-elements within the promoter region. *PkCPR* was found to be an inducible isoform of CPRs. MeJA, SA, UV-B and 2,4-D up-regulated the expression of *PkCPR* significantly and the mRNA level peaked at different time periods after the treatment. As shown in our earlier studies (Bhat et al. 2013), MeJA, SA and 2,4-D were found to increase the metabolic flux of picroside I and picroside II after 24 h of elicitor treatment. This is in agreement with our present results wherein there is an increase in the expression of *PkCPR* upon treatment with elicitors. CPRs have been found to responsive to various elicitors in some previous studies as well. For example, *A. thaliana* genome has two CPR genes, ATR1 and ATR2; while ATR1 is constitutively expressed, ATR2 is inducible by environmental stimuli, such as wounding and light treatments (Mizutani and Ohta 1998). In parsley, however, only one of the two CPRs isolated is responsive to pathogen infection (Koopmann and Hahlbrock 1997). Similarly, in case of *W. somnifera* two paralogs of CPRs have been reported and only one being inducible in nature in response to exogenous elicitor treatments (Rana et al. 2013). It has been contended that plants deploy distinct CPR isoforms to cope with the high reductive demand for the P450-mediated reactions in stressed conditions (Ro et al. 2002). In plants, the expression of P450s is highly regulated but can be affected by different external factors such as wounding, UV light and elicitor treatment. A simultaneous induction of CPR has been detected, presumably to ensure a sufficient supply of reducing equivalents to drive the P450 reactions (Reichhart et al. 1980). Moreover, CPR genes can be induced by various

environmental factors such as light, infection, mechanical wounding, low temperature and some signal substances. In general, elicitor exposure induces expression of CPR but in species with more than one paralog of CPR, only one of the paralogs is induced (Koopmann and Hahlbrock 1997; Ro et al. 2002; Meijer et al. 1993). In *P. crispum*, only one of the paralogs present showed increased expression when the plants were exposed to UV irradiation and wounding, and in *A. thaliana* one paralog was reported to be induced following wounding (Koopmann and Hahlbrock 1997; Mizutani and Ohta 1998). The constitutively expressed and non-inducible paralogs serve processes in primary metabolism and in constitutive synthesis of bioactive natural products whereas the inducible paralogs play a role in plant adaptation and defence reactions. Plants respond differently to irradiation with low or high doses of UV-B, either by stimulating protection mechanisms or by activating repair mechanisms to cope with different types of stresses. The most common protective mechanism against potentially damaging irradiation is the biosynthesis of UV absorbing compounds (Brosché et al. 1999). These secondary metabolites, mainly phenolic compounds, flavonoids, and hydroxycinnamate esters accumulate in the vacuoles of epidermal cells in response to UV-B irradiation and attenuate the penetration of the UV-B range of the solar spectrum into deeper cell layers with little effect on the visible region. Accumulation of the UV-B-absorbing pigments is one of the ways by which plants alleviate the harmful effect of UV-B light (Caldwell et al. 1983).

Crystal structures of membrane proteins are notoriously hard to obtain, and CPR has been no exception. In order to better understand the structural complexity of *PkCPR* protein, a comparative modelling of three-dimensional structure was performed using PHYRE2 server. A crystal structure of solubilized rat CPR at 2.6-Å resolution has been previously obtained following removal of the N-terminal anchor sequence by trypsination. Interaction between CPR and P450s is thought to be based on electrostatic interactions between a negatively charged region near the bound FMN cofactor and a positively charged indentation near the heme of the P450 enzyme. The ability of the CPRs from different species shows functional complementarity, indicative of the highly conserved nature of interacting domains. Together, this makes CPR an ideal bio-brick in synthetic biology based on the use of the “share your parts” principle to secure electron transfer between designed enzyme complexes.

To recapitulate our findings, we successfully identified an inducible NADPH–CPR from *P. kurrooa*. The full length ORF was expressed in *E. coli* and the protein was fully functional in reducing cytochrome *c*. We also isolated its upstream regulatory *cis*-elements, and studied the expression levels of *PkCPR* in different tissues of *P. kurrooa*. In addition, sequence homology and phylogenetic analysis classified *PkCPR* as a class II CPR. Moreover, transcript-profiling under

MeJA, SA, 2,4-D and UV-B elicitions displayed inducible nature of *PkCPR* which fully corroborated with the identified *cis*-elements. This work provides one of the basic foundations for creating designer circuitries both *in planta* as well as in engineered microbial systems. For homologous modulation and metabolic pathway intensification, we have already developed an efficient *Agrobacterium*-mediated genetic transformation system for *P. kurrooa* (Bhat et al. 2012b). Furthermore, we also tend to understand the regulatory role by co-expression of cloned CPR with a key P450 monooxygenase, geraniol 10 hydroxylase (Genbank Acc. No. HM187585) that we have already characterized.

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