Mol Biol Rep
DOI 10.1007/s11033-012-2147-1

Expression pattern of fifteen genes of non-mevalonate (MEP) and mevalonate (MVA) pathways in different tissues of endangered medicinal herb *Picrorhiza kurroa* with respect to picrosides content

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Received: 24 June 2012 / Accepted: 3 October 2012

Abstract *Picrorhiza kurroa*, has become an endangered medicinal herb due to excessive utilization, therefore it necessitates the understanding of biology and molecular basis of major chemical constituents i.e. Picroside-I (P-I) and Picroside-II (P-II). Estimation of P-I and P-II in different tissues of *P. kurroa* showed that shoots contain only P-I whereas P-II is present only in roots. Differential conditions with varying concentrations of P-I (0–27 μg/mg) and P-II (0–4 μg/mg) were selected. Four genes of MEP pathway; DXPS, ISPD, ISPE, MECPS and one gene of MVA pathway PMK showed elevated levels of transcripts in shoots (57–166 folds) and stolons (5–15 folds) with P-I contents 0–27 μg/mg and 2.9–19.7 μg/mg, respectively. Further HDS and DXPR genes of MEP pathway showed higher expression ~9–12 folds in roots having P-II (0-4 μg/mg). The expression of ISPH and ISPE was also high ~5 folds in roots accumulating P-II. GDPS was the only gene with high transcript level in roots (9 folds) and shoots (20 folds). Differential biosynthesis and accumulation of picrosides would assist in regulating quality of plant material for herbal drug formulations.

Keywords Mevalonate pathway · Non-mevalonate · *Picrorhiza kurroa* · Picroside-I (P-I) · Picroside-II (P-II)

Abbreviations

DXPS	1-deoxy-D-xylulose 5-phosphate synthase
ISPD	2-C-methylerythritol 4-phosphate cytidyl transferase
ISPE	4-(cytidine-5′-diphospho)-2-C-methylerythritol kinase
MECPS	2-C-methylerythritol-2, 4-cyclophosphate synthase
PMK	Phosphomevalonate kinase
GDPS	Geranyl diphosphate synthase

Introduction

Picrorhiza kurroa Royle ex. Benth. is an important medicinal herb (Family: Scrophulariaceae) mainly found in the Western Himalayan region, between 3,000 and 5,000 m elevation [9, 22, 7, 24, 1]. It is valued as a hepato-protective, immunomodulator, anti- periodic, stomachic, anti-oxidant, anti-helmintic, anti-inflammatory, cardio-tonic, laxative, carminative, expectorant, etc. [9, 58, 52, 29, 28]; Bhatt et al. [6, 17, 41]. Aqueous rhizome extracts of *P. kurroa* have shown hepatoprotective and antioxidant properties on CCl_4 induced liver toxicity in albino rats [59], antioxidant and anti-neoplastic activities [44] and a iridoid glycoside (RLJ-NE-299A) isolated from the roots was an effective immuno modulator specifically to improve

Electronic supplementary material The online version of this article (doi:10.1007/s11033-012-2147-1) contains supplementary material, which is available to authorized users.

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Published online: 14 October 2012

macrophage function during infections [49]. Recently rhizome extracts of *P. kurroa* have also shown to possess antimalarial activity [50]. Standardized iridoid fractions of *P. kurroa*, e.g. kutkin and Picroliv consist of glucosides, picroside-I and kutkoside in a ratio of 1:2 and other minor glycosides [51, 2]. Picroliv, which was launched as a herbal drug formulation, is prepared from a standardized iridoid fraction containing 60 % of Picroside-I and Kutkoside in a 1:1.5 ratio [15, 3, 11];). Picroliv has also been shown to have immunostimulating effect in hamsters and helps to prevent infections [42, 18]. There are several other commercially available drug formulations consisting of Picroside-I and Picroside-II, e.g. Katuki, Zandu Pharma works Ltd. (Mumbai, India) having Picroside-I (1.29 %) and Picroside-II (1.16 %), [5]. The Picroside-I (P-I) and Picroside-II (P-II) are, therefore, two important chemical constituents present in *P. kurroa* that have therapeutic importance and are major components of several herbal drug formulations [14]. Proper concentration of P-I and P-II is, therefore, required for the preparation of herbal drug formulations from *P. kurroa* with high quality and desired pharmacological efficacy. *P. kurroa* based herbal drug formulations are prepared mainly from the rhizomes of 3–4 years old plants. However, biology of picrosides biosynthesis and accumulation is not known.

The *P. kurroa* propagates vegetatively through stolons, which initially emerge as a young bud, grow to a mature stolon and then eventually into a rhizome with independent shoots and roots [37] (Fig. 1). *P. kurroa* plants are uprooted from their natural habitat for obtaining mature rhizomes, however, along with all other young propagules also get uprooted, thereby, disrupting the natural propagation of *P. kurroa*. The increasing national and international demand for *P. kurroa* raw material coupled with limited cultivation has made it a critically endangered plant species [39, 43]. The plant produces relatively small amounts of picrosides and that too in the rhizomes, which necessitate efforts to increase the yield of picrosides. However, lack of understanding about the biology and molecular basis of P-I and P-II biosynthesis and accumulation impedes perusal of a systematic genetic improvement programme in *P. kurroa*.

P-I and P-II are iridoid derivatives of monoterpenes [53]. The isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP) condense to form geranyl diphosphate (GPP), the precursors of monoterpenes [12]. The biosynthesis and accumulation of terpenoids is controlled at the molecular level by structural and regulatory genes in different plant species [40]. The non-mevalonate (MEP) and mevalonate (MVA) biosynthetic pathways lead to the biosynthesis of DMAPP (dimethyl allyl pyrophosphate) and IPP (isopentenyl pyrophosphate), which are the building blocks of GPP the starting material for the biosynthesis of P-I and P-II (Fig. 1), but it has been also shown that some monoterpenes in strawberry (Fragaria spp.) have an exclusive MVA origin [20]. Two different biosynthetic routes can synthesize both DMAPP and IPP: 1-deoxy-D-xylulose 5-phosphate/2-C-methyl-D-erythritol 4-phosphate (DXP/MEP) pathway in plastids and/or the classical mevalonate (MVA) pathway in cytoplasm [26]. Plants use both MEP and MVA pathways for isoprenoid biosynthesis, although they are localized in different compartments [33, 45]. Furthermore, the MEP pathway is essential for plastidial isoprenoid biosynthesis in plants [46]. The MEP pathway starts with the formation of 1-deoxy-D-xylulose 5 phosphate (DXP) from D-glyceraldehyde 3-phosphate and pyruvate by the catalytic action of a 1-deoxy-D-xylulose 5-phosphate synthase [56]; Bouvier et al. [8] and then, DXP is converted into IPP by a series of enzymes. Total 15 genes, 8 of MEP pathway, 6 of MVA pathway, and IPPI convert DMAPP or IPP into GPP in monoterpenoids biosynthesis [61]. Out of 15 genes, sequences for 10 genes were available in the GenBank but not for the remaining 5 genes. The expression of 2 genes *hmgr* and *dxs* was shown to be upregulated in *P. kurroa* vis-à-vis picrosides biosynthesis in in vitro plantlets exposed to different light and temperature regimes [31]. Both these genes are reported as rate limiting steps in MVA and MEP pathways in different plant species, which was considered as an assumption that the same genes might be limiting the biosynthesis of P-I and P-II in *P. kurroa* [31]. However, ascertaining the role of particular gene (s) in P-I and P-II biosynthesis would require expression analysis of all 15 genes of MEP and MVA pathways vis-à-vis P-I and P-II biosynthesis and accumulation in different tissues and organs of *P. kurroa.* The current study reports (a) Ascertaining differential biosynthesis of P-I and P-II in different tissues/organs of *P. kurroa* (b) cloning 5 genes, ISPD, MECPS, HDS, HMGS and PMK of MEP and MVA pathways in *P. kurroa* (c) expression analysis of 15 genes of MEP and MVA pathways in different tissues in relation to P-I and P-II contents.

Materials and methods

Differential biosynthesis of P-I and P-II

Previous studies have shown that *P. kurroa* shoots grown in tissue culture at 25 °C (TCS-0) do not accumulate P-I whereas shoots grown at 15 °C (TCS-6) contain P-I content (6 µg/mg) [54]. *P. kurroa* shoots from plants growing in nursery (Sairopa; 4,500 m altitude, 31°38′–31°54′N and 77°20′–77°45′E) were collected at two time intervals i.e. July (FGS-12) and September (FGS-27). The roots of *P. kurroa* grown in tissue culture, both at 15 °C and 25 °C, did not contain P-II, whereas roots from nursery grown plants (UR-4) contained P-II. The stolons represent important propagules in vegetative propagation as well as a

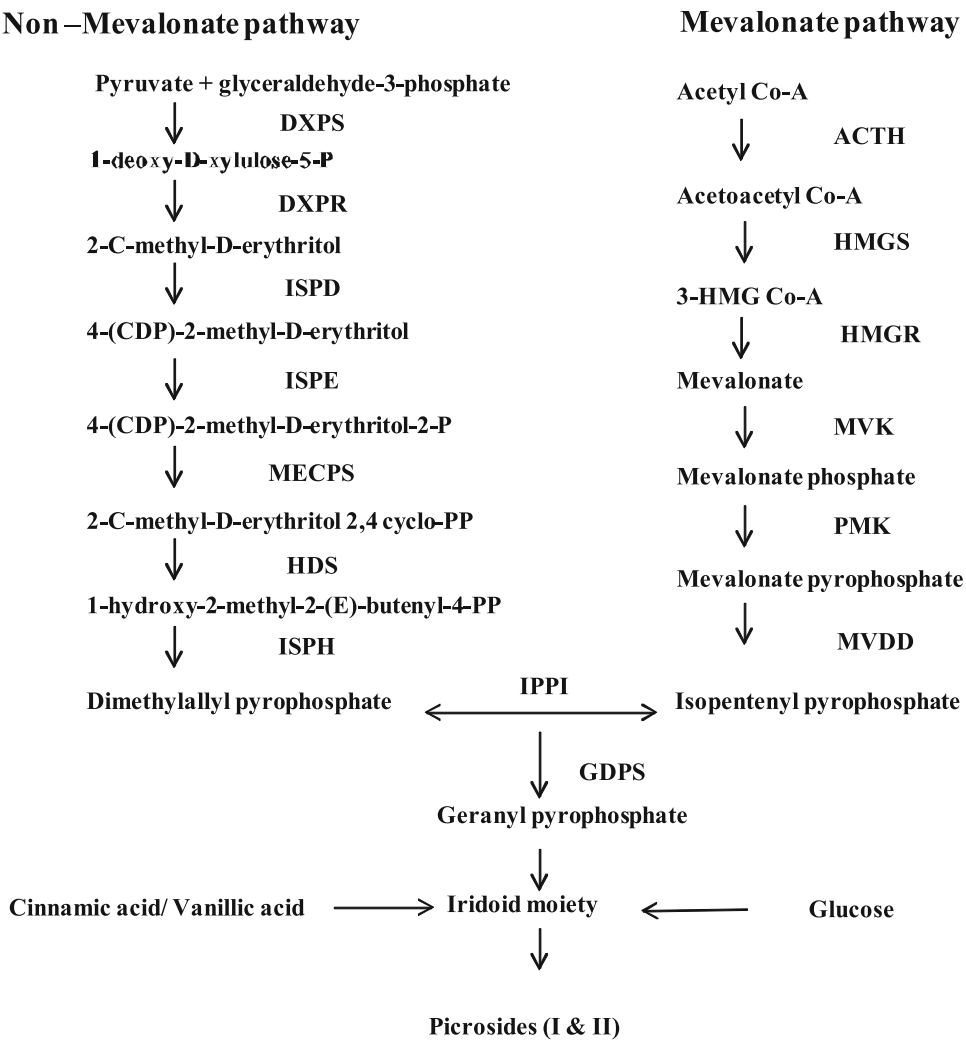

Fig. 1 Schematic pathway for picrosides biosynthesis (adapted from Mahmoud and Croteau [34]. MVA pathway: *ACTH* acetoacetyl-CoA thiolase, *HMGS* 3-hydroxy-3-methylglutaryl-CoA synthase, *HMGR* 3-hydroxy-3-methylglutaryl-CoA reductase, *MVK* mevalonate kinase, *PMK* phosphomevalonate kinase, *MVDD* mevalonate diphosphate decarboxylase, *IPPI* isopentenyl pyrophosphate isomerase, *GDPS* gerenyl diphosphate synthase. MEP pathway: *DXPS* 1-deoxy-D-xylulose 5-phosphate synthase, *DXPR* 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *ISPD* 2-C-methylerythritol 4-phosphate cytidyl transferase, *ISPE* 4-(cytidine-5′-diphospho)-2-C-methylerythritol kinase, *MECPS* 2-C-methylerythritol-2,4-cyclophosphate synthase, *HDS* 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, *ISPH* 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase

transition to rhizome formation in *P. kurroa*. Two types of stolons i.e. underground and aerial are formed in *P. kurroa*. Therefore, stolons differing w.r.t growth, development, source of plant-material and the environment of their formation and growth were selected to estimate the status of P-I and P-II biosynthesis and accumulation. Three types of stolons were considered; underground young stolons (UStY; 1 year), underground mature stolons (UStM; 2 yrs.) and aerial stolons (ASt; 1 year). The Mature rhizomes (MRz; 3 yrs.) were collected from the nursery grown (Sairopa; 4,500 m altitude, 31°38′–31°54′N and 77°20′–77°45′E) plants of *P. kurroa.* All samples were immediately stored at −80 °C for further analysis in the quantification of P-I and P-II and isolation of total RNA.

Preparation of samples for P-I and P-II analysis

The samples were thoroughly washed under running tap water, segregated into shoots, stolons, rhizomes and roots. The fresh samples were ground to fine powder using a pestle and mortar in liquid nitrogen. Powdered samples (100.0 mg) were extracted in 10.0 mL 80 % HPLC grade methanol. The samples were then vortexed to mix properly and extracted overnight at room temperature 25 °C. Samples were centrifuged at 10,000 rpm for 15 min. and the supernatant was filtered through 0.22-µm filter (Millipore) and used for HPLC analysis.

Quantification of picrosides

The quantification of P-I and P-II was carried by reverse phase (HPLC Waters 515) through C18 (5 µm) 4.6 × 250 mm Waters Symmetry Column using PDA detector (Waters 2996). The filtrate was diluted 10× and 20× and injected into above-mentioned column. Two solvent systems were used for running the test samples i.e. Solvent A (0.05 % trifluoro-acetic acid) and Solvent B (1:1 methanol/acetonitrile mixture). Solvents A and B were used in the ratio 70:30 (v/v). The column was eluted in isocratic mode with a flow rate of 1.0 mL/min. The P-I and

P-II were detected at absorbance of 270 nm wavelength. The cycle time of analysis was 30 min. at 30 °C. The compounds were identified on the basis of retention time and comparison of UV spectra with the authentic standard from ChromaDex, Inc.

Isolation of genomic DNA and total RNA

Genomic DNA was isolated from leaves of *P. kurroa* following the protocol of Murray and Thompson [38]. Total RNA was isolated from *P. kurroa* samples by using Raflex RNA isolation kit (GeNeiTM) by following manufacturer's instructions. The quality of RNA was checked by 1 % (w/v) ethidium bromide-stained agarose gel and through the absorbance spectrum at wavelengths 260 nm and 280 nm.

Cloning and sequencing of MEP and MVA pathways genes in *P. kurroa*

The nucleotide and protein sequences for 5 genes (ISPD, MECPS, HDS, HMGS and PMK) were retrieved from different plant species in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and the multiple sequence alignments (MSA) were done to identify conserved sequence regions. Though the extent of sequence similarity was low in coding regions of genes, short patches of conserved sequences were identified. Primer pairs were designed from the conserved regions of gene sequences (Table 1) and tested on genomic DNA and cDNA of *P. kurroa.* For rest of the 10 genes the nucleotide sequences were retrieved from *P. kurroa* cds available at GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and primers pairs were designed using Primer 3 (Table 2).

For amplification, the PCR was performed on 30 ng genomic DNA and cDNA separately with varying amount of primer pairs, Mg^{2+} dNTPs and Taq DNA polymerase. Amplification programs included 94 °C for 3 min, 30 cycles of 94 °C for 30 s, annealing temperature (50–65 °C) for 45 s, 72 °C for 2 min and a final extension of 7 min at 72 °C. 10 µl of each PCR product was mixed with 2 µl of 6 × gel loading dye (0.2 % bromophenol blue, 0.2 % xylene cyanol dye and 30 % glycerol) and electrophoresed in a 2 % agarose gel prepared in 0.5 × Tris borate-EDTA (TBE) buffer. The gels were analyzed using the gel documentation system Alpha Imager EP (Alpha Innotec Corp., USA). The PCR products were cloned in pGEMT vector (Promega) and sequenced. Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/Blast) calculated the identities of sequence similarities. Primer pairs for expression analysis were designed from *P. kurroa* gene sequences (Table 2).

Expression analysis of MEP and MVA pathway genes through quantitative PCR (qPCR)

The cDNA was prepared from 5 µg of RNA (RNA was treated with 2U of DNase I), reverse transcribed by using M-MuLV reverse transcriptase (GeNei TM) with oligo-dT primer. To study gene expression, equal sample quantities were verified by measuring the amount of RNA with a spectrophotometer. The qPCR was performed using gene specific primers (Table 2) in triplicate on a CFX96 system (Bio-Rad Laboratories; Hercules CA) with the iScript one step RT PCR kit (Bio-rad). The PCR protocol was as follows: denaturation for 5 min at 94 °C, followed by 40 cycles each of denaturation for 20 s at 94 °C, annealing for 30 s at 50–65 °C, followed by one elongation step for 20 s at 72 °C.

Statistical analysis

The heat map demonstrating the level of expression of different genes w.r.t various tissues was generated using R-language from triplicate data. Whisker plots were made for qRT-PCR data to determine folds expression of picrosides biosynthetic pathway genes in different tissues of *P. kurroa* by using MINITAB (v14).

Table 1 Primer sequences* used to clone five genes of mep and mva pathways in *p. Kurroa*

Gene	Forward primer	Reverse primer	Annealing temperatures (°C)	Fragment size (bp)
HMGS	5′-GATGGHGCAAGYAAAGGRAARTAYA-3′	5′-GGRTATTCACTNGCAAGHTTGGGC-3′	54	350
PMK	5′-TGGMWGTRGTKGCBTCKGCKCCKGG-3′	5′-GTMARAGGSAGWCCACRHGCTTCAA-3′	58	350
ISPD	5′-GAGAAAAGTGTHTCTGTGRTTCTTYG-3′	5′-RATNACCTGWGGWGTYTGCATTTCC-3′	56	510
MECPS	5′-ATCTATAGCGGCAAACCTACAC-3′	5′-ACTTTAGAGAGGGATGGAGGG-3′	57.1	350
HDS	5′-ATGCCYTTTAAGGAYCTKGCAACWG-3′	5′-GGAGCACCACCRACATAHCCAAART-3′	58	510

[a] R = A/G; M = A/C; W = A/T; Y = C/T; S = C/G; K = G/T; D = A/G/T; H = A/C/T; B = G/C/T; N = A/T/G/C

Table 2 Primers used in qRT-PCR analysis

Genes/ accession number	Forward primer	Reverse primer	Fragment size (bp)	Annealing temperatures (°C)
26S	5′-CACAATGATAGGAAGAGCCGAC-3′	5′-CAAGGGAACGGGCTTGGCAGAATC-3′	500	58
ACTH/ DQ347964	5′-AGTGTTACTAGAGAGGAGCAGGACA-3′	5′-CCTAGACCTTCATCCTTATCAACAA-3′	110	50
HMGS	5′-GATGGTGCAAGAAAAGGCAACTAGA-3′	5′-GGATATTCACTGGCAAGATTGGGCT -3′	110	54
HMGR/ DQ347962	5′-CGTTCATCTACCTTCTAGGGTTCTT-3′	5′-GACATAACAACTTCTTCATCGTCCT-3′	100	60
MVK/EU590911	5′-ATTAACTCTGAGTATGACGGGTCTG-3′	5′-GAGAGCCCATTTATTTAGCAACTC-3′	110	50
PMK/JQ950335	5′-TGGATGTTGTCGCATCAGCACCTGG-3′	5′-GTAATAGGCAGTCCACTCGCTTCAA-3′	100	58
MVDD/ EU590912	5′-GTAACTCTGGATCCTGACCACCT-3′	5′-TAATACCCCCTCTTTTTCATCCTC-3′	110	54
IPPI/EF421829	5′-TCTCCTATTCACTGTAAGGGATGTT-3′	5′-ACCACTTAAACAAGAAGTTGTCCAC-3′	110	54
GDPS/AY866498	5′-GATATATGTTCTGAGGGAATGGATG-3′	5′-ATACACCTAGCGAAATTCCTCAACT-3′	110	55
DXPS/EU561005	5′-ACATTTAAGTTCAAGTCTGGGAGTG-3′	5′-ATGTGCACTCTCTTCTCTTTTAGGA-3′	110	55.9
DXPR/DQ347963	5′-GGAGGAACTATGACTGGTGTTCTT-3′	5′-CAGGTCATAGTGTACGATTTCCTCT-3′	110	54.9
ISPD/JQ950336	5′-GAGAAAAGTGTATCTGTGCTTCTTAG-3′	5′-AATAACCTGCGGTGTATGCATTTCC-3′	150	56
ISPE/EF199769	5′-TTCATCTAGATAAGAAGGTGCCAAC-3′	5′-CCTCTACCAGTACAATAAGCAGCTC-3′	110	55
MECPS	5′-ATCTATAGCGGCAAACCTACAC-3′	5′-ACTTTAGAGAGGGATGGAGGG-3′	110	57.1
HDS/JQ950334	5′-ATGCCATTTAAGGAACTTGCAACAG-3′	5′-GGAGCACCACCAACATATCCAAATT -3′	110	58
ISPH/EF199770	5′-CATACTGGGTTGACAGTGATGTAAG-3′	5′-TAAGGACATCTTCAACAGCCTTATC-3′	110	57.2

ACTH acetoacetyl-CoA thiolase, *HMGS* 3-hydroxy-3-methylglutaryl-CoA synthase, *HMGR* 3-hydroxy-3-methylglutaryl-CoA reductase, *MVK* mevalonate kinase, *PMK* phosphomevalonate kinase, *MVDD* mevalonate diphosphate decarboxylase, *IPPI* isopentenyl pyrophosphate isomerase, *GDPS* gerenyl diphosphate synthase, *DXPS* 1-deoxy-D-xylulose 5-phosphate synthase, *DXPR* 1-deoxy-D-xylulose 5-phosphate reductoisomerase, *ISPD* 2-C-methylerythritol 4-phosphate cytidyl transferase, *ISPE* 4-(cytidine-5′-diphospho)-2-C-methylerythritol kinase, *MECPS* 2-C-methylerythritol-2,4-cyclophosphate synthase, *HDS* 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase, *ISPH* 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase

Results and discussion

Differential biosynthesis and accumulation of P-I and P-II

P-I and P-II contents in *P. kurroa* shoots grown in tissue cultures at 25 °C (TCS-0) showed negligible amounts of P-I content (0.01 µg/mg) compared to tissue culture shoots grown at 15 °C (TCS-6) with 6 µg/mg of P-I content. No P-II was detected in the tissue culture shoots grown at 25 °C and 15 °C. Shoots (FGS-27) collected from nursery grown plants of *P. kurroa* in the month of September showed high P-I content (27 µg/mg) compared to shoots (FGS-12) of the same plants collected in the month of July with relatively lower P-I content (12 µg/mg) (Fig. 2; Table 3). The underground young stolons (UStY) from nursery grown *P. kurroa* contained relatively low amounts of P-I (2.9 µg/mg) and P-II (2.7 µg/mg) compared to underground mature stolons (UStM) from the same *P. kurroa* plants, which contained significantly higher amounts of P-I (6.0 µg/mg) and P-II (7.0 µg/mg). The higher amounts of P-I and P-II in mature stolons may be

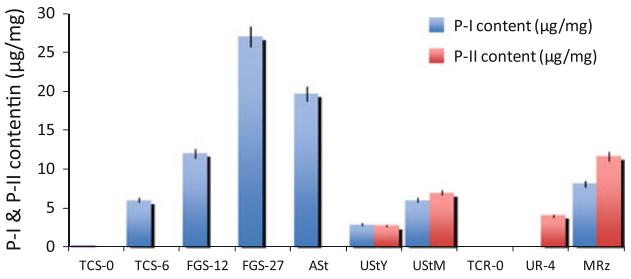

Fig. 2 Picroside-I and Picroside-II contents in different growth and developmental stages of *P. kurroa*. *TCS-0* tissue culture shoots at 25 °C, *TCS-6* tissue culture shoots at 15 °C, *FGS-12* field grown shoots (July), *FGS-27* field grown shoots (September), *ASt* aerial stolon, *UStY* underground stolon young, *UStM* underground stolon mature, *TCR-0* tissue culture roots, *UR-4* field grown roots, *MRz* mature rhizome

due to age difference. The aerial stolons (ASt) contained only P-I (19.7 µg/mg) and no P-II. The higher amount of P-I in aerial stolons is analogous to shoots, which also contain only P-I. The nursery grown mature rhizome (MRz) contained both P-I (8.1 µg/mg) and P-II (11.6 µg/mg). The roots derived from tissue culture grown plants of *P. kurroa* grown at either 25 °C (TCR-0) or 15 °C (TCR-0) contained no P-I and P-II, whereas roots (UR-4) from nursery grown plants contained P-II (4 µg/mg) (Fig. 2; Table 3). The estimation of P-I and P-II in different tissues and developmental stages of *P. kurroa* resulted in the identification of differential conditions for the biosynthesis and accumulation of P-I and P-II. Shoots with a progressive increase in P-I content from 0 to 27 µg/mg and roots with 0 and 4 µg/mg of P-II were identified. The underground stolons provided conditions with a progressive increase in the contents of both P-I and P-II. The higher amounts of P-I content in nursery grown shoots as compared to tissue culture shoots can be attributed to the influence of environmental factors such as altitude, temperature, light, radiations (UV), etc., which is in accordance with work done by Katoch et al. [30] and Kawoosa et al. [31]. It has also been reported that the, resperine content in *Rauwaolfia serpentina* and camptothecin content in *Nothapodytes nimmoniana* are influenced by the climatic factors [32].

The detection of P-II only in roots of nursery grown plants compared to roots in tissue culture grown plants of *P. kurroa* is probably due to the origin of roots, because roots in nursery grown plants originate mostly from the underground mature stolons or the mature rhizomes, whereas roots in tissue culture grown plants originate directly from the shoot base because there is no stolon or rhizome formation in tissue culture shoots. However, why this difference in origin of roots influences the biosynthesis of P-II remains to be investigated. The biosynthesis and accumulation of secondary metabolites do occur differentially in different cell types, tissues and organs and is influenced by their developmental status. For example the cell type-specific localization of protoberberine alkaloid biosynthesis and accumulation are temporally and spatially separated in roots and rhizomes of *T. flavum* [47]. The biosynthesis and accumulation of flavonoids (rutin) occur differentially in different developmental stages of *Fagopyrum* species [19].

The estimation of P-I and P-II contents in different tissues and organs of *P. kurroa,* therefore, suggests that the biosynthesis of P-I occur predominantly in the shoots and aerial stolons, whereas on the other hand P-II is biosynthesized in the roots or may be in the underground stolons. The preferential accumulation of P-I in shoots and that of P-II in roots is in accordance with the previous reports [55].

Cloning additional MEP and MVA pathway genes in *P. kurroa*

Three genes, ISPD, MECPS and HDS of MEP pathway and two genes, HMGS and PMK of MVA pathway were cloned through comparative genomics from *P. kurroa* using degenerate primers designed from the conserved gene sequence regions (Table 1). Degenerate primers yielded 2 amplicons of $\approx$ 510 bp for HDS and ISPD and a single amplicon of $\approx$ 350 bp for PMK, MECPS and HMGS. The amplicons were sequenced and those with identity to genes from other plant species were selected for designing primers for gene expression analysis (Table 2).

Differential expression of MEP and MVA pathway genes in different tissues with respect to P-I content

Most of the genes of MEP and MVA pathways showed relatively high expression in *P. kurroa* tissues/organs containing P-I. Four genes (DXPS, ISPD, ISPE and MECPS) of MEP pathway and one (PMK) of MVA pathway showed elevated levels of transcript in shoots and stolons compared to roots and rhizomes. The expression of DXPS increased from 11 to 57 folds with a corresponding increase of P-I from 6.0 to 27.0 µg/mg in shoots of *P. kurroa,* respectively (Figs. 3a, 4; Additional File 1). DXPS is the first enzyme of MEP pathway and plays an important role in the regulation of this pathway [10, 16]. The accumulation of monoterpenes in *Moscato bianco* berries as well as of artemisinin in *Artemisia annua* also correlates with DXPS expression [40, 4]. The fourth gene MECPS of MEP pathway showed very high increase in its transcript (56-folds) in shoots with P-I (6.0 µg/mg) compared to shoots with no P-I content. The transcript of MECPS increased to 166.7-fold in shoots containing P-I (27.0 µg/mg), which was the highest fold increase in the expression of a gene among all other genes (Figs. 3a, 4; Additional File 1). The higher expression level of MECPS

Table 3 Growth and developmental stages of *P. kurroa* showing differential biosynthesis and accumulation of P-I and P-II

Sample	Source	P-I content (μg/mg)	P-II content (μg/mg)	
TCS-0	Tissue culture	0.01	–	
TCS-6	Tissue culture	6.0	–	
FGS -12	Nursery (July)	12.0	–	
FGS-27	Nursery (September)	27.0	–	
ASt	Green house	19.7	–	
UStY	Nursery (July)	2.9	2.7	
UStM	Nursery (July)	6.0	7.0	
TCR-0	Tissue culture	–	–	
UR-4	Nursery	–	4.0	
MRz	Nursery	8.1	11.6	

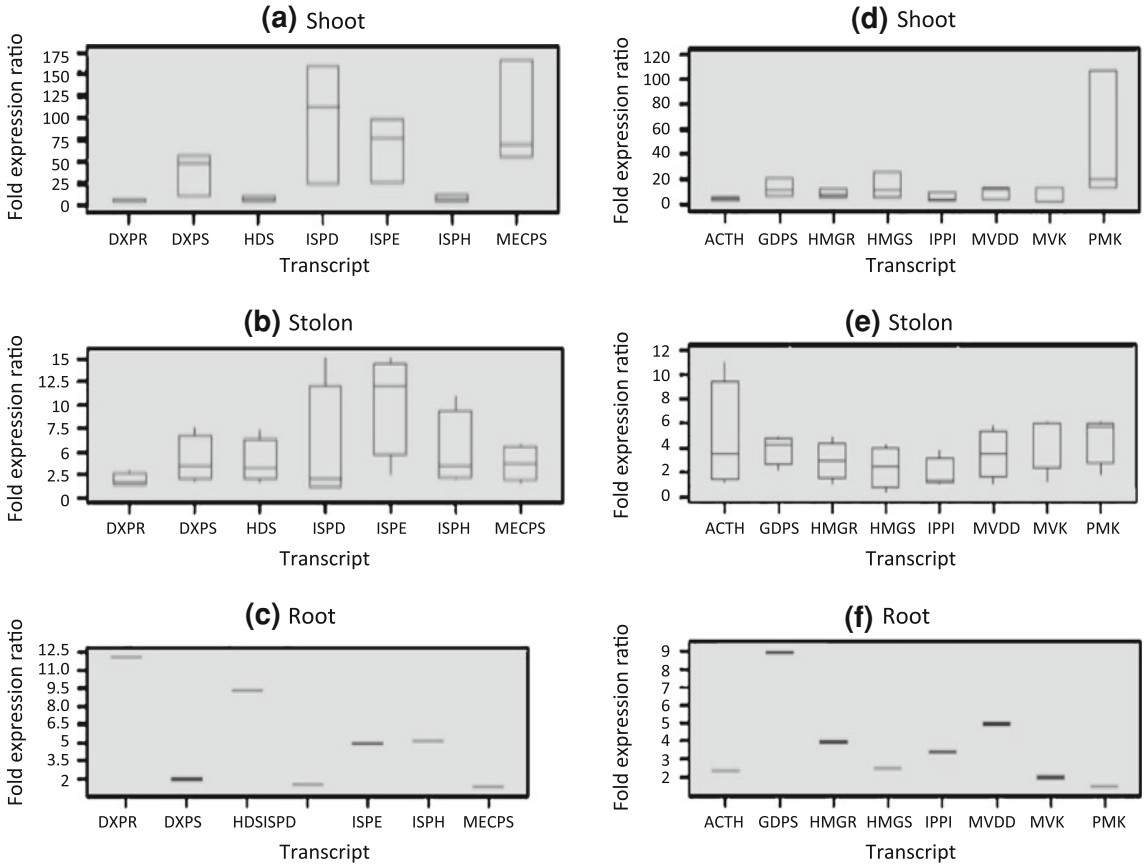

Fig. 3 Expression patterns of MEP (**a**–**c**) and MVA (**d**–**f**) pathway genes in different tissues/developmental stages of *P. kurroa*

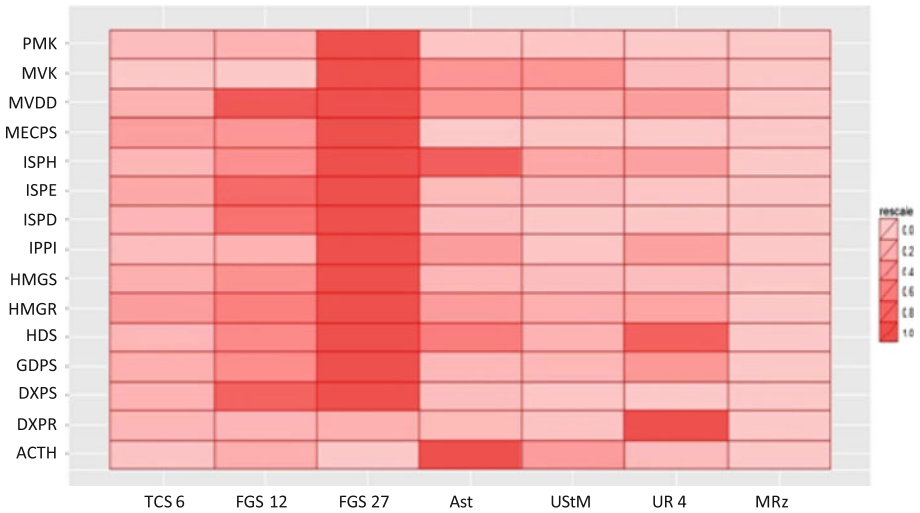

Fig. 4 A representative heat map demonstrating the differential expression pattern of genes involved in MEP and MVA pathway from different tissues/developmental stages. The fold change in expression of these genes is calculated from data in triplicate

has been associated with higher content of taxol in bark and leaves than roots and stem of *Taxus media* [35]. The expression of ISPD and ISPE increased to almost equal folds (25×) in shoots with 6 µg/mg of P-I compared to no P-II. However, the ISPD transcript went up to 160-folds in shoots with 27 µg/mg P-I compared to ISPE with 99 folds increase (Figs. 3a, 4; Additional File 1). The 4 genes of MEP pathway showed elevated expression in shoots (Fig. 3a), but their expression was relatively low in aerial stolons and underground mature stolon (Fig. 3b) with P-I contents of 19.7 µg/mg and 6 µg/mg, respectively. Only one gene, PMK out of 6 genes of MVA pathway showed increase in its transcript to 12-folds reaching to 107.7-folds in shoots (Fig. 3d) with P-I contents 6.0 µg/mg and

27.0 µg/mg. Relatively high expression of MEP pathway genes in shoots as compared to aerial stolons and underground mature stolon of *P. kurroa* may be due to the fact that MEP pathway enzymes are localized in plastids, particularly in chloroplasts [34, 48, 23]. In petals of *Snapdragon* flowers the MEP pathway provides IPP and DMAPP for the plastidial monoterpenes biosynthesis and operates only in light in the rhythmic manner controlled by the circadian clock [13]. The role of chloroplast in the biosynthesis of P-I was also suggested through tissue culture experiments in *P. kurroa* where no P-I was detected in the callus cultures derived from leaves whereas P-I was detected in shoots regenerated from the same callus cultures [54]. Furthermore, it was also confirmed in *Arabidopsis thaliana* by spectral counting approach that all enzymes of the MEP pathway reside in the stroma [25]. The expression of 5 genes (DXPS, ISPD, ISPE, MECPS, HMGS and PMK), which showed higher expression with P-I content mainly in shoots and to a lesser extent in the stolons, showed no change in there transcript abundance in the mature rhizomes (Fig. 4; Additional File 1). Rather the amounts of transcripts of those 5 genes were similar to other 10 genes of MEP and MVA pathways in the mature rhizome, thereby showing that the P-I is only biosynthesized in the shoots.

Picroside-II biosynthesis is controlled by differential expression of MEP pathway genes

Expression analysis of 15 genes of MEP and MVA pathways showed that only 2 genes, DXPR and HDS of the MEP pathway had elevated expression levels in roots with P-II content. The transcripts of DXPR and HDS increased to 12.12 and 9.4 folds, respectively in roots containing P-II (4.0 µg/mg) compared to roots with no P-II (Fig. 3c; Additional File 2). Although the expression of ISPH and ISPE was also high (5-fold) in roots accumulating P-II but the expression of both these genes was also high in aerial stolons which only contained P-I whereas no P-II (Figs. 3b, c). The DXPR gene serves as a significant control point, because it is the first committed step of the MEP pathway of terpenoid biosynthesis [57]. Positive correlations between isoprenoid accumulation and DXPR transcript levels are reported in gramineous plants [60, 21]. The DXPR expression is also known to be positively correlated with the isoprenoid biosynthesis in peppermint [36] and diterpenoid tanshinone accumulation in hairy roots of *Salvia miltiorrhiza* [62]. The GDPS gene, which codes for geranyl diphosphate synthase converts IPP to GDP, also showed positive correlation with the biosynthesis of P-I and P-II. GDPS was the only gene with high transcript levels in both the roots and shoots. GDPS expression was 9-fold in roots with P-II (4.0 µg/mg) and 20-fold in shoots with P-I (27 µg/mg), (Figs. 3d, f) which is analogous in *Salvia miltiorrhiza*, where GDPS gene was expressed at higher levels in leaves and roots and lower in stems correlating with the biosynthesis of tanshinones [27].

Conclusions

The present research led to the cloning of five genes of terpenoid biosynthesis pathway and also analyzed the expression of fifteen genes in relation to P-I and P-II content in *P. kurroa*. Relatively higher expression of 4 genes of MEP and one gene of MVA pathway in shoots compared to stolon, roots and rhizomes showed positive correlation with picrosides content. Differential conditions for the biosynthesis and accumulation of major medicinal compounds, P-I and P-II of *P. kurroa*, would be useful to regulate the quality of plant material to be used in the preparation of herbal drug formulations. The key genes with elevated expression vis-à-vis P-I and P-II contents are suitable targets for using in genetic improvement of *P. kurroa* for enhanced production of picrosides in different tissues. It was also concluded from the P-I and P-II contents in different tissues of *P. kurroa,* that the biosynthesis of P-I occur predominantly in the shoots and aerial stolons, whereas on the other hand P-II is biosynthesized in the roots or may be in the underground stolons and then picrosides are transported and stored in the rhizomes where the expression of fifteen genes of the terpenoid pathway did not show any increase vis-à-vis picrosides content, but the molecular mechanism of transport remains to be investigated.

Acknowledgments The authors are thankful to the Department of Biotechnology, Ministry of Science & Technology, Govt. of India for a grant to RSC. We are also thankful to Himalayan Forest Research Institute, Panthaghati, Shimla, India for providing plant material of *P. kurroa.*

Conflict of interest Authors declare that they do not have any conflict of interest.

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