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ORIGINAL ARTICLE

# ABC transporters mined through comparative transcriptomics associate with organ-specific accumulation of picrosides in a medicinal herb, *Picrorhiza kurroa*

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## Abstract

*Picrorhiza kurroa* Royle ex Benth is a valuable medicinal herb of North-Western Himalayas due to presence of two major bioactive compounds, picroside-I and picroside-II used in the preparation of several hepatoprotective herbal drugs. These compounds accumulate in stolons/rhizomes; however, biosynthesized in different organs, viz., picroside-I in shoots and picroside-II in roots. As of today, no information exists on what transporters are transporting these metabolites from shoots and roots to the final storage organ, stolon, which ultimately transforms into rhizome. The ATP-binding cassette (ABC) transporters are reported to transport majority of secondary metabolites, including terpenoids in plants, therefore, we mined *P. kurroa* transcriptomes to identify and shortlist potential candidates. A total of 99 ABC transporter-encoding transcripts were identified in 3 differential transcriptomes, PKSS (shoots), PKSTS (stolons), and PKSR (roots) of *P. kurroa*, based on in silico comparative analysis and transcript abundance. 15 of these transcripts were further validated for their association using qRT-PCR in shoots, roots and stolon tissues in *P. kurroa* accessions varying for picroside-I and picroside-II contents. Organ-specific expression analysis revealed that *PkABCA1*, *PkABCG1*, and *PkABCB5* had comparatively elevated expression in shoots; *PkABCB2* and *PkABCC2* in roots; *PkABCB3* and *PkABCC1* in stolon tissues of *P. kurroa*. Co-expression network analysis using ABC genes as hubs further unravelled important interactions with additional components of biosynthetic machinery. Our study has provided leads, first to our knowledge as of today, on putative ABC transporters possibly involved in long distance and local transport of picrosides in *P. kurroa* organs, thus opening avenues for designing a suitable genetic intervention strategy.

**Keywords** *Picrorhiza kurroa* · Picroside-I and picroside-II · ABC transporters · Transcriptome · Gene expression

## Introduction

*Picrorhiza kurroa* is an endangered perennial Himalayan medicinal plant belonging to Plantaginaceae family. The plant has high trade value due to the occurrence of biologically active compounds, picroside-I and picroside-II which are differentially present in shoots, roots, and stolons, having potent hepatoprotective property (Shitiz et al. 2015). Herbal formulations of *P. kurroa* have been used for the treatment of asthma, malaria, chronic diarrhoea, jaundice, and liver disorders (Bhardwaj et al. 2021). Picroside-I and picroside-II are monoterpenoids produced as secondary metabolites in *P. kurroa*. Monoterpenoids contain a broad group of compounds that are synthesized through the phenylpropanoid/shikimate pathways in plants (Tohge and Fernie 2017).

Several studies have shown that plant metabolites tend to accumulate in specific organs, instead of being equally distributed in all tissues (Wang et al. 2019). These metabolites migrate from source organ where they are synthesized to the sink organ where they are found in higher concentrations while passing through several membranes. For instance, in *Nicotiana* species, root tissues serve as the source organ where nicotine biosynthesis occurs which is transported

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through aerial parts and accumulates within the vacuoles of leaves (sink organ) (Kato et al. 2015).

Various cellular and biochemical factors regulate biosynthesis and accumulation of secondary metabolites both temporally and spatially in plant organs (Yang et al. 2012). The secondary metabolites are transported through either long distance or short distance depending on the sink and the source organ. Secondary metabolites in plants are known to transport through various mechanisms such as diffusion or via membrane transporters (Shitan 2016). Plant cells are highly compartmentalized; thus, metabolite transporters present on the vacuole and/or plasma membrane are required to facilitate the movement of metabolites across cell membranes. Membrane transporters in plants are often found in critical locations to govern whole pathways thus play a crucial role in biosynthesis and metabolite accumulation. These locations are the entry and exit points, mainly present on the membranes such as plasma membrane, vacuolar membrane, or other organelle membranes, which allow the transport of metabolites in and out of the cell. Secondary metabolites and their precursors are transported by the membrane transporters (Gani et al. 2021). For instance, *CjABCB2*, ABC B family transporter located on plasma membrane has been reported to transport berberine from root tissue (source organ) to the rhizome (sink organ) in *C. japonica* (Shitan et al. 2013). Similarly, *AtABCC*, ABCC family transporter located on tonoplast has been reported to mediate vacuolar transport of anthocyanins in *A. thaliana* (Behrens et al. 2019).

Significant progress has been made in understanding biosynthetic pathways of picroside-I and picroside-II in *P. kurroa* such as identification of key enzymes, gene paralogs, transcription factors, miRNAs, and kinases (Pandit et al. 2013a; Sharma et al. 2016; Vashisht et al. 2016, 2018; Kharb and Chauhan 2021). Recently, our group has identified putative acyltransferases which catalyze the last step of picroside-II biosynthesis through acylation of vanilloyl coA and catalpol (Kharb et al. 2022).

Our previous studies have shown that picroside-I is synthesized in shoots, while picroside-II is synthesized in roots and both are transported to accumulate in stolons/rhizomes (Sood and Chauhan 2010; Pandit et al. 2013b; Shitiz et al. 2015) (Fig. 1). However, transporters involved in transport of picroside-I from shoots to the rhizomes and picroside-II from roots to the rhizomes, as well as intracellular/ vacuolar storage mechanism of picroside-I and picroside-II in different tissues, is yet to be identified.

ATP-binding cassette (ABC) transporters are known to transport secondary metabolites between intercellular tissues, such as roots and shoots. In a plant cell, vacuole membrane possesses ABC transporters, channels, and pumps, facilitating metabolite transfer. ABC transporters help to transport plant metabolites into the vacuoles of plant cells

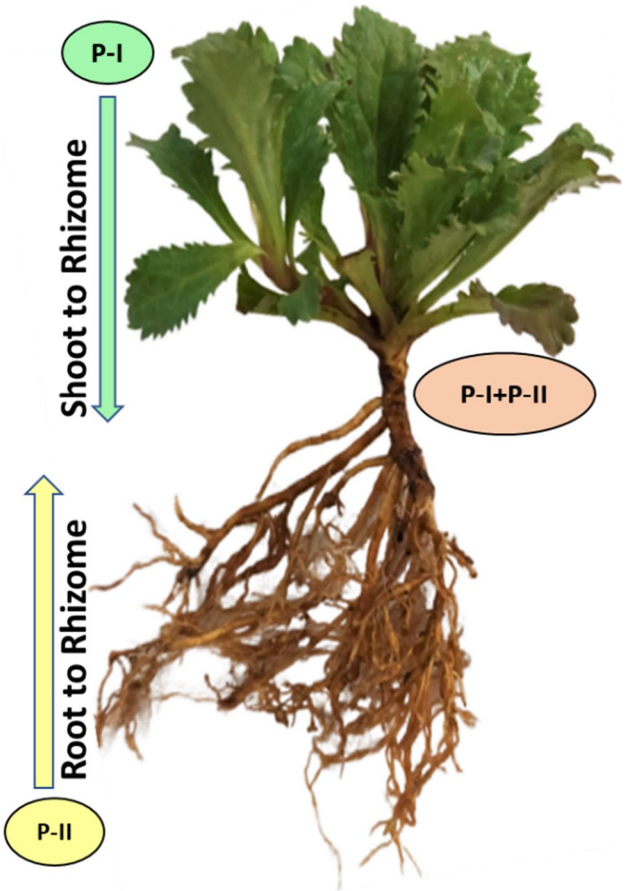


**Fig. 1** Picroside-I (P-I) and picroside-II (P-II) transport from source organ to sink organ in *Picrorhiza kurroa*

(Shitan and Yazaki 2020). For instance, *ZmMRP3* belonging to ABCC family has been reported as vacuolar anthocyanin transporter in *Zea mays* (Goodman et al. 2004), *CsABCC4* belonging to ABCC family has been reported using the transportomic approach to transport crocins in *Crocus sativus* (Saffron) (Demurtas et al. 2019), and *VvABCC1* in *Vitis vinifera* functions as anthocyanidin 3-O-glucosides transporters (Francisco et al. 2013). ABC family constitutes the most common transporters found in cell membranes of plants, animals, bacteria, and fungi to translocate different substrates (Rees et al. 2009). ABC family is one of the largest transporter proteins family, categorized into nine subfamilies ABCA–ABCI out of which ABCH is not found in plants and ABCG is found only in plants and fungi (Banasiak and Jasiński 2022). ABC transporters have nucleotide-binding domain (NBD) in addition to highly conserved motifs; Walker A (GXG-K-[ST]), Walker B ([RK]- X3-G-X3-L-[hydrophobic]3) motifs, and ABC signature motif ([LIVMFY]-S-[SG]-G-X3-[RKA]-[LIVMYA]-X-[LIVMF]-[AG]). They also have transmembrane domains (TMDs), in addition to NBDs which are composed of numerous

hydrophobic α-helices. There are four key domains in a functional ABC transporter, viz., two NBDs and two TMDs. The two NBDs work together to bind and hydrolyse ATP, which acts as a driving force for the transport of metabolites, while the TMDs participate in substrate recognition (Gani et al. 2021). The classification of ABC transporter family is according to their sequences and TMD structures (Xiao et al. 2021).

ABC transporters are involved in a variety of processes, including plant growth, nutrition, and development, as well as plant response to abiotic and biotic stresses and plant-environment interaction (Guo et al. 2022). Thus, investigating the role of ABC transporters in transport of secondary metabolites is of immense interest for directing their accumulation in specific organs.

Secondary metabolites such as anthocyanins, terpenoids, alkaloids, flavonoids, and carotenoids are transported across the membranes via ABC transporters. For instance, in *C. japonica*, benzylisoquinoline alkaloid berberine is synthesized in roots and is transported to rhizomes by an ABCB-type transporter (Shitan et al. 2003). Similarly, *AaPDR3* is reported to transport a sesquiterpenoid named β-caryophyllene in trichomes of *A. annua* (Fu et al. 2017). Transportation of diterpene compound, sclareol, in *Nicotiana* species is mediated by a PDR member (*NpABC1*) (Jasiński et al. 2001).

In the present study, we have described the first analysis of ABC transporters superfamily in *P. kurroa* transcriptomes followed by their validation through transcript per million (TPM) values, expression analysis by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and co-expression network analysis to identify not only the potential ABC transporters associated with transport of picroside-I and picroside-II but also unravelling additional components of biosynthetic machinery.

## Materials and methods

### Collection of plant material

*P. kurroa* Royle ex benth plants were collected from Regional Station of the Himalayan Forest Research Institute (HFRI), Kullu, Himachal Pradesh, India, at an elevation of 2193 m. The plants collected were washed with water and were segregated tissue wise. Shoots, roots, and stolons were labelled as PKSS, PKSR, and PKSTS, respectively. Samples were maintained at − 80 °C for their use in future experimentation. *P. kurroa* accessions selected for further qRT-PCR analysis are detailed in Table 1.

### Mining of potential ABC transporters from NGS transcriptome datasets

For identification of potential ABC transporters that are possibly associated with transport and accumulation of picroside-I and picroside-II in *P. kurroa*, three transcriptome datasets, viz., PKSS (2.7% picroside-I), PKSTS (1.7% picroside-I + 0.99% picroside-II), and PKSR (0.4% picroside-II) differing for picroside contents were mined computationally for sequences that have putative ABC transporter function. The transcripts were further shortlisted based on their TPM values.

### Comparative analysis of putative ABC transporters from NGS transcriptome datasets

In silico comparative analysis of ABC transporters-encoding transcripts was performed using three transcriptome datasets to identify potential ABC transporters relating to picroside-I and picroside-II transport and accumulation. BLASTn tool was used to extract sequences from various combinations of three transcriptome datasets for comparative analysis of mined ABC-encoding transcripts. ABC transporters unique in shoots were shortlisted for picroside-I transport and those unique in roots were shortlisted for picroside-II. Similarly, transporters unique to stolons were shortlisted for accumulation of both picroside-I and picroside-II. The candidate transcripts with higher TPM values were shortlisted for qRT-PCR verification. The detailed experimental strategy is described in Fig. 2.

### Isolation of total RNA and cDNA synthesis

RNA was extracted from three tissues of *P. kurroa*: shoots, roots, and stolons using total RNA isolation kit (Takara) using

**Table 1** Accessions shortlisted for qRT-PCR analysis based on differential picroside(s) contents in different tissues/organs of *Picrorhiza kurroa*

| Tissue/organ | Accessions (high picroside(s) %) | Accessions (low picroside(s) %) |
|---|---|---|
| Shoots | PKS54 (7.3% picroside-I) | PKS08 (0.25% picroside-I) |
| | PKS78 (5.46% picroside-I) | PKS25 (0.15% picroside-I) |
| Roots | PKR84 (2.68% picroside-II) | PKR08 (0.07% % picroside-II) |
| | PKR82 (2.37% picroside-II) | PKR57 (0.18% picroside-II) |
| Stolons | PKST67 (7.36% picroside-I + picroside-II) | PKST43 (0.07% picroside-I + picroside-II) |
| | PKST43 (0.12% picroside-I + picroside-II) | PKST03 (0.10% picroside-I + picroside-II) |

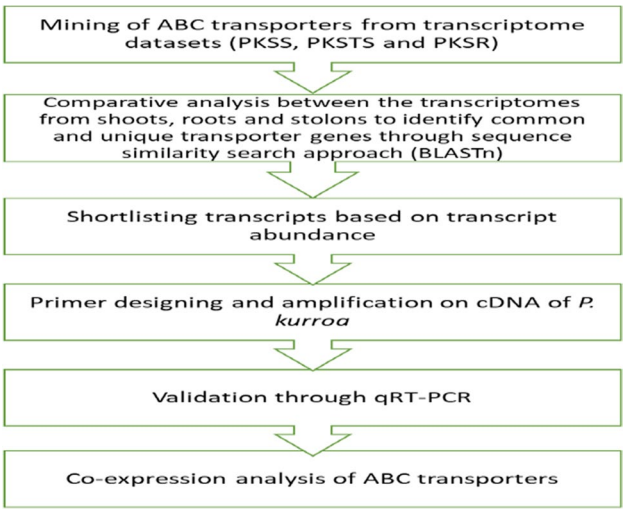


**Fig. 2** Experimental strategy for identification and shortlisting of ABC transporters in transcriptomes of *Picrorhiza kurroa*

the manufacturer's protocols. Quality of RNA was analyzed on 1% (w/v) ethidium bromide-stained agarose gel and was quantified in NanoDrop spectrophotometer (Thermo Scientific) by measuring absorbance at 260 nm wavelength and purity at 260/280 nm ratio. First-strand complementary DNA (cDNA) was synthesized from 1 µg of RNA using cDNA synthesis kit (Takara) as per the manufacturer's instructions. cDNA concentration for every sample was adjusted to equal volume for qRT-PCR analysis.

## qRT-PCR verification

The qRT-PCR analysis of 15 ABC transporter genes was performed on different tissues; shoot, root, and rhizome of *P. kurroa* accessions to evaluate the association of a particular transporter with picroside content. Primer 3 (v.0.4.0) software was used to design primers for qRT-PCR (supplementary Table 1). qRT-PCR reaction was performed in a CFX96 system (Bio-Rad Laboratories) with the Hi-SYBR master mix including Taq Polymerase (Hi-media) using gene-specific primers in triplicates. For analyzing variation in gene expression level, cDNA in equal quantities (100 ng) were taken for each sample to perform qRT-PCR, and protocol was as follows: denaturation at 95 °C (3 min), 95 °C (30 s), a corresponding annealing temperature 52–57 °C (45 s), and extension 72 °C (30 s) in 35 cycles. The reactions were performed in three biological and technical replicates with 26srRNA gene as an internal control for normalization.

## Picroside-I and picroside-II extraction and HPLC analysis

Accessions of *P. kurroa* stored at − 80 °C were estimated further for their picroside-I and picroside-II contents in shoots, roots, and stolons using reverse-phase high-performance liquid chromatography (RP-HPLC). *P. kurroa* tissues were ground to fine powder using liquid nitrogen. To 100 mg of the powdered samples, 10 mL of 80% HPLC-grade methanol was added. The contents were extracted by incubating overnight at ~ 25 ℃. Samples were centrifuged and filtered through 0.22 µm syringe filter for RP-HPLC analysis (Pandit et al. 2013b). A RP-HPLC system with Sunfire C-18 column (4.6 × 250 mm, 5 µm, Waters) and photodiode array detector (Waters 2998) was utilized for measuring of picroside-I and picroside-II at 280 nm. The mobile phase at isocratic mode consisted of solvent A 0.05% trifluoroacetic acid (TFA) in water (Merck, USA) and solvent B acetonitrile/methanol (Merck, USA) in 1:1 ratio. Sample volume of injected in HPLC run was 20 µL at 30 °C for 30 min at flow rate of 1.0 mL $min^{-1}$. Extraction and quantification of samples were performed in biological as well as technical triplicates (Kumar et al. 2016b). Standards of picroside-I (G-9923) (https://www.sigmaaldrich.com/IN/en/product/sigma/g9923) and picroside-II (G-0174) (https://www.sigmaaldrich.com/IN/en/product/sigma/g0174) with purity of 98% were obtained from Sigma. Retention time in the HPLC runs was observed at 21.0 ± 0.5 min for picroside-I and 10.5 ± 0.5 min for picroside-II.

## In silico domain analysis and subcellular localization of ABC transporters

The shortlisted ABC transporters were further checked for domain/motif similarity with functionally characterized transporters in different plant species. This was validated by aligning transcripts with the draft genome of *P. kurroa* (Sharma et al. 2021b) resulting in identification of genome sequence contigs. The sequence contigs were further annotated using GenScan (http://hollywood.mit.edu/GENSCAN.html) for predicting genes and proteins. The predicted proteins encoding ABC transporters were further analyzed for domain analysis using Pfam 35.0 (Mistry et al. 2021). Furthermore, Cell Ploc2 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) was used to verify cellular location of the candidate protein sequences.

## Co-expression network analysis

The strategy defined in our previous study was applied to build gene co-expression networks for three different transcriptomes of *P. kurroa* (Sharma et al., 2021a, b). GENIE3 was used to generate individual co-expression network for every transcriptome (Huynh-Thu et al. 2010) with parameter of "ensemble" with K value 7, and number of ensembles 50. Every relationship between transcripts in the form of a link list had a connectivity threshold of > 0.005. Three separate linked lists representing three transcriptome samples

were created. The gene co-expression linked list was used to extract linked pairs of putative function related to ABC transporters, which were then displayed using Cytoscape Network Visualization software (Shannon et al. 2003). The Cytoscape software's network analyzer was used to determine the degree of each node in the network. For comparison of transcript abundance amongst co-expressed organ-specific sub-networks, TPM values of nodes were included as an effective addition to the generated network graphs. The size in the network represents the degree of freedom, and the color represents expression in the form of TPM value.

## Results

### Identification of ABC transporters in *P. kurroa* transcriptomes

ABC transporters were mined from PKSS, PKSR, and PKSTS datasets of *P. kurroa* transcriptomes. In total 99 transcripts encoding transporters were identified that witnessed tissues/organ-specific expression, out of which 9 were ubiquitously expressed in all three tissues, 39 were expressed in PKSS, 23 in PKSR, and 37 in PKSTS. Distribution of ABC transporters was computed to be 10% in PKSTS as compared to PKSS and PKSR with 9% and 6%, respectively (Fig. 3). The ABC transporters were differentially distributed in shoots, roots, and stolons of *P. kurroa.* In shoot, a total of 14 ABC B family members were observed in contrast to 6 for ABC F family. Similarly, in stolons, only three ABC F family members were discovered, compared to 15 ABC B family (Fig. 4).

Abundance of common transcripts encoding ABC transporters was calculated in the form of TPM values. Transcripts having high TPM value were selected for comparative analysis for the identification of candidate transporters associated with picroside-I and picroside-II contents. TPM values were determined for 99 ABC transporters using Salmon tool. Transcript abundance for commonly present ABC transporters ranged from 0.16 to 44.20 in PKSS, 0.44 to 25.43 in PKSR, and 0.68 to 60.18 in PKSTS datasets. Transcripts pknode_807 showed highest expression in PKSS (44.20 TPM value), pknode_74 in PKSR (25.43 TPM value), and pknode_738 in PKSTS (60.18 TPM value). The ABC transporter transcripts with higher transcript abundance were further shortlisted for validation through qRT-PCR to assess their association with picroside-I and picroside-II accumulation.

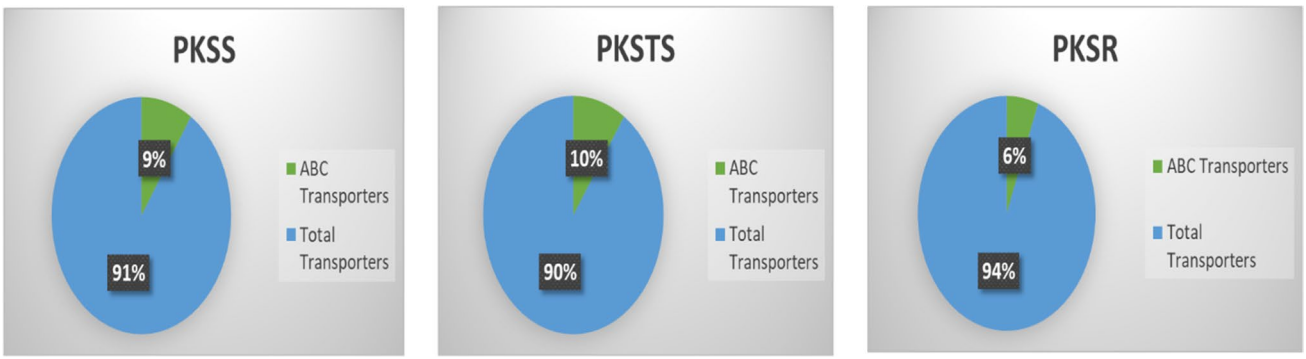


**Fig. 3** Proportion of ABC transporters among PKSS, PKSR, and PKST transcriptomes of *Picrorhiza kurroa*

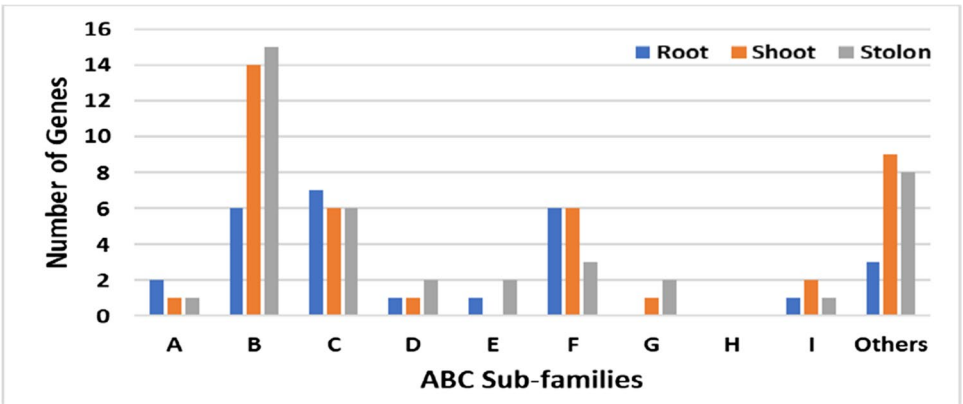


**Fig. 4** Distribution of ABC transporters among different subfamilies in transcriptomes of *Picrorhiza kurroa*

## Identification of common and unique ABC transporters

In this study, a detailed comparative analysis of ABC transporters in the transcriptomes of three tissues of *P. kurroa*, i.e., PKSS, PKSR, and PKSTS was carried out to identify candidate ABC transporters possibly involved in transport of picroside-I and picroside-II. Thirty-three ABC transporters were found to be commonly present in PKSS and PKSTS transcriptomes while 6 and 7 transporters were uniquely present in PKSS and PKSTS, respectively. Similarly, 16 ABC transporters genes were commonly present in PKSR and PKSTS transcriptomes while 8 and 21 transporters genes were uniquely present in PKSS and PKSTS, respectively. The unique ABC transporters identified in shoots transcriptomes of *P. kurroa* were considered possibly associated with the transport of picroside-I from shoots to stolons/rhizomes. Similarly, unique ABC transporters genes in roots transcriptome may be responsible for the transport of picroside-II from roots to stolons/rhizomes. The common transcripts among tissues might suggest their involvement in the transport of some common primary or secondary metabolites.

## Expression analysis of candidate ABC transporter genes among *P. kurroa* accessions varying for picrosides

The relative expression of 15 shortlisted ABC transporter genes was analyzed using qRT-PCR in shoots, roots, and stolons tissues of *P. kurroa* varying for picroside-I and picroside-II contents. *P. kurroa* accessions PKS 54 (7.31% fresh weight picroside-I) and PKS 78 (5.46% fresh weight picroside-I) showed higher accumulation of picroside-I whereas PKS 08 (0.25% fresh weight picroside-I) and PKS 25 (0.15% fresh weight picroside-I) showed less accumulation of picroside-I content in shoots. For picroside-II, accessions PKR 84 (2.68% fresh weight picroside-II) and PKR 82 (2.37% fresh weight picroside-II) showed higher accumulation of picroside-II whereas PKR 57 (0.18% fresh weight picroside-II) and PKR 08 (0.07% fresh weight picroside-II) showed lesser amount. Similarly, in stolons, PKST 67 (7.36% fresh weight picroside-I + picroside-II) and PKST47 (7.15% fresh weight picroside-I + picroside-II) showed higher accumulation of both the compounds (picroside-I + picroside-II) whereas PKST 43 (0.12% fresh weight picroside-I + picroside-II) and PKST03 (0.10% fresh weight picroside-I + picroside-II) accumulated in less amount for both the compounds. Overall, these accessions provided contrasting content phenotypes of picroside-I and picroside-II, therefore, were used to pinpoint association of 15 transporters responsible in transport of picrosides.

According to gene expression patterns, 4 transcripts—*PkABCA1*, *PkABCG1*, *PkABCB5*, and *PkABCB4*—were highly expressed in high picroside-I content accessions (PKS 54 and PKS 78) with relative expression ranging from 1.11 to 8.98 in comparison to shoots of low-content accessions, PKS 08 and PKS 25 (Fig. 5A). Similarly, for high picroside-II content in roots (PKSR 84 and PKSR 82), 3 genes—*PkABCB2, PkABCF3*, and *PkABCC2*—exhibited higher relative expression ranging from 4.21 to 10.98 (Fig. 5B). In stolon tissues of PKST 64 and PKST 47 having high picroside-I and picroside-II contents, 3 genes—*PkABCB3*, *PkABCC1*, and *PkABCF1*—exhibited higher relative expression ranging from 2.32 to 5.34 in comparison to accession having low picroside-I and picroside-II in stolons (PKST 43 and PKST 03) (Fig. 5C). By contrast, 2 genes—*PkABCF5* and *PkABCB6*—showed low expression levels in high picroside-I content shoots (PKS 54 and PKS 78). For picroside-II in roots, *PkABCB1* and *PkABCF2* exhibited low expression level PKR 84 and PKR 82. In stolon tissues, *PkABCE1* exhibited low relative expression in PKST 67 and PKST 47.

Furthermore, expression level of 15 genes was checked in all tissues of *P. kurroa.* Accession PK 82 exhibiting relatively high content in all the 3 tissues (picroside-I: 2.26%, picroside-II: 2.37%, and picroside-I and picroside-II: 3.36% in shoots, roots, and stolons, respectively). The 6 genes—*PkABCB4*, *PkABCA1*, *PkABCG1*, *PkABCB5*, *PkABCF5*, and *PkABCB6*, expressed high in the shoots while *PkABCB2*, *PkABCF3*, and *PkABCC2* expressed in roots of PK 82. Moreover, 5 genes, *PkABCB3*, *PkABCE1*, *PkABCF2*, *PkABCC1*, and *PkABCF1*, showed high expression in stolons rather than in other tissues of PK 82 (Fig. 6).

## In silico domain/motif analysis and sub-cellular localization of candidate ABC transporters

The predicted proteins from genome sequence contigs corresponding to shortlisted ABC transporters-encoding transcripts were tested for presence of signature domains and motifs. The proteins shared significant similarity with transmembrane domain and nucleotide binding domain of ABC transporters in Pfam analysis. Also, the functionally characterized ABC transporters—*VvABCC1* in *Vitis vinifera*, *NtPDR1* in *Nicotiana tabacum*, and *CjABCB2* in *Coptis japonica*—shared domain/motif similarity with the shortlisted ABC transporter transcripts. Additionally, in silico analysis for the sub-cellular localization of the putative ABC transporter proteins using Cell Ploc2 suggested that these proteins are membrane localized as detailed in Table 2.

## Co-expression network analysis to capture other components of picroside biosynthetic machinery

### Sub-network module specific to picroside-I in shoots

PKSS network module had 107 nodes and 213 edges. Among all the identified ABC transporters, *ABCB* and *ABCC* genes

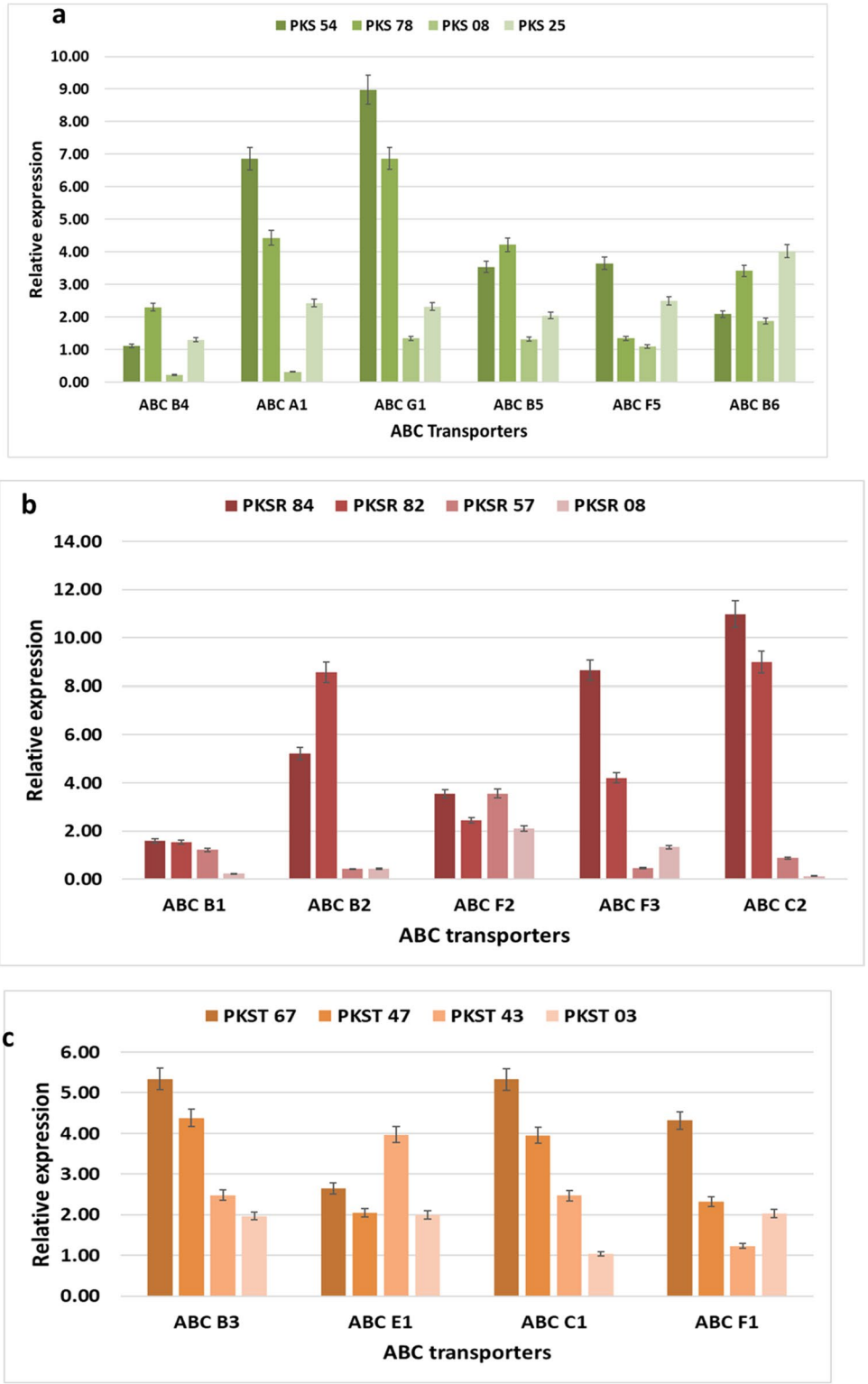


**Fig. 5** Relative expression of ABC transporters in *Picrorhiza kurroa* accessions (**A**) PKS 54 and PKS 78 vs PKS08 and PKS 25 varying for picroside-I content (PKS 54- 7.3%, PKS 78-5.46%, PKS 08-0.25% and PKS 25-0.15%) in shoots (**B**) PKR 84 and PKR8 2 vs PKR08 and PKR 57 varying for picroside-II content (PKR 84-2.68%, PKR 82-2.37%, PKR 08-0.07%, and PKR 57-0.18%) in roots (**C**) PKST 67 and PKST 47 vs PKST 43 and PKST 03 varying for picroside-I + picroside-II contents (PKST6 7-7.36%, PKST 43-0.12%, PKST 43-0.07%, and PKST 03-0.10%) in stolons

were characterized as major hubs with degrees of freedom 50 and 26, respectively, showing strong interconnections with co-expressed genes in the network (Fig. 7). ABCB and ABCC are the main hubs connected with kinases, transcription factors; *MYB4* and *WRKY71* showing strong influence with the transporters. Some important metabolic enzymes such as NADH dehydrogenase, glutathione-S-transferase, serine threonine-protein phosphatase, fructose-bisphosphate aldolase, 3-phosphoshikimate 1-carboxyvinyltransferase, and ketol-acid reductor-isomerase showed co-expressed

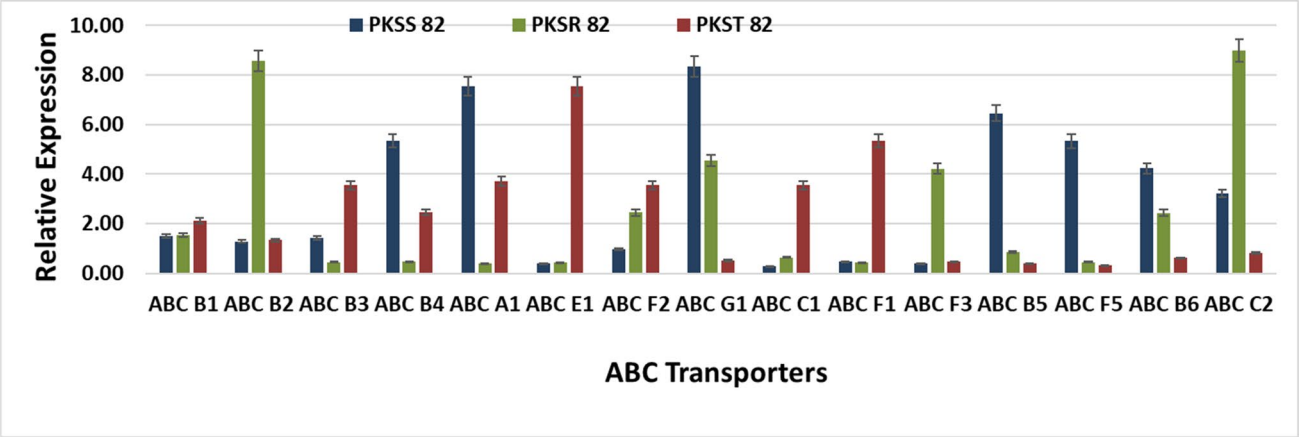


**Fig. 6** Relative expression of ABC transporters in shoots (PKSS), stolons (PKSTS), and roots (PKSR) of *Picrorhiza kurroa*

**Table 2** In silico sub-cellular location of candidate ABC transporter proteins using Cell Ploc2

| Transporter | Sub-cellular location using CELL PLOC 2.0 |
|---|---|
| *ABCB2* | Cell membrane |
| *ABCB5* | Cell membrane |
| *ABCC1* | Chloroplast |
| *ABCG1* | Cell membrane, chloroplast |
| *ABCB3* | Cell membrane |
| *ABCB4* | Cell membrane |

interaction with ABCB family of transporters. Also, ABCB subfamily members were interlinked with other members suggesting that these transporters play an important role in transport of metabolites. *STK* has a role in maintaining the metabolic balance between different terpenoids (Sharma et al. 2021a). Moreover, ABCG transporter gene co-expression network might provide additional clue to picroside-I accumulation and transport via gene–gene co-expressed interactions.

### Sub-network module specific to picroside-II in roots

PKSR network module had 75 nodes and 141 edges. Among all identified ABC transporters, ABCB and ABCC genes were characterized as major hubs with degrees of freedom 26 and 25, respectively, showings co-expression with genes according to the network analysis (Fig. 8). The ABC hub was found to be associated with kinases, transcription factors, and key metabolic enzymes such as hexokinase, glucose-6-phosphate 1-dehydrogenase and an important biosynthetic enzyme, i.e., serine acetyltransferase, which catalyzes the last step of picroside biosynthesis (Kumar et al. 2017a). This enzyme was co-expressed with ABCB suggesting its role in picroside-II biosynthesis in roots of *P. kurroa*. ABCC family members had co-expressed linkage with Zn fingers which regulate secondary metabolites biosynthesis in plants (Vashisht et al. 2016) that might influence picroside-II biosynthesis by regulating the enzymes of their biosynthetic pathway.

### Sub-network module specific to combined picroside-I and picroside-II in stolons

PKST network module had 94 nodes and 190 edges. Among all the identified ABC genes, ABCB and ABCC genes were characterized as hub genes with degree of freedom 54 and 23 showing strong interconnections with co-expressed genes according to the co-expressed network analysis (Fig. 9). ABCB, ABCG, and ABCC were in the center or main hub connected with serine threonine-protein kinases, sulfate transporter, magnesium transporter, and some ion channels for transport of metabolites. Also, certain metabolic enzymes such as arogenate dehydratase prephenate dehydratase, aminotransferase, E3 SUMO-protein ligase, cellulose synthase, acyl-coenzyme A oxidase, and *4-coumarate-CoA ligase* that catalyzes 4-coumarate to 4-coumaroyl-CoA showed connections in this network module.

## Discussion

Significant work has been done in deciphering biosynthesis pathways of picrosides in *P. kurroa* (Shitiz et al. 2015; Sharma et al. 2016; Kumar et al. 2017b), but transporters involved in their transport and accumulation were not yet explored. In present study, we addressed this question by utilizing transcriptomes data of *P. kurroa* by shortlisting ABC transporters-encoding transcripts, determining TPM value of each transcript, correlating expression with

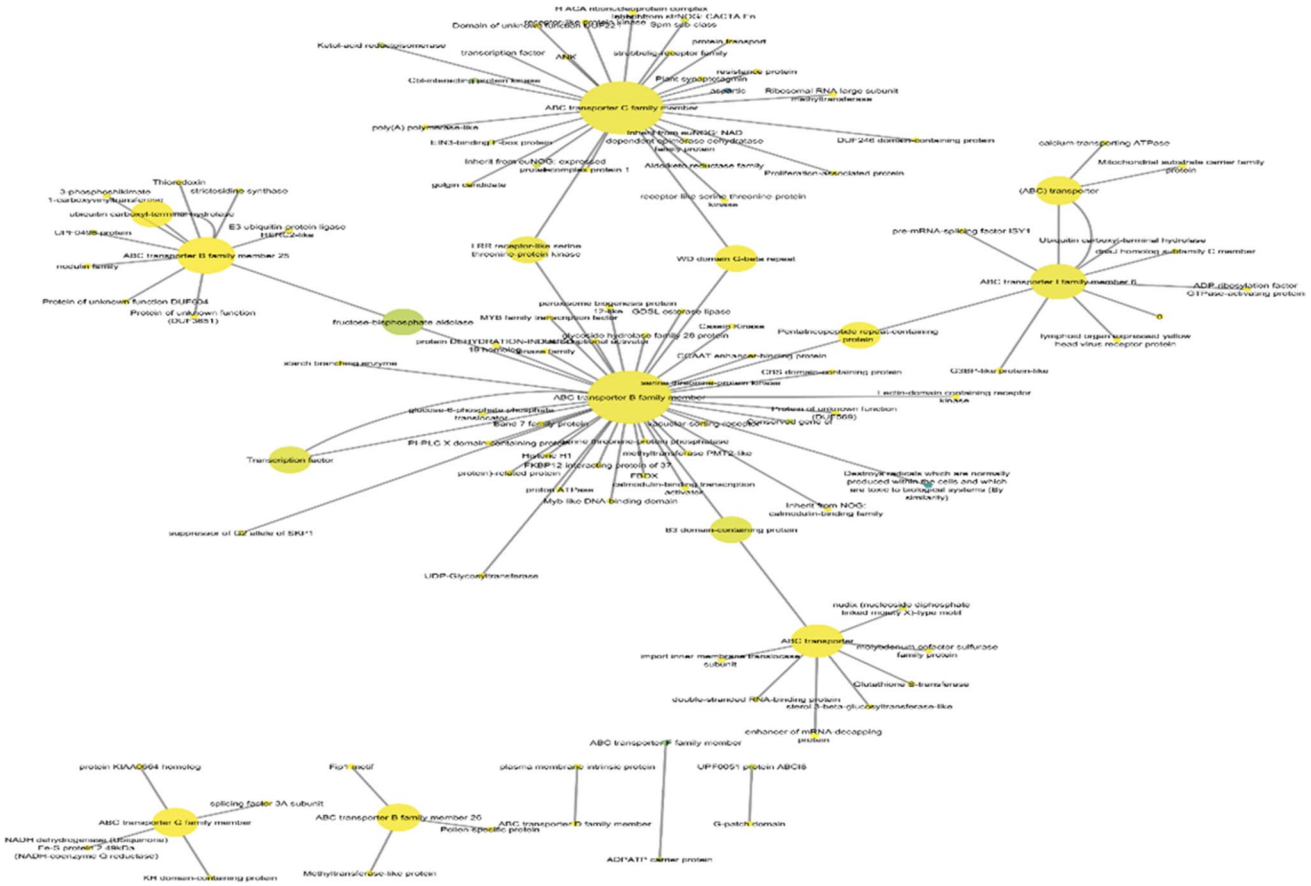


**Fig. 7** ABC family sub-network module constructed from *Picrorhiza kurroa* shoot-derived transcriptome (PKSS)

**Fig. 8** ABC family sub-network module constructed from in *Picrorhiza kurroa* root-derived transcriptome (PKSR)

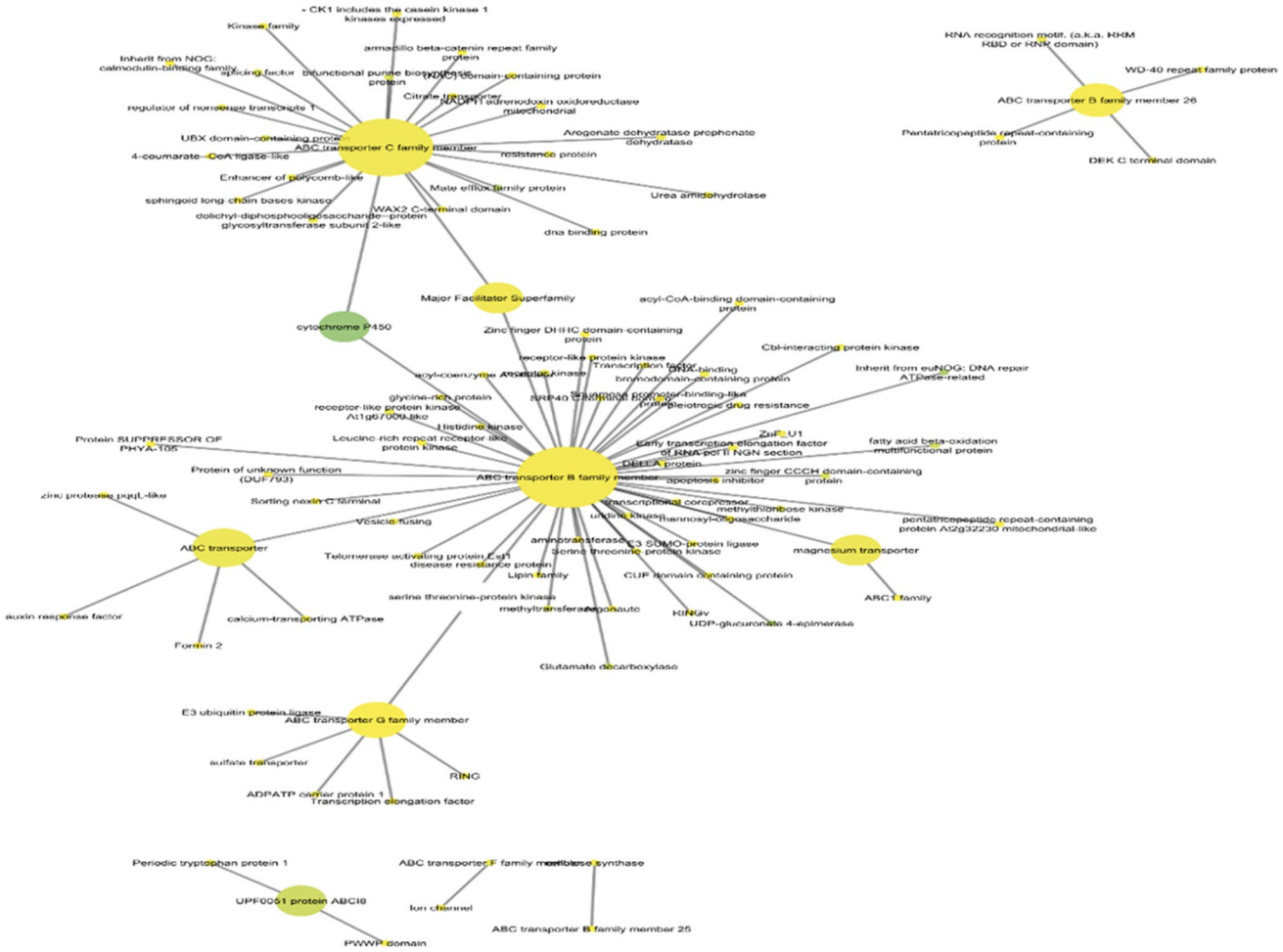


**Fig. 9** ABC family sub-network module constructed from in *Picrorhiza kurroa* stolon-derived transcriptome (PKST)

picrosides content among accessions of *P. kurroa* differing in picroside-I and picroside-II contents in different organs followed by validation through qRT-PCR and co-expression network analysis. Comparative analysis among transcriptomes was done using BLASTn and TPM values were measured to correlate the expression of ABC transporters with the contents of picrosides. A total of 99 ABC transporters identified in various organs, shoots, roots, and stolons of *P. kurroa* were further reduced to six ABC transporter genes by utilizing a combinatorial approach of expressional analysis and co-expression network analysis. In the study, ABCB family members were more abundant; in contrast, no member of ABCH family was identified. Moreover, we shortlisted *PkABCB4*, *PkABCB5*, and *PkABCG1* showing higher expression in shoots whereas *PkABCB2*, *PkABCF3*, and *PkABCC2* showed relatively higher expression in roots suggesting their key role in picroside-I and picroside-II transport and accumulation. *PkABCC1*, *PkABCB3*, and *PkABCF1* showed higher expression in stolons suggesting their role in transport and accumulation for both picrosides, picroside-I and picroside-II. Furthermore, the putative ABC transporters showed similarity with the functionally characterized ABC transporters—*VvABCC1* in *Vitis vinifera*, *NtPDR1* in *Nicotiana tabacum*, and *CjABCB2* in *Coptis japonica*—which are reported to play role in transport of anthocyanin, diterpene, and berberine, respectively. These transporters had TMD and NBD domains which were also identified in in our shortlisted ABC transporters. In addition to identification and shortlisting of potential transporters, we also utilized co-expression network analysis and captured components such as serine-threonine kinases, *Myb* and *WRKY* transcription factors, enzymes, and other transporters linked to the hubs giving more clarity on the source and sink correlation between organ/tissue in *P. kurroa*. It is apparent that understanding of both intracellular and intercellular movement of intermediates, precursors, and final plant metabolites is a pre-requisite for effective metabolic engineering. The molecular basis of biosynthesis and inter/intra-cellular transport of secologanin—an

iridoid glycoside and an important precursor to several monoterpenoid indole alkaloids (MIAs)—has been studied in *Catharanthus roseus* (Larsen et al. 2017). This medicinal herb is rich in several MIAs, and the pathway for MIA is localized in many subcellular compartments of at least four different types of cells. Before being exported to the cytosol, geraniol is synthesized in the plastids of internal phloem-associated parenchyma and transformed to loganic acid, which is then converted to secologanin, an iridoid glycoside in the cytoplasm of the epidermis cells. Secologanin is exported from apoplast to cytosol via *CrNPF2.4*, *CrNPF2.5*, and *CrNPF2.6* transporters localized in the plasma membrane. *Strictosidine synthase*, which catalyzes the formation of strictosidine from tryptamine and secologanin, is localized in the lumen of vacuole of epidermal cells in leaves. Strictosidine is then transported from the vacuolar lumen to the cytosol by *CrNPF2.9* acting as potential iridoid glucoside importer (Larsen et al. 2017; Shitan and Yazaki 2020). Thus, as an analogy can be speculated between the transport of secologanin and the picrosides as both are iridoid glycosides as well as biosynthesized and transported to different cell types. Our previous studies have reported that picroside-I (Sood and Chauhan 2010; Pandit et al. 2013b) is biosynthesized in leaf cells whereas picroside-II in root cells (Shitiz et al. 2015; Kumar et al. 2017a) and both finally accumulate in stolons/rhizomes (Pandit et al. 2013a; Kumar et al. 2017b). Picroside biosynthesis is a combinative route involving mevalonate (MVA), mevalonate-independent/methylerythritol phosphate (MEP), shikimate/phenylpropanoid, and iridoid pathway modules (Kumar et al. 2016a, 2017b). Picrosides are iridoid glycosides with 10-carbon molecules, belonging to the group of monoterpenoids and are synthesized from a 5-carbon precursor, isopentenyl pyrophosphate (IPP), and its functional isomer, dimethylallyl pyrophosphate (DMAPP). These two precursors are biosynthesized from MVA and MEP pathways, respectively. MVA pathway occurs in cytosol, and MEP pathway occurs in plastids (Mahmoud and Croteau 2001). Subsequently, IPP and DMAPP are condensed to form a 10-carbon product geranyl pyrophosphate (GPP), which is considered as a precursor of monoterpenoid (Boncan et al. 2020). GPP undergoes sequences of oxidation and cyclization to form backbone of picrosides, catalpol. Cinnamic acid and vanillic acid from phenylpropanoid pathway undergo acylation with catalpol (iridoid moiety) to form picroside-I and picroside-II (Shitiz et al. 2015). However, which of the shortlisted ABC transporters are involved in inter/intracellular transport of pathway intermediates or even the picrosides remain to be investigated.

ABC transporter family members perform diverse functions such as providing resistance through exporting various hydrophobic metabolites across the plasma membrane of the cell (Do et al. 2018). Among all ABCs, ABCG subfamily of proteins have lipophilic compounds and terpenoids functioning as their substrates. Also, several evidences report that ABCG transporters are localized in the plant plasma membrane (Dhara and Raichaudhuri 2021). In *N. plumbaginifolia*, a plasma membrane-localized ABCG transporter *NpPDR1* is induced by diterpenoids sclareol and sclareolide (Jasiński et al. 2001). In *S. polyrrhiza*, *SpTUR2*, a PDR5-like ABC transporter was reported to transport a diterpene sclareol (Van Den Brûle et al. 2002). ABCG members are also reported to transport different plant metabolites. For instance, *AtABCG29* has been characterized to transport p-coumaryl alcohol, a monolignol involved in lignin biosynthesis (Alejandro et al. 2012) and *AtABCG25* transports abscisic acid (ABA) in *A. thaliana* (Kuromori et al. 2010). Similarly, *CrTPT2* mediates efflux of catharanthine to the surface of leaves in *Catharanthus roseus* (Yu and De Luca 2013). Therefore, identification of *PkABCG1* in shoots might imply its involvement in the transport of picroside-I from shoots to other parts of the plant.

ABCC subfamily members are mostly located on tonoplast of vacuoles in a cell. In *Crocus sativus*, *CsABCC4* functions as a vacuolar transporter for crocins (Demurtas et al. 2019). *AtABCC2/AtMRP2*, another ABCC member localized on vacuolar membrane has been reported to play role in xenobiotic detoxification, Cd/Hg/As resistance and chlorophyll catabolite transport (Park et al. 2012; Shoji 2014). There is possibility that *PkABCC1* might be responsible for short distance vacuolar transport in stolon tissues of *P. kurroa.*

ABCB (*MDR*) subfamily members are reported to be present in plasma membranes of cells. In *C. japonica*, *CjABCB1/ CjMDR1* and *CjABCB2* are reported to transport an alkaloid, berberine from roots to rhizomes (Shitan et al. 2013). Thus, *PkABCB2*, *PkABCB4*, and *PkABCB5* might play a role in transport of picroside-I and picroside-II from one tissue to another in *P. kurroa.*

We suggest that cloning and functional characterization of shortlisted transporters-encoding genes can be undertaken through various molecular techniques such as knockout/ knockdown through RNAi/CRISPR/VIGS; tissue-specific expression through transcriptional fusions with reporter genes; sub-cellular localization by in situ hybridization, FISH, fluorescent fusions and expression of transporters in single cell systems of yeast mutants, *Xenopus* oocyte cells, plant cell cultures (BY2, Arabidopsis cell lines), etc. For instance, VIGS suppression of *CrTPT2* expression in developing seedlings and mature plants resulted in increased catharanthine–vindoline dimers within the leaves of *C. roseus* (Yu and De Luca 2013). In several studies, functioning of the transporters in the intracellular environment has been done by expression of transporters in different host systems, for example

mutant yeast strains and oocytes of *Xenopus* (Tang et al. 2020). For example, *VvABCC1*, an ABC transporter was found to be localized to the tonoplast in the exocarp cells of *Vitis vinifera*, involved in the cotransport of anthocyanidin 3-*O*-glucosides and GSH when heterologously expressed in yeast (Francisco et al. 2013). Additionally, in *N. tabacum*, overexpression of *NtPDR1* in *N. tabacum* BY-2 cells, followed by its purification and reconstitution into liposomes, validated the role of *NtPDR1* in transport of diterpene and sesquiterpenes (Pierman et al. 2017; Nogia and Pati 2021).

This study has thus not only identified putative ABC transporter genes that might play role in the transport and accumulation of picrosides but has also identified other components of biosynthetic machinery such as transcription factors, enzymes, kinases, and other transporter genes associated with these metabolites. The ABC transporters were further shortlisted and validated by qRT-PCR not only among different organs, varying for picroside-I and picroside-II contents but also among *P. kurroa* accessions (populations varying for picroside-I and picroside-II contents) to rule out possibility of differential expression of transporters due to developmental stage of different organs. Thus, the knowledge of ABC transporters further advanced through molecular functional characterization for their inter/intracellular localization can immensely help in understanding the molecular basis of picroside transport and accumulation so that a defined genetic strategy can be implemented to modulate picroside content in organ-specific manner in the economically important medicinal herb, *P. kurroa*.

## Conclusion

In current study, ABC transporters associated with the differential accumulation of two major iridoid glycosides, picroside-I and picroside-II, were identified in a medicinal herb *P. kurroa*. We are reporting shortlisted ABC transporters- encoding transcripts coupled with co-expressed network analysis among three transcriptome datasets, PKSS, PKSR, and PKST derived from shoots, roots, and stolons (rhizomes) of *P. kurroa*. Combined expression and network analyses identified two genes of ABCB family (*PkABCB4* and *PkABCB5*) and one ABCG member *PkABCG1* as potential candidates possibly involved in picroside-I transport and accumulation. Similarly, *PkABCB2*, *PkABCB3*, and *PkABCC1* have been identified as lead candidates for picroside-II in *P. kurroa.* Thus, transporters identified in the ABCC, ABCG, and ABCB subfamilies might be involved in the transport and accumulation of picrosides in *P. kurroa.* Furthermore, functional analysis of the shortlisted ABC transporters might provide a platform for enhancing picroside-I and picroside-II content in *P. kurroa.*

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**Author contribution** The work presented here was carried out in collaboration between all authors. Rajinder S. Chauhan and Roma Pandey defined the research theme and co-designed the experiments, wrote the manuscript, and discussed analyses. Ashish Sharma has done in silico network analysis. Hemant Sood has given useful suggestions for conducting research work. Roma Pandey has carried out the laboratory experiments and analyzed the data.

## Declarations

**Ethical approval and consent of participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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