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Identification, validation, and expression of ABC transporters in *Podophyllum hexandrum* and their role in podophyllotoxin biosynthesis

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Abstract

Podophyllum hexandrum Royle is an important medicinal herb of North-Western Himalayas, and podophyllotoxin, being its major metabolite, has been used extensively in the preparation of several anticancer drugs. Podophyllotoxin accumulates in rhizomes; however, no information exists on the role of ATP-binding cassette (ABC) transporters *vis-à-vis* podophyllotoxin content. The present study reports identification, validation, and expression analysis of ABC transporter genes from *P. hexandrum*. Total 252 ABC transporter genes were identified as unigenes out of which 22 were further validated using real time qPCR in different tissues of varying podophyllotoxin content. Differential expression analysis and Pearson's correlation coefficient revealed two candidate genes *PhABC6* and *PhABCIII* having a positive correlation with the podophyllotoxin content. *PhABCIV* showed the highest expression in rhizomes (20.53-folds compared to shoots) suggesting its possible role in transport and accumulation of podophyllotoxin.

Additional key words: gene expression, transcriptome.

Introduction

Podophyllum, a genus belonging to a family *Berberidaceae*, is commonly represented by two species, *Podophyllum hexandrum* (Bankakri) and *Podophyllum peltatum*. Since ancient times the extracts of dried rhizomes of *Podophyllum* species have been utilized as medicine (Moraes *et al.* 2002). *Podophyllum* spp. are the source of highly valued podophyllotoxin, which is a potent antiviral and anticancerous agent (Van Uden *et al.* 1989, Canel *et al.* 2000, 2001). The Indian species *P. hexandrum* has been reported to contain approximately three times more podophyllotoxin than *P. peltatum* (Kumar *et al.* 2015).

Podophyllotoxin belongs to a lignan group of secondary metabolites. Lignans are a diverse and ubiquitously distributed group of compounds that are derived from the phenylpropanoid pathway / shikimic acid pathway. The biosynthetic route leading to the formation of podophyllotoxin remains to be elucidated completely, till date; only 26 genes are known; they catalyze 33 steps in the podophyllotoxin biosynthetic pathway up to the intermediate compound pluviatolide (Marques *et al.* 2013, Kumar *et al.* 2015).

Several biochemical and cellular factors, contributing at different levels in the biosynthetic and storage process, affect accumulation of secondary metabolites at their final sites of storage in plants. Owing to a highly compartmentalized nature of plant cells, the entrance and exit points of metabolic pathways frequently involve membrane passages of solutes. Transport proteins are often located at strategic positions to control whole pathways, therefore exert a significant role in biosynthesis and accumulation of metabolites. Several lines of evidence suggest that synthesis of lignins occurs in cytoplasm and they are stored in vacuoles in plant cells (Kunze *et al.* 2002). The storage of podophyllotoxin and its precursor 6-methoxypodophyllotoxin as glucosides has been confirmed inside vacuoles in *Linum* cell cultures (Kuhlmann *et al.* 2002). Vacuoles possess a large number

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Abbreviations: ABC - ATP-binding cassette; miRNA - microRNA; FPKM - fragments per kilobase of transcript per million mapped reads; NGS - next-generation sequencing; PERL - Practical Extraction and Report Language; Pfam - protein family database; qPCR - quantitative polymerase chain reaction; RSEM - RNA sequences by expectation maximization; TFs - transcription factors.

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of ATP-binding cassette (ABC) transporters, channels, and pumps in their membranes, which facilitates transport of metabolites across them. However, no information exists on the types of ABC transporters involved in transport of podophyllotoxin in *P. hexandrum*.

The ABC transporters are known to transport secondary metabolites into the vacuoles of plant cells (Yazaki *et al*. 2005). They constitute one of the largest protein families that are present ubiquitously from bacteria to humans (Martinoia *et al.* 2002). The function of ABC proteins in plants was originally identified as transporters of phytotoxic xenobiotics into vacuoles (Martinoia *et al.* 1993), however, since then, several studies on their functionality in plants have been carried out, and these proteins are now known to be involved in a range of diverse processes including plant growth, nutrition and development, response to abiotic stresses, pathogen resistance, and interaction of plants with environment (Yazaki *et al.* 2008). The role of ABC proteins in transport of secondary metabolites within plants is of particular interest for directing future efforts in enhancing content of useful metabolites.

The ABC transporters in plants can be classified into subfamilies ABCA, ABCB, ABCC, ABCD, ABCE, ABCG, $ABCG_{WBC}$ group and $ABCG_{PDR}$ group, *etc.* (Sanchez-Fernandez *et al.* 2001, Andolfo *et al.* 2015). The ABCA, ABCB, ABCC, ABCG, and ABCGWBC group of ABC transporter subfamilies were thought to be involved in transport of various terpenoids, alkaloids, and lignins (Kang *et al.* 2011).

Biosynthesis of secondary metabolites is cell/tissue specific, *e.g.*, shikonin biosynthesis in vesicles of the endoplasmic reticulum of *Lithospermum erythrorhizon,* picrosides in different tissues of *Picrorhiza kurroa*, *etc.*, but finally they are mostly stored in vacuoles (Tabata 1996, Pandit *et al.* 2012). Similarly, podophyllotoxin biosynthesis is suggested to occur in different tissues/organs and stored in vacuoles of *Podophyllum* species (Kumar *et al.* 2015).

Next-generation sequencing (NGS) analysis can facilitate the elucidation of pathways, identification and characterization of ABC transporter genes, microRNAs (miRNAs), transcription factors (TFs), pathway mapping, construction of graphical connectivity diagrams, *etc*. (Bhattacharyya *et al.* 2013, Pal *et al.* 2015). Therefore, assembled transcriptomes of *P. hexandrum* were utilized in the identification and characterization of ABC transporter genes. Further, validation and expression analysis of identified ABC transporter genes were performed through real time quantitative polymerase chain reaction (qPCR) using different tissue/organs of *P. hexandrum* varying in podophyllotoxin content. Pearson's correlation analysis between metabolite content and expression analysis suggests an important role of ABC transporter genes in podophyllotoxin accumulation. Furthermore, a repertoire of ABC transporters has been provided to the scientific community at the following link: https://sites.google.com/site/combiogroup/data-access for future endeavours.

Materials and methods

The plants of *Podophyllum hexandrum* Royle were procured from the Himalayan Forest Research Institute, the Regional Station, Kullu, Himachal Pradesh, India, and maintained in plastic pots in a greenhouse with controlled environmental conditions (a 14-h photoperiod, an irradiance of 1 400 μmol m^{-2} s^{-1}, a temperature of 25 ± 2 °C, and a relative humidity of ≈ 80 %) at the Jaypee University of Information Technology, Waknaghat, Solan, India. Shoot, root, and rhizome tissues of 3-d-old plants were harvested, frozen in liquid nitrogen, and stored at -80 °C for quantification of podophyllotoxin and expression analysis through real time qPCR.

P. hexandrum assembled transcriptome data (2, 27, and 885 contigs) were downloaded from the medicinal plants transcriptomics database (http://medplants.ncgr.org/, Marques *et al.* 2013) and the transcriptomes were mined and analyzed for ABC transporters by using in-house developed Practical Extraction and Report Language (*PERL*) scripts. The protein family database (*Pfam*) (pfam.xfam.org) was used for identification of ABC transporters in the transcriptomes of *P. hexandrum*. Transcripts encoding ABC transporters in *P. hexandrum* were mined by searching the transcriptomes against all available ABC transporters sequences. For phylogenetic reconstruction of identified ABC transporters, the protein sequences of known ABC transporters characterized in model plant species were downloaded from the *NCBI* Genbank database. Alignment of the sequences was carried out by *CLUSTALX* and a final tree was constructed through the *MEGA 6* software using the maximum likelihood method (Tamura *et al.* 2013).

A comparative analysis between the transcriptomes from shoots and rhizomes of *P. hexandrum* was done in order to identify common and unique ABC transporter genes through a sequence similarity search approach. An e-value threshold with 10^{-5} was used while performing *BLASTN* for similarity search with a cut off value equal to or more than 70 %. In-house developed *PERL* scripts were used for *BLASTN* analysis.

The transcript abundance of ABC transporter genes in the *P. hexandrum* transcriptomes was calculated by an RNA-seqences by expectation maximization (*RSEM*) approach using the fragments per kilobase of transcript per million mapped reads (FPKM) parameter of the *RSEM* package. The *RSEM* estimates transcript abundance based on the mapping of RNA-seq reads to the assembled transcriptome. All the parameters were kept default in the query option.

The total RNA was isolated from *P. hexandrum*

tissues (shoots, roots, and rhizomes) by using an *RNeasy* mini kit (*Qiagen*, Hilden, Germany) by following the manufacturer's instructions. The first-strand complementary DNA (cDNA) was prepared from 2 µg of the total RNA using a *Verso* cDNA synthesis kit (*Thermo Scientific*, Waltham, USA) as per manufacturer's instructions. The *Primer 3 v.0.4.0* software was used to design primers for real time qPCR (Table 1 Suppl.).

The real time qPCR analysis of 22 ABC transporter genes was performed on shoot, root and rhizome tissues of *P. hexandrum*. The PCR reaction was performed in a *CFX96* system (*Bio-Rad Laboratories*, Hercules CA, USA) using gene-specific primers (Table 1 Suppl.). The real time qPCR protocol was as follows: 94 °C (3 min), 94 °C (30 s), a corresponding annealing temperature (45 s), 72 °C (20 s) in 40 cycles. All real time qPCR reactions were done in three biological replicates with the *Elongation factor-1 alpha* gene [a left primer sequence (5'-3')-TGAAAGGTTCGAGAAGGAAG, a right primer sequence (5'-3')-TCCTTAGGCATGATTGTCAC] as an internal control for normalization. The transcript abundance of each gene was calculated using the comparative Ct value method.

Different tissues of *P. hexandrum* were also used for quantification of podophyllotoxin content through reverse phase high-performance liquid chromatography (HPLC). The quantitative analysis was performed on the HPLC system *Waters 515* equipped with a corresponding pump, autosampler, photodiode-array detector and *Spherisorb*® C18 (4.6 × 250 mm, 5 µm) reverse phase column. The protocol performed for podophyllotoxin quantification was adopted as described in our previous study (Kumar *et al*. 2015). The podophyllotoxin content in different tissues was analyzed at UV (wavelength 210 nm) using the *Empower-2* software. An authentic podophyllotoxin standard (*Chroma Dex*, Irvine, CA, USA) was used for quantification of podophyllotoxin content in different tissues.

The statistical analysis have been performed using *MS Excel 2007*.

Results

Total 252 putative ABC transporters were identified in the transcriptome of *P. hexandrum* by searching protein sequences corresponding to the full complement of ABC transporters through the *Pfam* database (Fig. 1 Suppl.). The identified transporter genes were suggested to be involved in transport of primary and secondary metabolites in *P. hexandrum.* We further identified five functionally validated transporter genes, three from *Arabidopsis thaliana* (*PhABCI*, *PhABCII,* and *PhABCIII*), one from *Vitis vinifera* (*PhABCIV*), and one from *Nicotiana plumbaginifolia* (*PhABCV*) in the transcriptomes of *P. hexandrum*. These transporter genes are assumed to play a role in transport of secondary metabolites in *P. hexandrum* as has been demonstrated in other plant species. Phylogenetic analysis of the identified ABC transporters was performed through the *MEGA6* software using the functionally characterized ABC transporters retrieved from model plant species such as *Arabidopsis thaliana*, *Nicotiana plumbaginifolia, Zea mays*, and *Medicago truncatula.* The ABC transporter genes of *P. hexandrum* showed a high homology with well characterized genes in model plants, which further confirm the biological functions of identified ABC transporters in *P. hexandrum* (Fig. 1 Suppl).

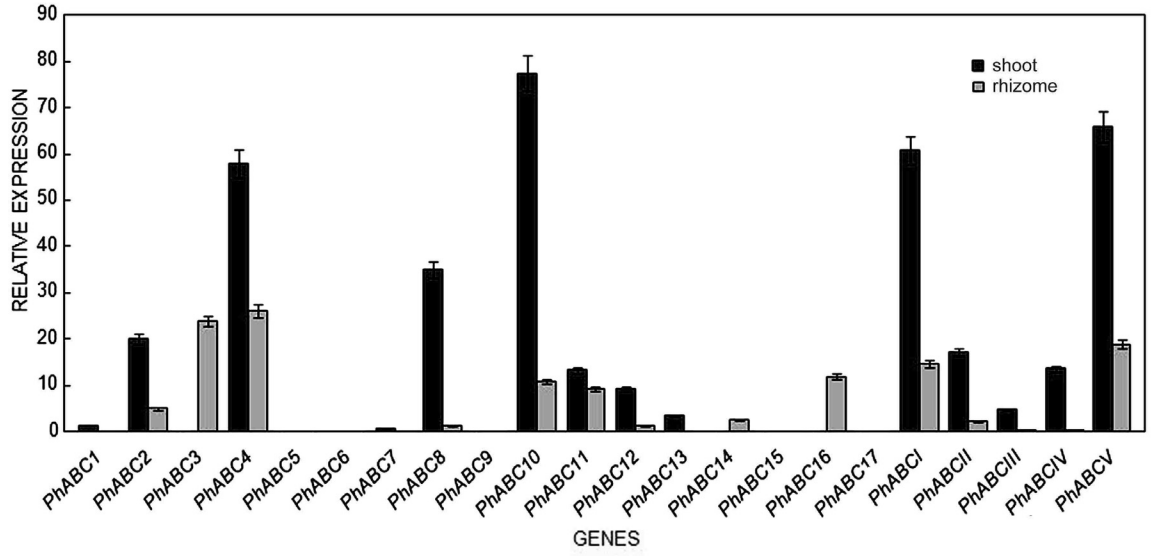

Fig. 1. The relative expression of transporters in shoots and rhizomes of *Podophyllum hexandrum* analyzed by fragments per kilobase of transcript per million mapped reads based approach. *Error bars* indicate percentage error (5 %).

Comparative analysis was done for identification of common and unique ABC transporter genes in the transcriptomes from shoots and rhizomes of *P. hexandrum*, and 249 ABC transporter genes were found commonly present in shoot and rhizome transcriptomes whereas 16 transporter genes were uniquely present. Out of these 16, 12 genes were present only in shoots and 4 genes in rhizomes (Fig. 1 Suppl). The common ABC transporter genes in shoots and rhizomes might be responsible for transport of metabolites existing in both the tissues.

Transcript abundance was determined for 252 ABC transporters through the *RSEM* approach using in-house developed *PERL* scripts. The transcript abundance for commonly present ABC transporter genes ranged from 0.41 to 252.71 FPKM in shoots and 0.06 to 402.88 FPKM in rhizomes (Fig. 1 Suppl). Transcript abundance for uniquely present genes in shoots and rhizomes of *P. hexandrum* was also analyzed. It ranged from 0.00 to 11.96 in rhizomes and from 0.00 to 0.71 in shoots. The ABC transporter genes with a higher transcript abundance were further selected for validation through real time qPCR to assess their association with podophyllotoxin accumulation (Fig. 1).

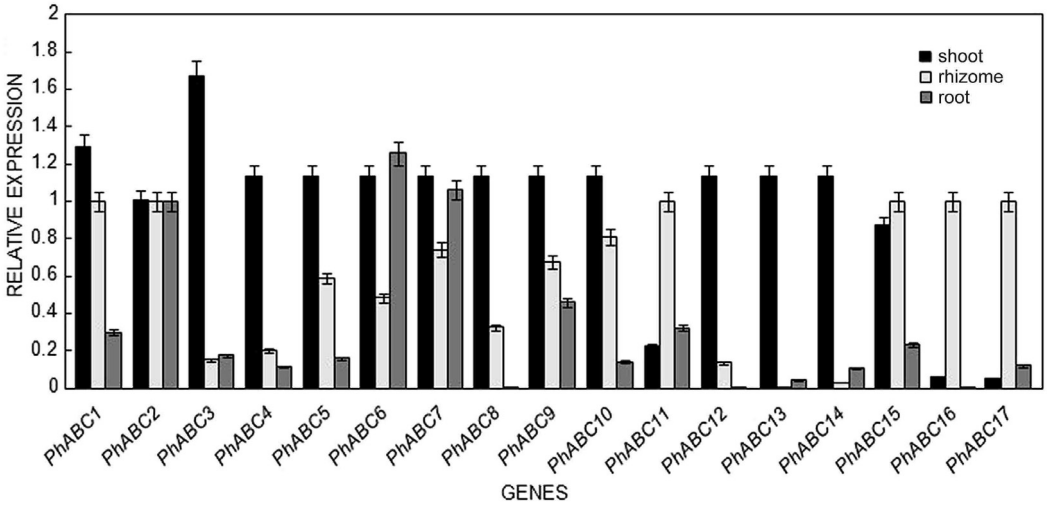

Fig. 2. The relative expression of transporter genes in shoots, roots, and rhizomes of *P. hexandrum* analyzed by real time qPCR. *Error bars* indicate percentage error (5 %).

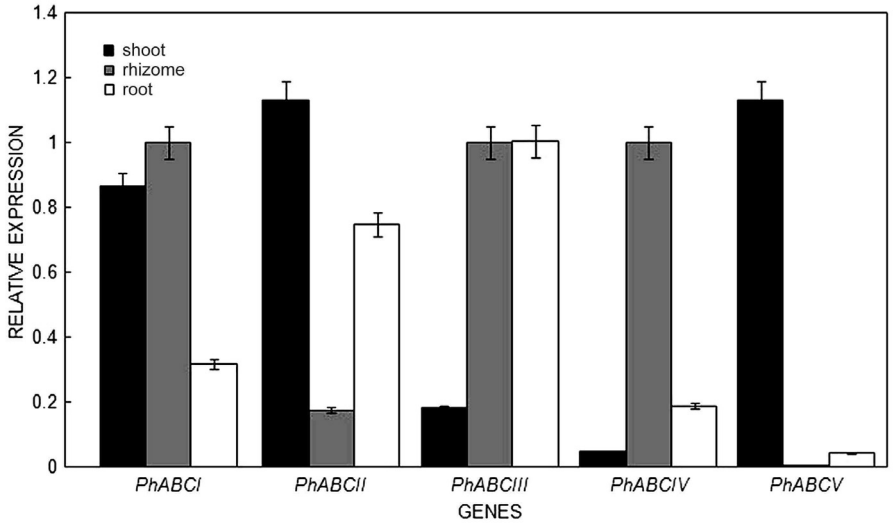

Fig. 3. The relative expression of orthologous transporters *PhABCI*, *PhABCII*, *PhABCIII* from *Arabidopsis thaliana, PhABCIV* from *Vitis vinifera,* and *PhABCV* from *Nicotiana plumbaginifolia*, in shoots, roots, and rhizomes of *P. hexandrum,* analyzed by real time qPCR. *Error bars* indicate percentage error (5 %).

The highest amount of podophyllotoxin was detected in rhizomes (3.02 % of fresh mass) followed by roots (1.28 %) and shoots (0.005 %) (Table 2 Suppl.). The podophyllotoxin content differed in different tissues/ organs, thereby providing differential conditions to correlate the transcript abundance of ABC transporters with the podophyllotoxin content in *P. hexandrum*.

Seventeen ABC transporter genes were selected for

validation through real time qPCR on the basis of a higher transcript abundance in shoots, roots, and rhizomes varying for podophyllotoxin content. The real time qPCR-based relative transcript abundance of transporter genes ranged from 0.002 to 1.673 in shoots, roots, and rhizomes of *P. hexandrum*, respectively (Fig. 2). The gene expression pattern observed through two different approaches was in agreement with each other. The *PhABC3*, *PhABC6*, and *PhABCIV* transporter genes showed the highest transcript abundance 1.673, 1.25, and 1.0, respectively, in shoots, roots, and rhizomes of *P. hexandrum*. Most of the ABC transporter genes showed higher transcript abundance in shoots suggesting their role in transporting macro- and micro-molecules in shoots. Moreover, expression of five functionally characterized transporter genes (*PhABCI*, *PhABCII*, *PhABCIII*, *PhABCIV*, and *PhABCV*) from *Arabidopsis thaliana, Vitis vinifera*, and *Nicotiana plumbaginifolia* were also evaluated through real time qPCR across different tissues/organs of *P. hexandrum.* Expression analysis reveals that *PhABCII* and *PhABCV* showed a higher transcript abundance in shoots and *PhABCIII* in rhizomes of *P. hexandrum* (Fig. 3).

Discussion

The ABC transporters are known as the largest family of transporters of various macro- and micro-molecules across plant membranes (Theodolou *et al.* 2000, Holland *et al.* 2003). These transporters have a broad substrate specificity and a number of ABC proteins have been implicated in transport of secondary metabolites in different plant species. Most of the identified transporters belonging to the ABC family include ABCB/MDR (multi drug resistance), ABCC/MRP (multidrug resistance protein) and ABCG/PDR (pleiotropic drug resistance) (Table 3 Suppl.). The transporters have a significant role in various physiological and biochemical processes in plant systems (Jeong *et al.* 2014). The role of transporters is not fully understood due to a limited availability of desired radiolabeled compounds requisite to decipher transport mechanisms. There is no report available on identification of ABC transporter genes and dissecting their role in secondary metabolite biosynthesis in *P. hexandrum*. Therefore, the transcriptomes of *P. hexandrum* were mined to identify and validate ABC transporter genes.

The ABC transporter genes were identified as 252 unigenes in the transcriptome of *P. hexandrum*. They were further classified into nine sub-families such as ABCA, ABCB, ABCC, ABCD, ABCE, ABCF, ABCG, ABCI, and others (Fig. 2 Suppl.). The phylogenetic analysis of the ABC transporters shows a high homology with the functionally characterized genes of *Nicotiana plumbaginifolia, Medicago truncatula, Arabidopsis thaliana*, *Zea mays, etc.* Clustering ABC transporter genes with the well characterized genes of model plant species further confirmed the function of the identified ABC transporters in *P. hexandrum* (Fig. 2 Suppl). Out of 252, 22 genes were selected for validation on the basis of transcript abundance to gain insights into regulation of podophyllotoxin biosynthesis. Transcript abundance for 22 ABC transporter genes was checked by two approaches, FPKM and real time qPCR. Expression analysis of 22 ABC transporter genes revealed 14 genes showing a higher expression in shoots as compared to roots and rhizomes of *P. hexandrum* suggesting their major role in transport of various macro- and micro-molecules in shoots (Fig. 1 Suppl.). Shoots are the primary organs for synthesis of sugars and starch (Huber *et al.* 1989). Expression of ABC transporters in shoots might be due to the their involvement in transporting sugars, amino acids, peptides, polyamines, metal ions, *etc.*, to rhizomes (the storage organs of *P. hexandrum*). The expression pattern observed by the two approaches was similar. Further, in order to find out suitable candidate genes correlating with podophyllotoxin content in *P. hexandrum,* Pearson's correlation coefficient was determined. Two genes, namely *PhABC6* and *PhABCIII*, showed a positive correlation with podophyllotoxin content (Table 2 Suppl.). These genes were suggested to be potential candidates for generation of transgenic plants with enhanced podophyllotoxin content.

Furthermore, common and unique ABC transporter genes were also found in the shoot and rhizome transcriptomes of *P. hexandrum*: 239 ABC transporter genes were found common in shoots and rhizomes of *P. hexandrum.* Similarly, 16 genes (3 in rhizomes and 12 in shoots) were uniquely present in *P. hexandrum*. Transcript abundance analysis for unique genes revealed transcript medp_podhe_95366 with the highest abundance with 11.96 FPKM value in *P. hexandrum*. Similarly, transcript medp_podhe_20101112|14082 showed the highest abundance (402.88 FPKM) among commonly present ABC transporter genes in shoots and rhizomes of *P. hexandrum* (Fig. 1 Suppl.). Previously, unique and common genes have been identified in plant species, such as *Jatropha curcas*, *Ricinus communis*, *Arabidopsis thaliana*, *etc*., having a role in plant defense systems (Armisen *et al.* 2008, Sood *et al.* 2014). The genes commonly present in shoots and rhizomes of *P. hexandrum* may be involved in transport of common metabolites. The uniquely present genes in rhizomes might be of much more interest as they were suggested to be involved in transport of specific compounds in *P. hexandrum*.

Till date, only 22 ABC transporter genes are known and have been functionally characterized in different tissues/organs of *Arabidopsis thaliana* having diverse roles such as cutin and pollen exine formation, plastid lipid formation, auxin transport, fatty acyl-CoA import to peroxisomes, cuticle formation, stomata regulation,

abscisic acid import, metal/metalloid tolerance, folate transport, chlorophyll catabolite transport, kanamycin tolerance, and biotic and abiotic stress tolerance (Kang *et al.* 2011). Out of these 22 ABC transporters genes from *Arabidopsis*, 3 genes were found to have orthologs in the transcriptome of *P. hexandrum*, and the orthologs of 2 ABC transporter genes from *Vitis vinifera* and *Nicotiana plumbaginifolia* were also identified (Fig. 2 Suppl.). The identified ABC transporter genes were further validated, and their expression patterns were evaluated across different tissues/organs of *P. hexandrum* using real time qPCR in order to correlate their expressions with podophyllotoxin content (Fig. 3). *PhABCII* and *PhABCV* showed a higher transcript abundance in shoots and *PhABCIII* in rhizomes of *P. hexandrum* (Fig. 3). The expression pattern of *PhABCV* was consistent with previous reports where this gene was highly expressed in shoots of *Nicotiana plumbaginifolia* having a role in terpenoids secretion (Grec *et al.* 2003). Similarly, *PhABCII* showed a higher expression in shoots where it was involved in transportation of metabolites, xenobiotics, folates, conjugates of chlorophyll catabolite, phytohelatins, *etc*., in *Arabidopsis thaliana* (Raichaudhuri *et al.* 2009, Song *et al.* 2010).

Podophyllotoxin biosynthesis occurs in roots/ rhizomes of *P. hexandrum*; however, the transport mechanisms of podophyllotoxin remain to be elucidated. Podophyllotoxin is mainly stored in vacuoles in rhizomes of *P. hexandrum*. The ABC transporters were thought to be involved in transporting podophyllotoxin and its derivatives from different compartments to vacuoles of rhizomes. Several questions need to be answered such as how many transporters are involved in transportation of podophyllotoxin. An attempt has been made by providing preliminary information on ABC transporter genes through their identification and validation in different tissues/organs of *P. hexandrum.* The ABC transporters are well known for transporting various secondary metabolites including lignins, flavonoids, terpenoids, *etc*., across the plasmalemma and tonoplast (Sibout and Hofte 2012). To gain insights into podophyllotoxin biosynthesis and accumulation, we also evaluated the expression status of ABC transporters genes in roots and rhizomes with respect to shoots of *P. hexandrum* (Fig. 1 Suppl.). Five genes, namely *PhABC11*, *PhABC16*, *PhABC17*, *PhABCIII*, and *PhABCIV* showed a higher expression ranging from 4.45-folds to 20.53-folds in rhizomes and roots as compared to shoots. *PhABCIV* showed the highest expression (20.53-folds) in rhizomes suggesting its major role in accumulation and transport of podophyllotoxin into rhizomes of *P. hexandrum*. *PhABCIV* is well characterized for transport of anthocyanidin 3-*O*-glucosides into vacuoles in *Vitis vinifera* (Francisco *et al.* 2013).

The identified ABC transporter genes are thought to be involved in podophyllotoxin accumulation as their transcript abundance correlated with podophyllotoxin content (Table 2 Suppl). However, the functional validation of these highly correlated genes remains to be deciphered. The present study has set-up a new platform by providing ABC transporter gene sequences to a scientific community for further research. This study would help in elucidating the physiological and biological functions of ABC transporters in *P. hexandrum*.

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