DIFFERENTIAL EXPRESSION OF PINORESINOL-LARICIRESINOL REDUCTASE GENE IN RELATION TO PODOPHYLLOTOXIN ACCUMULATION IN DIFFERENT PLANT ORGANS OF ENDANGERED ANTICANCER SPECIES *PODOPHYLLUM PELTATUM*

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Abstract

Pinoresinol-lariciresinol reductase (*PLR*) is a key enzyme in podophyllotoxin (PTOX) biosynthesis pathway, which catalyzes the conversion of pinoresinol into secoisolariciresinol, a central precursor for the PTOX biosynthesis. *Podophyllum peltatum* is an important anticancer species, and PTOX content is obviously different from various plant organs. Therefore, investigation of *PLR* gene expression facilitates the understanding of the molecular mechanisms of the variation of the PTOX content in different organs from *P. peltatum* as well as its biosynthesis mechanisms, suggesting that it is an important gene for metabolic engineering of the PTOX. The PTOX was found in all the test organs (root, rhizome, petiole, fruit, leaf and flower), with significant differences among them ($p<0.05$). The highest PTOX content ($2.8018 \pm 0.23\%$) was found in rhizome, which was 30 times higher than the lowest PTOX content in fruit. Furthermore, tissue expression profile showed that *P. peltatum PLR* (*PpPLR*) was expressed in all the test organs except for the flower. The highest and lowest expression level was found in rhizome and leaf, respectively. The expression profile of *PpPLR* was inconsistent with the PTOX content variation in the test organs except for the rhizome, suggesting that the PTOX biosynthetic organ is not necessarily its storage organ. After the initial synthesis, PTOX can be transferred to its storage organ, such as the rhizome.

Key words: *Podophyllum peltatum*, *PLR*, Expression profile, RP-HPLC, Podophyllotoxin.

Introduction

Plants are natural sources of important ingredients in medicinal development for human healthy. *Podophyllum peltatum* belongs to the Berberidaceae family, and aryltetralin lignan is its key natural product. Also, aryltetralin lignan has been demonstrated to reduce the occurrence of breast and prostate cancers by regulating steroidal hormone synthesis (Adlercreutz & Mazur, 1997). The podophyllotoxin (PTOX), one kind of aryltetralin lignin, possesses strong antitumor and antihyperlipidemic activities, which is well documented *In vitro* and *In vivo* researches (Farkya *et al.*, 2004; Westcott & Muir, 2003; McCann *et al.*, 2005; Bylund *et al.*, 2005). Furthermore, the PTOX is the initial raw material for the semi synthesis of the anticancer medicines etoposide and teniposide (Stahelin & von Warhurg, 1991; Canel *et al.*, 2000; Ajani *et al.*, 1996; Ekstrom *et al.*, 1998; Wexler *et al.*, 1996). Simultaneously, fresh and more water-soluble analogs of etoposide with more utility have been generated, and are experiencing clinical trials (Imbert, 1998; Mross *et al.*, 1996). The PTOX is commercially extracted from the rhizomes of *Podophyllum* species, and *P. peltatum* is predominant in the aspect of the PTOX content compared with other *Podophyllum* species or other plant species that also produces PTOX (Jackson & Dewick, 1984; van Uden *et al.*, 1989). As one of the native species to high mountains and sub-mountains regions of the Himalayas, *P. hexandrum*, which was proclaimed in risk of extinction in 1989 due to the indiscriminate harvesting in their natural habitat (Farkya *et al.*, 2004; Foster, 1993). With a combination of increased demand along with impaired availability of the raw material, researchers are motivated to explore the substitution for PTOX-source (Jackson & Dewick, 1984; van Uden *et al.*, 1989; Choi *et al.*, 1998;

Heyenaga *et al.*, 1990; Bhattacharyya & Chattopadhyay, 2015). Recent work has demonstrated that the leaves of the American mayapple, *P. peltatum*, could also produce PTOX (Canel *et al.*, 2000). This finding has stimulated the use of *P. peltatum* (roots and rhizomes), as functional medicaments with enhanced lignin levels. This provides a great chance for the taming of the mayapple as well as its growth as a high-valued crop for small-scale peasant in the United States.

It is known that *P. peltatum* is a rhizomatous herb growing in community groups in the oak-hickory forests all over the eastern of the United States and the southern of the Canada. It has whitish nodding flowers with parts in whorls of three between palmately dissected peltate leaves, flowering from March to May, with fruits ripening from May to August. Ripe fruits are edible (Meijer, 1974; Morazs *et al.*, 2000). Since 1820 the plant has been recognized as being of medicinal value officially in USA (Meijer, 1974; Morazs *et al.*, 2000). The significant pharmacological characteristics as well as physiological action of lignans in planta have caused multitudinous studies in regard to the accumulation and biosynthesis of these metabolites (Seidel *et al.*, 2002; Ford *et al.*, 2001; Sicilia *et al.*, 2003). Lots of researchers have put forward the biosynthetic pathway of PTOX. It is speculated that a dirigent protein-assisted coupling of E-coniferyl alcohol leads to (-)-pinoresinol which is transformed to (+)-secoisolariciresinol by way of the action of pinoresinol–lariciresinol reductase (*PLR*) (Fig. 1) (Lau & Sattely, 2015; Xia *et al.*, 2000). Secoisolariciresinol is dehydrogenated by secoisolariciresinol dehydrogenase (*SDH*) to give matairesinol (Xia *et al.*, 2001; Arneaud & Porter, 2015). Matairesinol is regarded as a precursor of some crucial secondary metabolites such as PTOX in *Podophyllum* (Broomhead *et al.*, 1991).
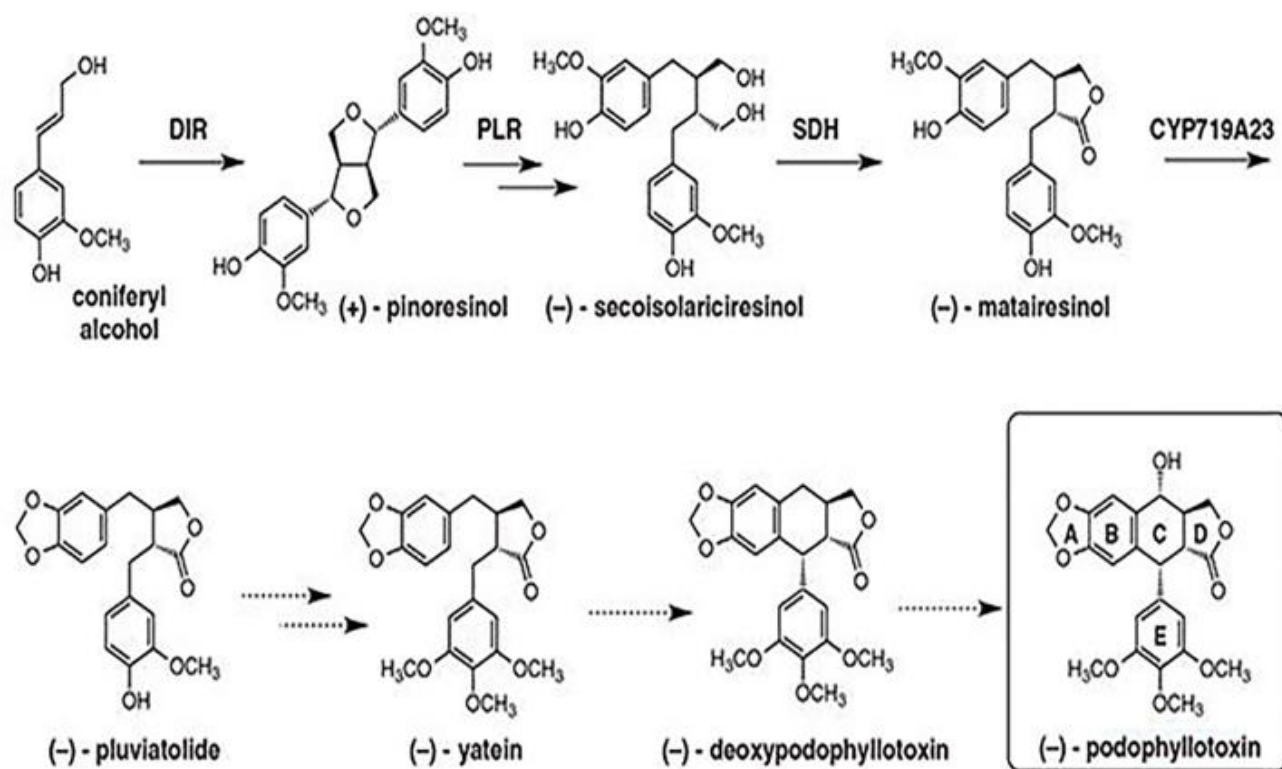
Fig. 1. Biopathway of podophyllotoxin (Lau & Sattely, 2015, Science).

P. peltatum, in spite of being a high PTOX-producing and endangered plant, is poorly studied about the clarification of the PTOX biosynthetic pathway, especially with respect to the biosynthetic pathway from matairesinol to PTOX which is commonly considered to occur through pluviatolide, yatein and deoxy-podophyllotoxin (Farkya *et al.*, 2004; Jackson & Dewick, 1984; Wankhede *et al.*, 2013; Javadian *et al.*, 2017). In *Linum flavum*, a cytochrome P450 monooxygenase, deoxy-podophyllotoxin 6-hydroxylase, is considered to catalyze the procedure of deoxy-podophyllotoxin to PTOX (Molog *et al.*, 2001), nevertheless, very little information about P450 is available. In addition, a few genes encoding enzymes such as secoisolariciresinol dehydrogenase (*SDH*) (Xia *et al.*, 2001; Arneaud & Porter, 2015) and dirigent protein oxidase (*DPO*) (Xia *et al.*, 2000; Davin *et al.*, 1997) have been cloned and characterized. Thus, *PLR* that catalyzes the key steps of the intermediate formation in the PTOX synthesis pathway (Fig. 1) could be a pivotal enzyme for PTOX synthesis (Dinkova-Kostova *et al.*, 1996; Wankhede *et al.*, 2013; Javadian *et al.*, 2017; Rahman *et al.*, 1990; Katayama *et al.*, 1993; von Heimendahl *et al.*, 2005). Presently, cloning work of the gene encoding *PLR* has been performed from the congeners species of *Podophyllum* such as *Sinopodophyllum hexandrum* (former name is *Podophyllum hexandrum*) (Wankhede *et al.*, 2013) and other plant species such as *Forsythia intermedia, L. album* and *L. usitatissimum* (Dinkova-Kostova *et al.*, 1996; von Heimendahl *et al.*, 2005). *PhPLR* demonstrates that a 936-bp-long open reading frame encoding a protein of 311 amino acids with the calculated molecular weight of 34.8 kDa and pI of 6.64. However, it is still imaginary that the accurate localization and timing of synthesis of the PTOX, which demand further research. As for the spatio-temporal

regulation of the key genes expression concern to the lignan synthesis in *P. peltatum*, there is few published reports. Previous data could generate message holding out the suppose on the function of such compounds in planta as well as verify the *PLR* participates in the PTOX synthesis of *P. peltatum*. In order to further clarify the function of *PLR* in *P. peltatum*, we investigated its spatio-temporal expression profile of *PLR* gene and PTOX content difference in different plant organs of *P. peltatum*. For this purpose, the quantitative RT-PCR technology is employed to analyse expression of the *P. peltatum PLR* (*PpPLR*) gene. The *PLR* gene with a ACTIN gene was isolated and cloned, and content of PTOX was measured concomitantly. The current work would provide a foundation to understand the potential molecular mechanisms of PTOX content variance in different plant organs and PTOX biosynthesis in *P. peltatum* and implement their metabolic engineering for high-yield PTOX.

Materials and Methods

Plant materials and biochemicals: Plant materials were purchased from Rakuten Market, Tokyo, Japan during the April ~ August, 2018. Fresh plant organs including roots, rhizomes, leaves, petioles, fruits and flowers were sampled from healthy *P. peltatum* plants. A part of plant samples were placed separately into liquid nitrogen for RNA extraction at once, and the remainders were naturally drought in the shade at room temperature before being grounded into a fine powder for the PTOX analysis. Voucher specimens were identified by Professor De-hua Qin from Henan University of Science and Technology (HaUST). The partial samples were deposited into the Herbarium of HaUST (HaUST0005461-0005469), the others were stored in the Sample Collection Center of HaUST.

In the present study, primers for *PLR* gene were designed according to the known *PLR* sequence (accession No. ABY75535) (https://www.ncbi.nlm.nih.gov/protein/ABY75535). The oligonucleotides for *PLR* gene and ACTIN gene amplification were synthesized by Invitorgen (Tokyo, Japan). QIAGEN RNeasy Plant Mini Kit (20) was purchased from Qiagen (Co., Ltd., Japan), and RNA isolation reagent and other reverse transcriptase were bought from Takala (Co., Ltd., Japan). RACE Kit was purchased from Clontech (CA, USA). The authoritative standard sample of PTOX and chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Preparation of standards and sample solution: A small amount of pulverized plant organs (1.000 g) was clear of chlorophyll by incubation in 95% ethanol and extracted with methanol (20 mL) for one hour under supersonic conditions. The number of extractions is three times. Combined the extraction solutions, filtered, evaporated in vacuo, and diluted with methanol in a 10 mL volumetric flask. Filtered the extracted solution through a 0.22 μm PTFE syringe for RP-HPLC analysis. Simultaneously, the standard stock solutions of PTOX (0.1 mg mL^{-1}) was prepared in methanol for the identification. The experiment was performed in triplicate.

Quantification of PTOX: A vacuum degasser, SPD-10Avp detector (LC-10ATvp, Shimadzu Co., Japan) and Class-VP workstation assemble an liquid chromatography (LC) system, which was used to obtain the chromatograms and UV spectra (Zhou *et al.*, 2008; Liu *et al.*, 2015). Chromatographic separation was carried out with a C18 diamonsil reversed phase chromatographic column (250 mm×4.6 mm, Inertsil ODS-3, 5 μm, Shimadzu, Japan), and a gradient solvent system consisting of 0.1% distiled-water (solvent A) and methanol (solvent B). The procedure of gradient elution was showed in Table 1. A flow rate of 0.8 mL min^{-1} was used. UV detection was performed at 290 nm and the column temperature kept at 35.0 ℃. The injection amount of test sample was set at 15 μL. Chromatogram profile generated in the 145 min-elution time was employed because no peaks were observed after 145 min. The standard solution was used to the calibrate a standard curve using an external standard method. The analyses were carried out in triplicate.

RNA extraction: Total RNAs were extracted from various *P. peltatum* organs according to the manual of Qiagen RNA Plant Mini Kit (Co., Ltd., Japan) and on-column RNAse free DNAse treatment (Qiagen). After collection, total RNAs were stored at -80°C for the further usage.

Synthesis of cDNAs of *PpPLR*: Single-stranded cDNAs were synthesized from 0.5 μg μL^{-1} of total RNAs with an oligo(dT)$_{20}$ primer that were reversely transcribed according to the manufacturer's protocol of the SMART rapid amplification of cDNA ends (RACE) cDNA amplification kit (Clontech, USA). After RNaseH treatment, the single-stranded cDNA mixture used as templates and a pair of primers (fppplr: 5'-TTTCCGC TAGCTTCTACGAT-3' and rpplr: 5'-AGGACCGAGA GTACTTTCCA-3') was applied for polymerase chain reaction (PCR) amplification of the *PpPLR* fragments. The PCR reaction was performed by denaturation of the

cDNA at 94°C for 3 min followed by 30 cycles of amplification (94°C for 45 s, 57.5°C for 45 s and 72°C for 1 min) and by extension at 72°C for 6 min. Amplification of the *PpPLR* fragment was subcloned into the pGEM T-easy vector by sequencing, and the fragments was verified in other plants containing *PLR* genes (*Sinopodophyllum hexandrum, Forsytha intermedia* and *L. usitatissimum*) by blast-n search in NCBI database.

Table 1. Gradient elution procedures for quantification of podophyllotoxin.

Time/min	A%	B%
0	15	30
4	15	30
25	24	40
45	24	40
60	32	50
75	32	50
95	55	70
140	55	70
145	70	30
145	stop	—

Table 2. Procedure of semi-quantitative RT-PCR.

Temperature/℃	Time/Sec
94	60
94	30
60	30
72	60
4	Stop

Quantitative RT-PCR: First-strand cDNA was synthesized using the following method. 14.0 μL reaction system in 15 mL tube was composed of 10.0 μL DEPC-treated water, 1.0 μL 10 mM dNTPMix, 1.0μL 50 mM oligo(dT)$_{20}$ and aliquots of 2 μL 0.5 μg μL^{-1} total RNA for each sample. After heated this reaction system at 65.0℃ for 5 min and placed it on the ice for 1 min, and then added 4 μL of 5×First-Standard Buffer, 1 μL 0.1 M DTT and 1 μL of Super Script Ⅲ RT into this reaction system, finally the reaction system was carried on the PCR instrument in accordance with the following protocol. First of all, the reaction system was heated at 50.0℃ for 60 min, and then the current temperature was changed into 70℃ for 15 min, finally 80 μL of TE was added into the PCR products in the tube and stored at -20℃.

Briefly, 40.0 μL reaction system consisted of 23.6 μL of dH$_2$O, 3.2 μL of 10 Mm dNTPMix, 4.0 μL of *PLR*F1 forward primer, 4.0 μL of *PLR*R1 reverse primer, 1.0 μL of first-strand cDNA and 0.2 μL of TaKaRa ExTaq. cDNA fragments were amplified for 40 cycles on the PCR instrument. The operating procedure was showed in Table 2. In order to standardize the amount of mRNA in each PCR reaction, PCR amplification of ACTIN was employed as a control. Using gene-specific primers ACT-F1 forward primer (5'-GAYTCBGGWGATGGTGT BWSYCAYA-3') and ACT-R1 reverse primer (5'-RTCRTAYTCNBM CTTBGMDATCCAC-3') performed the amplification under the same conditions as described in Table 2. It was repeated three times for the RT-PCR reaction.
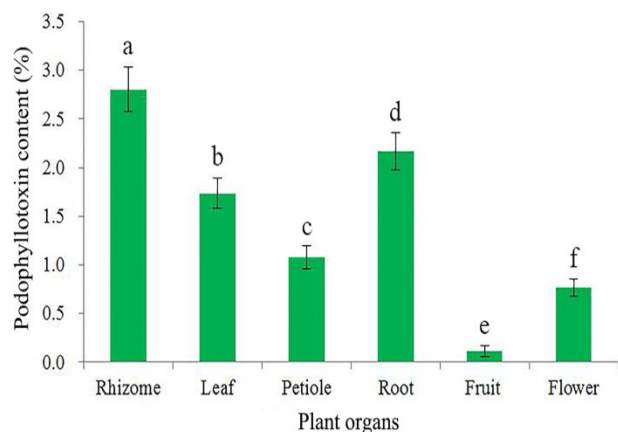
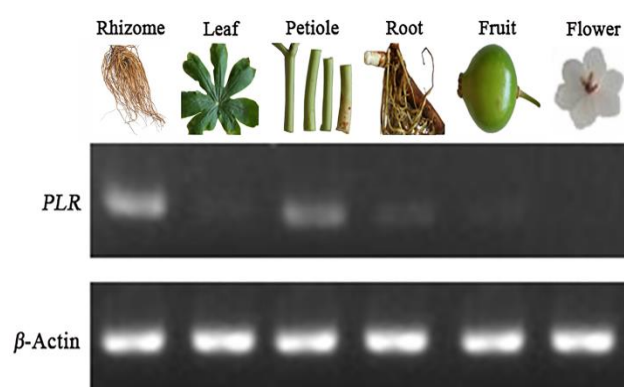Fig. 2. The podophyllotoxin content in various plant organs of *Podophyllum peltatum*.

Fig. 3. Expression profile of *PpPLR* gene in various plant organs of *Podophyllum peltatum*.

Statistical analysis: CLUXTALX was used for multiple alignment analysis of the *PLR* amino acid sequences (Thompson *et al.*, 1997). Homology-based structural modeling of the sequence was performed by Swiss-Model (Arnold *et al.*, 2006). All data indicated by means ± SD (standard deviation) were compared by one-way analysis of variance. The results were considered significant at *p*<0.05.

Results and Discussion

PTOX content variance in different plant organs of *P. peltatum* by RP-HPLC: The contents of PTOX in different plant organs of *P. peltatum* were quantified by RP-HPLC (Fig. 2). The PTOX could be detected in all the test organs from *P. peltatum* including root, rhizome, leaf, petiole, flower and fruit, which is consistent with the previous results about PTOX (Canel *et al.*, 2000; Giri & Narasu, 2000; Maqbool *et al.*, 2004; Moraes *et al.*, 2002; Canel *et al.*, 2001). It was the first time that PTOX was found in the flowers and fruits of *P. peltatum*. PTOX content level of six test organs from *P. Peltatum* successively were rhizome (2.8018 ± 0.23%) > root (2.1638 ± 0.19%) > leaf (1.7357 ± 0.16%) > petiole (1.0762 ± 0.12%) > flower (0.7645± 0.09%) > fruit (0.1131±0.06%). The PTOX content has significant differences at *P* level of 0.05 among six different tissues. Rhizomes of *P. peltatum* had the highest PTOX content, which agreed with the results described by Liu *et al.*,

(2004). The root, leaf, petiole and flower had the middle-level content of PTOX, whereas fruits had the lowest value. Comparing with rhizomes, the content of PTOX of fruits was 30 times lower than it. In the present study, the fruit samples are at mature period and the PTOX content is relatively low at the mature fruit samples, possibly indicating a rapid transformation of these precursors (4'-5'dimethylthujaplicatin and yatein) to dexypodophyllotoxin and then to 6'-methoxypodophyllotoxin, only a few conversions from dexypodophyllotoxin to podophyllotoxin. This indicates that the rhizomes are probably the storage organs for PTOX in *P. peltatum.*

The PTOX does not distribute equally in different plant organs, which is similar to the famous anti-tumor taxol in Yews and the ginkgolides in Ginkgo. For example, it is richer in the older bulks of Yews than that in any other organ for the taxol (Wani *et al.*, 1971). Comparing with other tissues of Ginkgo, there is more Ginkgolides in roots (Carrier *et al.*, 1998). That may be because the fact that functional genes in the secondary metabolic pathways are always differentially expressed due to the different developmental stages and environmental factors influence. According to the present data information, we also suggested that various gene expression in PTOX synthesis pathway resulted in their activity difference such as *PpPLR* gene allowing the difference of PTOX synthesis and accumulation.

cDNA of *PpPLR*: Two reverse gene-specific primers (5'-GSP and 5'-NGSP) were designed on the basis of the known *PLR* sequences (accession No. ABY75535). Two special PCRs were conducted, afterwards, a single band of approximately 500 bp was obtained and verified by sequencing. The BLASTN searching results indicated that the 500 bp cDNA fragment has strong homology with *PLR* genes of other plant species, such as *P. hexandrum* and *F. intermedia*. These powerfully indicated that it has obtained the core fragment of *PpPLR*, which offered necessary and sufficient sequence information to separating the cDNA ends of *PpPLR* by RACE.

Quantitative RT-PCR monitoring of the endogenous *PpPLR* gene expression: The expression of *PpPLR* gene in various organs of *P. peltatum* was investigated by quantitative RT-PCR (Fig. 3). It chose 40 PCR amplification cycles as it permitted the best results, enabling simultaneous observation of transcription of the ACTIN gene and the *PpPLR* gene. The PCR products had the prospective size of *PpPLR*, and there are ACTIN control transcripts in all the samples. The results showed that the *PpPLR* expression was found in rhizome, petiole, root, fruit and leaf but at different levels. However, the expression level was absent in isolated flowers samples. No amplified gene product was discovered in the flower with the PCR amplification conditions used, indicating that the flower lacked visible *PpPLR* transcription activity. The constitutive ACTIN gene had the almost equal expression level in all the tested tissues. The leaf had the lowest level expression of *PpPLR*. The middle-level expression of *PpPLR* was discovered in petiole, root and fruit, and their expression profiles were at the analogous levels. The highest level expression of *PpPLR* was discovered in

rhizomes (Fig. 3). The tissue expression profile results proved that *PpPLR* was not a house-keeping gene (constitutive gene) in *P. peltatum*, however, probably a developmental regulatory gene that is susceptible to external environmental factors. It just like other genes associated with secondary metabolic pathways, for example, the loganic acid methyl transferase (*LAMT*) gene in S-adenosyl-L-methionine biosynthesis pathway in *Catharanthus roseus* (Murata *et al.*, 2008).

Temporal and spatial expression of the *PpPLR* gene: Several cDNAs encoding flax monolignol and lignan-specific biosynthetic enzymes were reported in a previous researches, and the *PLR* gene was included, in order to monitor their expression about the biological stress response in flax cell suspension (Attoumbre *et al.*, 2006; Hano *et al.*, 2006). This gene and the heterologously expressed enzyme have been showed in previous reports (Wankhede *et al.*, 2013; Javadian *et al.*, 2017; Rahman *et al.*, 1990; von Heimendahl *et al.*, 2005; Katayama *et al.*, 1993; Dinkova-Kostova *et al.*, 1996).

The usability of gene sequences and semi-quantitative gene expression corresponding to the lignan-specific biosynthetic enzyme *PLR* provided the temporal and spatial regulations to study the formation of PTOX. This pathway of lignan biosynthesis had been researched in ligneous species such as *Pinus taeda* and *F. intermedia* through in situ hybridization (Kwon *et al.*, 2001). These outcomes indicated that *PLR* gene was expressed in the secondary xylem. However, non-ligneous species did not have the related data. Also, the previous studies concerned with gene cloning and biochemistry of the *PLR* protein, its expression profiles were rarely reported. In this work, we tried to define the expression pattern of the *PLR* gene correlated with the accumulation of PTOX in different plant organs of *P. peltatum*. This study aimed to (1) investigate the PTOX content differences in various plant organs of *P. peltatum*, (2) check which plant organ the *PpPLR* gene expression takes place in, (3) validate that the *PpPLR* gene is a pivotal gene in the biosynthesis pathway of PTOX, and (4) reveal the potential molecular mechanism for PTOX content differences in different plant organs.

In the present study, we used RT-PCR to monitor the transcription activity of *PpPLR* gene and established the tissue expression profile for *PpPLR* gene. The results demonstrated that the expression occurred only in five of six plant organs, including rhizomes, petiole, root, fruit and leaf. The highest level expression of *PpPLR* was found in rhizomes, whereas expression in leaf was the lowest. In the flower, expression of *PpPLR* was not observed. It could be ascribed to the starting of dehydration, evaporation as well as the associated reduction in metabolic activity characterized by the stage of development (Haughn & Chaudhury, 2005). We concluded that *PpPLR* is a developmentally regulated gene, similar to some functional genes participated in other secondary metabolic pathways. For instance, the *H6H* gene encoding the committed-step enzyme of scopolamine biosynthesis specifically expressed in the roots of *Atropa belladonna* (Suzuki *et al.*, 1999) and the ADS gene encoding amorpha-4,11-diene synthase regulating artemisinin biosynthesis is only expressed in

the glandular secretory trichome of *Artemisia annua* (Olsson *et al.*, 2009). In addition, in situ hybridization and promoter–reporter experiments showed that the BAN gene encoding dihydroflavonol reductase was localized primarily in *Arabidopsis thaliana* seed coats (Devic *et al.*, 1999). The same case of gene expression involved in PTOX synthesis pathway was mainly observed in rhizome organ of *P. peltatum*.

Relationship between *PpPLR* gene expression profile and the PTOX content differences in various plant organs: The *PpPLR* gene tissue expression profile and the PTOX content among different plant organs were compared and analyzed (Figs. 2 and 3). In the rhizomes of *P. peltatum*, *PpPLR* gene expression was at the highest level while the PTOX content was also the highest. *PpPLR* gene expression and the PTOX content have positive correlations. The *PpPLR* gene expression pattern is inconsistent with the content profile of PTOX among different test plant organs except for the rhizome. Similarly, Kim *et al.*, (2013) found that the *MYB* transcription factors regulated the biosynthesis of glucosinolate (GSL) in different Chinese cabbage organs (*Brassica rapa* ssp. pekinensis). The results showed that the expression of *MYB28* and *MYB29* in the stem was significantly different from that in other plant organs, however, the total GSL contents of different plant organs were arranged in an ascending order: old leaves <root <stem< young leaves<flower <seed. In addtion, Lan *et al.*, (2010) used the similar species Tibet Dysosma (*Dysosma tsayuensis* Ying) as tested materials, and studied the influence of secoisolariciresinol dehydrogenase (*SDH*) gene expression variations involved in PTOX biosynthetic pathway on PTOX contents in various plant organs of Tibet Dysosma. The results demonstrated that the expression levels of *Dysosma tsayuensis SDH* (*DtSDH*) were inconsistent with the content of PTOX in the six test plant organs. That is reasonable because the synthesis, transportation and storage of secondary metabolites were widespread in plant kingdom, which is a complicated and integrative system. In consequence, the biosynthetic organ of secondary metabolite is not always its storage organ.

Conclusions

The PTOX content evaluation, specific *PpPLR* gene expression pattern in different plant organs, and their correlations were investigated in the present work. The anticancer lignan PTOX is accumulated mainly in the rhizome of *P. peltatum*. The *PpPLR* gene is most actively expressed in the rhizomes of *P. peltatum* among the six test plant organs. In the rhizome of *P. peltatum, PpPLR* gene expression pattern match entirely with the accumulation profile of PTOX. None of the correlation for *PpPLR* gene expression pattern and the PTOX content profile was established in any other test plant organ except for the rhizome, suggesting that PTOX synthesis most likely occurs at the rhizome. The rhizome is probably the synthesis and storage location for PTOX. It also confirmed the key function of *PLR* enzyme in PTOX synthesis in *P. peltatum*. Therefore, investigation of the relationship between PTOX accumulation and *PLR* gene

expression in various plant organs may be helpful in understanding the molecular mechanism of the PTOX content variance in different plant organs, as well as the PTOX biosynthesis. This research also offers a crucial gene of interest for metabolic engineering of the PTOX.

Acknowledgments

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