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ISOLATION AND CHARACTERIZATION OF A GENE ENCODING FARNESYL DIPHOSPHATE SYNTHASE FROM *Panax vietnamensis* Ha et Grushv.

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ABSTRACT

Panax vietnamensis Ha et Grushv. is a species of the genus *Panax* native to Central Vietnam, containing a family of triterpene saponins named ginsenosides. This group of biomolecules possesses valuable therapeutic properties against cancer, hepatitis, diabetes, inflammation as well as stress and anxiety. Farnesyl diphosphate synthase (FPS) is a key enzyme participating in the ginsenoside biosynthesis pathway. In this study, a *FPS* gene from *P. vietnamensis* (*PvFPS*) was isolated and characterized. The *PvFPS* cDNA contained an open reading frame of 1032 bp, encoding a polypeptide chain of 342 amino acid residues. Nucleotide sequence comparison showed that *FPS* was highly conserved among most species, with two Aspartate-rich motifs responsible for product chain length determination strongly sustained. *PvFPS* was closely related to those of the same genera and order and differed from those from other kingdoms. *PvFPS* expression was detected at a greater level in root tissues than in leaves in all ages. Our findings provided information concerning the properties of a crucial gene in the ginsenoside biosynthesis, thus enhancing our understanding of this important pathway.

Keywords: *Panax vietnamensis*, ginsenoside biosynthesis, triterpenoid saponins, farnesyl diphosphate synthase, farnesyl pyrophosphate synthase, gene expression.

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INTRODUCTION

The *Panax* genus belongs to the Araliaceae family, comprising approximately 12 recognized species (Shin et al., 2015; Zhang et al., 2015). The word "*Panax*" takes its roots from "Panceae", a Greek term meaning "all-healing". Accordingly, species of genus *Panax*, generally called "ginseng", is used as sacred herbal medicine in many Asian nations as well as some other countries, namely the United States or Russia. Among this genus, *Panax vietnamensis* Ha et Grushv. is distributed the furthest to the south of Asia, specifically on Ngoc Linh mountain and other highland areas in Central Vietnam. Also known as "sam Ngoc Linh" or Vietnamese ginseng (VG), it was first used by Sedang ethnic people to treat severe illnesses and improve physical health.

Studies have confirmed that *P. vietnamensis* possesses many valuable medicinal properties against toxic effects as well as positive impacts against some serious diseases. Banskota et al. (2005) stated in their research that VG can reduce fatigue, enhance physical strength, improve appetite, and mitigate psychological or stress-related disorders in the body. In addition, this plant can hinder and prevent cancer, diabetes, hepatitis, anemia, and arteriosclerosis (Banskota et al., 2005). In more recent studies, VG showed anti-inflammatory activities by inhibiting excessive activation of the nuclear factor-kappaB (NF-κB) signaling pathway (Jeong et al., 2015). It also expressed protective effects on porcine kidney cells from cisplatin-induced damages (Vu-Huynh et al., 2019). *P. vietnamensis* extract was proven to promote the growth and differentiation in neural stem cells harvested from murine fetuses (Do et al., 2019). Most of these pharmacological features stem from the activities of therapeutic biomolecules called ginsenosides.

Ginsenosides are triterpenoid saponins found only in the *Panax* genus. These constituents fall into four categories based on the aglycone moieties they possess, i.e., protopanaxadiol (PPD), protopanaxatriol (PPT), oleanolic acid, and ocotillol (OCT) types (Zhang et al., 2015). Apart from PPD and PPT saponins similar to *Panax ginseng* (Korean ginseng, KG), VG contains an exceptionally high amount of OCT-type ginsenosides, such as majonosides R1 and R2, vina-ginsenosides R1 and R2 (Jeong et al., 2015). Especially, majonosides R2 (MR2) has been proven to play strong roles in many of VG's pharmaceutical effects (Banskota et al., 2005; Jeong et al., 2015). Therefore, studies on MR2 and other saponins in VG are necessary and highly valuable.

Farnesyl diphosphate synthase, or farnesyl pyrophosphate synthase, is a rate-limiting enzyme with a crucial role in one of the first steps in ginsenoside biosynthesis, catalyzing the condensation of isopentenyl diphosphate (IPP) with geranylgeranyl diphosphate (GPP) and dimethylallyl diphosphate (DMAPP) into farnesyl diphosphate (FPP) (Koyama et al., 1994). Despite abundant researches on gene *FPS* in *P. ginseng* and other species of genus *Panax*, up until now, there were no in-depth studies on this gene in *P. vietnamensis*. In this study, we aim to isolate *FPS* from VG and explore its expression pattern in different ages and tissue types.

MATERIALS AND METHODS

Plant materials

Leave and root samples of 1-year-old, 4-year-old, and 11-year-old *P. vietnamensis* were randomly collected from Tak Ngo Ginseng Farm in Ngoc Linh Ginseng Center of Nam Tra My district (coordinates: 15.0094 N; 108.0258 E) and from Tra Linh Medicinal Plant Center of Ngoc Linh Ginseng Center of Quang Nam (coordinates: 15.0311 N; 107.9791 E) with permission. After cleaning, plant samples were cut into small pieces, frozen in liquid nitrogen, and stored at -80 °C until RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted from each plant tissue using the RNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's protocol. The isolated RNA

was treated with RNase-free DNase (Thermo Fisher Scientific, USA), followed by purification on RNA-purification column (Qiagen, USA), and finally collected by ethanol precipitation. RNA quality and quantity were then validated by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and 1.5% agarose gel electrophoresis.

The mRNA fragments were used as templates to synthesize cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol.

Reverse transcription PCR (RT-PCR)

A pair of *FPS*-specific primers (forward, 5' - TCCTCATCTCACCGCTCTTTC - 3'; reverse, 5'- GATACAGACAACAACTT CCCC - 3') was designed using OligoAnalyzer™ Tool by Integrated DNA Technologies (IDT) (https://www.idtdna.com/pages/tools/oligoanalyzer) and BioEdit software, then synthesized by Macrogen, Inc. (South Korea). The RT-PCR reactions were conducted in a 20 µL volume containing 15.15 µL of nuclease-free water, 0.15 µL of DreamTaq DNA polymerase and 2 µL of 10X DreamTaq buffer (Thermo Fisher Scientific, USA), 0.3 µL of 2 mM dNTPs (Sigma, USA), 0.2 µL of each primer at the concentration of 10 µM, and 2 µL of cDNA template. PCR was performed using Mastercycler Pro Gradient S (Eppendorf, Germany) as followed: initial denaturation at 94 ºC for 2 min, followed by 40 cycles of denaturation for 45 s at 94 ºC, annealing for 30 s at 58 ºC, extension for 1 min at 72 ºC, then a final step of elongation at 72 ºC for 2 min. The PCR products were checked by gel electrophoresis with agarose gel 1.2% using FastGene 100 bp DNA Marker (Nippon Genetics, Germany) as a product size reference, then stored at 4 ºC.

Real-time PCR (RT-qPCR)

A pair of *FPS*-specific primers was designed using BioEdit and OligoAnalyzer™ Tool as followed: forward, 5' - CGATGATCCTGCCTCTGTAGC - 3'; reverse, 5' - GTACGGCTTGGCTCGGATGAG - 3'. The primer pair was designed to satisfy the requirements for RT-qPCR, theoretically yielding a product of 136 bp. House-keeping gene *Elongation factor 1-gamma* (*EF 1-γ*) was used as an internal standard. *EF 1-γ*-specific primer pair was as followed: forward, 5' - ATCGCATTAAAGAGAGCACTAGG - 3'; reverse, 5' - CATGGTCCAAAAATATCT CTCTACG - 3', yielding a theoretical product of 145 bp. Both primer pairs were synthesized by Macrogen, Inc. (South Korea). Each reaction was conducted in a 10 µL volume containing 2.6 µL of nuclease-free water, 0.2 µL of each primer at the concentration of 10 µM, 5 µL of 2x SensiFAST SYBR No-ROX Mix (Meridian Bioscience, Inc., USA), and 2 µL of cDNA template. The qPCR reactions were carried out using LightCycler® 96 System (Roche Molecular Systems, Inc., Switzerland) under the following conditions: preincubation at 95 ºC for 5 min, followed by 37 cycles of 3-step amplification including 95 ºC for 15 s, 65 ºC for 10 s, 72 ºC for 2 s. Each reaction was conducted in triplicates. The PCR products were checked by gel electrophoresis with agarose gel 2%.

Bioinformatic analysis

The raw data of *FPS* sequencing were analyzed using BioEdit software. Ambiguous bases at the beginning and the end of the sequence were discarded, and questionable bases were replaced with special IUPAC notations (Dixon et al., 1986). The deduced nucleotide sequence was searched for homologous mRNA sequences using the Nucleotide Basic Local Alignment Search Tool (BLASTN) on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn). We used ClustalW Multiple Alignment Tool with default gap penalties to align *PvFPS* with previously registered mRNA sequences of *FPS* in other species of genus *Panax*, and verify the questionable bases based on the reference sequences from NCBI.

The amino acid sequence was translated using BioEdit and confirmed using the

Translate tool by ExPASy (https://web.expasy.org/translate/). The deduced amino acid sequence was searched for homologous protein sequences using the Nucleotide Basic Local Alignment Search Tool (BLASTN) on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn). The protein properties were then analyzed using ProtParam (https://web.expasy.org/protparam/). SMART (http://smart.embl-heidelberg.de/) and Conserved Domain Search Tool based on the Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) were used to examine special domains of the protein.

Based on the sequence alignments, a phylogenetic tree was constructed using the Maximum Likelihood method with the Tamura 3-parameter substitution model in the MEGA X software. Bootstrap analysis with 1,000 replicates was performed to assess confidence levels for the branches. The nucleotide sequences used for this construction were presented in Table 1.

Table 1. Nucleotide sequences used for phylogenetic tree construction

Accession no.	Source species	Accession no.	Source species
X75789	*Arabidopsis thaliana*	J05262	*Homo sapiens*
HM219226	*Aralia elata*	AB053486	*Humulus lupulus*
AF461050	*Bos taurus*	X76026	*Kluyveromyces lactis*
HQ123429	*Bupleurum chinense*	AY083165	*Malus x domestica*
X84695	*Capsicum annuum*	AF384040	*Mentha x piperita*
AY787627	*Centella asiatica*	AF309508	*Mus musculus*
JQ178346	*Eleutherococcus senticosus*	MW447137	*Oliveria decumbens*
D00694	*Escherichia coli*	DQ087959	*Panax ginseng*
AY389818	*Ginkgo biloba*	KP684141	*Panax japonicus*
Y12072	*Gossypium arboreum*	DQ059550	*Panax notoginseng*
KU942523	*Hedera helix*	GQ401664	*Panax quinquefolius*
AF019892	*Helianthus annuus*	KT936527	*Panax sokpayensis*
AY135188	*Hevea brasiliensis*	GFP67485	*Saccharomyces cerevisiae*

Data analysis

Experiments were performed in triplicates. Data were shown in the form of means ± SEM (standard error of the mean) and compared using a Student t-test with a significant difference at $P < 0.05$.

RESULTS AND DISCUSSIONS

Isolation of a *PvFPS* gene

Previously, from our transcriptome sequencing data of *Panax vietnamensis*, the unigene encoding FPS was identified and optimized for clear reads as presented in Fig. 1A. Even though it has been proven that FPS is encoded by a small gene family in several species including a closely related *Panax ginseng* (Cunillera et al., 1996; Hemmerlin et al., 2003; Wang et al., 2004; Kim et al., 2010; Cao et al., 2012), we only detected one in *P. vietnamensis*.

The *PvFPS* cDNA (GenBank accession number MZ272019) was 1141 bp long including untranslated regions, with a putative open reading frame (ORF) of 1032 bp, starting at position 16 with the start codon ATG and ending at position 1048 with the stop codon TAA. It encodes a farnesyl diphosphate synthase of 342 amino acids. Similar ORF length and polypeptide chain length were found in multiple other plant species, including *P. ginseng*, *Arabidopsis thaliana*, *Centella asiatica*, and *Withania somnifera* (Cunillera et al., 1996; Hemmerlin et al., 2003; Wang et al., 2004; Kim et al., 2005, 2010; Schmidt et al., 2007; Gupta et al., 2011; Cao et al., 2012; Xia et al., 2019).

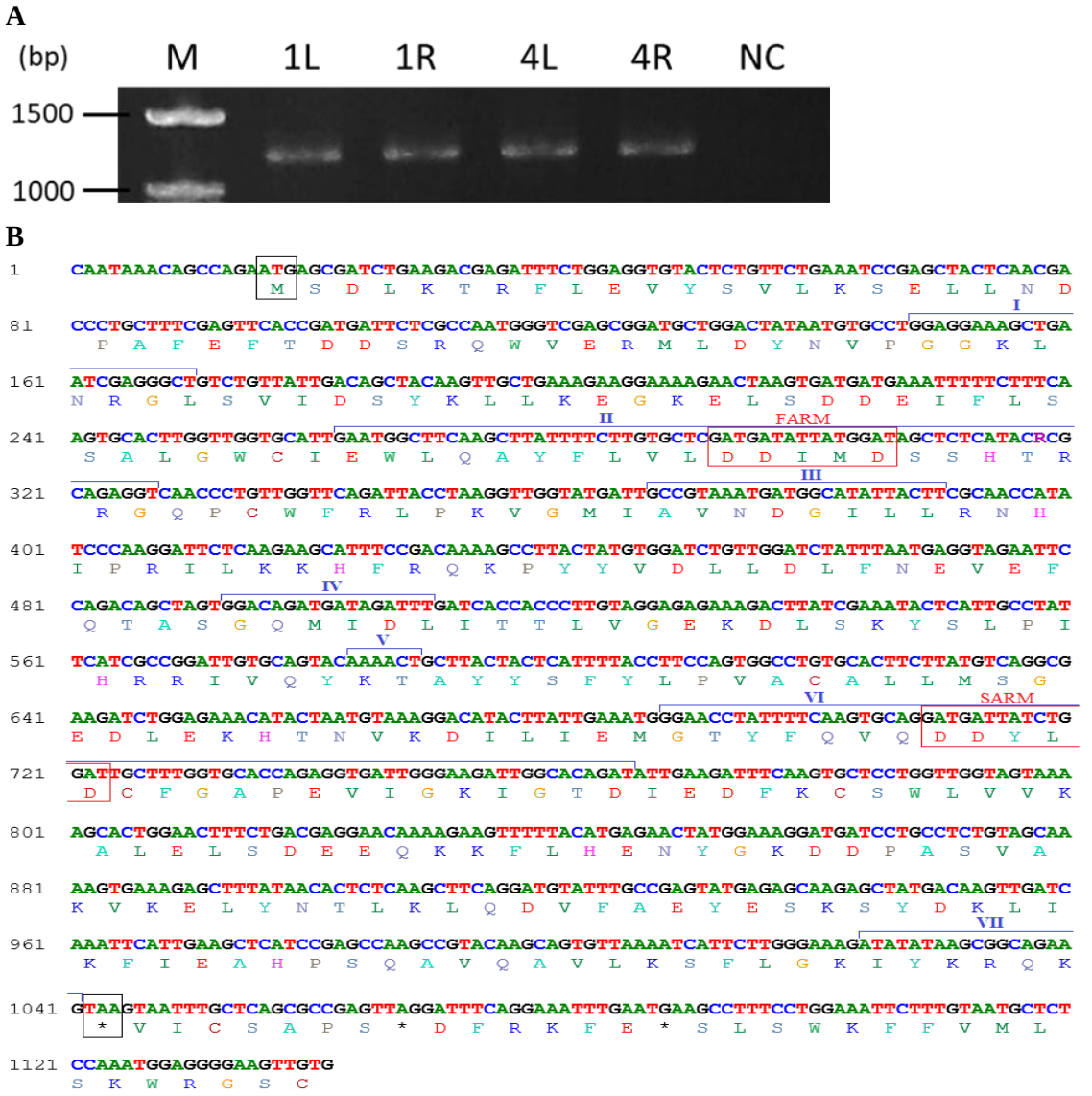

Figure 1. (A) Successful isolation and amplification of PvFPS extracted from 1L, 1-year-old leaves; 1R, 1-year-old roots; 4L, 4-year-old leaves; 4R, 4-year-old roots. M, FastGene 100 bp DNA Marker (Nippon Genetics, Germany); NC, negative control. The electrophoresis bands are at a suitable size with no non-specific PCR products. (B) Nucleotide and amino acid sequence of a PvFPS gene. ORF ranges from 16-1048. Each amino acid residue is displayed under the second nucleotide of its respective codon. The position of the first nucleotide on each line is shown on the left. Black boxes highlight the start and stop codons of the ORF. Blue brackets represent conserved regions from I to VII. Red boxes highlight the Aspartate-rich active sites. FARM, first Aspartate-rich motif; SARM, second Aspartate-rich motif

Protein analysis

Analysis on ProtParam estimated the molecular weight of a matured PvFPS protein to be 39.63 kDa, with an isoelectric point (pI) of 5.52. The molecular weight fell within the suitable spectrum for an individual FPS subunit, which was reported

in previous research to range from 32 kDa to 44 kDa (Szkopińska & Płochocka, 2005). Both the molecular mass and the pI of our PvFPS were similar to that of FPSs from *Panax notoginseng*, *Artemisia tridentata*, *Ginkgo biloba*, and more (Hemmerlin et al., 2003; Wang et al., 2004; Kim et al., 2005, 2010; Schmidt et al., 2007; Gupta et al., 2011; Cao et al., 2012; Xia et al., 2019). Among the amino acid composition, Leucine (L) accounted for the highest percentage (12.6%), followed by Lysine (K; 9.1%) and Aspartate (D; 7.9%). In total, the number of positively charged residues (Arg + Lys) was 44, while the number of negatively charged residues (Asp + Glu) was 51. The protein was considered stable with an instability index (II) of 37.60. No distinct domains such as transmembrane domains or repeats were confidently detected by SMART, consistent with previous studies (Xia et al., 2019).

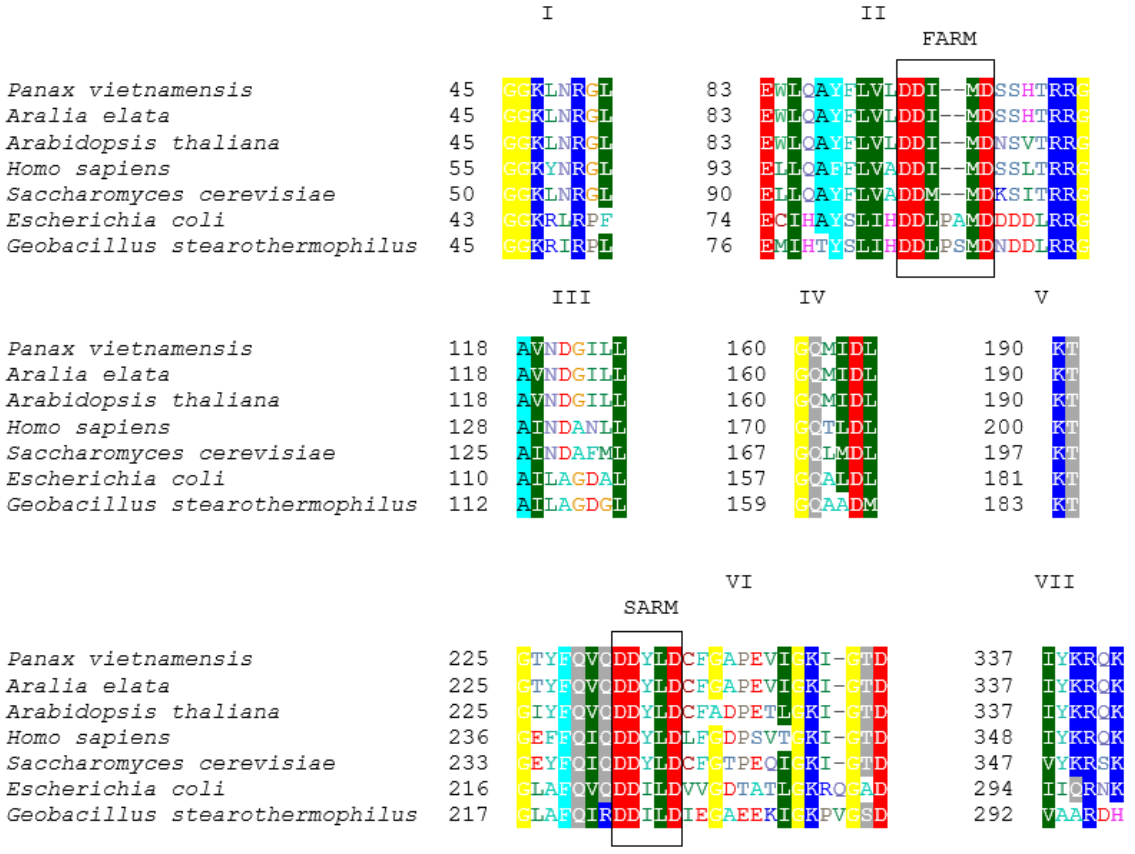

Figure 2. Amino acid sequence alignment of seven conserved regions of FPS from different species. Numbers to the left specify the positions of the first displayed residue. FARM, first Aspartate-rich motif; SARM, second Aspartate-rich motif

According to previous studies, *FPS* exists as homodimers with seven conserved regions throughout the amino acid sequence. Two highly conserved regions named first Aspartate-rich motif (FARM) with the sequence motif DDXX(XX)D (D as Aspartate, X as other amino acids) and second Aspartate-rich motif (SARM) with the motif DDXXD were designated as two opposite active sites responsible for C-C bond formation and chain length determination of the farnesyl pyrophosphate (FPP) product (Szkopińska & Płochocka, 2005). Alignment of the PvFPS protein sequence with *FPS* of

other species showed that all seven conserved regions on *FPS* of *P. vietnamensis* exhibited high similarity with those of *A. elata, A. thaliana, H. sapiens*, and other species. On the *FPS* polypeptide sequence of *P. vietnamensis*, FARM starts from position 93 to 97 and SARM from position 232 to 236, with all D residues remaining conserved (Fig. 1B, Fig. 2). Search results on CDD confirmed that conserved domains II and VI are Aspartate-rich active sites with catalytic residues, responsible for crucial functions including substrate-Mg^{2+} binding and chain length determination.

SOPMA results showed that the secondary structure of PvFPS consists of 63.74% α-helix, 3.22% β-turn, 25.44% random coil, and 7.60% extended strand (Fig. 3). An analogous structural motif was found in *FPS* protein from *G. biloba*, with a fold made of multiple α-helices connected by sheets and loops (Wang et al., 2004). Szkopińska and Płochocka (2005) also reported the same structural features under the term "terpenoid synthase fold", which further confirmed that PvFPS was structurally highly similar to *FPS* from other species.

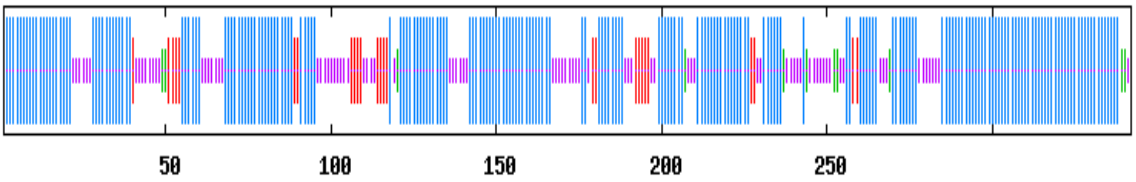

Figure 3. Secondary structure prediction of PvFPS protein. Blue depicts α-helix, green depicts β-turns, purple depicts random coils, and red depicts extended strands

Homology analysis

Using the NCBI Nucleotide BLAST tool, we compared the similarity between *PvFPS* with reference *FPS* sequences from other species. *PvFPS* showed a high similarity rate of at least 98% with five other *Panax* species, namely *P. quinquefolius* (KC524468), *P. sokpayensis* (KT936527), *P. notoginseng* (KC953034), *P. ginseng* (DQ087959), and *P. japonicus* (KP684141).

Phylogenetic tree construction based on the *FPS* gene revealed that the sequences from different kingdoms form clearly separate clusters, further confirming that all *FPSs* evolved from one ancestor gene into three distinct groups of plant, fungi, and animal *FPSs* (Cao et al., 2012). All six species from the *Panax* genus clustered into one group, which shared a close relationship with *Aralia* and other genera from the same Apiales order. According to the calculated branch distance, *PvFPS* closely resembled those derived from plants and significantly differed from *FPS* from fungi and animals. This motif is similar to the studies on GbFPS from *Ginkgo biloba* and EpFPS from *Euphorbia pekinensis* (Wang et al., 2004; Cao et al., 2012), both of which compared the amino acid sequences of *FPS*. This suggested that apart from highly conserved regions, the difference in the nucleotide sequences of *FPS* from distant species is certainly substantial, and this difference could be observed on both nucleotide and amino acid levels. Expected clustering and high bootstrap values indicated that this phylogenetic tree reached sufficient reliability. However, additional data should be required to confirm our results as the reference database was relatively poor.

Differential expression of *FPS* in different organs and ages of *P. vietnamensis*

To investigate the level of expression of *PvFPS* in different tissues throughout the years, RT-qPCR was applied on cDNA samples extracted from 1-year-old, 4-year-old, and 11-year-old *P. vietnamensis* leaves and roots. Overall, *PvFPS* was expressed in both tissue types at all ages investigated. Transcripts were detected relatively low in leave tissues, but varied through different stages in roots.

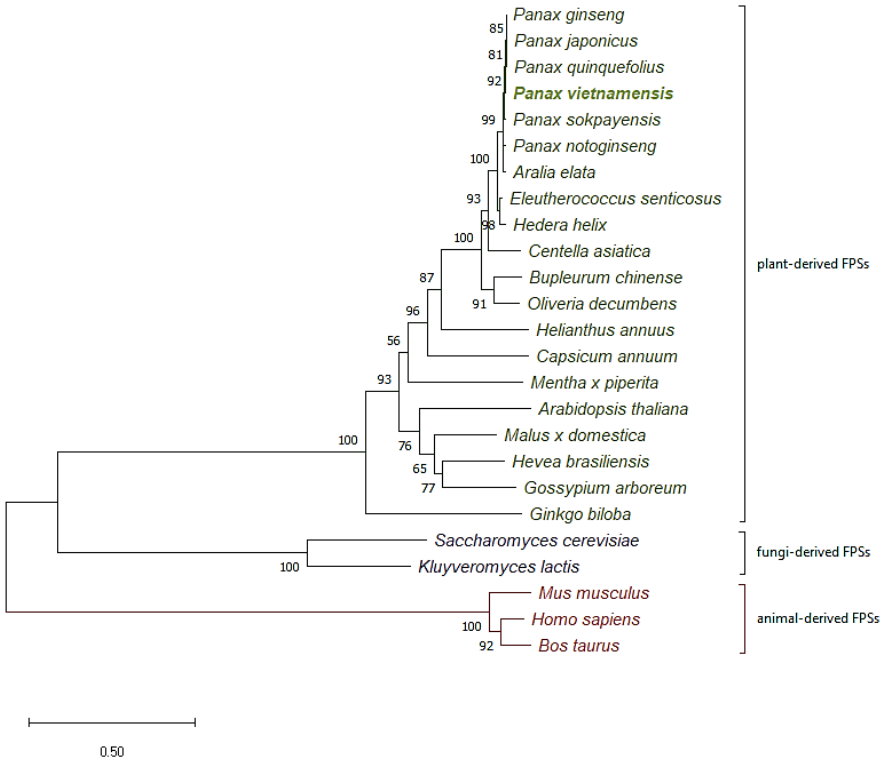

Figure 4. Phylogenetic tree constructed based on the nucleotide sequences of PvFPS and reference sequences retrieved from NCBI databases. Green indicated plant-derived FPSs; purple for fungi-derived FPSs; and red for animal-derived FPSs. Numbers by the branches indicate bootstrap values

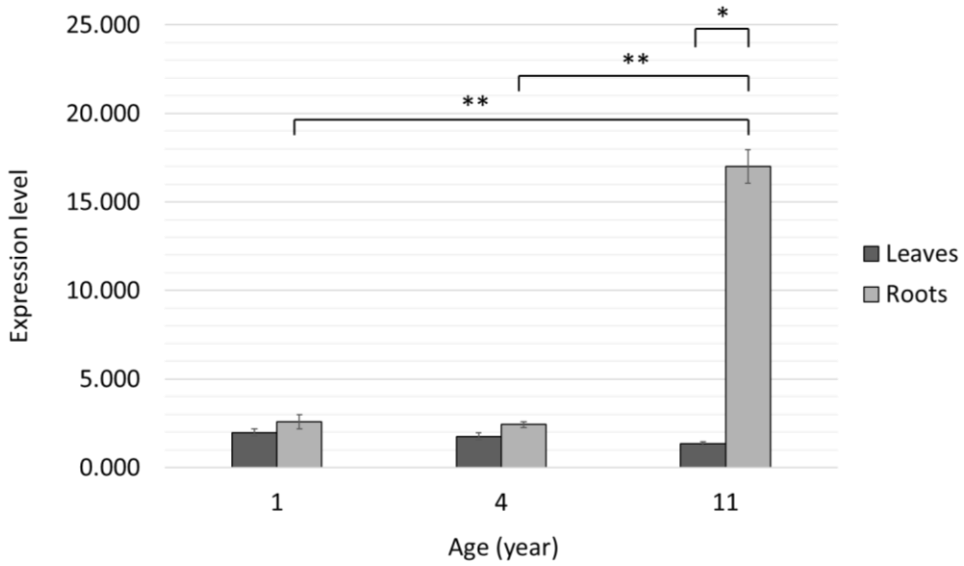

Figure 5. Expression analysis of PvFPS in leaves and roots tissues at different ages by quantitative RT-PCR. Experiments were performed in triplicates. Data was shown as the mean value ± standard error of the mean (SEM). * $p \leq 0.002$, ** $p \leq 0.0001$

In 1- and 4-year-old *P. vietnamensis*, we observed no significant differences between leaf and root tissues. In 11-year-old plants, the expression in roots was 12.47 times higher than that in leaves. *PvFPS* expression in 11-year-old roots was also significantly higher than that in 1- and 4-year-old roots. An especially high level of *PvFPS* expression in the 11-year-old root tissues suggested that at this age, ginsenoside might be abundantly synthesized and stored in roots. The expression of *PvFPS* in leaves did not show relevant fluctuations between the three ages.

CONCLUSION

FPS plays an important role in the biosynthesis pathway of ginsenosides, the valuable bioactive molecules in *P. vietnamensis*. In this study, we isolated the gene, analyzed the sequence using bioinformatics tools, and investigated its expression pattern. Its nucleotide sequence resembled those of other plants, especially those of the *Panax* genus. Conserved Aspartate-rich active sites indicated similar catalytic activities to FPSs from other species. *PvFPS* expression in 11-year-old roots was significantly higher than that in 1- and 4-year-old roots. *PvFPS* was detected 12.47 times higher in roots than in leaves of 11-year-old *P. vietnamensis* samples, while little significant differences were observed between the two tissue types at the age of 1 and 4. With these results, we hope to contribute to the overall knowledge of *P. vietnamensis* and ginsenosides.

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