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CLONING AND CHARACTERIZATION OF THREE SQUALENE EPOXIDASE GENES IN *PANAX VIETNAMENSIS* VAR. *FUSCIDICUS*, A RARE MEDICINAL PLANT WITH HIGH CONTENT OF OCOTILLOL-TYPE GINSENOSIDES

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Abstract

Panax vietnamensis var. *fuscidiscus*, is a new variety of *Panax. vietnamensis*, which was first found in Jinping County, the southern part of Yunnan Province, China. It is also one of the most prized medicinal plants used in traditional ethnic minority medicine systems. This species contains higher content of ocotillol-type saponin, especially majonoside R_2, which make this plant particularly suitable for identification of the *SE* genes responsible for the biosynthesis of ginsenosides. Three cDNAs encoding *SE* were cloned from *P. vietnamensis* var. *fuscidiscus*, and their molecular characterizations were investigated. The tissue-specific expression patterns of the three *SE* genes were analyzed by RT-qPCR. The transcription levels of these genes in Methyl jasmonate (MeJA)-treated leaves of *P. vietnamensis* var. *fuscidiscus* were also estimated by RT-qPCR. *PvfSE*, *PvfSE2* and *PvfSE3* differed in predicted membrane-spanning helices. Phylogenetic analysis grouped these *SE* into three different clades. The three *PvfSE* isoforms were all most highly expressed in leaves. Moreover, they exhibited different response patterns under MeJA induction. The three *PvfSE* isoforms may play different roles in sterol or ginsenoside biosynthesis in this herb This report is the first attempt to clone and expression analysis of *SE* from *P. vietnamensis* var. *fuscidiscus* and provides a new foundation for further understanding the role of *SE* in the biosynthesis of ginsenosides.

Key words: *Panax vietnamensis* var. *fuscidicus*, Ginsenoside, Ocotillol-type saponin, Gene expression, MeJA induction.

Introduction

Ginsenosides are a special group of triterpenoid saponins, which is the primary bioactive components of *Panax* species (Araliaceae family), and exhibit diverse pharmacological properties on the central nervous, endocrine, cardiovascular and immune systems (Ng, 2006; Wang *et al*., 2006; Christensen, 2009; Qi *et al*., 2011; Radad *et al*., 2011). There are four basic aglycones of ginsenosides: protopanaxadiol, protopanaxatriol, ocotillol and oleanolic acid. The commonly used Panax species, included ginseng (*P. ginseng*), American ginseng (*P. quinquefolium*) and sanchi ginseng (*P. notoginseng*) mainly contain protopanaxadiol-type and protopanaxatriol-type ginsenosides, and the other species, like *P. japonicus* and *P. zingiberensis* contain large amounts of oleanane-type ginsenosides (Zhu *et al*., 2004). Interestingly, high content of ocotillol-type ginsenosides only were found in one species, Vietnamese ginseng (*P. vietnamensis*) (Yamasaki, 2000; Tran *et al*., 2002), particularly is majonoside R_2 which exhibited anti-tumor-promoting activity and hepatocytoprotective activity (Konoshima *et al*.,1998; Konoshima *et al*.,1999; Tran *et al*., 2001).

The first committed step in triterpenoid biosynthesis is the cyclization of 2,3-oxidosqualene, which came from squalene oxidized by squalene epoxidase (*SE*, EC:1.14.99.7). In ginseng species, 2,3-oxidosqualene is cyclized by dammarenediol-II synthase (DDS) (Kushiro *et al*., 1997; Tansakul *et al*., 2006) to produce dammarenedil-II, which is further converted to protopanaxadiol by protopanaxadiol synthase (PPDS), a cytochrome P450 enzyme (CYP716A47) , and protopanaxadiol is further converted to protopanaxatriol by protopanaxatriol synthase (PPTS, CYP716A53v2) (Han *et al*., 2013). In contrast, 2,3-oxidosqualene is cyclized by β-amyrin synthase (bAS) to produce β-amyrin, which is further converted to oleanolic acid by β-amyrin 28-oxidase (β-A28O, CYP716A52v2) (Han *et al*., 2013) (Fig. 1). These aglycons will be to ginsenosides catalyzed by uridine diphosphate glycosyltransferases (UGTs) (Achnine *et al*., 2005; Augustin *et al*., 2012; Naoumkina *et al*., 2010). Recently, two UGTs (PgUGT74AE2 and PgUGT94Q2) also were characterized in *P. ginseng* which involved the biosynthesis of ginsenoside Rg_3 and Rd (Jung *et al*., 2014). In conclusion, the biosynthesis of protopanaxadiol, protopanaxatriol and oleanolic acid are fully elucidated and their aglycons and some ginsenosides can produced through yeast fermentation (Jung *et al*., 2014; Dai *et al*., 2014). Despite the pharmacological importance of ocotillol-type ginsenosides, little is known about their biosynthesis (Christensen, 2009).

Based on the molecular structure of ocotillol, we presumed that there are two pathways for the biosynthesis of ocotillol: in pathway A, 2,3-oxidosqualene is further epoxidized to 2, 3; 22, 23- dioxidosqualene (DOS) by SE (Shan *et al*., 2005), followed by cyclization and hydroxylation to produce ocotillol. In pathway B, ocotillol might be biosynthesized via epoxidation of the double bond at C-24-C-25 of protopanaxatriol (Christensen, 2009). Both pathways need SE or its ortholog which epoxidized the similar double bonds of squalene or protopanaxatriol (Fig. 1). Previous studies had shown that SE normally produce 2,3-oxidosqualeneare biosynthesized via the insertion of an oxygen atom into the linear squalene molecule and some SE genes have been characterized in *P. ginseng* and *P. notoginseng* (Han *et al*., 2010; Niu *et al*., 2014). Now, we

also supposed that the SE or its homologous may participate in other epoxidation in two putative ocotillol-type ginsenosides biosynthetic pathway. Obviously, the more detailed understanding of SE genes involved in ocotillol-type ginsenosides biosynthesis is essential to metabolic engineering and synthetic biology.

P. vietnamensis var. *fuscidiscus* is a new variety of *P. vietnamensis*, which accumulated ocotillol-type ginsenosides, majonoside R_2, even higher that in *P. vietnamensis*. For elucidating the molecular mechanism of biosynthesis of ocotillol-type ginsenosides, the transcriptome of *P. vietnamensis* var. *fuscidiscus* root was sequencing by Illumina HiSeq™ 2000, many putative genes involved in ginsenosides biosynthesis were discovered (The manuscript submitted to *BMC Genomics*). In the Illumina dataset, 15 unigenes annotated to SE were found, based on their sequences three SE genes were cloned and characterized, and their tissue-specific expression patterns were detected using reverse transcription quantitative real-time PCR (RT-qPCR). To explore the effect of MeJA on the regulation of expression of these cloned *SE* genes, the transcription levels of these genes in Methyl jasmonate (MeJA)-treated leaves of *P. vietnamensis* var. *fuscidiscus* were also estimated by RT-qPCR. Our findings are an important foundation for further understanding of the mechanism of higher production and storage of ginsenosides, and also could provide opportunities to engineer microorganisms for the *de novo* production of ginsenosides.

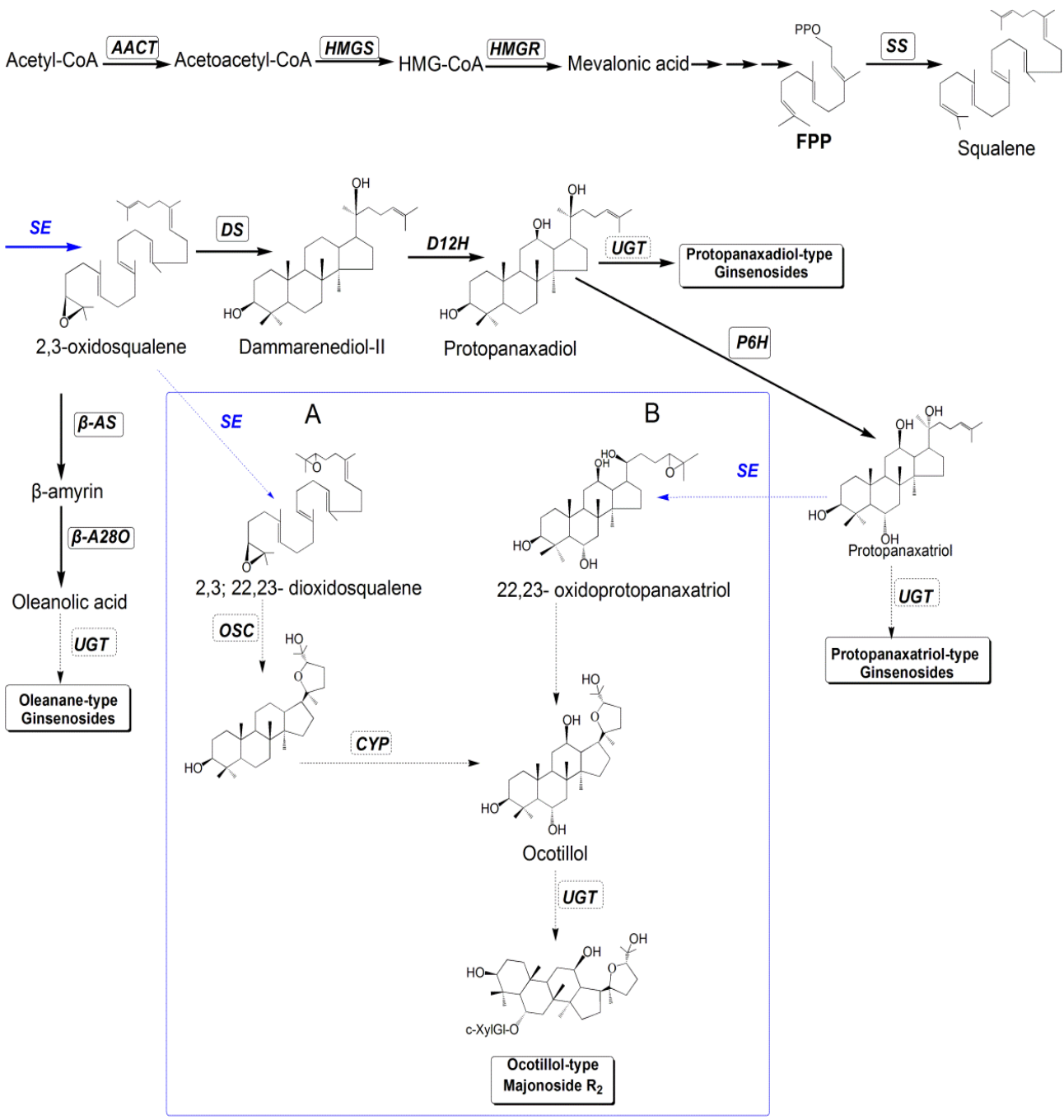

Fig. 1. Putative biosynthetic pathway of triterpene saponin in *P. vietnamensis* var. *fuscidiscus*. Two proposed pathways (A and B) for the biosynthesis of ocotillol-type saponins.

Fig. 2. The photographs of *P. vietnamensis* var. *fuscidiscus*. (**A**) The aerial parts of this plant. (**B**) The rhizome of this plant.

Materials and Methods

Plant material and RNA extraction: Three-years-old *P. vietnamensis* var. *fuscidiscus* plants (Fig. 2) were collected from Jinping County, Yunnan province, southwest of China (Latitude: 22° 47' 38"N, Longitude: 103° 2' 22"E, Altitude: 1690 m), and then twenty wild-grown plants were transplanted into 5 L plots and grown in good environmental for one month. After morphological and molecular identification according the reference (Zhu *et al*., 2003), the root, lateral root, stem, leaf samples were collected separately from three randomly selected plant individuals. These samples were used for *SE* gene cloning and tissue-specific expression analysis.

Three randomly selected *P. vietnamensis* var. *fuscidiscus* plants were used for MeJA treatment. MeJA Induction was conducted by spraying the entire leaf with a solution of 250 µM MeJA (Sigma, Aldrich, USA), with addition of ethanol at 0.25 %. All treated leaves were covered with a transparent plastic bags and the leaves were harvested after 3h, 6h and 24h treatment. Control leaves were sprayed with 0.25 % ethanol and harvested at 0h. All the above samples were stored at −80 °C until use.

The total RNA of all samples was extracted by Trizol Kit (Promega,USA), following RNA purification by RNeasy MiniElute Cleanup Kit (Qiagen, Hilden, Germany), according to the manufacture's protocol.

Full-length cDNA cloning: Approximately 1 µg of total RNA from mixed root samples were reverse transcribed to synthesize first strand cDNA using oligo dT primer and a PrimeScript™II 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. The RT-PCR products were used as template for cloning of *PvfSE1*, *PvfSE2* and *PvfSE3*. The full-length cDNA sequences of *PvfSE1*, *PvfSE2* and *PvfSE3* were obtained from *P. vietnamensis var. fuscidiscus* transcriptome data (NCBI SRA database accession No. SRA146484), and their sequence informations were used to design specific primers (Table 1) using Primer3 (http://primer3.ut.ee/). PCRs were conducted in a total reaction volume of 25 µL, containing 1 µL of cDNA, 0.5 µM of each of the forward and reverse primers, 200 µM of dNTPs, 5 µL of 5×Q5 Reaction Buffer, and 0.25 µL of Q5 High-Fidelity DNA polymerase (NEB, Beijing, China). PCR was performed using the following protocol: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 59°C for 1 min, 72°C for 5 min, with a final 10 min extention at 72°C.

The PCR products were electrophoretically separatedon a 1% agarose gel, ligation into the pMD19-T vector (TaKaRa, Dalian, China) and were then sequenced using an ABI 3730XL sequencer(Applied Biosystems, Foster City, USA).

Bioinformatics analysis: The Open reading frame (ORF) were detected using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The nucleotide sequence was translated using Translate tool (http://web.expasy.org/translate/). Predictions of physicochemical properties of the deduced amino acid sequence were performed using ProtParam (http://web.expasy.org/protparam/). The Hydrophobicity analysis was performed using ProtScale (http://web.expasy.org/protscale/). The transmembrane domains were were predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) web tools. The secondary structures of deduced amino acid sequences were determined by SOPMA (http://npsa-pbil.ibcp.fr/). Structural and functional domains were identified in deduced protein sequence by SMART tool (http://smart.embl-heidelberg.de/). Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to conduct the homology sequence searches. Multiple sequence alignments was done by Clustal X with the default parameters. For the phylogenetic analysis, a neighbor-joining tree was constructed using MEGA5.0, and 1000 replicates were used for bootstrap test.

RT-qPCR analysis: Total RNA was extracted individually from lateral root, root, stem, leaf, and from MeJA treated leaves of *P. vietnamensis* var. *fuscidiscus* using Trizol Kit (Promega,USA) following the manufacturer's protocol. Approximately 1 µg RNA from each sample was used to generate first-strand cDNA fragment using SuperScript III (Invitrogen, USA). Gene-specific primers were designed base on the gene sequences using Primer3 software. All gene-specific primers used for the RT-qPCR analysis were showed in Table 2. The quantitative reaction were

performed in a 20 μL volume composed of 1 μL of cDNA, 0.5 μLof each primer, and 12.5 mL 2x SYBR premix Ex taqTM (Takara, Japan) in 7500 Fast Real-Time PCR Systems (Applied Biosystems, USA). PCR amplification was performed under the following conditions: 2min at 95°C, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 70°C for 45s. Three technical replications were performed for all quantitative PCRs. The β-actin was chosen as an internal control for normalization. The relative changes in gene expression levels were calculated using the 2- $\triangle\triangle^{Ct}$ method.

Table 1. Gene-specific primers used for gene cloning.

Gene	Primers
PVfSE1 L:	GTCGCTCGCATTGTTCATCA
PVfSE1 R:	TCTGTGGAACGAGGATTGCA
PVfSE2 L:	ATTCATGGAACTCGGGAGGA
PVfSE2 R:	AGTTGTCTGTCTGCTCTTGAGA
PVfSE3 L:	AACGTCCAACCAACACCATG
*PVfSE3*R:	TCGCCCGTGACTGATATTCA

Table 2.All gene-specific primers used for the RT-qPCR analysis.

Gene	Primers
PVfSE1 L:	GTCTGAACCCTCGTCCACTGA
PVfSE1 R:	GAAATCAATCTGGCTCCAATCC
PVfSE2 L:	CGGTGGAGGAATGACTGTTG
PVfSE2 R:	CACTGGCTTGCGAAGAGTGTA
PVfSE3 L:	GTGGCTATCTATGGCGTTGG
*PVfSE3*R:	CAGGAACAGTTGCAGGGAAG

Results

Cloning and sequence analysis of *PvfSE1, PvfSE2* and *PvfSE3*: In our previous study, transcriptome sequencing and analysis of *P. vietnamensis* var. *fuscidiscus* has been completed by our research group. Numerous *SE* candidate gene sequences were identified from our transcriptome database (accession number: SRA146484) based on a search for standard gene names and synonyms in the functional annotations of the unigenes, and then these sequence informations were used to design primers for the cloning of the full-length cDNA encoding *PvfSE*s. Using the gene-specific primers, we eventually obtained the full-length cDNA of three *SE* genes, namely, *PvfSE1*, *PvfSE2* and *PvfSE3*. The nucleotide and the deduced amino acid sequence of the cloned *PvfSE* genes were listed in Table 3. Three full-length cDNAs of *PvfSE1*, *PvfSE2* and *PvfSE3* obtained in this study have been deposited in the Genbank database (accession numbers: KJ946467, KJ946468 and KJ946469).

The cDNA sequence of *PvfSE1* containing a 1,581bp open reading frame (ORF) encoding a 536 amino acid residues with molecular weight of 57.57 kDa and predicted isolelectric point (pI) of 8.28. The cloned full-length cDNA of *PvfSE2* including a 1,446 bp ORF encoding a deduced protein of 544 amino acid residues. The deduced amino acid sequence of *PvfSE2* had a pI of 9.11 and a calculated molecular mass of about 60.06 kDa. The cDNA sequence of *PvfSE3* comprising a 1,611bp ORF. *PvfSE3* codes for a residues of 537 amino acid residues with a predicted molecular mass of 59.09 kDa and predicted isolelectric point (pI) of 8.38.

Analysis of the predicted protein sequence: All the three cloned *PvfSE* genes were selected for thorough characterization. The Hydrophobicity analysis done by Kyte-Doolittle web tools, Kyte–Doolittle Protein Hydrophobicity Plots analysis showed that the deduced amino acid sequence of *PvfSE1* contained a typical hydrophobic region in the N-terminus (Fig. 3A). In contrast, a typical hydrophilic region could be found in the N-terminus of the deduced amino acid sequence of *PvfSE2* and *PvfSE3* (Fig. 3B, C). Besides, *PvfSE1*, *PvfSE2* and *PvfSE3* are all have a typical hydrophobic region in the C-terminus, and this region have a role in anchoring the enzyme to the ER membrane. In general, we suggests that *PvfSE1*, *PvfSE2* and *PvfSE3* are all transmembrane protein.

The transmembrane regions were predicted by TMHMM Server v.2.0. Result of TMHMM2 program shown that *PvfSE1* contain 3 putative transmembrane helices at positions 5-24 aa, 456-478 aa, and 485-503 aa (Fig. 4A), *PvfSE2* contain 3 putative transmembrane helices at positions 13-32 aa, 476-495aa, and 502-519 aa (Fig. 4B), and *PvfSE3* contain 4 putative transmembrane helices at positions 20-42 aa, 68-90aa, 466-488 aa, and 495-512 aa (Fig. 4C). In previous studies, *SE* was thought to be bound to the endoplasmic reticulum, the presence of transmembrane helices were required for anchoring the protein to the endoplasmic reticulum membrane (Sakakibara *et al*., 1995). *PvfSE1*, *PvfSE2* and *PvfSE3* differed in predicted membrane-spanning helices, likely contributing to formation of metabolic channels specific for sterol or ginsenoside biosynthesis (Han *et al*., 2010). Hence, our results were not unexpected, and in accordance with previous studies (Han *et al*., 2010; Niu *et al*., 2011; He *et al*., 2008).

Secondary structure analysis of protein was performed using SOPMA program. The result showed that the putative *PvfSE1* contained 32.32% of alpha helices, 31.94% of random coils, 23.76% of extended strands and 11.98% of beta turn. The putative *PvfSE2* comprises of 34.01% alpha helices, 32.72% random coils, 22.79% extended strands and 10.48% beta turns. the deduced *PvfSE3* contained 34.89% of alpha helices, 33.96% of random coils, 21.46% of extended strands and 9.70% of beta turn. The alpha helices and random coils constituted interlaced domain of the main part of the secondary structure of these putative *PvfSE*s.

Table 3. The nucleotide and the deduced amino acid sequence of the three *PvfSE* isoforms.

Gene	Nucleotide sequence	The deduced amino acid sequence
PVfSE1	ATGGAGGAACACTACGTTTTAGGATTAATTCTGGCTTCTCTGTTAGGTTTTCTT TTGGTTTACGCGTTGTTTTTCAAGAAAAATGACCGAAGATATTCCGTAGAGGC CGTTAAGAGCAACACCGCGACCACAACCACCGCCATTAACGGGGAATGCAGA TCCAGGAACGGCGCCGGAGACGATGTTGATGTTATTATCGTAGGTGCCGGCGT TGCCGGTGCGGCGCTCGCTCACACGCTCGGGAAGGATGGGAGACGGGTACAT GTGATTGAAAGAGACTTGACAGAACCGGATAGAATTGTCGGAGAATTATTACA ACCAGGTGGCTACCTGAAGTTGATTGAGTTGGGACTGGAAGATTGTGTGGAA GAAATTGATGCTCAAAGAGTGTTTGGTTATGCTCTTTTCAAGGATGGAAAGAA TACTCGGCTATCTTATCCTTTGGAGAAATTTCACTCGGATGTGTCTGGGAGGA GCTTTCATAATGGTAGATTCATACAGAGGATGCGTGAGAAAGCTGCAACCCTT CCCAATGTCCAAATGGAGCAAGGAACTGTCACATCTTTGCTTGAAGAAAATG GAACCATTAAAGGTGTACAATACAAGACTAAGACTGGTGAAGAAATGAATGC GTATGCACCTCTAACCATTGTATGTGATGGCTGCTTTTCAAACTTGCGACGTAA CCTTTGCAGTCCAAAGGTGGATGTGCCTTCTTGTTTTGTTGGTTTGGTCCTTG AGAACTGCAAACTACCACATGCAAATCATGGACATGTTATCTTAGCAGACCCA TCACCCATCTTGTTTTATCCCATCAGTAGCACAGAGATCCGCTGTTTGGTTGAT GTTCCTGGTCAAAAGGTGCCTTCCATTTCAAATGGCGAAATGGCCACATATTT GAAGACCGTGGTTGCTCCCCAGATTCCTCCAGAGCTGCATGATGCCTTCATAG CCACAGTTGAAAAAGGAAATATTAGGACAATGTCCAACAGAAGCATGCCAGC TGCTCCTCACCCTACTCCAGGGGCTTTGCTGATGGGGGATGCATTCAACATGC GCCACCCTTTAACTGGTGGAGGAATGACTGTTGCACTTTCTGATATTGTTGTTC TACGTAATCTTCTAAGGCCTCTCCGTGACATGAATGATGCATCTACCCTATGCA AATACCTCGAATCCTTTTACACATTGCGTAAGCCTGTGGCATCCACCATCAATA CGCTGGCAGGTGCTCTATACAAGGTGTTTTGTGCCTCCCCTGATCAAGCTAGG AAAGAAATGCGCGAGGCATGCTTCGACTATCTTAGCCTTGGGGGTTTTTGTTC AGAAGGACCAGTATCCTTGCTCTCTGGTCTGAACCCTCGTCCACTGAGTTTGG TTGTCCATTTCTTTGCCGTAGCAATATTTGGCGTTGGCCGCTTGCTACTGCCAT TCCCCTCACCCAAACGAATGTGGATTGGAGCCAGATTGATTTCAAGTGCTTCC GGAATCATTTTCCCCATAATCAAGGCAGAAGGATTTAGGCAGATGTTCTTCCCT GCGACAGTACCTGCATATTACAGAGCTCCTCCTGTCTGGTGA	MEEHYVLGLILASLLGFLL VYALFFKKNDRRYSVEAV KSNTATTTTAINGECRSRN GAGDDVDVIIVGAGVAGA ALAHTLGKDGRRVHVIER DLTEPDRIVGELLQPGGYL KLIELGLEDCVEEIDAQRVF GYALFKDGKNTRLSYPLEK FHSDVSGRSFHNGRFIQRM REKAATLPNVQMEQGTVT SLLEENGTIKGVQYKTKTG EEMNAYAPLTIVCDGCFSN LRRNLCSPKVDVPSCFVGL VLENCKLPHANHGHVILA DPSPILFYPISSTEIRCLVDV PGQKVPSISNGEMATYLKT VVAPQIPPELHDAFIATVEK GNIRTMSNRSMPAAPHPTP GALLMGDAFNMRHPLTGG GMTVALSDIVVLRNLLRPL RDMNDASTLCKYLESFYT LRKPVASTINTLAGALYKV FCASPDQARKEMREACFD YLSLGGFCSEGPVSLLSGL NPRPLSLVVHFFAVAIFGVG RLLLPFPSPKRMWIGARLIS SASGIIFPIIKAEGFRQMFFP ATVPAYYRAPPVW
PVfSE2	ATGGAACTCGGGAGGAGTTACAGGGAGAATGATGAGTATTTTTTGATGTTTGC TGCTACGTTCCTTTTTGGGTTTGTTCTGTACTTGTTTACTTTGAGGAGGAGGAG GAGGAGGAGGAGAGAGAAAAAAGGTGGTGCTGGCTCCATGGAGATTATTAAT GGAGCATATAAGTTGACTAGTAGCTCTGAAGTTAACGGTCATTGCACCCCGGA GGATATCGCCGGAAGTTCTGATGACGTCATCATTGTCGGTGCCGGCGTTGCCG GTTCTGCACTTGCTTACACGCTTGCCAAAGATGGCCGGAGAGTTCATGTAATC GAAAGAGACTTGACTGAGCAAGACAGAATTGTTGGTGAACTTCTACAGCCAG GAGGGTATTTAAAATTGGTTGAATTAGGCCTTGAGGATTGTGTTAATGAAATTG ATGCACAAAGAGTTTTCGGATATGCCCTTTACATGGATGGCAAAAATACTAGG CTTTCTTATCCCCTGGAGAAATTCCATGCAGATGTAGCAGGAAGAAGCTTTCA TAATGGCCGCTTCATTCAACGGATGAGGGAAAAAGCTGCATCTCTTCCAAATG TACGAATGGAGCAAGGGACTGTGATATCTCTGGTTGAGCAAAAAGGAACTGT AAAAGGGGTGCGATACAAAACAAAAAATGGCCAAGAAATGTCGGCAGCATAT GCTCCCCTGACAATTGTTTGTGATGGtTGTTTTTCGAATCTTCGGCATTCCCTCT	MELGRSYRENDEYFLMFA ATFLFGFVLYLFTLRRRRR RRREKKGGAGSMEIINGAY KLTSSSEVNGHCTPEDIAGS SDDVIIVGAGVAGSALAYT LAKDGRRVHVIERDLTEQD RIVGELLQPGGYLKLVELG LEDCVNEIDAQRVFGYALY MDGKNTRLSYPLEKFHAD VAGRSFHNGRFIQRMREK AASLPNVRMEQGTVISLVE QKGTVKGVRYKTKNGQE MSAAYAPLTIVCDGCFSNL RHSLCNPKVDVPSCFVGLI

	GCAATCCCAAGGTGGATGTGCCGTCGTGTTTTGTTGGTTTGATTTTGGAAAATA	LENIDLPHINHGHVILADPS
	TTGATCTTCCACATATAAACCATGGCCATGTTATTCTAGCAGATCCTTCTCCAAT	PILFYKISSTEIRCLVDVPGQ
	CTTGTTTTATAAAaTTAGCAGCACCGAAATTCGCTGTTTAGTTGATGTGCCTGG	RVPSIANGELAHYLKTSVA
	ACAAAGAGTGCCTTCTATTGCTAATGGTGAATTGGCTCATTATTTGAAGACTTC	PQIPPELYKSFIAAIDKGQT
	AGTAGCTCCTCAGATTCCACCTGAGCTATACAAGTCTTTTATAGCAGCAATAGA	KTMPNRSMPADPHPTPGA
	CAAAGGGCAGACAAAAACAATGCCAAACAGAAGCATGCCAGCTGATCCTCAT	LLLGDAFNMRHPLTGGGM
	CCCACTCCAGGTGCACTTCTTTTGGGGGATGCTTTCAATATGAGGCATCCCTTA	TVALSDIVLIRDLLRPLRDL
	ACCGGTGGAGGAATGACTGTTGCTCTGTCGGACATTGTTCTGATACGTGATCT	HDSSTLCKYLESFYTLRKP
	TCTTAGGCCATTACGCGATCTCCATGACTCATCAACCCTATGCAAATATCTTGA	VASTINTLAGALYKVFCAS
	ATCCTTTTACACTCTTCGTAAGCCAGTGGCATCGACTATAAACACATTGGCCGG	PDKARQEMRDACFDYLSL
	TGCGCTATACAAAGTGTTTTGCGCATCACCTGACAAAGCAAGGCAGGAAATG	GGICSEGPIALLSGLNPRPM
	CGCGACGCGTGCTTTGATTACCTGAGCCTCGGAGGGATTTGTTCAGAAGGGC	SLFFHFFAVAIYGVGRLLIPF
	CAATTGCTCTTCTTTCTGGCCTAAATCCCCGCCCAATGAGTCTGTTCTTCCACT	PSPRKIWLGARLISGASGIIF
	TCTTTGCTGTGGCTATTTATGGCGTTGGCCGCTTATTGATTCCATTTCCTTCACC	PIIKSEGVRQMFFPATVPAY
	AAGAAAAATATGGCTTGGAGCTAGATTGATTTCGGGGGCATCTGGAATTATTTT	YRAPPITK
	CCCTATCATAAAGTCTGAAGGAGTTAGGCAAATGTTTTTCCCAGCAACTGTTC	
	CGGCATACTACAGAGCTCCTCCCATTACTAAATAA	
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	GGACGTCATCATCGTCGGCGCCGGGGTTGCTGGCTCTGCTCTTGCTTATACGC	DSNGYYTPENIAGSTDVIIV
	TTGCCAAAGATGGCCGGAGAGTTCATGTAATTGAGAGGGACTTAACTGAGCA	GAGVAGSALAYTLAKDGR
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	AATTTCATTCGGATGTTGCTGGAAGAAGCTTTCATAACGGCCGTTTTGTTCAA	TRLSYPLEKFHSDVAGRSF
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	TTtCGAaTCTCcGTCGCTCCcTCTGCAaTCcCAaGgTGgAGgTGCcTtCGTGTTTtGTt	LTIVCDGCFSNLRRSLCNP
PVfSE3	GgTTTGATTTTGGAAAATAtTGATCTTCCACATATAAaCCATGGCCATGTCAtTTT	KVEVPSCFVGLILENIDLPH
	AGCAGATCCTTCTCCGATCTtGtTTTATAAAATTAGTAGTACCGAGATTCGCTGT	INHGHVILADPSPILFYKISS
	TTGGTTGATGTGCCTGGACAaGAgGTGCCTTCTATTTCTAATGGGGAATTGGCT	TEIRCLVDVPGQEVPSISNG
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	ATAGCAGCAGTTGACAAAGGAAACATTAGAACCATGCCAAACAGAAGCATGC	YNSFIAAVDKGNIRTMPNR
	CAGCCGATCCTCATCCAACTCCGGGTGCACTTCTATTAGGGGATGCTTTCAATA	SMPADPHPTPGALLLGDAF
	TGCGCCATCCTTTAACCGGCGGGGGAATGACAGTGGCTCTGTCCGATATTATC	NMRHPLTGGGMTVALSDII
	TTGATCCGGGATCTTCTTAGACCTTTACGCGATCTCCATGACTCATCAACCCTC	LIRDLLRPLRDLHDSSTLC
	TGTAAATATCTCGAATCCTTTTACACCCTTCGTAAGCCCGTGGCATCTACTATA	KYLESFYTLRKPVASTINTL
	AATACATTGGCAGGTGCACTTTATAAAGTTTTTTGTGCATCACCTGATAAAGCA	AGALYKVFCASPDKARQE
	AGGCAAGAAATGCGCAATGCGTGTTTTGATTATCTGAGCCTCGGAGGAATTTG	MRNACFDYLSLGGICSQGP
	TTCCCAAGGGCCAATTGCTTTACTTTCTGGCCTAAACCCGCGTCCAATTAGCCT	IALLSGLNPRPISLFLHFFAV
	ATTTCTCCACTTCTTTGCCGTGGCTATCTATGGCGTTGGCCGCTTGTTGATTCCT	AIYGVGRLLIPFPSPKRMW
	TTTCCTTCACCAAAACGAATGTGGCTCGGCGCTAGATTGATTTTGGGTGCATC	LGARLILGASGIIFPIIKSEG
	TGGAATTATTTTCCCTATTATAAAGTCAGAAGGACTTCGACAAATGTTCTTCCC	LRQMFFPATVPAYYRAPPIH
	TGCAACTGTTCCTGCTTACTACCGAGCTCCCCCCATTCACTAA	

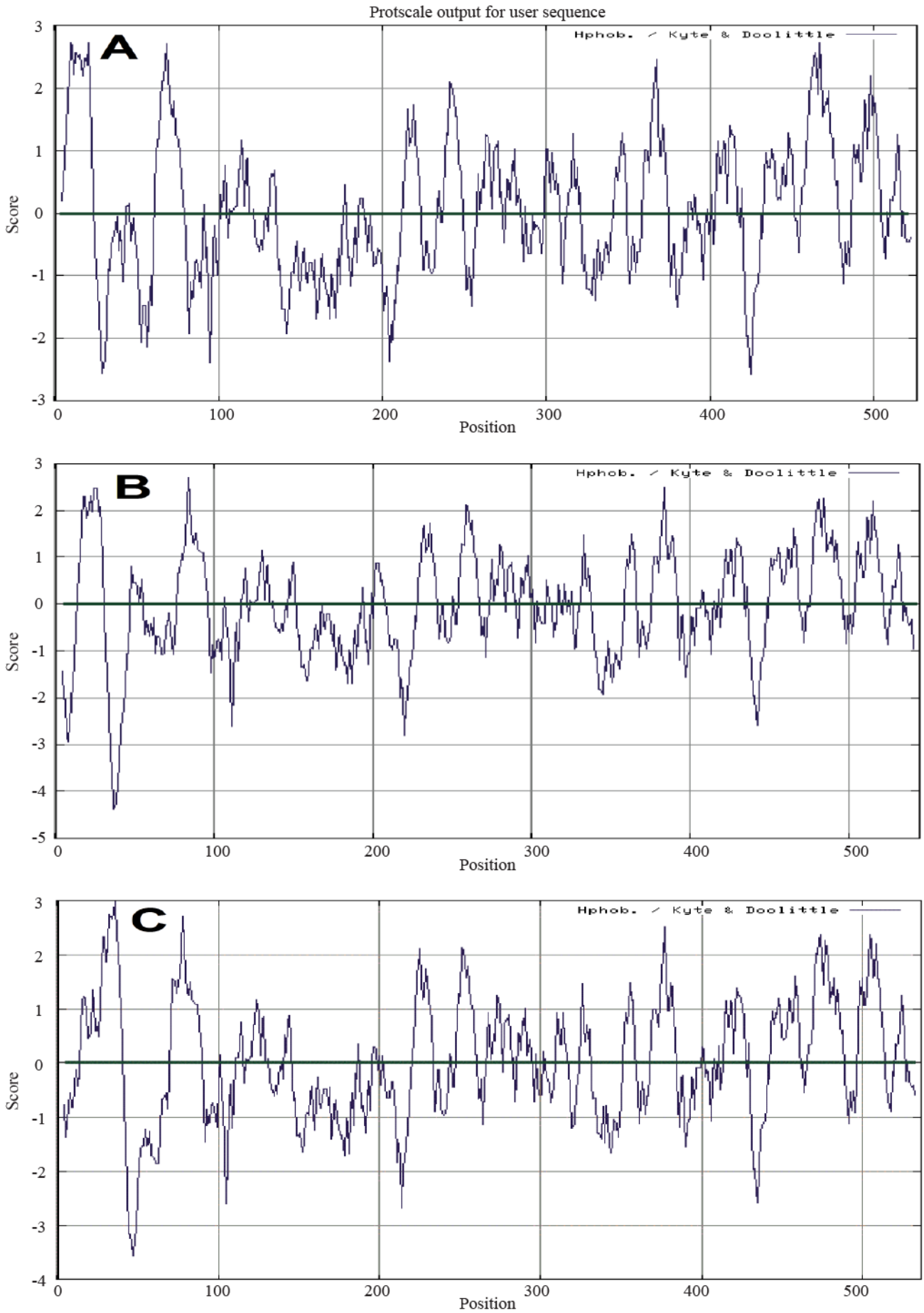

Fig. 3. Kyte-Doolittle hydrophobicity plots of *PvfSE1* (A), *PvfSE2* (B) and *Pvf SE3* (C). A window of 9 aa was used.

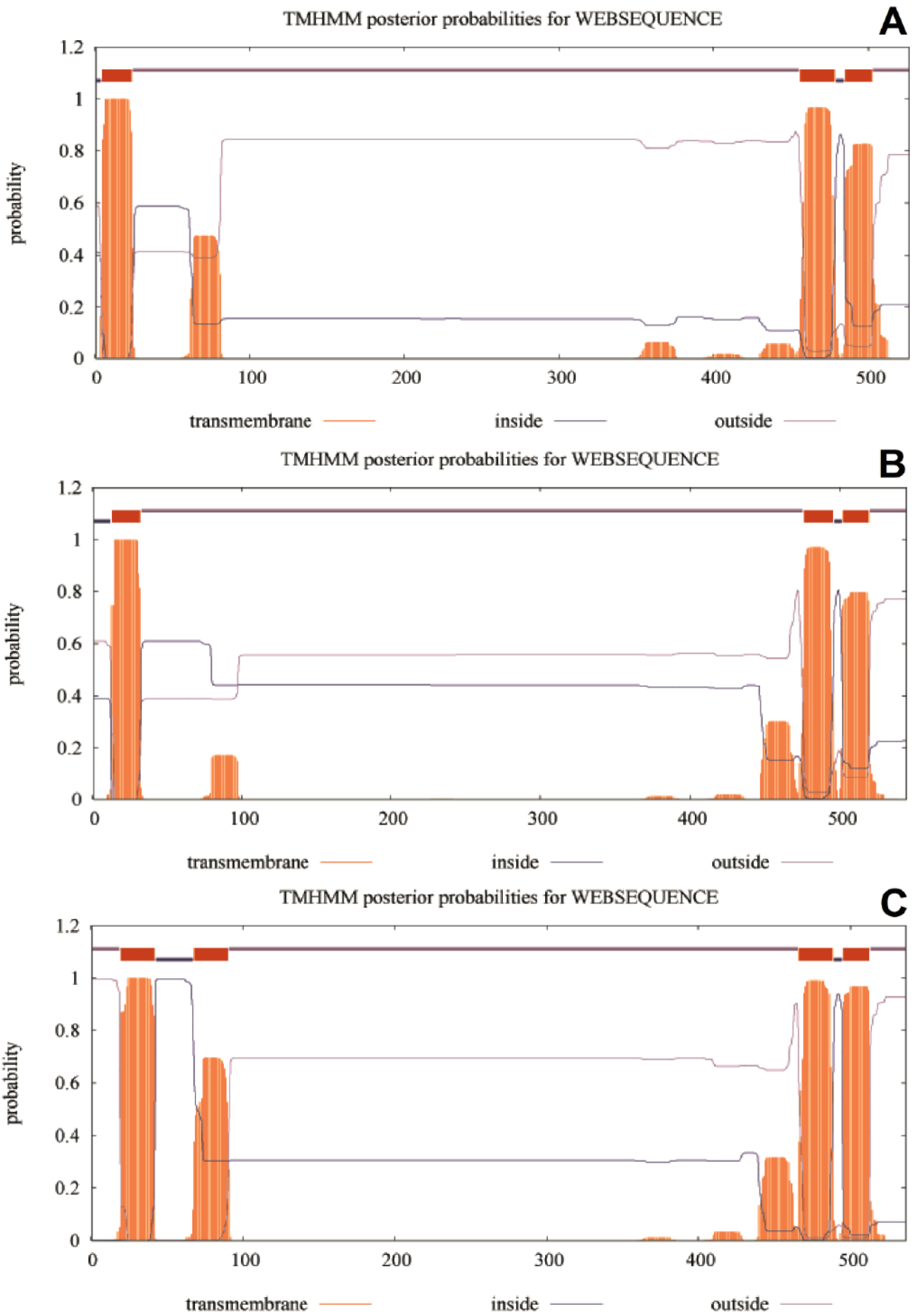

Fig. 4. Prediction of the transmembrane regions of *PvfSE1* (A), *PvfSE2* (B) and *Pvf SE3* (C) amino acid sequence.

Table 4. Confidently predicted domains.

Gene	name	start	end	E-value
PvfSE1	signal peptide	1	22	N/A
	FAD_binding_3	61	406	3.50E-20
	FAD_binding_2	63	95	0.00014
	GIDA	63	234	0.000075
	NAD_binding_8	66	95	0.000045
	SE	212	485	3.20E-124
PvfSE2	transmembrane region	13	32	N/A
	Thi4	71	118	0.000042
	FAD_binding_3	77	423	6.60E-17
	DAO	79	114	4.70E-07
	FAD_binding_2	79	111	0.000018
	GIDA	79	252	0.0000042
	NAD_binding_8	82	116	0.0000026
	SE	229	502	2.40E-125
PvfSE3	low complexity	3	9	N/A
	transmembrane region	20	42	N/A
	Pfam:Thi4	61	112	0.000022
	Pfam:FAD_binding_3	71	416	6.50E-18
	Pfam:DAO	73	108	4.60E-07
	Pfam:GIDA	73	245	0.0000064
	Pfam:FAD_binding_2	73	105	0.000018
	Pfam:NAD_binding_8	76	110	0.0000025
	Pfam:SE	222	495	3.80E-127

The conserved domains was analyzed by SMART online tool, our results show that these deduced amino acid sequences all include two typical high conserved domains for *SE* (Ruckenstuhl *et al*., 2005): the flavin adenine dinucleotide (FAD) binding domain which is a key enzyme site in the sterol or ginsenoside biosynthetic pathway, and a second domain typically referred to squalene epoxidase function domain (Fig. 5A, B). This suggested that there is a high degree of sequence conservation in certain key regions. Besides, some other conserved domains were also been found in these deduced amino acid sequences, such as the nicotinamide adenine dinucleotide (NAD) binding domain, glucose-inhibited division protein A (GIDA) domain, D-amino acid oxidase (DAO) function domain, etc (Table 4). In previous studies, it is believed that squalene epoxidase (*SE*) is an essential flavin adenine dinucleotide (FAD)-dependent monooxygenase in sterol or ginsenoside biosynthesis (Ruckenstuhl *et al*., 2008), all of them are microsomal enzymes, the enzymatic activity depends on the presence of molecular oxygen and a reducing cofactor, NADPH or NADH, and they are stimulated by FAD (Favre & Ryder, 1996; Favre & Ryder, 1997; Gomelsky & Klug, 2002). No doubt, our results support those already found in the previous studies.

PvfSE1 and *PvfSE2* showed 71.50% identity at the nucleotide level and 75.05% identity at the amino acid level. *PvfSE1* and *PvfSE3* showed 69.90% identity at the nucleotide level and 75.60% identity at the amino acid level. *PvfSE2* and *PvfSE2* exhibited a high degree of identity with each other, 80.72% at the nucleotide level and 81.44% identity at the amino acid level, respectively. The identities obtained in all the pairwise comparisons were lower at DNA than at amino acid level, indicating that the highest sequence variation was at the third codon positions (Infante *et al*., 2008). It is noteworthy that the N-terminal region of amino acid sequences show high discrepancy between *PvfSE1*,*PvfSE2* and *PvfSE3*, whereas amino acid residues in the C-terminal region exhibited a high level of sequence identity among all *SE* proteins. Alignment of the amino acid sequences of the three *PvfSE* isoforms with *SE* isoforms from other plants, full-length alignment result showed that the three *PvfSE* isoforms shares high similarity (87%) with the *SE* gene from other *Panax* plants (Fig. 5B), and revealed that the sequences of *PvfSE1*,*PvfSE2* and *PvfSE3* are highly conserved at amino acid level.

Phylogenetic analysis: To evaluate the phylogenetic relationship between *PvfSE*s with other other plant *SE*, a phylogenetic tree was constructed (Fig. 6). Phylogenetic analysis grouped these *SE* into three main clades. *PvfSE3* was closely related to two *SE* genes of *P. ginseng*, followed by three *SE* genes of *P. notoginseng*. *PvfSE2* was clustered together with the remaining *SE* sequences of *P. ginseng* and *P. notoginseng*, and formed another subgroup. Interestingly, *PvfSE1* was clustered together with *SE* sequences of non-*Panax* plants, forming a distinct subgroup, and more closely related to the *SE* of *Theobroma cacao* than to those of other plants. Our results suggested that although the three *PvfSE* isoforms shares high identity (83%) with each other, they may have different biological functions in *P. vietnamensis* var. *fuscidiscus*.

Expression characteristics of *PvfSE1*, *PvfSE2* and *PvfSE3* at different tissues: In order to examine the tissue-specific expression of the three *PvfSE* isoforms in *Panax vietnamensis* var. *fuscidiscus*, we evaluated their relative mRNA expression levels by RT-qPCR in different tissues (lateral root, root, stem, leaf). The expression patterns of these *PvfSE* isoforms were shown in Fig. 7A. The transcripts of the three *PvfSE* isoforms were produced in all tested tissues, but the expression patterns varied among the different tissues. The highest abundances of *PvfSE1* mRNA were found in the stems, while the highest abundances of *PvfSE2* and *PvfSE3* mRNA were in the leaves. *PvfSE1* was highly expressed in the stems; moderately expressed in the leaves; weakly expressed in the roots and lateral roots. *PvfSE2* with the strong expression in leaves, moderate expression in roots, weak expression in stems and lateral roots. *PvfSE3* mRNA accumulated preferentially in the leaves and stems, weakly accumulated in lateral roots and roots, and moreover that in lateral root and roots were less highly than that in the stem and leaves. As expected, the transcript abundances of the three *PvfSE* isoforms were all lower in lateral roots, whereas all the three *PvfSE* isoforms were strong expression in leaves. The expression level of *PvfSE2* was at least 8.2 fold higher in the roots as compared to that of *PvfSE1* and *PvfSE3*. *PvfSE1* expression in stems was 29.5 fold and 5.7 fold higher than that of *PvfSE2* and *PvfSE3*, respectively. Our data supports that leaves and stems are the main site for synthesizing the precursors of sterol or ginsenosides in this plant, and ginsenosides probably were modified and stored in roots and lateral roots. According to the above results, indicating that the three *PvfSE* isoforms may play different roles in sterol or ginsenoside biosynthesis, *PvfSE1* and *PvfSE3* may participate in the epoxidation reaction to form 2,3-oxidosqualene, and *PvfSE2* may related to the last epoxidation reaction in the putative pathway A and B (Fig. 1). However, further studies on the biological function of the three *PvfSE* isoforms are needed.

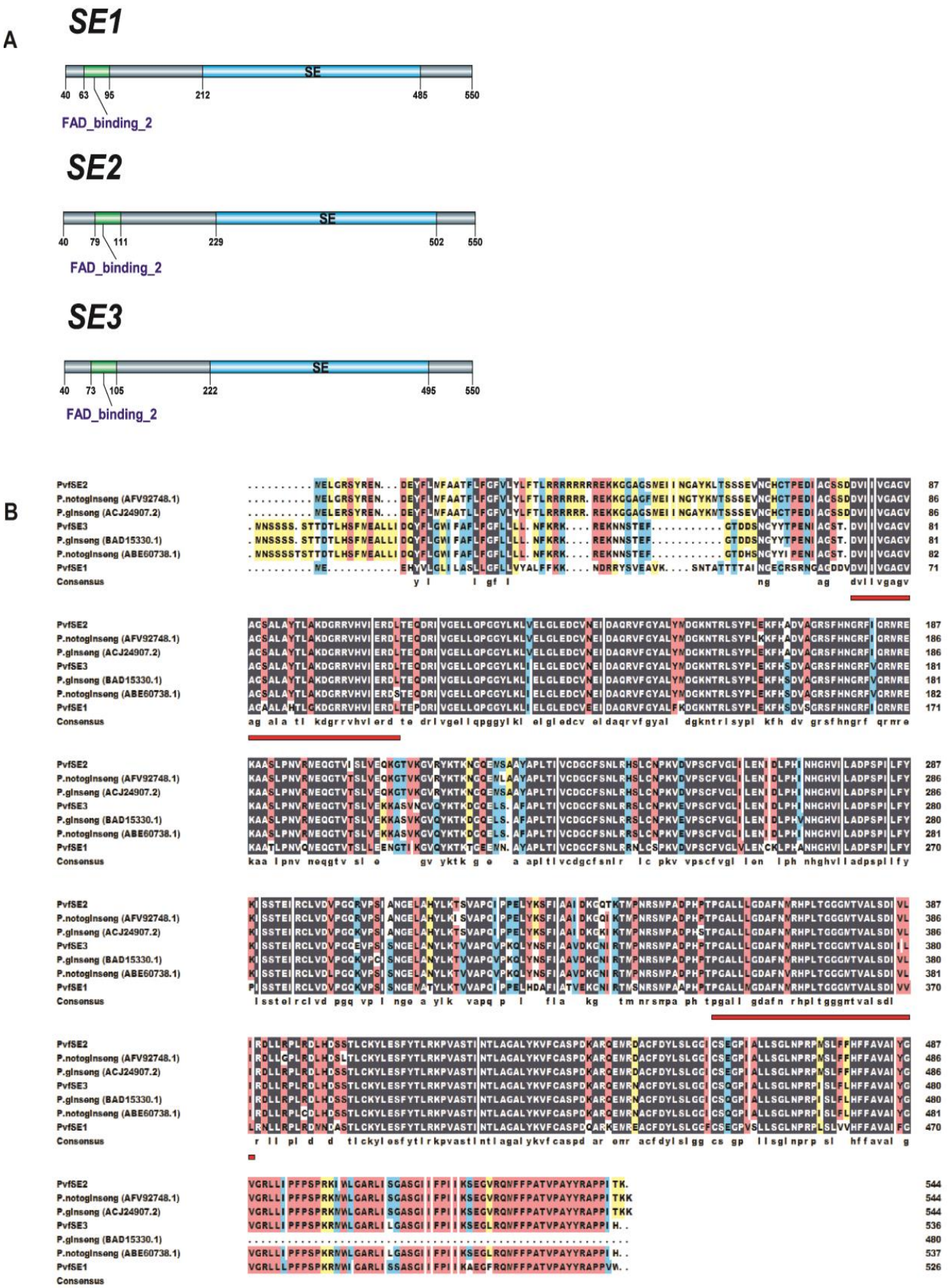

Fig. 5. Analysis of of deduced amino acid sequences of *PvfSE1*, *PvfSE2* and *Pvf SE3*. (A) The conserved domains of *PvfSE1* , *PvfSE2* and *Pvf SE3*. (B) Comparison of putative amino acids sequence of *PvfSE1* , *PvfSE2* and *PvfSE3* with the most closely related *SE*s from other plants. Identical amino acid residues are black. Red indicates 50% or more identity among all the aligned sequences. The bold red colored lines under amino acid sequences indicate the predicted flavin adenine dinucleotide (FAD) binding domains.

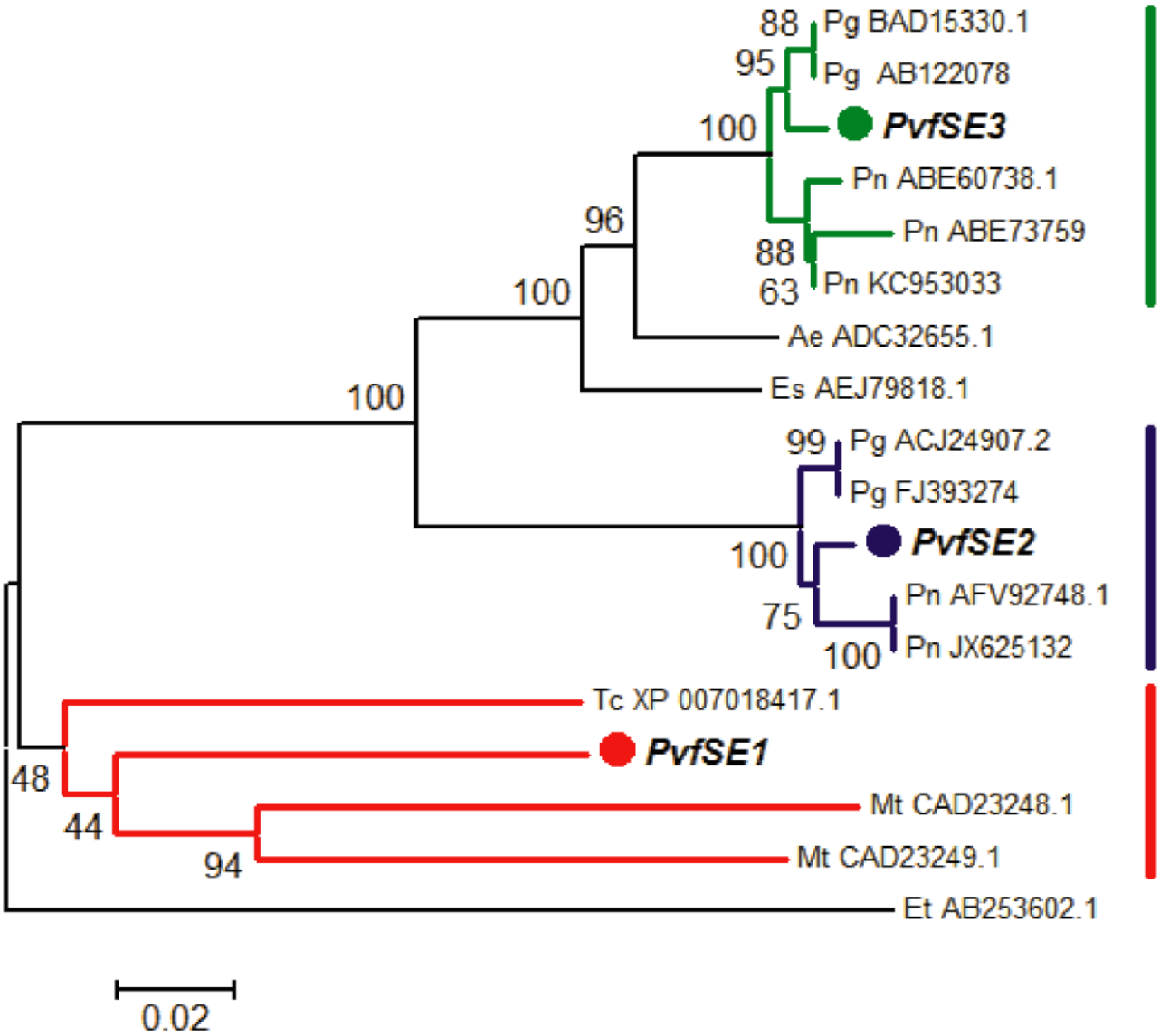

Fig. 6. Phylogenetic tree of *the three PvfSE isoforms* (bold italic letters) and other plants *SE*s. The species and GenBank accession numbers of the sequences are as follows: *Panax ginseng* (ACJ24907.2), (BAD15330.1), (AB122078), and (FJ393274); *Panax notoginseng* (ABE60738.1), (AFV92748.1), (ABE73759), (KC953033) and (JX625132); *Arabidopsis thaliana* (NP 197803.1); *Aralia elata* (ADC32655.1); *Eleutherococcus senticosus* (AEJ79818.1); *Euphorbia tirucalli* (AB253602.1); Medicago truncatula (CAD23248.1) and (CAD23249.1); *Saccharomyces cerevisiae* (AAA34592.1); *Theobroma cacao* (XP 007018417.1).

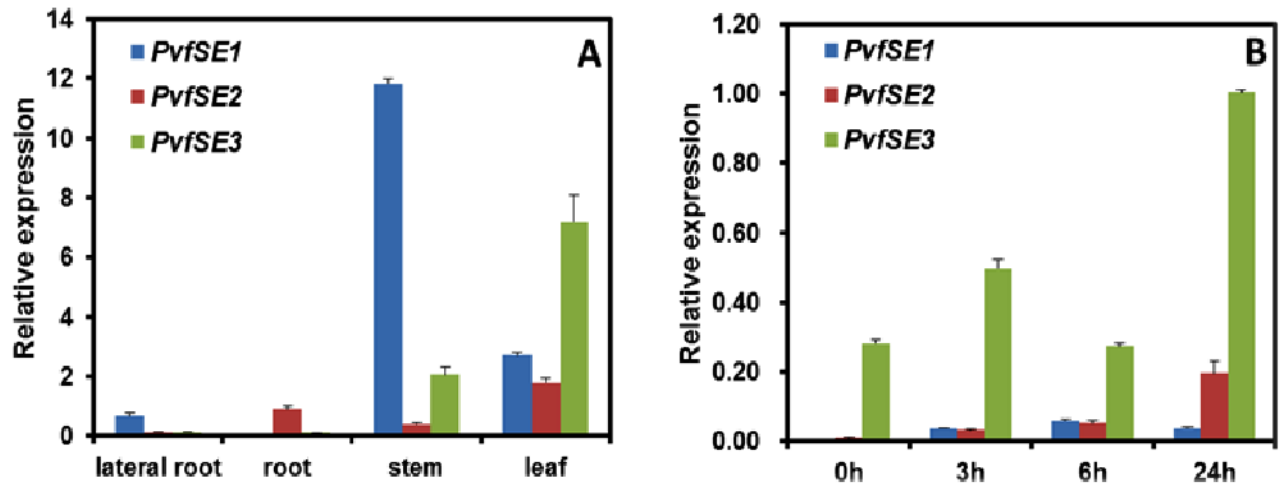

Fig. 7. Expression analysis of *PvfSE1, PvfSE2 and Pvf SE3* using RT-qPCR. (A) Relative expression levels of *PvfSE1, PvfSE2 and PvfSE3* in diffrent tissues of *P. vietnamensis* var. *fuscidiscus*. (B) Relative expression levels of *PvfSE1, PvfSE2 and PvfSE3* in *P. vietnamensis* var. *fuscidiscus* leaf tissues after 3, 6 and 24 hours of MeJA treatment.

Expression of *PvfSE1*, *PvfSE2* and *PvfSE3* in response to MeJA: Methyl jasmonate as an abiotic elicitor and key signaling molecule stimulates the biosynthesis of many secondary metabolites (Gundlach *et al*., 1992). Previous studies (Suzuki *et al*., 2002, Han *et al*., 2006, Bae *et al*., 2006) also show that MeJA strongly activates the biosynthesis of triterpene saponins in many species.The expression of the three *PvfSE* isoforms were detected under the induction of MeJA. Fig. 7B shows the stimulating effect of MeJA on the three *PvfSE* isoforms expression level in *P. vietnamensis* var. *fuscidiscus* leaves. The expression patterns of the three *PvfSE* isoforms were different in response to MeJA treatment. *PvfSE1* transcripts were only weakly induced by MeJA. *PvfSE2* expression was rapidly and strongly induced, reaching a peak (19.5-fold increase) after 24h MeJA treatment. The expression of *PvfSE3* was increased at 3h, and decrease at 6h, then increased reaching a high expression level at 24h (3.5-fold increase). In previous study (Han *et al*., 2010), MeJA treatment enhanced the expression of *PgSQE1* (GenBank accession No. AB122078) but not that of *PgSQE2* (GenBank accession No. FJ393274). It is also reported that *PgSQE1* plays a regulatory role in saponin biosynthesis, and *PgSQE2* gene positively regulates sterol production (Han *et al*., 2010; Niu *et al*., 2014). In the present study, *PvfSE3* was closely related to *PgSQE1* (Fig. 6), and we also found that the transcription patterns of *PvfSE3* in response to MeJA treatment was similar to that of *PgSQE1*. In contrast, despite *PvfSE2* share high sequence similarity with *PgSQE2* (Fig. 6), they exhibited different response patterns under MeJA induction, and suggested their regulation are different in different *Panax* plants. According to the above analysis, we suggested that *PvfSE3* may be mainly involved in ginsenoside biosynthesis in this species, while *PgSQE2* might be mainly involved in phytosterol biosynthesis in *P.vietnamensis* var. *fuscidiscus*. These observations above strongly suggest that *PvfSE1* may have some novel biological functions in the sterol or ginsenoside biosynthesis pathway in this herb. A related study is under way to investigate the function of the three *PvfSE* isoforms.

Conclusions

In this study, we have cloned and characterized a full-length cDNA of three *SE* gene from *P.vietnamensis* var. *fuscidiscus*, which will provide useful information on the genes involved in the biosynthesis of ginsenosides. Our results showed that the three *PvfSE* isoforms exhibited different tissue-specific expression patterns, which will be helpful to aid understanding of the mechanism of ginsenoside biosynthesis. *PvfSE* 2 and *PvfSE* 3 transcripts were strongly induced by MeJA, and this finding will facilitate us to uncover molecular induction mechanism for further improving ginsenosides biosynthesis in other *Panax* plants.

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