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Molecular characterization of lipoxygenase genes and their expression analysis against biotic and abiotic stresses in *Panax ginseng*

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Abstract Lipoxygenase (LOX) belongs to a family of non-heme-iron-containing fatty acid dioxygenases that are widely distributed in plants and animals. LOX involved in the synthesis of jasmonic acid and six-carbon (C6) volatiles which is necessary for plant growth and responses to a wide range of biotic and abiotic stresses. We have isolated and characterized LOX cDNA clones from *Panax ginseng* Meyer. From their deduced amino acid sequences, two diverse classes of 9-LOX (LOX1, LOX2, and LOX3) and 13-LOX (LOX4, LOX5) are defined in *P. ginseng*. A GenBank Blast X search revealed that the deduced amino acid of *PgLOXs* share a high degree of homology with LOX from other plants and mammals especially in three distinct motifs; motif1 harboring iron binding regions, motif2 and motif3. Chloroplast localization was predicted for PgLOX5. *PgLOXs* displayed organ-specific expression, highly expressed in aerial parts of the plant such as 3-year old flower, stem and leaf tissues. *PgLOXs* mRNAs were elevated strongly by bacterial infection. Expression of *PgLOXs* was differentially induced in ginseng not only by mechanical damage and methyl jasmonate but also after exposure to withholding water. Ginseng 13-LOXs positively respond to wounding that may involve in production of C6 volatiles and jasmonic acid at the wounded sites. However, the higher expression of *PgLOX3* by water deficit and 82 % of the nucleotide sequence identity with the EST from severe drought-stressed leaves of Populus (CU229089.1) at +6371 bp downstream of *PgLOX3* genomic DNA structure can suggest drought tolerance role for *PgLOX3*. Ginseng LOX genes have different expression pattern which may suggest different specific function against various environmental stresses.

Keywords Abiotic stress · Biotic stress · Gene expression · Jasmonic acid · Lipoxygenase · *Panax ginseng*

Abbreviations

AOC	allene oxide cyclase
AOS	allene oxide synthase
EST	expressed sequence tag
HPL	hydroperoxide lyase
13-HPOD	(13S)-hydroperoxyoctadecadienoic acid
9-HPOD	(9S)-hydroperoxyoctadecadienoic acid
IBA	indole-3-butyric acid

Kwi-Sik Bae and Shadi Rahimi contributed equally to this work.

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JA	jasmonic acid
LOX	lipoxygenase
MJ	methyl jasmonate
OPDA	(9S,13S)-12-oxo phytodienoic acid
Pst	Pseudomonas syringae pv tomato strain DC3000
PUFAs	polyunsaturated fatty acids
RT-PCR	reverse transcriptase-polymerase chain reaction.

Introduction

Panax ginseng Meyer, a perennial herbaceous plant from Araliaceae family, is one of the most commonly utilized medicinal plants. Due to long cultivation period of ginseng, various ecological stresses might affect the plant and this condition makes this highly valued medicinal plant more susceptible to environmental stresses. However, plants activate distinct defense responses which are mediated by a complex hormonal network to adapt with adverse conditions during their growth and development. Jasmonates belong to the oxygenated compounds known as oxylipins are essential signaling molecules in response to environmental changes as well as growth and development. Oxylipin biosynthesis is initiated by the function of LOX enzyme (EC1.13.11.12) which is ubiquitously occurring in plant and mammals and catalyzed hydroperoxidation of polyunsaturated fatty acids. Interestingly, LOX-derived oxylipins are involved in wound healing and defense processes in plants, while in mammals they are involved in inflammation, asthma and heart disease (Andreou and Feussner 2009).

Two different isomer products of 13-HPOD and 9-HPOD are synthesized by introduction of molecular oxygen either at carbon atom 9 or at carbon atom 13 of hydrocarbon backbone by the function of plant LOX which can justify their role as 13-LOX and 9-LOX, respectively (Wasternack 2007). LOX function can change from 13–to 9-LOX by a change in histidine residue of substrate binding site (Hornung et al. 1999). 9-LOXs are a subfamily of proteins which share high amino-acid sequence identity (~60 %) to one another, but 13-LOXs are sharing only ~35 % sequence identity among themselves (Andreou and Feussner 2009; Park et al. 2013). The discrete pathways are proposed for 13-LOX and 9-LOX which can lead to numerous types of oxylipins. 13-HPOD can be further metabolized into jasmonates in plants. 13-LOX and 9-LOX can be differentiated by their gene expression patterns and subcellular localizations (Shen et al. 2014). LOX form gene families ranging from six in Arabidopsis to at least 18 genes in potato which differently localized in microsomal membranes (Feussner and Kindl 1994), plasma membranes (Nellen et al. 1995), and lipid bodies (Feussner and Kindl 1992) of cucumber cotyledons, cytosol and vacuole of soybean leaves (Grayburn et al. 1991; Stephenson et al. 1998), envelope of leaf chloroplasts in spinach, barley, tomato, potato or Arabidopsis (Blée and Joyard 1996; Feussner et al. 1995; Heitz et al. 1997; Royo et al. 1996). Different LOXs may have different function in plants. There are different four major fates for the LOX-derived hydro (pero) xy PUFAs in plants (Feussner and Wasternack 2002). First is hydroperoxide isomerase pathway which converts fatty acid hydroperoxides to epoxy- or dihydrodiol polyenoic fatty acids, second is AOS pathway which forms unstable allene oxides further metabolized to OPDA by an AOC. Third is the HPL pathway oxidatively cleaves the hydrocarbon backbone of fatty acid hydroperoxides and forms short chain aldehydes (C6- or C9-). Fourth one is the DES pathway forms divinyl ethers such as colneleic acid or colnelenic acid. Ketodiene-forming pathway, epoxy alcohol synthase pathway and reductase pathway are other reactions for hydro (pero) xy PUFAs metabolisms which is less studied.

Since JA will be rapidly accumulated in response to wounding or herbivore attack to confer direct defenses at the site of attack and systemic defense signaling (Howe and Jander 2008), the characterization of LOX as the critical protein catalyzed the initial step of JA pathway will help us to understand the plant defense mechanism in response to ecological stresses. Despite of the importance of LOX, there is a dearth of genetic evidence for the function of this defensive gene in the ginseng plant. On the one hand, weeds compete with ginseng to absorb water and nutrients and on the other hand, due to the long cultivation period, ginseng is frequently exposed to bacterial infection and herbivores. In these stressful conditions, ginseng root growth will be affected and it can decrease the worth of this economically important plant.

In this report, we attempt to identify the five LOX genes in *P. ginseng* and understand the biological significance of these genes in the initial step of plant

interaction with pathogens, wounding and in drought condition. In addition, the expression of these LOX genes at different organs was examined. The present study examined the phylogenetic relationship of ginseng LOXs and that of other organisms, to contribute to the understanding of the possible role of ginseng LOXs.

Materials and methods

Identification of the LOX genes from the *P. ginseng* EST database

To identify genes from the EST library, we used the EST library constructed from *P. ginseng* (Sathiyamoorthy et al. 2009). Homologous sequences of LOX ESTs were searched against the GenBank databases using a BLASTX algorithm. We identified and selected the LOX genes based on open reading frames encoding the specific protein via the BlastX program (NCBI BlastX program). Clustal X with default gap penalties was used to perform multiple alignments of LOXs isolated from *P. ginseng* and previously registered in other species. A phylogenetic tree was constructed by the neighbor-joining method, and the reliability of each node was established by bootstrap methods using MEGA6 software. The protein properties were estimated using ProtParam (Gasteiger et al. 2005) and the hydropathy value was calculated by the method described by Kyte and Doolittle (1982). Identification of conserved motifs within LOXs was accomplished with MEME (Bailey et al. 2009). Chloroplast localization was inspected by ChloroP (Emanuelsson et al. 1999) and proposed signal peptide is predicted from PSORT (K. Nakai, Tokyo University, http://psort.ims.u-tokyo.ac.jp/). Other database also used to analyze the *PgLOX* genes, such as MotifScan (http://myhits.isbsib.ch/cgi-bin/motif_scan), HMMTOP (http://www.enzim.hu/hmmtop), SOPMA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html).

Plant materials and growth conditions

P. ginseng adventitious roots were collected from Ginseng Bank, Kyung Hee University and grown for 1 month in liquid Murashige & Skoog medium (Murashige and Skoog 1962) supplemented with 2 mg L^{-1} IBA at 25 °C. The roots were maintained by regular subculture every 4 weeks. Different organs of 3-year old healthy ginseng plants (leaf, stem, flower bud, and root) were collected from Ginseng Bank, Korea.

Stress treatment

To investigate the response of the *PgLOX* genes to MJ elicitor, 100 μM MJ, was applied to 30-day-old subcultured adventitious roots, and harvested at 6, 12, 24, 48, 72 h after treatment. The *P. ginseng* seeds used in the present study were obtained from the Korean Ginseng Resource Bank, South Korea. The 4- week-old ginseng seedlings germinated in in vitro condition on solidified MS media (Duchefa biochem, Netherland) with 3 % sucrose, supplemented with gibberellic acid (10 mg L^{-1}) at 25 °C under a 16 h photoperiod and used for the treatment studies. To induce drought stress, plants were exposed to dehydration by withholding water. Ginseng plants were inoculated with the virulent Pseudomonas syringae pv tomato (Pst) strain DC3000. The bacteria were grown at 28 °C in King's B medium containing rifampicin (50 mg L^{-1}). To study bacterial infection, bacterial suspensions (5×10^6 cfu mL^{-1}) were infiltrated into the ginseng leaves using a syringe. At appropriate time points, three independent leaves infiltrated with Pst were harvested. For wounding experiments, leaves were pricked with a needle (Hwang and Hwang 2010). For stress treatments, the plantlets were subjected to treatment for 6, 12, 24, 48 and 72 h. Control plants were grown at 25 °C under a 16 h photoperiod.

RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted from adventitious roots of *P. ginseng* using RNeasy mini kit (Qiagen, Valencia, CA, USA). For RT-PCR, 200 ng of total RNA was used as a template for reverse transcription using oligo (dT) $_{15}$ primer (0.2 mM) and AMV Reverse Transcriptase (10 U μL^{-1}) (INTRON Biotechnology, Inc., South Korea) according to the manufacturer's instructions. Real-time quantitative PCR was performed using 100 ng of cDNA in a 10-μL reaction volume using SYBR® Green Sensimix Plus Master Mix (Quantace, Watford, England). Gene-specific primers listed in Table 1, were used to perform quantitative RT-PCR. The thermal cycler conditions recommended by the manufacturer were used as follows: 10 min at 95 °C, followed 40 cycles of 95 °C for 10 s, 58 °C for 10 s, and 72 °C 20 s. The fluorescent product was detected at the last step of each cycle. Relative quantity of the *PgLOX* genes

Table 1 List of gene-specific primer sequences used for qRT-PCR analysis

Gene	Primers (5'–3')
PgLOX1	F: ATCAAGAGAGGGATGGCAGTTGA R: ATGGCCCTTTTCTCGGACTTCT
PgLOX2	F: TACTGATCGAGGACTACCCATATGCA R: CATTTTAGGCCATGCTTCAT
PgLOX3	F: ATGTTGCCATTTCTGCTCAAGTTC R: CAGATATCCAACCACGACAAGCA
PgLOX4	F: CAAGTCATTCTTACCATCTCAAAC R: CCTTTAGTGTGAACGATGTCTCCT
PgLOX5	F: TATGTGCCTAATCGTCCCACTCTCAT R: CCTGAACCAAGTACTCTTCATCTGG
Pgβ-actin	F: GTGATCTTACAGATAGCTTGATGA R: AGAGAAGCTAAGATTGATCCTCC

transcription level was performed using Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Sydney, Australia), and calculated using the comparative cycle threshold (CT) method according to the manufacturers' instructions for normalizing data. A constitutively expressed β-actin gene was used as internal reference. Three independent experiments were performed. The primer efficiencies were determined according to the method by Livak and Schmittgen (2001) to validate the ΔΔCt method. The observed slopes were close to zero, indicating that the efficiencies of the gene and the internal control β -actin were equal.

Results

Isolation and characterization of *PgLOX* genes

As the part of a genomic project to identify genes of the medicinal plant *P. ginseng*, a cDNA library consisting about 20,000 cDNAs were previously constructed. Five LOXs, designated *PgLOX1, PgLOX2, PgLOX3, PgLOX4,* and *PgLOX5* were isolated. Moreover, full genomic DNA sequence of *PgLOXs* was analyzed using the genomic DNA sequence retrieved from ginseng genome database (http://im-crop.snu.ac.kr/new/index.php). The corresponding characteristics of each PgLOXs are indicated in Table 2. As it was shown in Fig. 1a GenBank Blast search (http://www.ncbi.nlm.nih.gov/BLAST/) resulting in highly conserved N-terminus domain of PLAT/LH2 which is found in a variety of membrane or lipid associated proteins to possibly mediate interaction with lipids or membrane bound proteins and the C-terminus domain of LOX superfamily possessed by all the previously cloned plant and indicated that *PgLOX1, PgLOX2, PgLOX3, PgLOX4,* and *PgLOX5* belonged to the LOX family. Red underlined sequence showed the proposed signal peptide in Fig. 1 predicted by IPSORT program, showing that PgLOX5 had a transit peptide at its N-terminal which may suggest chloroplast localization. Multiple sequence alignment revealed that the N-terminal sequences differ among the LOXs in different plants, including five PgLOXs (Fig. 1). Plant LOX enzymes contain iron atom in their active site which coordinated by 5 five amino acids including three histidines, one asparagine and the carboxy group of the carboxy-terminal isoleucine. Two iron-binding regions were shown in green boxes in motif1. In addition, three conserved motifs were found by MEME analysis in all plant LOX isozymes and mammals (Fig. 2b) Three conserved motif sequences predicted by MEME are shown in Online Resource 1.

It was revealed that the deduced amino acid of *PgLOX1, PgLOX2 and PgLOX3* share a high degree of sequence homology with 9-LOX proteins encoded by *Actinidia deliciosa* (ABF60002.1) (identity 74 % & similarity 99 %), *Camellia sinensis* (ABW75772.2) (identity 79 % & similarity 100 %), *Capsicum annuum* (ABF19103.2) (identity 78 % & similarity 98 %), respectively. However, *PgLOX4* and *PgLOX5* share a high degree of sequence homology with chloroplastic 13-LOX proteins encoded by *Camellia sinensis* (ADO51752.1) (identity 66 % &similarity 100 %) and *Theobroma cacao* (XP_007045017.1) (identity 78 % & similarity 90 %), respectively. Clustal X and the MEGA6 Program were used for the construction of phylogenetic tree based on LOX amino acid sequences (Fig. 2). Moreover, the phylogenetic relationship is shown among LOXs from *P. ginseng* and *Arabidopsis thaliana* (Online Resource 2). Transmembrane helices of integral membrane proteins and localization of the N-terminus were predicted by HMMTOP and mentioned in Table 2. The hydrophobicity profile of the estimated 9- and 13-LOX proteins is shown in Fig. 3a, b. N-terminal peptides differ from each other but more hydrophilic amino acid composition similar to other LOXs were found in PgLOXs.

Secondary structure analysis and molecular modeling for PgLOXs were conducted by SOPMA (Geourjon and Deleage 1995) (Table 3). The secondary structure

Table 2 Characteristics of ginseng LOXs

Protein	Length[a]	Molecular mass[a]	pI[a]	Predicted localization[b]	Transmembrane helices[c]	localization of N terminus[c]
PgLOX1	815	94	5.4	–	–	Out
PgLOX2	860	97.7	5.8	–	–	Out
PgLOX3	868	99.3	6.5	–	–	Out
PgLOX4	762	86.6	5.2	–	–	Out
PgLOX5	908	103.4	8.2	Chloroplast	–	Out

[a] Length (number of amino acid residues), molecular mass (kDa), and isoelectric point (pI) of PgLOX proteins deduced from the open reading frames (Table 2). Values for predicted mature proteins are indicated in brackets

[b] Transit peptides of PgLOX proteins were predicted by the IPSORT

[c] Transmembrane helices of integral membrane proteins and localization of the N-terminus were predicted by HMMTOP

analysis revealed that PgLOX1 consists of 312 α-helices, 74 β-turns joined by 128 extended strands, and 301 randomcoils; PgLOX4 consists of 268 α-helices, 73 β-turns joined by 143 extended strands, and 278 randomcoils. PgLOX5 consists of 335 α-helices, 77 β-turns joined by 144 extended strands, and 352 randomcoils which is highly similar to the secondary structure of At13-LOX6 from *A. thaliana*, includes 346 α-helices, 62 β-turns joined by 140 extended strands, and 369 random coils; and At9-LOX from *A. thaliana*, made up of 335 α-helices, 70 β-turns joined by 149 extended strands, and 332 randomcoils. Similar secondary structure were found in PgLOX2 and PgLOX3 which consists of 297 α-helices, 90 β-turns joined by 146 extended strands, and 327 randomcoils and 294 α-helices, 95 β-turns joined by 141 extended strands, and 338 randomcoils, respectively.

Distribution of LOX genes transcript in different organs

To determine the expression of *PgLOX* genes at different various organs of ginseng, we performed quantitative RT-PCR expression analysis of *PgLOX* genes in 3-year-old leaf, stem, flower, and root samples (Fig. 4). The results indicate that these LOX enzymes are tissue-regulated showing *PgLOX1, PgLOX4,* and *PgLOX5* transcripts were highly detected in flower bud whereas relatively higher levels of *PgLOX2* and *PgLOX3* transcripts were observed in leaf and stem, respectively. Three-year-old root tissue contain lowest level of *PgLOX1, PgLOX4* and *PgLOX5* transcripts when *PgLOX3* transcriptome level appears to be same as that in leaf root tissue.

Expression of PgLOXs in response to MJ elicitor, wounding and bacterial infection

To determine the expression of the *PgLOX* genes in response to MJ, we performed quantitative RT-PCR analysis of *LOXs* in ginseng adventitious roots treated with 100 μM MJ (Fig. 5). MJ elicitor upregulated *PgLOX5* 6 h after treatment however, *PgLOX4* and *PgLOX3* were highly responsive 12 h and 72 h after MJ treatment. *PgLOX1, PgLOX2 and PgLOX3* expression were not responsive to mechanical damage. *PgLOX4* was highly upregulated 72 h after wounding treatment however, *PgLOX5* was showed higher transcript level at 48 h and then it was declined after 72 h.

To recognize pathogen can induce the transcriptome profile of *LOXs*, we infected 30-day old ginseng seedlings with *Pseudomonas syringe* pv tomato strain DC3000 suspension and checked the transcription pattern of these genes using quantitative RT-PCR analysis. The expression level of all *PgLOXs* showed similar pattern with maximum transcription at 48 h after bacterial infection followed by decreased expression 72 h after bacterial treatment. The mRNA level of *PgLOX2* was induced by pathogen attack however it was lower than other *PgLOXs*.

Expression of *PgLOXs* in response to drought stress

To investigate the possible role of LOXs in response to drought stress, plants were exposed to dehydration by withholding water. The expression patterns of *PgLOXs* at different time points after treatments were analyzed using real-time PCR. Among all *PgLOXs,* only *PgLOX3*

was responsive to water shortage. The expression profile of *PgLOX3* highly increased at 24 h of post treatment with drought and after that declined at 48 h and 72 h after treatment compared to the control (Fig. 5). The full genomic DNA sequence of *PgLOX3* has a length of 2,604 bp encoding 906 amino acids. This gene contains nine exons interrupted by the eight introns (Online Resource 3A). By carefully checking the genomic DNA structure of *PgLOX3,* a nucleotide sequence with 438 bp size was found at +6,371−+6,808 bp downstream of *PgLOX3* genomic DNA structure that shares the highest nucleotide sequence identity (82 %) with the EST from severe drought-stressed leaves of Populus (CU229089.1). A GenBank Blast X search revealed that the deduced amino acid of this sequence share a high degree of homology with Photosystem I reaction center subunit D-2 (PsaD2) from other plants (Online Resource 3B) which its Arabidopsis homolog (NP_171812.1) is highly expressed 24 h after drought treatment.

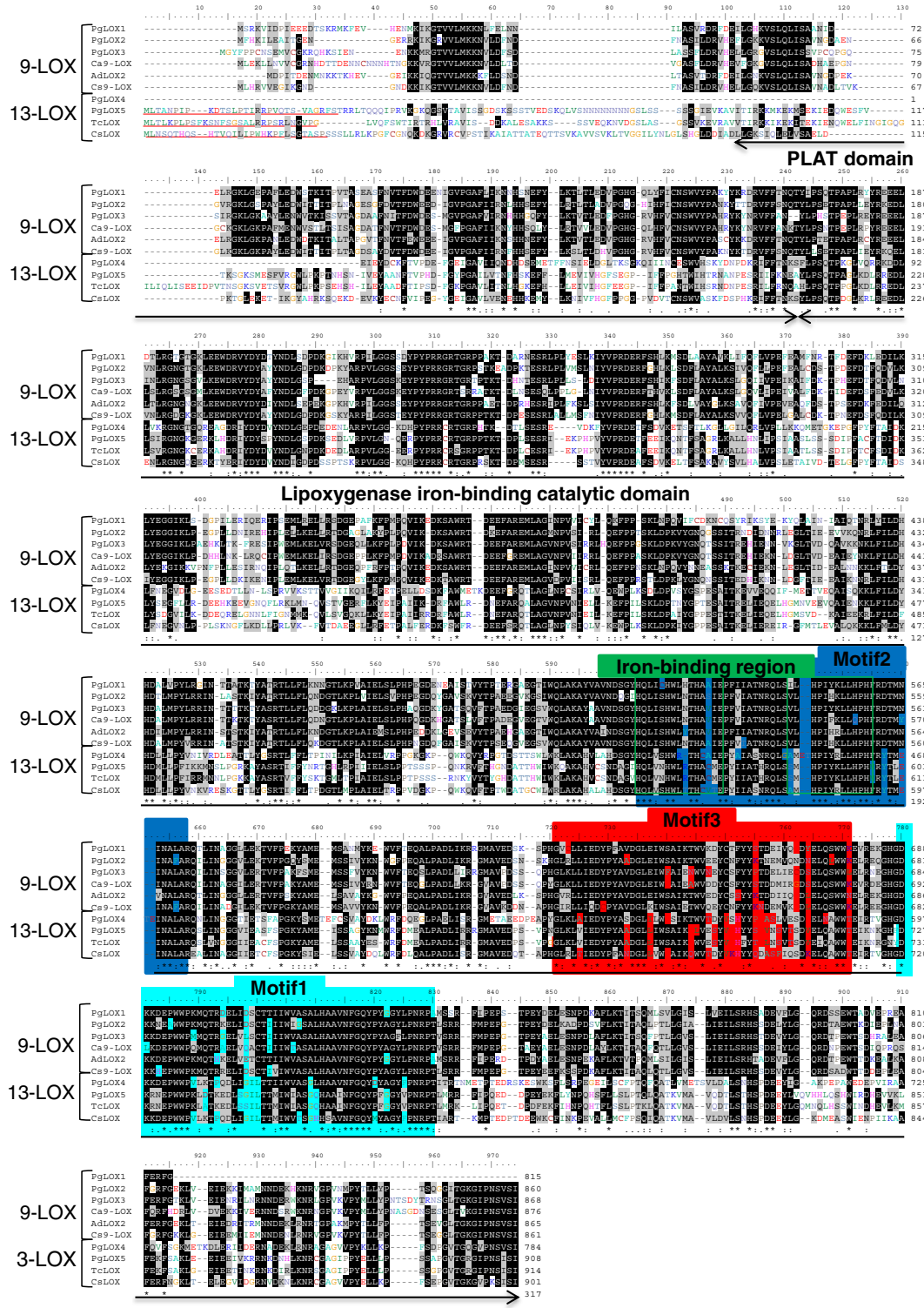
Fig. 1 Multiple amino acid sequence alignment of *PgLOXs* with ▶ those of proteins encoded by *Actinidia deliciosa* (ABF60002.1), *Camellia sinensis* (ABW75772.2), *Capsicum annuum* (ABF19103.2), *Camellia sinensis* (ADO51752.1) and *Theobroma cacao* (XP_007045017.1). A hyphen was inserted within the amino acid sequence to denote a gap. Shadow box indicates well conserved residues, * represents a conserved amino acid, and: represents a very similar amino acid. Red underlined sequences are the proposed signal peptide predicted by IPSORT program. The arrow indicates the conserved domains. Three conserved motifs obtained by MEME analysis. Two iron-binding regions are shown in green boxes

Discussion

In our experimental study, for the first time we have isolated and characterized the LOX genes in *P. ginseng*. These sequences were named *PgLOX* due to their high homology with the LOX sequences of other plants. It was revealed that the deduced amino acid of *PgLOX1, PgLOX2 and PgLOX3* shares a high degree of sequence homology with 9-LOX proteins however, *that of PgLOX4 and PgLOX5* shares a high degree of sequence homology with chloroplastic 13-LOX proteins. Phylogenic tree analysis of LOXs from different species suggested that the ginseng LOXs are classified into two groups of 9-LOX and 13-LOX with respect to their positional specificity of fatty acid oxygenation. Plant *LOX* genes encoded around 94–100 kDa protein sequences which contain two different domains. PLAT domain around 25–30 kDa at the N-terminus consists of b-barrel domain (Corbin et al. 2007) and possibly mediates interaction with lipids or membrane bound proteins. There are around 55–65 kDa domain at the C-terminus which conatins a-helices and includes catalytic site of enzyme (Andreou, and Feussner 2009). LOXs belong to a family of non-heme-iron-containing fatty acid dioxygenases that are widely distributed in plants and animals. Two iron binding regions were identified in PgLOX enzymes. PgLOX5 contain transit peptide at its N-terminal which directed the enzyme to the chloroplast. However, there was no homology among the N-terminals of the LOX. Surprisingly, N-terminal chloroplast transit peptide was not identified in 13-LOX ginseng LOX4. However, there are some examples for occurrence of enzymes within or at the plastid without transit peptide. Despite the lack of a putative chloroplast signal peptide in AOS sequences of barely, immunocytochemical analysis showed their chloroplast localization (Maucher et al. 2000). Those chloroplast proteins without transit peptide are located in outer envelope membrane of chloroplast which imported through the transit peptide-independent path (Keegstra and Cline 1999).

The temporal expression analysis from different organs demonstrated that *PgLOX* mRNAs were ubiquitously expressed in all organs that we examined though an expression level of *LOXs* is low in root tissue. *LOXs* were strongly expressed in aerial parts of the plant, especially in 3-year old flower, stem and leaf tissues. Since, JA is involved in regulation of pollen maturation, anther dehiscence, and flower opening (Scott et Al. 2004); we can suggest that higher expression of LOXs in ginseng flower will provide evidence for their possible role in producing JA during flower development.

Ginseng plants have evolved effective mechanisms to deal with the ever-changing environment during their growth and development. Understanding these mechanisms is helpful to improve stress tolerance in ginseng plant. In order to determine the molecular mechanisms of ginseng plant adapted to biotic and abiotic challenges over long years of life cycle, transcript profiling of *PgLOXs* in response to wounding, MJ supplementation, pathogen attack and drought was studied by quantitative real time-PCR analysis. *LOX* expression in plants is regulated in response to stresses. In our current study, ginseng *LOXs* transcript level exhibited different results. *LOX1, LOX2 and LOX3* classified as the 9-LOXs, were



not responsive to wounding which is in consistent with the previous report by Yang et al. (2012) showed the downregulation of cucumber LOX in response to wounding. Meanwhile, *LOX4* and *LOX5* which belong

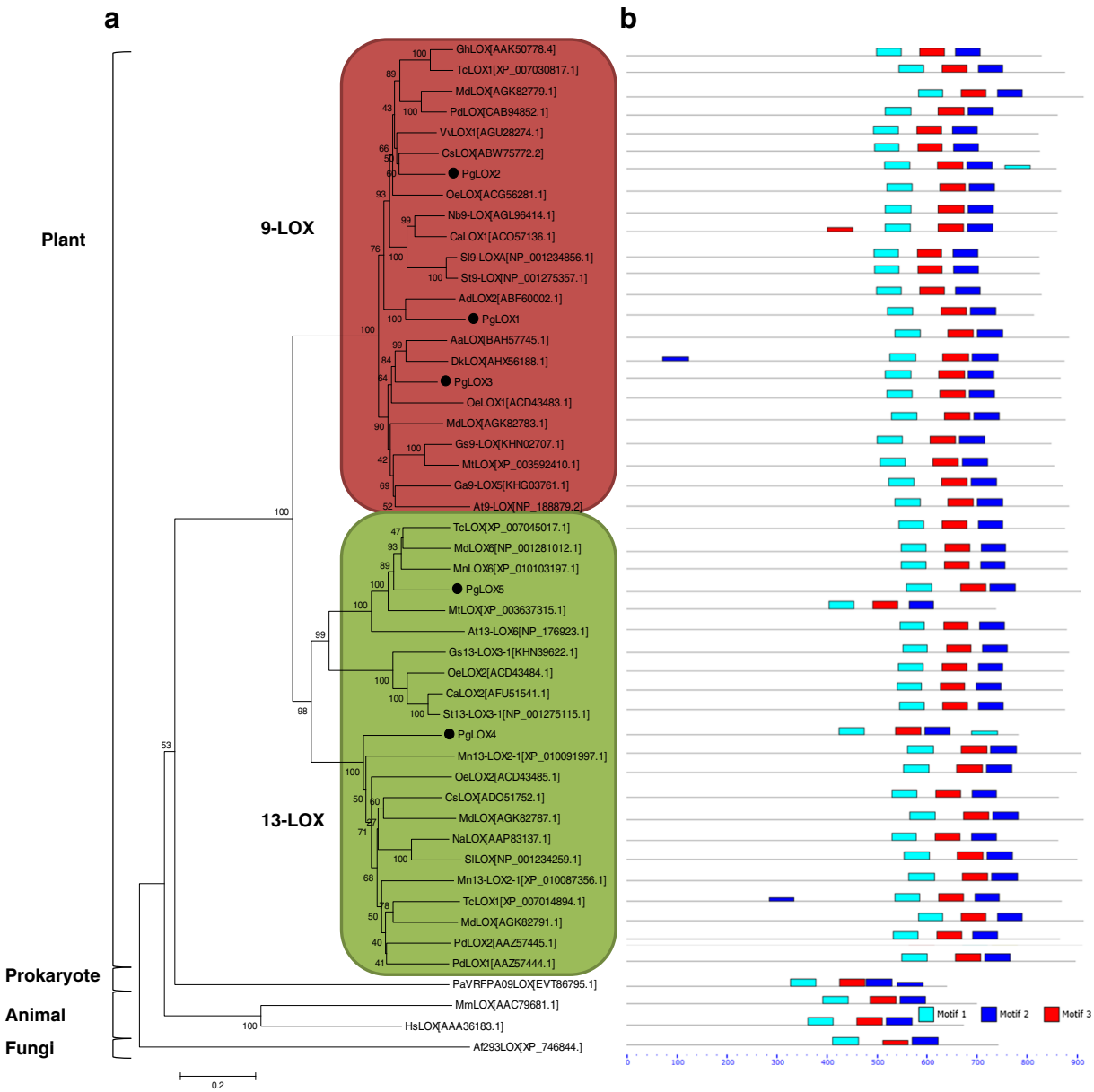


Fig. 2 Sequence homology analysis of PgLOXs with other LOX genes. **a** Phylogenetic relationships in LOX from various organisms including plant species. Database accession numbers are indicated in brackets. Distances between each clone and group are calculated with Clustal X program. 9-LOX and 13-LOX are shown in red and green boxes, respectively. **b** Organization of putative motifs in LOXs identified by MEME. Numbered color boxes represent different putative motifs Motifs 1, 2, and 3 are indicated by the mint (First), blue (middle), and red (last) boxes, respectively. Motif sizes are indicated at the bottom of the figure

to 13-LOXs, were highly induced in mechanically damaged ginseng seedlings and it can be correlated to their specific function in production of related oxylipins which can help to relieve the tension. Three potential fates for LOX-derived hydroperoxides were identified by Shen et al. (2014). One of them is JA pathway and its first step catalyzed by allene oxide synthase. The other one is biosynthesized C6 volatiles which initiated by oxidative cleavage of hydroperoxides through the action of HPL. The third pathway is the C5 compounds synthesis and in that LOX oxygenate hydroperoxides.

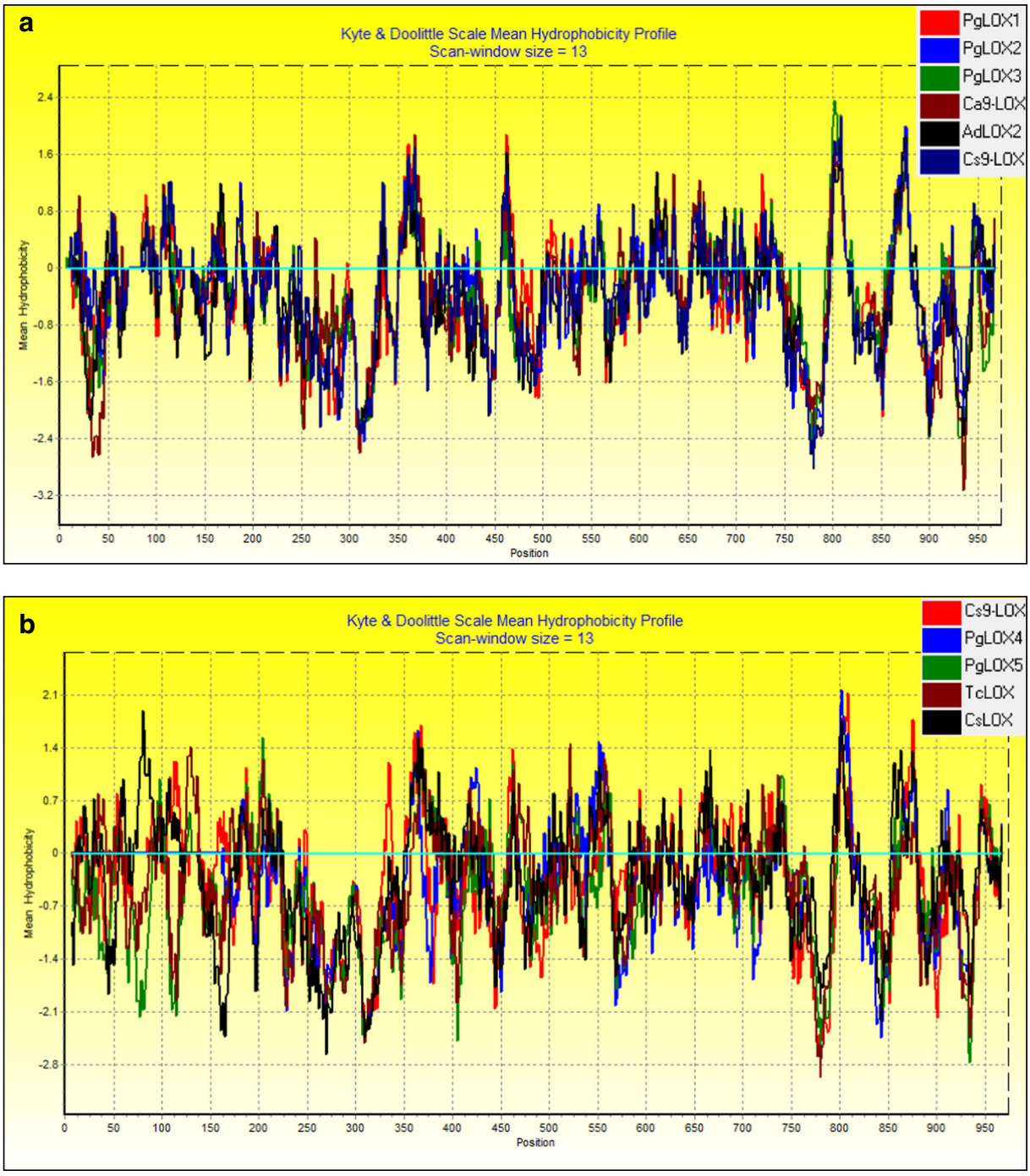


Fig. 3 Superimposed hydrophilicity profiles for 9- and 13-PgLOXs and homologous plant LOXs. Hydrophobic domains are indicated by positive numbers; hydrophilic domains are above the line and hydrophilic domains are below the line

LOX-derived C6 volatiles accumulated in wounded plants and acted as the signal molecule to induce defense and repair mechanisms on the damaged site (Bate and Rothstein 1998). Also, JA is an essential signaling oxylipin produced highly in wounded plants. There are six LOX genes in tomato (*Solanum lycopersicum*).

Table 3 Secondary structure predictions for PgLOXs and homologous plant LOXs

Protein	α-Helix	β-Turn	Extended strand	Random coil
PgLOX1	312	74	128	301
PgLOX2	297	90	146	327
PgLOX3	264	95	141	338
At9-LOX	335	70	149	332
PgLOX4	268	73	143	278
PgLOX5	335	77	144	352
At13-LOX6	346	62	140	369

TomloxA, *TomloxB*, and *TomloxE* encoded 9-LOXs and their functions are not related to C6 biosynthesis (Griffiths et al. 1999; Chen et al. 2004). However, other three chloroplast-localized LOXs of *TomloxC, TomloxD and TomloxF* involved in generation of JA and C6 volatiles (Chen et al. 2004).

Among all *PgLOXs,* only *PgLOX3, PgLOX4* and *PgLOX5* transcriptions were affected by 100 μM MJ in the adventitious roots of *P. ginseng* as suggesting that those *LOXs* were elicitor-responsive. In most plants, wound-responsive LOXs are induced by JA (Porta et al. 1999) and *PgLOX5* which was early responsive to mechanical damage, up-regulated earlier than *PgLOX4* by MJ. Endogenous JA biosynthesis can be stimulated by several elicitors and it is thought to be a transduction molecule for production of secondary metabolites (Gundlach et al. 1992). Several studies have reported that exogenously applied jasmonates could induce endogenous JA biosynthesis (Zhao et al. 2005). Our previous study (Rahimi et al. 2014) provided evidence that higher expression of LOX in ginseng roots preceded JA accumulation when elicited. These reports can provide support for our study that the higher induction of *PgLOX* genes can affect endogenous JA level and it can consequently change the secondary metabolite content by regulating expression level of secondary metabolite biosynthetic genes (Reviewed by Rahimi et al. 2015a; Rahimi et al. 2015b; Devi Balusamy et al. 2015) .

C6 volatiles and JA are the essential molecules for plant response to biotic stress, as it was already shown LOX derived (E)-2-hexenal released from infected leaves with bacteria strongly inhibit *Pseudomonas syringae* growth (Croft et al. 1993) and the aerial treatment of Arabidopsis with that induced expression of defensive genes (Bate and Rothstein 1998). Higher mRNA levels of *PgLOXs* during bacterial infection can suggest their role in response to biotic stress. LOX pathway can produce hydroperoxy, hydroxy and keto fatty acids upon infection by pathogen especially during the hypersensitive reaction via LOX action. It was suggested that these LOX pathway products can regulate pathogenesis-related and oxidative-stress-related genes as well as of cell death (Weber 2002). LOX-derived α-DOX pathway was induced by *Pseudomonas syringae* infection in Arabidopsis and α-DOX reaction products can be suggested to protect plant tissues from oxidative stress and cell death (De León et al. 2002).

Drought is a severe environmental stress which can cause significant reduction of crops yield. The effect of wounding on expression of LOX genes are well studied whereas less information related the response of LOX genes to water deficit is available. Expression analysis of *PgLOX3* in ginseng seedlings subjected to withholding water demonstrated that early stimulation of *PgLOX3* expression could correspond to an

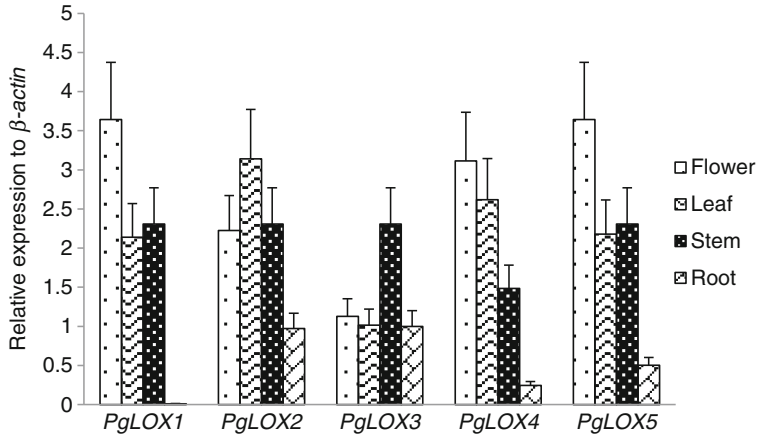


Fig. 4 Expression of *PgLOX* genes in specific organs of 3-year old ginseng. The error bars represent the standard error of the means of three independent replicates

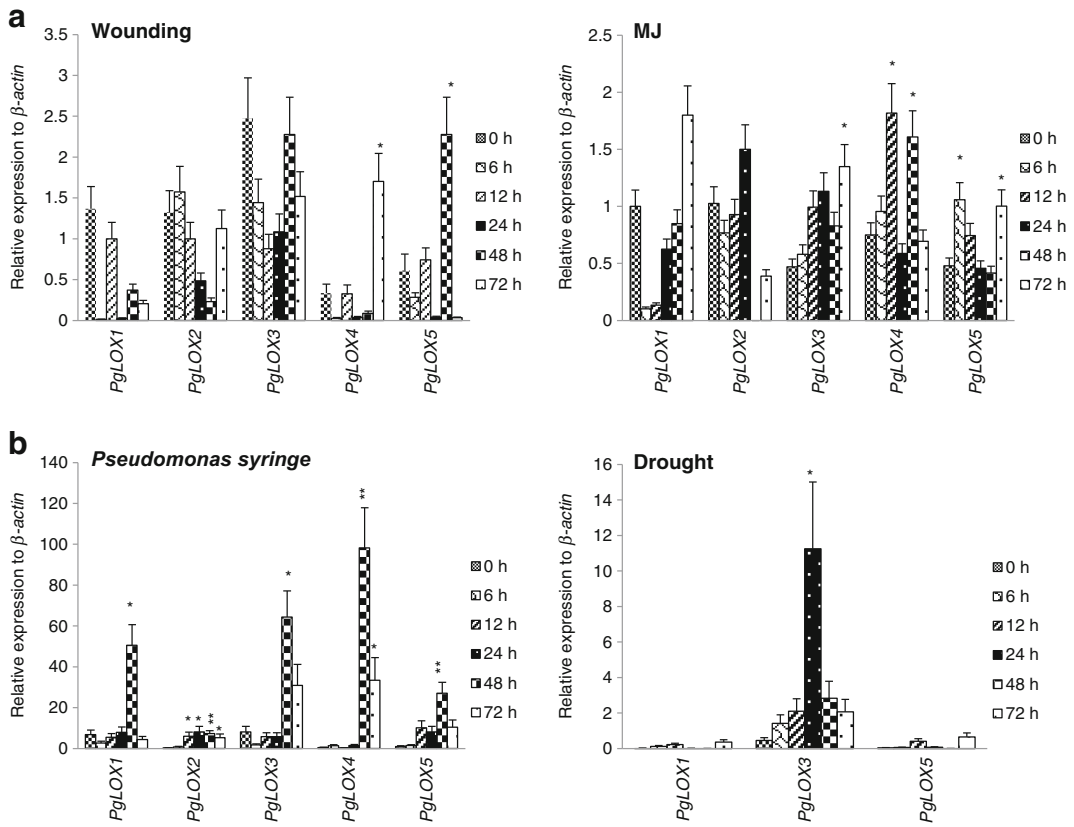


Fig. 5 **a** Inductive effect of MJ (100 μM) and wounding on expression of *PgLOX* genes in ginseng adventitious roots and 4-week old ginseng seedlings. **b** Effect of dehydration and infection with *Pseudomonas syringe* pv tomato strain DC3000 suspension on mRNA level of *PgLOX*. 30-day old ginseng adventitious roots were subject to MJ treatments. Four-week old ginseng seedlings were subject to these treatments. *β-actin* is an internal reference gene. The error bars represent the standard error of the means of three independent replicates and it was statistically analyzed and compared with control (*$P < 0.05$; **$P < 0.01$) using Student's *t* test

adaptive response to drought-stress. This can be correlated with the effect of water deficit on modification of membrane lipids and the immediate induction of LOX activity (Maccarrone et al. 1995). As it was already reported that C6 volatile virgin olive oil content and composition was affected by water deficit (Gómez-Rico et al. 2006; Servili et al. 2007; Stefanoudaki et al. 2009; Dabbou et al. 2011), it may also suggest that higher mRNA level of *LOX3* in drought treated seedlings affect the amount of those related oxylipins. The genomic DNA structure of *PgLOX3* revealed a nucleotide sequence with high identity with EST of severe drought-treated Populus encoded the *PgPsad2* protein which is localized at downstream of *PgLOX3* and this result can suggest that *PgLOX3* may involve in drought stress tolerance.

In conclusion, we can suggest a crucial role for *PgLOXs* in protection of ginseng plant under environmental

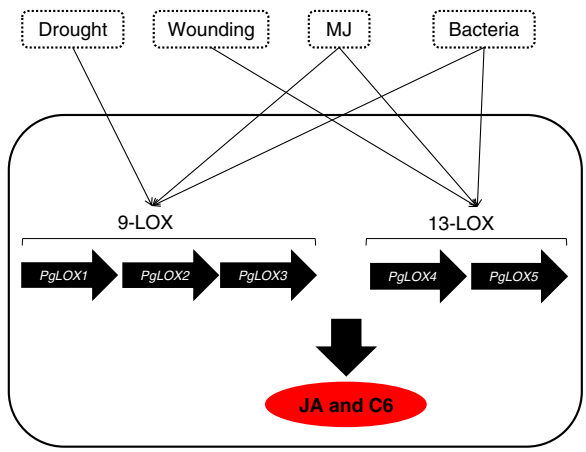


Fig. 6 Presumptive model for activation of *PgLOX* genes in response to drought, wounding, methyl jasmonate treatment and bacterial infection. Abbrevations: JA, jasmonic acid; LOX, lipoxygenase; MJ, methyl jasmonate

stresses. However, our results showed that ginseng *LOX* genes have different expression pattern which may suggest their different specific function against various environmental stresses (Fig. 6), hence further experiments on analysis of C6 volatiles and JA production in overexpressed and/or knockout lines of *PgLOXs* can answer the questions related to their function in response to wounding, MJ, bacterial infection and drought stresses. Less information related to the molecular characterization of LOX isoforms in ginseng is available hence; this study might provide support for more analysis of LOXs in ginseng. Further studies on *PgLOX* genes are needed for specific localization within the cell and substrate preferences to clarify the physiological functions of each LOX isozymes in ginseng.

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Compliance with ethical standards

Conflict of interest None.

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