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Research article

Leaf-specific pathogenesis-related 10 homolog, PgPR-10.3, shows *in silico* binding affinity with several biologically important molecules

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ABSTRACT

Background: Pathogenesis-related 10 (PR-10) proteins are small, cytosolic proteins with a similar three-dimensional structure. Crystal structures for several PR-10 homologs have similar overall folding patterns, with an unusually large internal cavity that is a binding site for biologically important molecules. Although structural information on PR-10 proteins is substantial, understanding of their biological function remains limited. Here, we showed that one of the PgPR-10 homologs, PgPR-10.3, shares binding properties with flavonoids, kinetin, emodin, deoxycholic acid, and ginsenoside Re (1 of the steroid glycosides).
Methods: Gene expression patterns of *PgPR-10.3* were analyzed by quantitative real-time PCR. The three-dimensional structure of PgPR-10 proteins was visualized by homology modeling, and docking to retrieve biologically active molecules was performed using AutoDock4 program.
Results: Transcript levels of *PgPR-10.3* expressed in leaves, stems, and roots of 3-wk-old ginseng plantlets were on average 86-fold lower than those of *PgPR-10.2.* In mature 2-yr-old ginseng plants, the mRNA of *PgPR-10.3* is restricted to leaves. Ginsenoside Re production is especially prominent in leaves of *Panax ginseng* Meyer, and the binding property of PgPR-10.3 with ginsenoside Re suggests that this protein has an important role in the control of secondary metabolism.
Conclusion: Although ginseng *PR-10.3* gene is expressed in all organs of 3-wk-old plantlets, its expression is restricted to leaves in mature 2-yr-old ginseng plants. The putative binding property of PgPR-10.3 with Re is intriguing. Further verification of binding affinity with other biologically important molecules in the large hydrophobic cavity of PgPR-10.3 may provide an insight into the biological features of PR-10 proteins.
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1. Introduction

Pathogenesis-related 10 (PR-10) proteins are ubiquitously distributed among plants, including monocots and gymnosperms [1–4]. Several PR-10 homologs exist within various species, and their crystal and solution structures have been reported previously [5–12]. PR-10 proteins share the same general folding pattern consisting of three α-helices and a seven-stranded antiparallel β-sheet [13]. These two elements, together with two short helices, form a hydrophobic cavity whose volume is unusually large for this protein size. Owing to its unusually large volume, the cavity in the structure of PR-10 proteins is assumed to have evolved to accommodate hydrophobic ligand binding [14,15]. PR-10 proteins are regulated by plant hormones, biotic stresses such as fungal and bacterial infections, and abiotic stresses such as salt and cold. However, biological functions of the proteins are not well understood. In *Panax ginseng* Meyer, three homologs of PgPR-10 proteins have been isolated, and one of them, PgPR-10.3, shows the highest sequence identity with the major celery allergen Api g 1 (PDB code: 2BKO) whose structure is well defined. Screening of possible binding molecules can provide clues as to how PR-10 proteins react against various biotic and abiotic stresses. There is supporting

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evidence for such a ligand-binding role, as shown by the following: (1) the structural similarity between PR-10 proteins and the steroidogenic acute regulatory protein-related lipid transfer domain of human MLN64 protein, which is a steroid-binding domain related to steroidogenic acute regulatory proteins involved in cholesterol translocation in human placenta and brain [16]; (2) the structural similarity to cytokinin-specific binding protein [12]; and (3) the crystal structure of Bet v 1 (a birch-pollen PR-10 protein) in complex with deoxycholate [9]. Deoxycholate is not a plant metabolite and therefore cannot be a natural PR-10 ligand, but it is noteworthy that it is structurally similar to brassinosteroids (BRs), which are plant steroid hormones. These observations prompted us to investigate whether ginseng PgPR-10.3 possibly interacts with sets of biologically important ligands. Full genomic DNA sequences were obtained using cDNA sequence as a template, and organ-specific expression patterns of *PgPR-10.3* in ginseng plants of different ages were examined. In this study, we addressed the biological significance of ginseng PR-10 based on tissue-specific expression and *in silico* ligand-binding affinity.

2. Materials and methods

2.1. Plant materials and growth conditions

Korean ginseng (*P. ginseng* Meyer) cv. "Chun-Poong" seeds were kindly provided by the National Institute of Horticultural and Herbal Science of the Rural Development Administration in Eumsung, Korea. Different organs of ginseng (stem, leaf, root, petiole, and rhizome) were harvested from 3-wk-old plantlets and 2-yr-old ginseng plants grown in soil at 25°C under a 16-h photoperiod.

2.2. Abiotic stresses and hormone treatment

Three-week-old ginseng plantlets were used for studying various abiotic stimuli and two defense-modulating plant hormones. The plantlets were placed on petri dishes by dipping the root into 60 mL of a solution containing 5mM salicylic acid (SA), 0.2mM jasmonic acid (JA), 100μM abscisic acid (ABA), 10mM H_2O_2, and 100mM NaCl. Methanol for JA and distilled water for SA, ABA, NaCl, and H_2O_2 were used as solvents. Cold stress was applied by placing the roots in tap water at 4°C. Treated plantlet samples were gathered at intervals of 1 h, 4 h, 8 h, 12 h, 24 h, and 48 h after treatment. The sampled plant materials were immediately frozen in liquid nitrogen and stored at −70°C.

2.3. Quantitative real-time PCR

Total RNA was extracted from frozen ginseng samples using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions with modification. Genomic DNA contamination was eliminated by treatment with the DNase I (Takara, Kyoto, Japan) for 1 h in 100 μL of reaction volume immediately before the washing step. Concentration of RNA was measured using a NanoDrop 2000 UV−Vis spectrophotometer (Thermo Scientific, Wilmington, DE, USA). To obtain the first strand of cDNA, 5 μg of total RNA was reverse transcribed with RevertAid Reverse Transcriptase (Thermo Scientific). Quantitative real-time PCR was carried out using the Thermal Cycle Dice real-time PCR system (Takara) in a 25 μL reaction volume using 12.5 μL of SYBR Premix Ex Taq (Takara) with the following gene-specific primers: 5′-CTG TTC AAG GGT TCT TTC-3′ (forward) and 5′-GGT GGT CTG TGT GAT GGT-3′ (reverse) for the *PgPR-10.2*, and 5′-GAG TAT CGA AAT TCT TGA-3′ (forward) and 5′-GTG GTA TTC TTC ATA ATG-3′ (forward) for *PgPR-10.3*. The housekeeping gene that encoded β-actin (DC03005B05) was used as a control and was amplified with the primers 5′-AGA GAT TCC GCT GTC CAG AA-3′ (forward) and 5′-ATC AGC GAT ACC AGG GAA CA-3′ (reverse). Thermal cycler conditions recommended by the manufacturer were used: initial denaturation at 95°C for 30 s followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. At the end of the PCR reaction, a melt curve was generated to evaluate the possibility of undesirable side products.

2.4. Retrieval and processing of molecules for interaction with PgPR-10 proteins

Molecules used for the ligand-docking study were retrieved from the KEGG database (*www.genome.jp/kegg/*): emodin (C10343), quercetin 3-O-glucoside (C05623), deoxycholic acid (C07880 + Na), kinetin (C08272), quercetin 3-(2G-xylosylrutinoside) (C10175), and ginsenoside Re (C008944). These small molecules were retrieved in the standard two-dimensional SDF (Structure Data File) format, converted into three-dimensional coordinates by applying the MMCF (Merck Molecular Force Field) force field, and subsequently minimized to the standard recommended value of 50 iterations for small molecules using the VLifeMDS suite (VLife Sciences, Pune, India).

2.5. Molecular docking study of PgPR-10 proteins

Docking of the retrieved molecules with the theoretical structure of PgPR-10 was studied using the AutoDock4 program (The Scripps Research Institute, La Jolla, CA, USA), keeping the ligands as flexible as possible and placing the grid at the interface region. Genetic algorithm docking was applied for 100 docking poses. All small molecules that bound PgPR-10 were examined for their binding energy and for specific interacting residues. The ligands were again docked with PgPR-10.3 using AutoDock4, keeping the ligand nonrotatable (frozen) by reducing the torsion-angle degrees of freedom to zero. The same protocol was followed for subsequent consecutive dockings. The PgPR-10.3 ligand-bound complex was then examined for specific and nonspecific residues.

3. Results and discussion

3.1. Gene sequence of PgPR-10.3

The full cDNA sequence of *PgPR-10.3* has been reported previously [4]. *PgPR-10.3* is the closest homolog to *PgPR-10.2*, which was isolated as the most abundant transcript, representing 2.3% of the total cDNA library constructed using 14-yr-old ginseng roots [1]. It also had the highest number of transcripts in the ginseng leaf [17]. To obtain the full-length genomic DNA sequence of *PgPR-10.3*, gene-specific primers encompassing the full cDNA sequence were utilized. The whole gene structure of *PgPR-10.3* was interrupted by one intron (Fig. 1A), which is similar to that of *PgPR-10.2* [1]. *PgPR-10.3* encodes 465 base pairs of nucleotides between the start and the stop codon, and its deduced protein contains 154 amino acid residues. The most conserved glycine-rich loop domain is preserved in a large family of highly homologous food and tree pollen allergens, even in distant homologs, e.g., *Vigna radiata* (Vr CSPB) and the phytohormone-binding protein MtPhBP (Fig. 1B, 2A). The glycine-rich loop with the sequence EG(D/N)GG(V/P)G(T/S) constitutes a signature motif of PR-10 proteins [13]. The PR-10 protein members are generally similar in terms of their small size and primarily acidic nature [18]. The deduced nucleotide sequence ranges from 154 to 163 residues with molecular masses around 17 kDa [13]. *PR-10* genes usually consist of two exons and one intron [13], and *PgPR-10.3* matches well with these general features of PR-10 proteins.

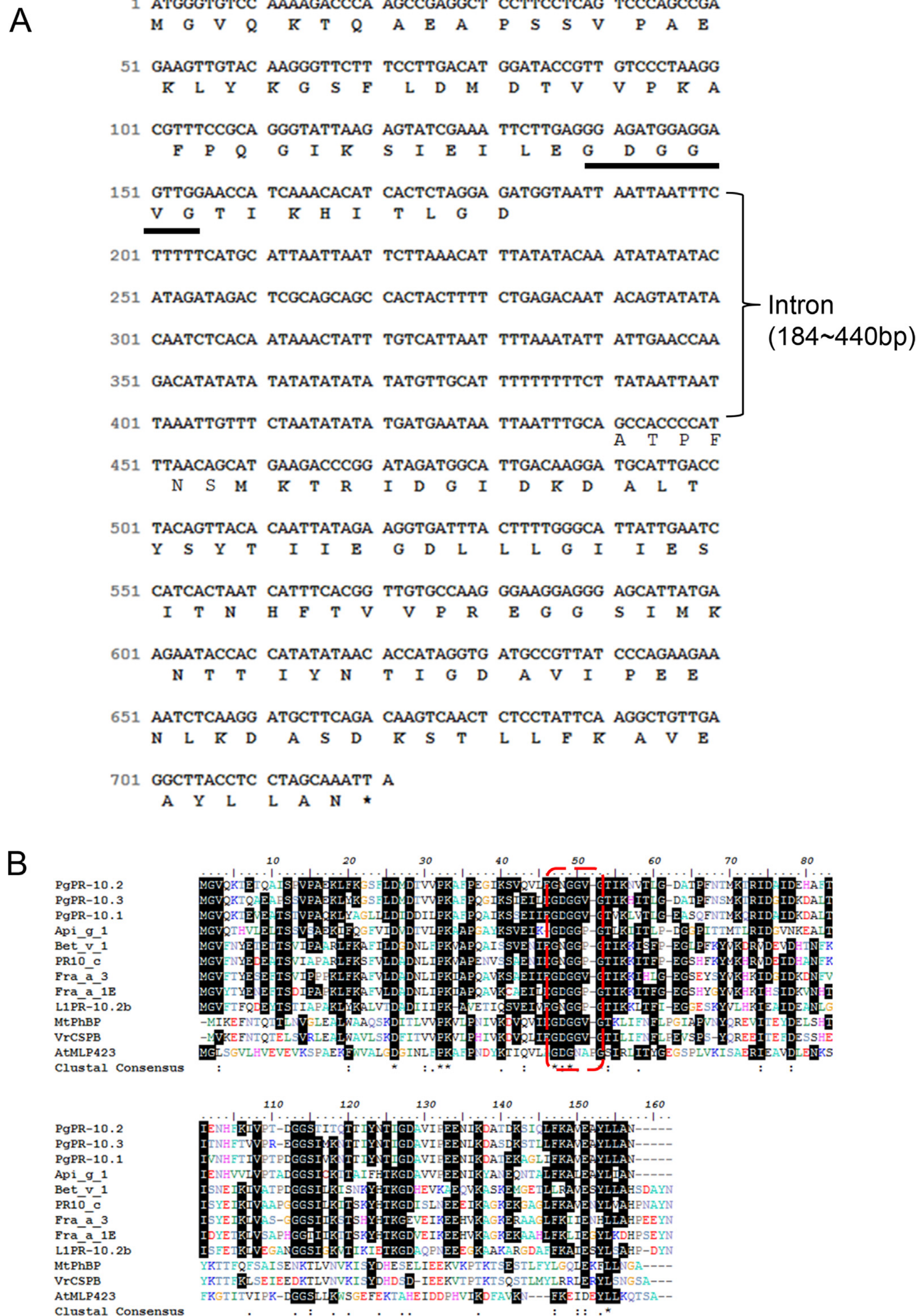

Fig. 1. Structure of *PgPR-10.3* and PR-10 homologs. (A) Genomic DNA sequence coding the full-length of cDNA. Capitalized letters indicate the translated regions, and the star indicates the stop codon. The internal intron region is indicated. The conserved glycine-rich domain is underlined. (B) Alignment of PgPR-10 proteins with close homologs. The red-dot-boxed domain represents a conserved motif (glycine-rich domain), known to be a functional domain in PR-10 proteins.

3.2. Three-dimensional structure of PgPR-10.3

With the sequence similarity and crystal structure information of the major celery allergen Api g 1 [10], the three-dimensional structures of PgPR-10.3 was constructed (Fig. 2A) [3]. The quality of the Ramachandran plot was reasonably good, compared with that of the template structure, supporting the reliability of the theoretical model. The average pair-wise root mean square fit of the Cα coordinates of these theoretical and experimental structures was 0.463 Å, indicating strong structural conservation among them and similarity in the structural folds. The overall folding pattern of PgPR-10.3 consists of seven antiparallel β-sheets curved around long α-helices (Fig. 2B) [3]. PR-10 proteins consist of approximately 160 amino acids and fold into a highly curved seven antiparallel β-

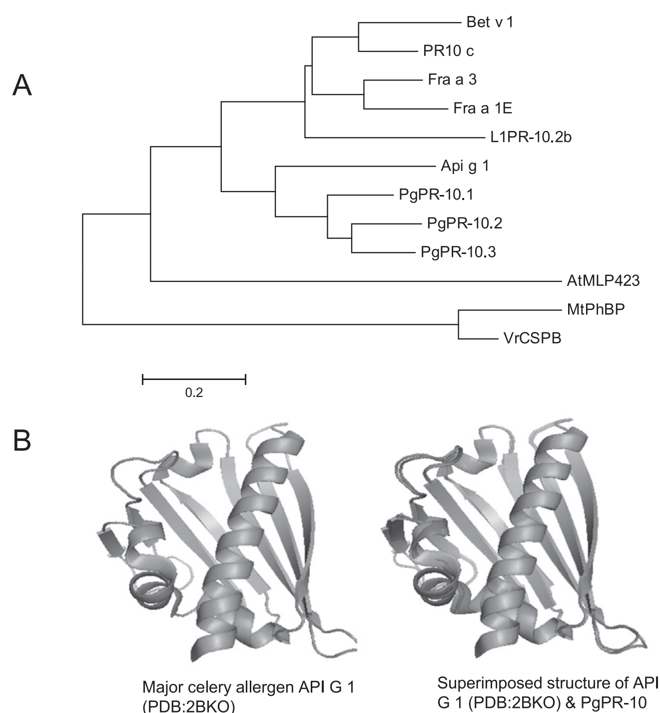

Fig. 2. Phylogenetic tree and canonical 3-D structure of PR-10 proteins. (A) Phylogenetic tree of PgPR-10 proteins with closely homologous proteins. The phylogenetic tree was constructed using the ClustalX program (neighbor-joining method): PgPR-10.1 (HQ171905), PgPR-10.2 (HQ171907), PgPR-10.3 (HQ171906), Api g 1 (P49372), L1PR-10.2b (2QIM_A). PR10-c (2122374C), Bet v 1(CAB02159), AtMLP423 (NM_102249), Fra a 3 (4C94_A), Fra a 1E (2LPX_A), MtPhBP (4Q0K_A), and Vr CSBP (BAA74451). (B) Stereo views of the predicted 3-D structures of PgPR-10 proteins. The theoretical structure of PgPR-10.3 protein was derived based on the structure of Api g 1(PDB code: 2BKO) as a template. 3-D, three-dimensional.

sheet (β-β7) along with two consecutive short α-helices (α1 and α2) and a long C-terminal helix (α3) [13,19]. These structural elements form an internal cavity that is able to bind to various physiologically relevant biological ligands [13]. Despite the accumulating structural information and the reported roles in many aspects of plant biotic and abiotic stress-related responses [2–4], the molecular function of PR-10 is still unclear. Recent studies suggest that the biological functions of PR-10 proteins involve the storage and transport of plant hormones and other small molecular ligands, including secondary metabolites [13,19,20]. *In silico* molecular ligand-binding studies based on the sequence and structural homology of PR-10 can shed light on possible molecular functions.

3.3. PgPR-10 proteins bind biologically important molecules

Owing to its unusually large volume, the cavity in the structure of PR-10 proteins, particularly in view of their small size, is assumed to have evolved for hydrophobic ligand binding [13–15]. Protein–ligand interaction studies with saturation transfer difference NMR show that birch PR-10c interacts with cytokinins, flavonoids, glycosides, sterols, and emodin [21]. L1PR-10.2 (PR-10 protein from yellow lupin) forms a complex with *trans*-zeatin in the crystal structure [14]. L1PR-10.2 was further shown to bind *N,N′*-DPU (*N,N′*-diphenylurea), the first synthetic cytokinin to be identified. Cytokinin-specific binding proteins from *V. radiata* (Vr CSBP), similar to plant PR-10 proteins, are also crystallized in complex with zeatin [12]. In this study, we tested whether PgPR-10.3 also structurally interacts with previously tested ligands. As shown in Fig. 3 and Table 1, PgPR-10.3 seems to interact with flavonoids, kinetin (a cytokinin), emodin, and deoxycholic acid. Binding of flavone and flavanone (naringenin) to birch Bet v 1 has been reported [22]. As flavonoids (often present as glycosides in plants) are located in the vacuole, PgPR-10 may function as a cytoplasmic flavonoid carrier into the vacuole. Emodin is a biologically active, naturally occurring anthraquinone derivative (1,3,8-trihydroxy-6-methylanthraquinone). It possibly acts as a feeding deterrent, an allelopathic compound, and an antimicrobial, and participates in seed germination and dispersal. The docking analysis of PgPR-10.3 with emodin suggests that PgPR-10 may also participate in the formation of hypericin. The high structural similarity between Bet v 1 and the steroidogenic acute regulatory protein-related lipid transfer domain of human MLN64 protein, a steroid-binding domain involved in cholesterol translocation, suggests, likely for the first time, that PR-10 is involved in the transport of steroid compounds. Pru av 1, a major cherry allergen similar to Bet v 1, was shown to bind the plant BR homocastasterone [7]. Based on the overlapping heteronuclear single quantum coherence spectra, it was concluded that Pru av 1 and Bet v 1 are probably steroid carriers [7]. Bet v 1 is a naturally occurring, hypoallergenic isoform of the major birch pollen allergen Bet v 1. Using the molecular replacement method, the crystal structure of Bet v 1l was found to be in a complex with deoxycholic acid [9]. Deoxycholic acid is structurally very similar to BRs, an important family of plant sterols. Docking experiments using PR10-c also indicated that it could bind sitosterol as well as the triterpene betulin, which gives color to birch bark [21]. In light of the fact that ginseng saponins (ginsenosides) are glucosides consisting of sugar and aglycones that share chemical structural similarity with plant sterols, the possible interaction of PgPR-10.3 with one of the ginseng saponins was established (Fig. 3). This docking result suggests the possible interaction of PgPR-10.3 with ginsenoside Re. This interaction needs to be confirmed in other biochemical and physiological ways based on the interacting residues (Table 1), but it is very likely and should be studied in the future.

3.4. Leaf-specific expression of PgPR-10.3

Differential expression patterns of *PgPR-10.1* and *PgPR-10.2* have been reported previously [4]. Both genes are expressed ubiquitously in most organs analyzed, with *PgPR-10.2* being more abundant in roots. To obtain differential expression patterns between *PgPR-10.2* and *PgPR-10.3,* quantitative reverse transcription (qRT) PCR was performed using ginseng plantlets and 2-yr-old ginseng plants. Owing to differential organogenesis in perennial ginseng plants [23], it is worthwhile to analyze expression patterns in ginseng plants of different ages. Overall, the expression was highest in roots and lowest in leaves, with intermediate transcripts in stems (Fig. 4A). However, once the whole organs are formed in 2-yr-old ginseng plants, the mRNA of *PgPR-10.3* is specifically restricted to leaves (Fig. 4B). This result indicates that the transcript levels of *PgPR-10.2* are consistently high in roots, whereas *PgPR-10.3* expressed in leaves as they age. It is also worth noting that, on average, the expression of *PgPR-10.2* in leaves is 86-fold higher than that of *PgPR-10.3.* This indicates that although the expression of *PgPR-10.3* is low in ginseng organs, it may have important biological impact, especially in mature ginseng leaves.

3.5. Differential transcript levels of PgPR-10.3 in response to abiotic stresses

Differential expression patterns of *PgPR-10.1* and *PgPR-10.2* in ginseng plantlets exposed to various abiotic stresses have been analyzed previously [4]. *PgPR-10.1* and *PgPR-10.2* expression was notably upregulated in response to NaCl treatment, and the

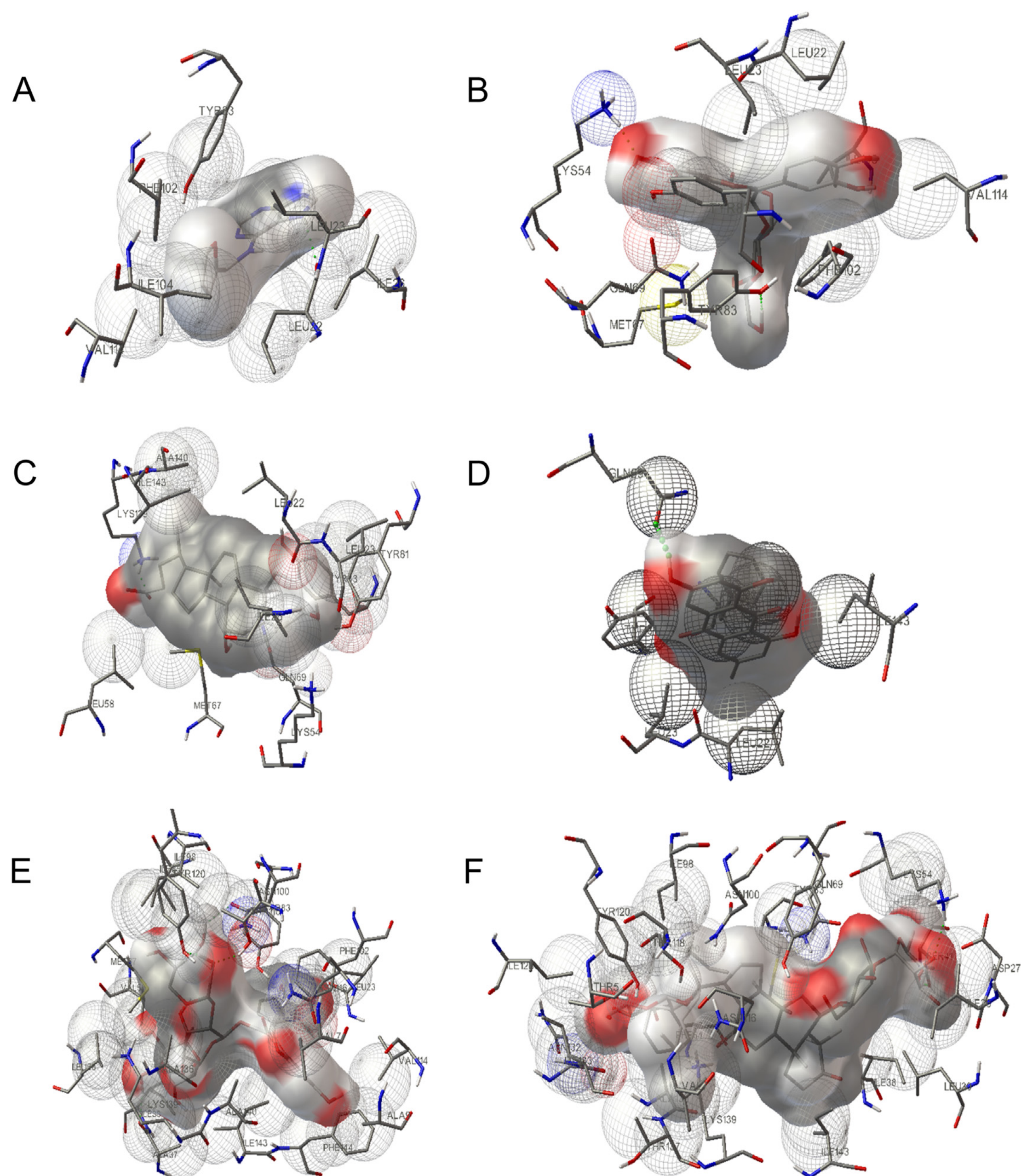

Fig. 3. Rigid ligand interaction study shows several possible PgPR-10.3-interacting ligands. The following ligands were tested: (A) kinetin (KEGG compound code: C08272), (B) quercetin 3-O-glucoside (KEGG compound code: C05623), (C) deoxycholic acid (KEGG compound code: C07880 + Na), (D) emodin (KEGG compound code: C10343), (E) quercetin 3-(2G-xylosylrutinoside) (KEGG compound code: C10175), and (F) ginsenoside Re (KEGG compound code: C08944).

Table 1
Interacting residues obtained from neuronal PgPR-10[a]

S. No.	Ligand	Energy (kcal/mol)	Residues on interaction
1	Kinetin	−6.03	LEU22, LEU23, VAL24, VAL114, TYR83, PHE102, ILE 104, ILE26
2	Quercetin 3-O-glucoside	−4.65	ILE143, PHE102, MET67, LEU22, LYS54, LEU23, GLU69, TYR81, TYR83, VAL114
3	Deoxycholic acid	−7.60	LYS139, MET67, ALA10, GLN69, ILE143, TYR81, LEU22, LEU58, TYR83, LEU23, LYS54, ILE26
4	Emodin	−5.08	PHE102, ILE143, LEU23, TYE81, PHE144, ALA140, LEU22
5	Quercetin 3-(2G-xylosylrutinoside)	−7.38	TYR118, ALA9, LEU58, ILE85, LYS139, ASN100, VAL114, TYR83, ILE38, ALA136, ILE 98, VAL56, VAL7, LEU23, ASN116, ILE143, ALA37, PHE102, ALA140, TYR120, PHE114, MET67
6	Ginsenoside Re	−15.16	LYS139, ASN100, LEU30, ALA136, ILE98, VAL7, TYR120, ASN132, GLN69, THR118, ILE143, SER41, LYS54, PHE64, ILE90, THR137, TYR83, MET67, ILE38, ASN116, ILE128, ASP27, THR5, ILE26, ILE133

[a] Flexible ligand docking was performed using AutoDock4. Energy calculations and interacting residues were selected using the built-in module in AutoDock4.

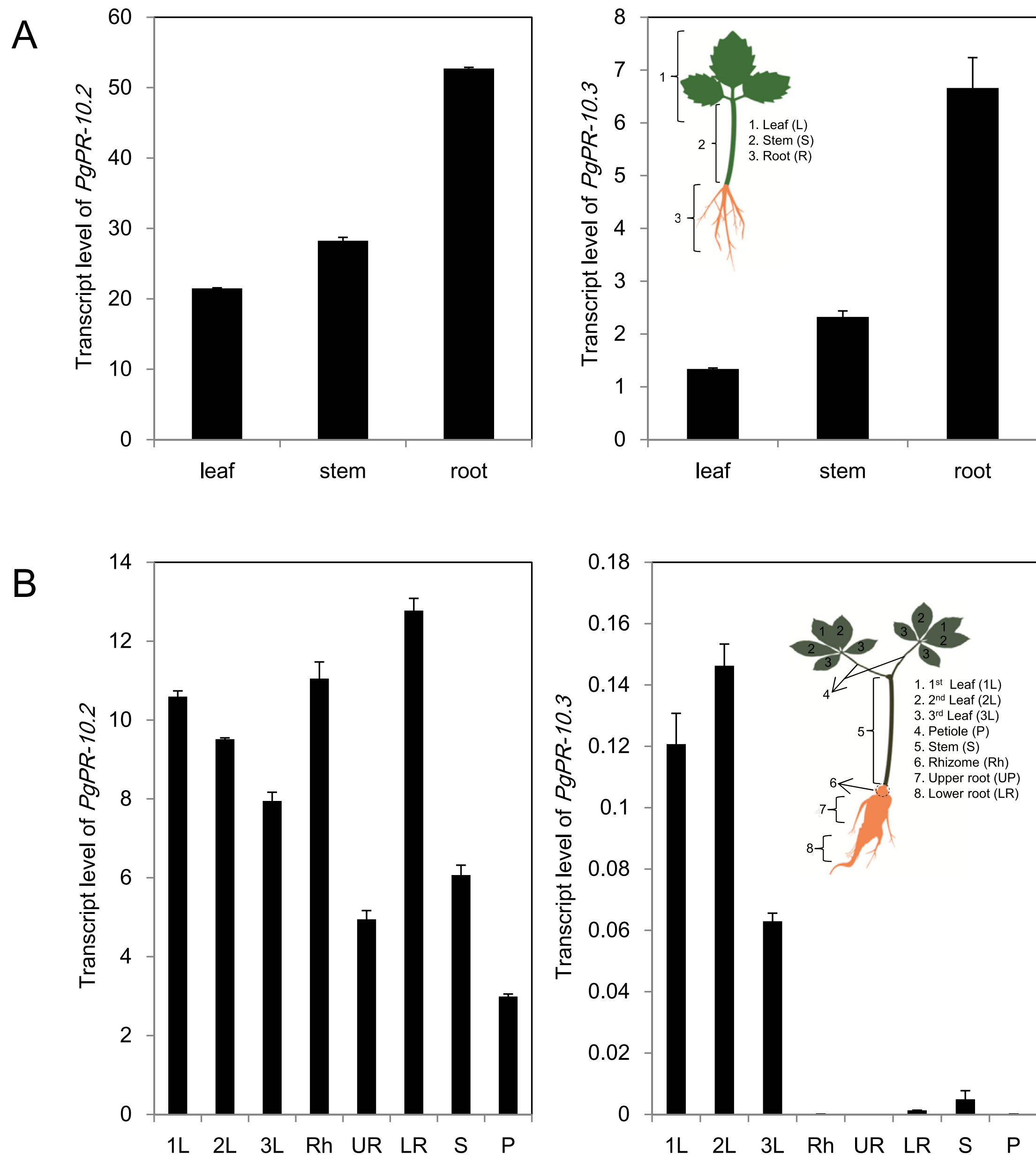

Fig. 4. Organ-specific expression patterns of *PgPR-10.2* and *PgPR-10.3* in ginseng plants of different ages. (A) Expression patterns in 3-wk-old ginseng plantlets. (B) Expression patterns in 2-yr-old ginseng plants. Differential expression patterns of *PgPR-10.2* and *PgPR-10.3* were evaluated using qRT-PCR with the cDNA of the stem, leaf, root, rhizome, and petiole. Exact organ nomenclatures for plantlets and 2-yr-old ginseng plants are indicated in the inset at the right-side of the data. The Ct value for *PgPR-10.2* and *PgPR-10.3* was normalized to the Ct value for β-actin, and was calculated relative to a calibrator using the formula $2^{-\Delta\Delta Ct}$. Data represent the mean ± SE for three independent replicates. qRT-PCR, quantitative reverse transcription polymerase chain reaction; SE, standard error.

resulting salt resistance was also showed [4]. To differentiate between the exact functional roles of the three isoforms of *PR-10* in ginseng, relative expression of *PgPR-10.3* was analyzed using quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) following exposure to several abiotic stresses such as cold (4°C), NaCl (100mM), H_2O_2 (10mM), ABA (100μM), SA (5mM), and JA (0.2mM). Overall, the transcript levels of *PgPR-10.3* increased, except for those after ABA treatment (Fig. 5), which suggests its accessory role in addition to both major *PgPR-10.1* and *PgPR-10.2* gene functions related to abiotic stresses. Two phytohormones, SA and JA, are well-known inducible defense modulators that act antagonistically. Thus, increased expression of *PgPR-10.3* induced by SA and JA (Fig. 5) shows that *PgPR-10.3* plays an independent role in a distinct defense system. It is also noteworthy that the mRNA levels of *PgPR-10.3* are increased markedly in response to NaCl (Fig. 5), similar to *PgPR-10.1* and *PgPR-10.2* [4]. Taken together, these results suggest that *PgPR-10.3* may play a major role in salt stresses and is involved in hormone, lipid, or ginsenoside transport in ginseng.

4. Conclusion

Several studies on binding of cytosolic PR-10 proteins to cytokinins including kinetin, *trans*-zeatin, and *N,N′*-DPU ([12,14,15,21] and this study) suggested that PR-10 acts as a reservoir for cytokinins, similar to other transport proteins that deliver them to their membrane receptor CRE1. It is noteworthy that PR-10 proteins from peach (*Prunus persica*), Pru p 1.01, bind zeatin in isothermal titration calorimetry, and this binding is related to RNA hydrolysis [24]. Mogensen et al [22] reported that birch Bet v 1 did not bind to

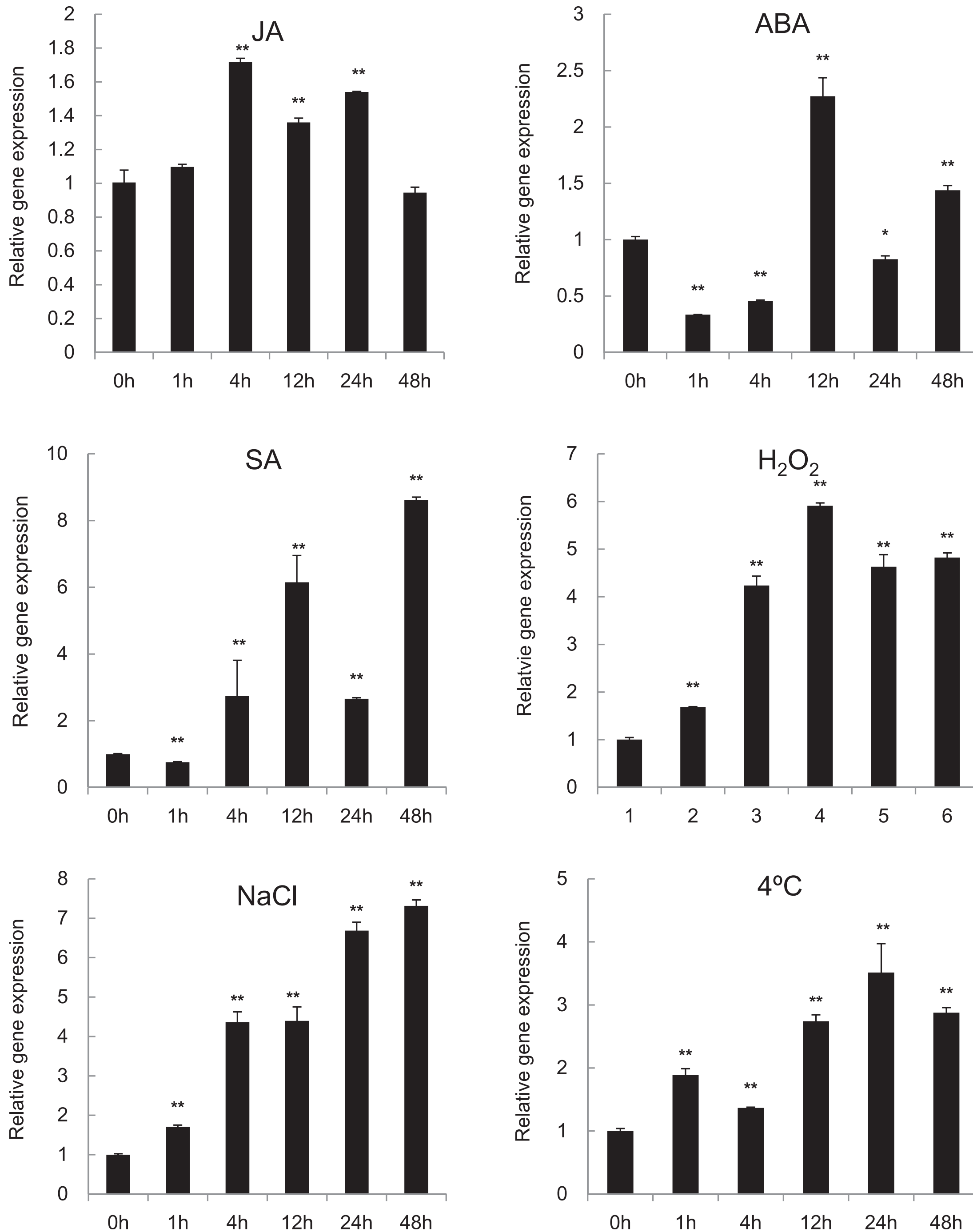

Fig. 5. Temporal expression patterns of the *PgPR-10.3* gene in response to abiotic stresses. Three-week-old ginseng plantlets were exposed to jasmonic acid (0.2mM), ABA (100μM), salicylic acid (5mM), H_2O_2 (10mM), NaCl (100mM), and chilling (4°C) for the indicated time intervals. β-Actin was used as a loading control. Data represent the mean ± SE for three independent replicates. Averages for treated samples were significantly different compared to the control. * $p < 0.05$. ** $p < 0.01$. ABA, abscisic acid; JA, jasmonic acid; SA, salicylic acid; SE, standard error.

indole-3-acetic acid or gibberellic acid, which suggests that these molecules are negatively interacting hormones. Structural similarity of deoxycholate and BRs, which are ubiquitous plant steroid hormones, suggests that PR-10 may also act as a phytosterol hormone carrier. Further indications that PR-10c is able to bind sitosterol as well as triterpene [21] allowed us to determine the binding property of PgPR-10s with ginsenoside Re, a class of steroid glycoside, and a triterpene saponin (Fig. 3, Table 1). As is the case with BRI1, which is a leucine-rich repeat receptor kinase localized to the plasma membrane and a critical component of a receptor complex for BRs, PR-10s may also deliver steroid molecules to the receptor. PR-10 proteins may interact with biologically important plant hormones and possibly other small molecules when they are exposed to biotic and abiotic stresses. Further biochemical analysis is definitely necessary to better understand the nature of PR-10 proteins. We can speculate that the PR-10 family might have evolved to participate in biologically important processes over the whole course of development by binding physiologically relevant

small molecules such as cytokinins or BRs. Cytokinins are structurally diverse and biologically versatile phytohormones, involved in the differentiation of the shoot meristem and the tissues of the root, mitosis, leaf formation and senescence, and chloroplast development [25]. BRs contribute to the regulation of cell division and differentiation, and participate in the control of overall developmental processes, leading to morphogenesis [26].

Conflicts of interest

All authors declare that they have no conflicts of interest.

Acknowledgments

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