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Molecular analysis of the annexin gene family in soybean

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Abstract

Annexins play a crucial role in plant development and response to environmental stimuli. In this study, a total of 23 annexin genes (*GmANN1 - GmANN23*) were identified from the soybean genome database and for two of them (*GmANN11* and *GmANN*14), complete cDNAs were cloned. *GmANN1 - GmANN23* encoded a set of predicted proteins which showed high similarity to other known annexins. Most *GmANN* genes contained four putative annexin repeats. Generally, a type II Ca^{2+}-binding site is found to exist in the first and fourth repeats. *GmANN1*, *10*, *11*, *12*, and *14* showed different organ-specific expression patterns. Furthermore, expression of these five *GmANN*s was significantly induced by drought and abscisic acid. Expression of four annexins (*GmANN1*, *11*, *12*, and *14*) was induced by cold and expression of three annexins (*GmANN1*, *11*, and *12*) responded to high salinity.

Additional key words: abscisic acid, cold, drought, gene expression, *Glycine max*, salinity.

Introduction

Annexins are soluble proteins capable of Ca^{2+}-dependent or Ca^{2+}-independent binding membrane phospholipids (Laohavisit and Davies 2011). Most annexins have four putative annexin repeats of around 70 amino acids. These conserved annexin repeats confer Ca^{2+}-binding activity and contain a consensus endonexin sequence referred to as K-G-X-G-T-{38 variable residues}-D/E. (Gerke *et al.* 2005). Annexins belong to a diverse and multifunctional family in animal cells where they are involved in a wide variety of essential cellular processes (Laohavisit *et al.* 2012). Compared to animal annexins, plant annexin function *in vivo* remains poorly understood.

In vitro studies have revealed five main protein activities for plant annexins. These functions include nucleotide phosphodiesterase (ATPase and GTPase) activity (McClung *et al.* 1994), cytoskeletal binding (Hayes 2004), regulation of complex saccharides (Andrawis *et al.* 1993), peroxidase activity (Gorecka *et al.* 2005), and ion transport (Hofmann *et al.* 2000, Laohavisit *et al.* 2010, 2012). These functions suggest that plant annexins are involved in a wide range of processes including exocytosis, cell elongation, cell wall synthesis, fruit ripening, auxin perception, nodulation, and response to environmental stimuli (Andrawis *et al.* 1993, Seals and Randall 1997, Cantero *et al.* 2006, Jami *et al.* 2008, Konopka-Postupolska *et al.* 2009, Lu *et al.* 2012). For example, in tobacco, an annexin-like protein, VCaB42, is associated with plant vacuoles and is correlated with the expansion of cells (Seals and Randall 1997). Annexin expression, abundance, and cellular positioning respond to cold, osmotic stress, salinity, drought, and ABA (Jami *et al.* 2008, Lu *et al.* 2012). For example, over-expression of mustard *AnnBj1* enhances drought and salt tolerance in transgenic tobacco and cotton plants (Jami *et al.* 2008, Divya *et al.* 2010). A recent study revealed that AnnAt1 and AnnAt4 function cooperatively in response to drought and salt stresses (Huh *et al.* 2010). These studies show that some annexins can be potential candidates for genetic engineering to enhance stress resistance of important crops.

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Abbreviations: ABA - abscisic acid; DAF - days after flowering; ORF - open reading frame; RT-PCR - reverse transcriptase polymerase chain reaction; UTR - untranslated region.

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Recently, a comparative analysis of annexin gene families in 16 sequenced genomes ranging from unicellular green algae to multicellular plants was performed and 148 annexins were identified (Jami *et al.* 2012a). However, only limited transcriptional analysis of annexin gene families have been performed in *Arabidopsis*, mustard, rice, and tomato (Clark *et al.* 2001, Cantero et al. 2006, Jami *et al.* 2009, 2012b, Lu *et al.* 2012). In this study, we identified 23 annexin genes (*GmANN*s) from the soybean genome database and further analyzed genomic organization, sequence features, and evolutionary relationship of these annexins. Five soybean annexins were selected to investigate organization of promoter regions and expression patterns in different organs as well as response to external stimuli.

Materials and methods

Plant cultivation and treatments: Soybean [*Glycine max* (L.) Merr. cv. HPXQD] were grown in a chamber maintained at temperature of 28 °C, relative humidity of 60 %, a 12-h photoperiod, and irradiance of 110 μmol m^{-2} s^{-1}. Young leaves, stems, and roots were collected from 2-week-old seedlings. Blooming flowers and developing seeds at 25 d after flowering (DAF) were collected from field-grown mature plants. All stress and chemical treatments were applied to 2-week-old seedlings. Dehydration was induced by submerging the roots of plants in 15 % (m/v) PEG 6000 solution. Cold treatment was applied by transferring plants to a growth chamber set to 4 °C. Salinity treatments were applied by submerging the roots in 150 mM NaCl solution. For abscisic acid (ABA) treatment, roots of plants were submerged in 100 μM aqueous solution of ABA. The leaves were collected at 0, 1, 6, and 24 h intervals for each treatment. All tissues were immediately frozen in liquid nitrogen and stored at -80 °C until use.

Isolation of *GmANN11* and *14* genes: Total RNA was isolated from leaves of 2-week-old seedlings using the *RNeasy* plant mini kit (*Qiagen*, Valencia, CA, USA) according to the manufacturer's instruction. The first strand of cDNA was obtained with total RNA using *M-MLV* reverse transcriptase (*Takara*, Otsu, Shiga, Japan). Genomic DNA was isolated from seedling leaves using plant DNA isolation kit (*Roche*, Mannheim, Germany) according to the manufacturer's instruction. The soybean genome database (http://www.phytozome.net) was searched using the *tBLASTn* program with the amino acid sequence of AnnAt1 (At1g35720) (Cantero *et al.* 2006) and a total of 23 full-length ORFs encoding annexin-like proteins were found (supplementary data S1, http://pleaserescueourplanet.blogspot.com/). The corresponding cDNA sequences were designated *GmANN1* - *GmANN23*, respectively. Among the five *GmANN*s (*GmANN1*, *10*, *11*, *12*, and *14*) that were used for expression analysis, *GmANN1*, *10*, and *12* have been cloned and submitted to *NCBI* Genbank by other research groups. However, *GmANN11* and *14* had not been isolated. Therefore, sequences *Glyma15g38060* and *Glyma13g26040* were used as templates to design primers for amplification of the full-length cDNA and DNA sequences of *GmANN11* and *14*, respectively. The primer pair for *GmANN11* are A11F and A11R. And the primer pair for *GmANN*14 are A14F and A14R (supplementary data S2, http://pleaserescueourplanet.blogspot.com/). Total cDNA and DNA of soybean were used as templates to amplify the full-length cDNA and DNA sequences of *GmANN11* and *14*, respectively. All PCR products were cloned into the pMD19-T vector (*Takara*) and then sequenced (*Invitrogen*, Grand Island, USA).

Multiple sequence alignment and phylogenetic analysis: Homology searches were performed using *BLASTn* with default parameters at *NCBI* (http://www.ncbi.nlm.nih.gov/blast/). Multiple sequence alignment of annexins was performed using *ClustalW* (http://www.ebi.ac.uk/clustalw/) and *BOXSHADE* (http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html). A total of 55 plant and animal annexins from the *NCBI* protein database were chosen for phylogenetic analysis (S1) and displayed using the *ClustalX v. 1.83* (Thompson *et al.* 1997) with default parameters. Phylogenetic analysis was based on the neighbour-joining (NJ) method using *MEGA v. 4.1* (Saitou 1987) with 1 000 bootstrap replications.

Expression analysis of *GmANN* genes: Total RNA and the first strand of cDNA were obtained from frozen tissues according to the previous methods (see above). The primer pairs A1-Q1 and A1-Q2, A10-Q1 and A10-Q2, A11-Q1 and A11-Q2, A12-Q1 and A12-Q2, A14-Q1 and A14-Q2, and T1 and T2 were used for the expression analysis of *GmANN1*, *10*, *11*, *12*, *14*, and soybean tubulin (AY907703), respectively (S2). These primer pairs amplify fragments with sizes of 269, 118, 218, 288, 178, and 189 bp for *GmANN1*, *10*, *11*, *12*, *14*, and tubulin, respectively. The following thermal cycle conditions were used: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 25 s, and 72 °C for 20 s. All reactions were performed in triplicate. Amplification efficiency of *GmANN* primers was tested using Ct slope method. Following PCR, a melting curve analysis was performed. Ct or threshold cycle was used for relative quantification of the input target number. Results from different groups were analyzed using relative expression software tool *REST 2009* (http://www.gene-quantifi-cation.de/rest-2009.html) to compare all samples of each group. The mathematical model used in the *REST* software is based on the PCR efficiencies (E) and the crossing point deviation between the samples (CP) (Pfaffl *et al.* 2002).

Results

Total of 23 full-length soybean ORFs were found to potentially encode annexins. We designated these sequences as *GmANN1* - *GmANN23*. Based on the phylogenetic analysis of annexins from various organisms (see below), we decided to choose *GmANN1*, *10*, *11*, *12*, and *14* as representatives of the annexin family for expression analysis in soybean. To obtain exact sequence information of *GmANN11* and *GmANN14*, we isolated full-length cDNA and DNA sequences of them. *GmANN11* cDNA contained an open reading frame (ORF) of 942 bp, a 5′-untranslated region (UTR) of 8 bp, and a 3′-UTR of 7 bp, whereas *GmANN14* cDNA contained an ORF of 1 110 bp, a 5′-UTR of 31 bp, and a 3′-UTR of 21 bp. These DNA sequnces were deposited in *NCBI* Genbank (acc. No. ADG59899 and ADG59900, respectively). At the nucleotide level, the coding regions of all *GmANN* genes shared 45.6 - 95.5 % identity (not shown). These *GmANN* genes were unequally distributed on 9 chromosomes. Among these chromosomes, chromosome 13 (Glyma13g) had 7 annexin genes. Comparison of genomic and cDNA sequences revealed that all introns were located in coding regions but the number and length of introns were extremely varied between different annexins. The shortest introns contained only 32 nucleotides, such as the third one of *GmANN17* and the fourth one of *GmANN21*. However, the longest intron contained 7 999 nucleotides, such as the third one of *GmANN7* (Fig. 1).

*GmANN*1 - *GmANN*23 encoded polypeptides consisting of 294 - 370 amino acid residues with total predicted molecular masses of 33.16 - 41.66 kDa and predicted pI values of 5.46 - 9.91 (supplementary data S3, http://pleaserescueourplanet.blogspot.com/). These deduced amino acid sequences showed 15.9 - 96.5 % identity to each other with the highest similarity found between *GmANN16* and *GmANN23* and the lowest between *GmANN14* and *GmANN21* (supplementary data S4, http://pleaserescueourplanet.blogspot.com/).

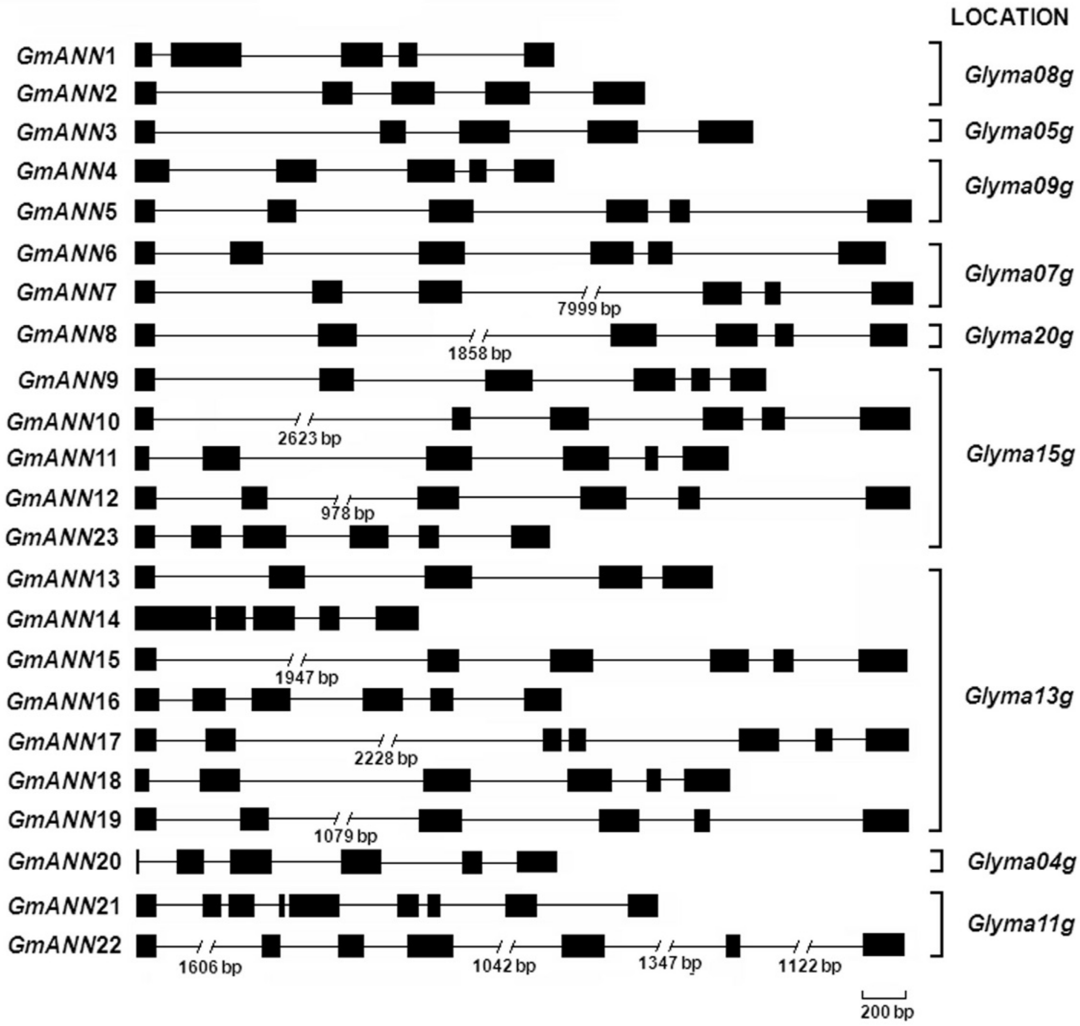

Fig. 1. Schematic diagram of genomic sequence of *GmANN* genes. Solid boxes and lines indicate exons and introns within *GmANN* genes, respectively. Chromosomes which contain soybean annexin gene are annotated. This illustration is drawn to scale.

Additionally, comparative multiple alignment of these deduced soybean annexins with other plant annexins obtained by *tBLASTn* searching in *NCBI* GenBank database showed sequence similarity ranging from 29 to 90 % (not shown).

Table 1. Relative expression of *GmANN1*, *10*, *11*, *12*, and *14* in different soybean organs relative to roots where expression was considered as 1. Expression was different from that in roots at $P < 0.05$ (*) or $P < 0.001$ (**).

	Stem	Leaf	Flower	Seed
GmANN1	2.08*	0.66	1.27	2.35**
GmANN10	3.90**	0.36	2.97*	0.43*
GmANN11	5.17	3.75*	3.77	0.02**
GmANN12	1.46**	2.56	1.42*	4.42**
GmANN14	0.12**	0.05*	0.44	4.42**

Alignment of amino acid sequences revealed that most soybean annexins contained four putative annexin repeats. The first and fourth repeats showed higher levels of conservation than the second and third repeats. Generally, the GXGT-(38 residues)-(D/E) motif, a type II Ca^{2+}-binding site (Moss and Morgan 2004), is found to exist in the first and fourth repeats but not in the second and third repeats of soybean annexins. However, the first repeats of GmANN8, 20, and 21, and the fourth repeats of GmANN4, 9, 11, and 18 do not have this motif. GmANN14 is essentially devoid of type II calcium binding sites. Additionally, some conserved residues involved in structural stability as well as salt bridge composition associated with channel function of animal annexins (Liemann *et al.* 1996) were found to exist in most soybean annexins (S4).

To determine the evolutionary relationship between GmANNs with other annexins, a phylogenetic tree was generated from the deduced amino acid sequences of 55 annexins from plants and animals (S1). Annexins were classified into two primary clades. The clade A included most of plant annexins, whereas the clade B contained eight plant annexins and six animal annexins. All animal annexins were grouped into a subclade in the lower clade (Fig. 2). In addition, most GmANNs had direct paralogs in the soybean genome (Fig. 2).

To understand the organization of the regulatory regions of *GmANN* genes, 2000 bp fragments upstream of the start codon of *GmANN1*, *10*, *11*, *12*, and *14* were identified from the soybean genome database and were designated *pGmANN1*, *10*, *11*, *12*, and *14*, respectively. The promoter motif search of *pGmANN* sequences was performed to reveal putative *cis* elements using online software *PlantPAN* (plant promoter analysis navigator; Chang *et al.* 2008) and *PlantCARE* (plant *cis*-acting regulatory element; Lescot *et al.* 2002). At least two TATA boxes appeared nearby the start codon indicating that *pGmANN* sequences are typical gene promoters. Furthermore, these *pGmANN* sequences had several common elements including the ABA response element (ABRE), the GT-1 box which partially mediates pathogen-induced and NaCl-induced expressions of the SCaM-4 (soybean calmodulin isoform) promoter (Park *et al.* 2004), and the MYB/MYC binding sites (MBS) which are involved in drought and ABA responses (Yamaguchi-Shinozaki and Shinozaki 1993). In addition, jasmonate response element (JARE) and elicitor responsive element (ElRE) were found in the regulatory regions of *GmANN1*, *10*, and *11* genes. The low temperature responsive element (LTRE) was found in the regulatory regions of *GmANN11*, *12*, and *14* genes. The ethylene responsive element (ERE) and salicylic acid response element (SARE) appeared in the *pGmANN1* and *11* but not in *pGmANN10*, *12*, and *14* sequences. The promoter regions of *GmANN* genes also have other *cis*-elements including auxin, heat, and drought responsive elements as well as TC-rich elements involved in defense and stress responses (supplementary data S5, http://pleaserescueourplanet.blogspot.com/) (Diaz-de-Leon *et al.* 1993).

To determine the tissue expression patterns of *GmANN*s, real time quantitative RT-PCR was performed using soybean mRNAs from different organs including

Table 2. Relative expression of *GmANN1*, *10*, *11*, *12*, and *14* under various stresses and ABA treatment. Expression was different at $P < 0.05$ (*) or $P < 0.001$ (**) from that at time 0 where expression was considered as 1.

Treatment	Time [h]	*GmANN1*	*GmANN10*	*GmANN11*	*GmANN12*	*GmANN14*
Drought	1	2.76**	1.08	5.41**	11.72**	0.88
	6	4.81**	0.96	2.00**	5.31**	1.87**
	24	59.96**	4.13*	1.08	7.05**	25.82**
NaCl	1	2.65**	0.60**	1.97	5.65*	0.76
	6	1.86*	0.88	0.40*	1.64*	1.32
	24	4.14	0.73	1.59	1.01	1.20*
Cold	1	0.65**	1.00	15.90**	5.11**	0.60**
	6	0.83	1.24*	37.58*	11.97**	0.51
	24	8.47*	1.14	2.28**	7.10**	3.45*
ABA	1	1.73*	1.78**	2.58	5.20**	2.16**
	6	0.96	1.00	1.42**	7.65**	0.80
	24	5.56**	1.69	4.09**	9.97*	5.55**

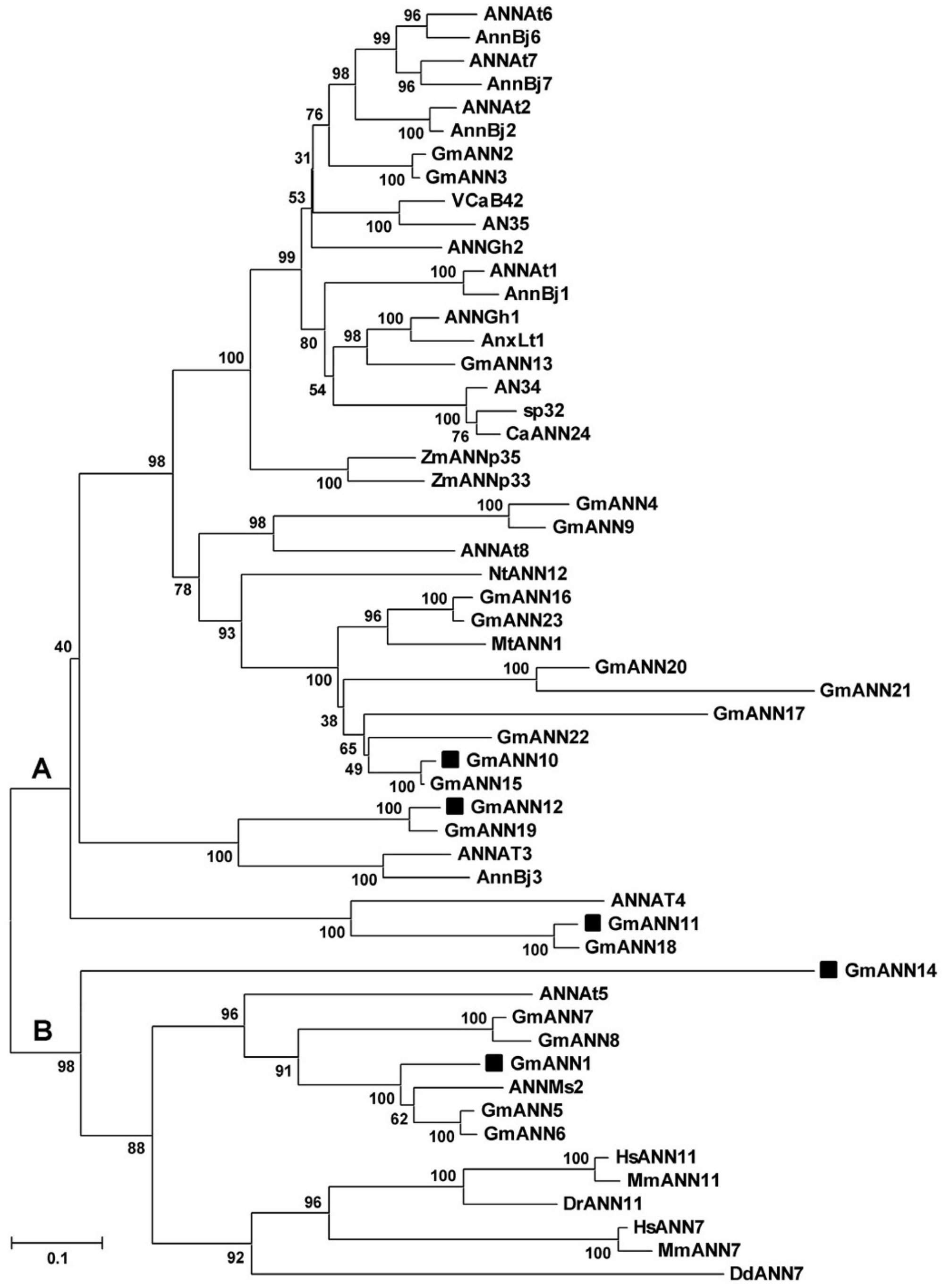

Fig. 2. Phylogenetic tree of annexins from various organisms. The numbers beside the branches represent bootstrap values (≥ 50) based on 1 000 replications. The *scale bar* indicates the relative amount of change along branches. Five GmANNs for expression analysis are marked by *solid quadrangles*.

roots, stems, leaves, flowers, and young seeds (25 DAF). The unique fragments of 269, 118, 218, 288, and 178 bp were obtained by PCR using the gene-specific primers for *GmANN1*, *10*, *11*, *12*, and *14*, respectively. Melt curve analysis revealed specific *GmANN1*, *10*, *11*, *12*, and *14* melting temperature peaks at 85, 82, 83, 87, and 84 °C, respectively (data not shown) indicating the quantitative assay results of *GmANN* gene expression are reliable. *GmANN* genes showed different organ-specific expression patterns. Transcripts of *GmANN1*, *10*, and *12* could be detected in all organs suggesting that these genes play essential roles in the soybean developmental process. *GmANN1*, *12*, and *14* were expressed at higher levels in developing seeds whereas *GmANN10* and *11* showed higher expression in seedling stems than in other tissues. Additionally, transcripts of *GmANN11* and *14* were hardly detected in developing seeds and seedling leaves, respectively (Table 1).

To determine the effect of environmental stresses on expression of *GmANN*s, the transcriptional levels of *GmANN1*, *10*, *11*, *12*, and *14* in seedling leaves under various stresses (drought, cold, and salinity) and ABA treatments were investigated by qPCR. Drought stress could significantly induce the expression of all *GmANN* genes at different time points. Salt stress caused an increased transcript level in *GmANN1*, *11*, and *12* but not in *GmANN10* and *14* during the course of treatment. All *GmANN* genes were induced by cold treatment at the different stages except for *GmANN10*. Moreover, the transcription of all five *GmANN* genes tested was significantly up-regulated by ABA treatment, especially at 1 and 24 h (Table 2).

Discussion

Previously, it was proposed that annexins in plants form a smaller, less diverse gene family than in vertebrates (Jami *et al.* 2008). However, recently, Jami *et al.* (2012a) identified 22 annexins from the soybean genomic database. Here, we found 23 non-redundant annexins including 22 members identified by Jami *et al.* (2012a). The additional one (*Glyma0903430*), named *GmANN4* in our study, is located on chromosme 9. Soybeans are thought to have arisen through an ancient allotetraploid event involving both chromosome doubling and chromosome loss followed by the subsequent diploidization of the genome (Danna *et al.* 1996). This may explain why so many annexin genes exist in the soybean genome and most genes have one or more paralogs. Notably, several soybean annexins, such as *GmANN10*, *15*, *16*, *17*, *20*, *21*, and *23*, have no orthologs in *Arabidopsis* (Fig. 2). These annexins probably represent the most recent lineage-specific gene duplications following *Magnoliidae-Rosidae* separation.

The number of introns per gene varied from 0 to 8 in 16 plant species studied by Jami *et al.* (2012a). Soybean annexins contained 4 - 8 introns (Fig. 1) whereas *Arabidopsis* annexins had 3 - 5 introns (Cantero *et al.* 2006). Both the soybean gene *GmANN21* (*Glyma11g21460*) and the grapevine gene *Vv03g02080* possessed 8 introns, the maximum number of introns seen in annexins which may be related to the fact that they are both from dicot species (Jami *et al.* 2012a). Soybean and *Arabidopsis* annexins shared five common intron occurrence sites (data not shown). Several soybean annexins showed intron loss or acquisition. For example, *GmANN4* lost the first conserved intron whereas both its paralog (*GmANN*9) and ortholog (*ANNAt8*) had 5 conserved introns (Cantero *et al.* 2006). The last three introns of *GmANN10* and *15* were located in novel sites not existing in any *Arabidopsis* annexin. *GmANN14* had four novel occurrence sites totally different from sites typically conserved (Fig. 1). How these intron-losses or acquisitions come into being remains unknown. They apparently increase the diversity in the genomic structure of plant annexins. This indicates there may be more diverse functions or regulatory mechanisms associated with the plant annexin family. Notably, two small introns located in coding sequences (CDS) of *GmANN17* and *21* contain only 32 bp (data not shown). The data from both *Arabidopsis* and *Drosophila* strongly suggests that the minimum intron size is about 80 bp within a CDS (Hong *et al.* 2006). However, minimum CDS intron size is 13 bp in 2 903 genes from 10 eukaryotes (Deutsch and Long 1999) and the number of small introns (20 - 59 bp) in *Arabidopsis* and rice is 257 and 1 034 (corresponding to 0.2 and 0.9 % of the total number of introns), respectively (http://www.plantgdb.org/ASIP/pub/Mini_Intron.php). Intron length has been found to affect gene expression including expression level and transcription time (Ren *et al.* 2006, Swinburne *et al.* 2008). Further studies are required to investigate the influence of those small introns on the expression of soybean annexins.

Most soybean annexins have the type II calcium-binding site in the first and/or fourth repeat which is consistent with other known plant annexins (Cantero *et al.* 2006, Jami *et al.* 2012a). However, GmANN14 is essentially devoid of type II calcium binding sites (S4). In addition, GmANN14 containing 370 amino acids (aa) is obviously longer than other plant annexins (< 350 aa) and has a unique fragment composed of 18 uninterrupted serine residues. This protein, therefore, shows extremely low identity (21 - 32 %) to other known annexins and was classified into a separate clade in the phylogenetic tree (Fig. 2). All these findings indicate that GmANN14 might have functions different from other plant annexins.

GmANN12 and its ortholog *ANNAt3* showed different organ-specific expression paterns (Table 1) (Clark *et al.* 2001). Previous studies showed that *AnnBj6* and *7* and their orthologs *AnnAt6* and *7* also exhibit different expression patterns (Clark *et al.* 2001, Jami *et al.* 2009). Actually, expression discrepancy between orthologs generally exists in other plant species. For example, the expression patterns of several potato *HyPRP* (hybrid proline-rich protein) genes do not correlate with those of their *Arabidopsis* orthologs (Dvořáková *et al.* 2007). All these findings indicate the functional diversification of those orthologs from different plants and the limits of extrapolation of knowledge gained by experiments in model plants towards target crop species (Dvořáková *et al.* 2007).

In mustard, all five annexins are classified as late response genes according to their expression profiles under different signaling molecules (Jami *et al.* 2009). However, expression of all *GmANN* genes showed rapid response to ABA (Table 2). Recently, a *Cynanchum komarovii* annexin, *CkANN*, is also found to quickly respond to environmental stimuli (Zhang *et al.* 2011). This discrepancy is probably attributed to different treatment intensity or different response time of these species to the same stress. Considering that some plant annexins are capable of forming Ca^{2+}-permeable channels in an oxidized membrane simulating ROS signaling conditions (Laohavisit *et al.* 2010), these soybean annexins might be the early response genes that are located upstream of the stress signal pathway.

In conclusion, our data suggest the conserved soybean annexins play a role in response to abiotic stresses. Considering that overexpression of *AnnAt1*, *AnnBj1*, and *BcFLC1* (Chinese cabbage *FLOWERING LOCUS C*) genes enhances the resistance to environmental stresses in plants (Jami *et al.* 2009, Zhang *et al.* 2011, Liu *et al.* 2013), it would be interesting to see whether the ectopic expression of *GmANN*s can enhance tolerance to abiotic stresses in plants.

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