Plant Omics Journal POJ

POJ 8(5):420-428 (2015) ISSN:1836-3644

Genome-wide analysis and functional identification of the annexin gene family in maize (*Zea mays* L.)

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Abstract

Annexins have previously been identified and characterized in *Arabidopsis* and rice. They compose a multigene family in plants. In this study, we indicated the isolation and characterization of maize annexin genes across a whole genome using bioinformatics, microarray and real-time PCR methods. A total of 12 members of this family were identified in the maize genome. The 12 maize annexins were distributed on eight maize chromosomes. Multiple alignment and motif display results revealed that most maize annexin proteins contained 1–4 annexin repeats. A phylogenetic analysis indicated the maize annexin gene family could be divided into four subfamilies. In brief, putative *cis*-elements involved in abiotic stress response, phytohormones, pollen-specific elements and seed development were observed in the promoters of maize annexin genes. Microarray data showed that the maize annexin genes had tissue-specific expression patterns in the maize developmental steps. The QRT-PCR analysis result indicated that all 12 genes were induced in the seedling leaves by PEG and NaCl.

Keywords Abiotic stress; Annexin genes; *cis*-elements; Expression behavior; Maize.
Abbreviations: ABA_abscisic acid; DAP_day after pollination; GAPDH_glyceraldehyde-3-phosphate dehydrogenase; ORF_open reading frame; PEG_polyethylene glycol.

Introduction

Annexins are an evolutionarily conserved, homologous, structurally related to superfamily of Ca^{2+}-dependent phospholipid-binding proteins. They exist in some prokaryotes and all eukaryotes with varying number across various organisms (Gerke et al., 2002; Laohavisit et al., 2011; Clark et al., 2012; Jami et al., 2012). Plant annexins were first identified in tomato (Boustead et al., 1989) and the first plant annexin multigene family was detected from the model plant *Arabidopsis* (Clark et al., 2001). In the past decade, studies on plant annexin multigene families were mostly focused on identification and characterization in rice, mustard and tomato (Jami et al., 2008, 2009, 2010; Laohavisit et al., 2011; Lu et al., 2012). Structurally, plant annexins consist of four annexin repeats of approximately 70 amino acids, and only the first and fourth repeats contain the conserved motif of GxGT-(38 residues)-D/E for calcium binding (Geisow et al., 1986). For plant annexins, there are five main *in vitro* protein activities. These functions include nucleotide phosphodiesterase (ATPase and GTPase) activity (McClung et al., 1994), peroxidase activity (Gorecka et al., 2005), cytoskeletal binding (Hayes et al., 2004), regulation of glucan synthesis (Andrawis et al., 1993) and transport (Hofmann et al., 2000; Laohavisit et al., 2009, 2010, 2012). These functions suggested that plant annexins are involved in a wide range of processes including reproductive and vegetative development and responses to environmental stimuli. Differential expression had been observed in the cell cycle (Proust et al., 1999), primary and lateral root development (Clark et al., 2001; Bassani et al., 2004), tuber enlargement (Sheffield et al., 2006), vasculature development (Clark et al., 2001), fiber elongation of cotton (Yang et al., 2008), cork formation (Soler et al., 2007), embryogenesis (Gallardo et al., 2003), pollen and seed germination (Buitink et al., 2006; Dai et al., 2006; Yang et al., 2007) and petal senescence and fruit ripeness (Bianco et al., 2009; Bai et al., 2010). Genetic and transgenic evidence indicated that annexins play a vital role both in abiotic and biotic stresses response (Lee et al., 2004; Jami et al., 2008; Konopka-Postupolska et al., 2009; Divya et al., 2010). For instance, the over-expression of mustard *AnnBj1* enhanced salt and drought tolerance in transgenic cotton and tobacco (Jami et al., 2008; Divya et al., 2010). A recent study indicated that *AnnAt1* and *AnnAt4* function cooperatively in response to drought and salt stresses (Huh et al., 2010). These studies indicated that some plant annexins could be vital candidates for genetic engineering to enhance stress resistance of crops. A recent study performed a genome-wide survey of annexin multigene families in 16 plant species and identified 149 genes. This study was mainly for comparative analyses, gene organization, duplications and structural analyses of the annexin protein (Jami et al., 2012). To indicate the functions of all maize annexin genes, we investigated transcript expression levels of the family genes in various maize tissues and in the seedling leaves under various abiotic stresses. The results presented in this study give an important reference for function studies of annexin family

genes in maize.

Results

Identification of annexin family genes in maize

After careful surveying the maize genome, 12 members were defined as *annexin* genes (Table 1). The maize annexin genes were named *ZmAnn*1 to 12, according to their order in chromosomes 1 to 10. The length of maize *annexin* proteins ranged from 178 aa (amino acids) to 394 aa. BLAST analysis against the Pfam database showed that all of them included the *annexin* domain (accession no. PF00191) (Table 1). The 12 maize *annexin* genes were distributed on eight maize chromosomes: chromosomes 1, 3, 4, 5 and 7, which contained one gene each; chromosomes 2 and 8 contained two genes; and chromosome 6 contained three genes (Fig. 1).

Based on the phylogenetic results (Fig. 1), five paralogs were identified in maize *annexin* genes including *ZmAnn1*/*ZmAnn9*, *ZmAnn2*/*ZmAnn10*, *ZmAnn4*/ *ZmAnn11*, *ZmAnn6*/*ZmAnn7* and *ZmAnn8*/*ZmAnn12* (Fig. 1). The full-length cDNA sequences were compared with the corresponding genomic DNA sequences to determine the numbers and positions of exons and introns within each maize *annexin* gene using GSDS (http://gsds.cbi.pku.edu.cn/chinese.php) (Guo et al., 2007). The number of introns per gene varied from 0 to 8 in 16 plant species (Jami et al., 2012). The genome structure of the maize *annexin genes* showed that most maize *annexins* have five exons (*ZmAnn3, 4, 6, 7, 8, 11* and *12*) (Supplementary Fig. 1).

Motif analysis and protein architecture

In vertebrates, the C-terminal region of these annexins proteins contains four internal repeats of 70 amino acids and a highly conserved protein sequence (GxGT-[38 residues]-D/E) for binding to type-II Ca^{2+}. The N-terminal region of vertebrate annexins is variable in length and possesses sites for post-translational modifications and protein–protein interactions (Gerke et al., 2002). In plant annexins, the N-terminal region was short and the type-II Ca^{2+}-binding residues were absent in repeats 2 and 3 (Clark et al., 1995). Our search in MEME database indicated that most of the maize annexin proteins contained four annexin repeats, except ZmAnn1, ZmAnn 9 and ZmAnn 11 (Supplementary Fig. 2).

Phylogenetic analysis

To investigate the evolutionary pattern and phylogenic relationships among *annexin* in maize (12 genes), rice (10 genes) and *Arabidopsis* (8 genes), the amino acid sequences of *annexin* genes were aligned using ClutalX (1.83) and a phylogenetic tree was constructed with the NJ method by MEGA4 (Fig. 2). All of the proteins fell into four clusters (I, II, III and IV). Cluster I contained 11 members (with four, three and four members of maize, rice and *Arabidopsis*, respectively). Cluster II included five members (with two, two and one members of maize, rice and *Arabidopsis*, respectively). Cluster III contained five members (with two, one and two members of maize, rice and *Arabidopsis*, respectively). Cluster IV had nine members (with four, four and one members of maize, rice and *Arabidopsis*, respectively).

Cis-element analysis

By searching the PLACE database, promoter regions (2 kb range B73 genomic DNA sequences upstream of translation start site) of maize *annexin* genes were analyzed. In this study, a series of *cis*-elements were found that were involved in abiotic stress responses, phytohormones, pollen-specific and quantitative seed development and germination such as ABRE (ACGTG), DRE (GCCGCC), LTRE (CCGAC) (Haberer et al., 2006), LTRE (Hamilton et al., 1998) (CCGAC) and SEF (RTTTTTR) (Allen et al., 1989) motifs (Table 2). An *in silico* sequence analysis showed that 12 annexin genes contain at least two of the five putative *cis*-elements.

Expression profiles of maize annexin family in different tissues and organs

To identify the spatial- and temporal-specific expression patterns of maize *annexin* genes, we explored microarray data that records the gene expression levels of 60 tissues from varying developmental stages of the maize (Sekhon et al., 2011). From the heat map, it can be concluded that all of the 12 detected genes are involved in numerous biological processes and expressed in almost all tissues, but their expression levels were distinct (Fig. 3). Most of them had higher expression in reproductive organs such as cob, tassel, pericarp, whole seed (DAP), endosperm (DAP) and embryo (DAP). The transcript level of *ZmAnn9* was detected at the higher levels in vegetative organs such as primary root, coleoptile, SAM, sheath, different leaves, internode and innermost husk. *ZmAnn8* had higher expression in R2_thirteenth leaf (approximately 10 cm section of the base of thirteenth leaf), whereas *ZmAnn5* was detected at the higher level in V7_tip of stage-2 leaf (approximately 5.0 cm tip of the stage-2 leaf) (Fig. 3).

Expression profiles of maize annexin genes under abiotic stresses

To check whether maize *annexin genes* were responsive to stresses in the seedling stage, real-time quantitative PCR (qRT-PCR) was conducted to analyze the transcriptional expression in all 12 genes in shoots at the three-leaf-stage treated by PEG and NaCl.

The results indicated that all of the 12 genes were induced in the seedling leaves by at least one of the two stresses applied (Fig. 4). Among them, three genes (*ZmAnn2*, *ZmAnn3* and *ZmAnn12*) were obviously induced by PEG stress with distinct patterns (Fig. 4a). For example, the transcript levels of *ZmAnn12* was increased at the early stage of PEG stress and then decreased, whereas the expression level of *ZmAnn2* and *ZmAnn3* increased gradually and reached the highest level at the late stage of the stress. *ZmAnn3, ZmAnn5 and ZmAnn6* were obviously induced by NaCl stress (Fig. 4b). *ZmAnn3 and ZmAnn6* were increased gradually and reached the highest level at 24 h after treatment, whereas *ZmAnn6* was increased at 6 h after NaCl treatment and then was decreased.

Discussion

Annexins are evolutionarily conserved, homologous, structurally related superfamily of Ca^{2+}-dependent phospho-lipid-binding proteins. In this study, 12 genes belonging to the annexins in maize were identified. Among the better-analyzed plant annexin families, *Arabidopsis* likely have eight annexins, rice at least 10 expressed annexins, and sorghum at least 10 annexins (Jami et al., 2012).

Table 1. List of *annexin* genes in maize.

Gene Name	Chr	ORF length	Genbank ID	Protein length	Protein ID
ZmAnn1	1	1098	KR822689	365	AC204530.4_FGP003
ZmAnn2	2	951	KR822690	316	GRMZM2G009136_P01
ZmAnn3	2	975	KR822691	324	GRMZM2G134502_P01
ZmAnn4	3	963	KR822692	320	GRMZM2G031040_P01
ZmAnn5	4	954	KR822693	317	GRMZM2G172834_P01
ZmAnn6	5	945	KR822694	314	GRMZM2G061950_P01
ZmAnn7	6	1023	KR822695	340	GRMZM2G064993_P01
ZmAnn8	6	1176	KR822696	391	GRMZM2G132442_P01
ZmAnn9	6	537	KR822697	178	GRMZM2G132461_P01
ZmAnn10	7	951	KR822698	316	GRMZM2G067752_P01
ZmAnn11	8	987	KR822699	328	GRMZM2G048763_P01
ZmAnn12	8	1185	KR822700	394	GRMZM2G034229_P01

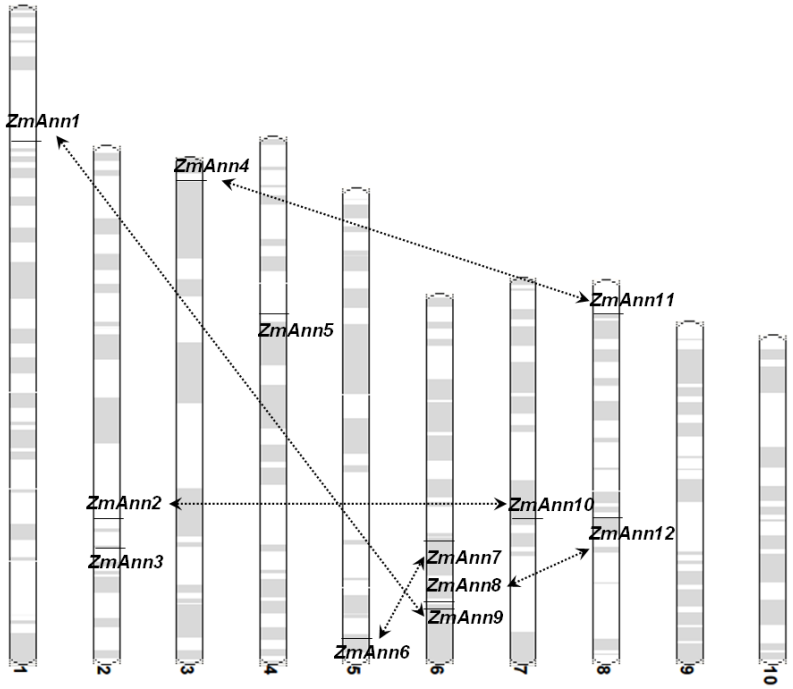

Fig 1. Chromosomal localization of maize *annexin* genes. Segmental duplicates (marked in dotted arrows), including *ZmAnn1*/*ZmAnn9*, *ZmAnn2*/*ZmAnn10*, *ZmAnn4*/ *ZmAnn11*, *ZmAnn6*/*ZmAnn7*, and *ZmAnn8*/*ZmAnn12*.

The genome structure of the maize annexin genes showed that most of them have five exons (Supplementary Fig. 1). Most rice and sorghum *XHS genes* also have five exons, but most *Arabidopsis XHS genes* had six exons (Jami et al., 2012). Our search in the MEME database resulted in 1–4 annexin repeats with different maize annexin proteins. Plant annexins have been detected in all organs and the localization and expression appear linked to growth and development (Mortimer et al., 2008). Cotton annexin *GhANN1* was up-regulated during fiber elongation (Andrawis et al., 1993). *Arabidopsis* and maize annexins have been detected in the root elongation zone (Carroll et al., 1998; Clark et al., 2005). Tomato and tobacco annexins were associated with the expansion of the tomato pericarp (Faurobert et al., 2007) and tobacco cells (Proust et al., 1999). Rice annexin genes were expressed differentially in the root and shoot tissues of young seedlings and were developmentally regulated in seeds (Jami et al., 2012). As in rice, the transcript levels of the twelve maize annexins all had different expression in reproductive and vegetative organs (Fig. 3). The differences in expression patterns of individual annexins suggested the vital spatial functional specificity for maize annexin genes.

Accumulation of evidence from numerous plant species, including *Arabidopsis* and rice, has shown that *annexin* genes were up-regulated by abiotic stress conditions (Laohavisit et al., 2011). However, no such information was available on the response of the annexin family genes in maize. We examined the expression patterns of the members of the maize annexin family genes in the seedlings in response to abiotic stresses salinity (NaCl) and drought (PEG).

Drought stress induced by PEG treatment caused an obvious increase (12-, 17- and 11-fold) in the transcript abundance of *ZmAnn2*, *ZmAnn3* and *ZmAnn12* compared to the untreated control (Fig. 4a). Similar to the expression pattern of salt treatment, the expression of *ZmAnn3* increased significantly by the PEG-induced drought stress (17-fold). There were reports of up-regulation of annexin gene expression in response to drought stress in *Arabidopsis* (Konopka-Postupolska et al., 2009), *Medicago* (Kovacs et al., 1998), rice seedlings (Gorantla et al., 2005) and wheat seedlings (Peng et al., 2009). A significant increase was induced in expression of three annexin genes (Fig. 4b), *ZmAnn3*, *ZmAnn5* and *ZmAnn6*, (7-, 4.5- and 4-fold, respectively) by exposure to NaCl, compared to control.

Table 2. Putative *cis*-elements in the 2 kb upstream promoter region of translation start site in maize annexin genes.

Name	ABRE ACGTG	DRE GCCGCC	LTRE CCGAC	SEF RTTTTTR	QEL AGGTCA
ZmAnn1	5	4	3	2	3
ZmAnn2	1	1	2	0	2
ZmAnn3	4	0	0	0	1
ZmAnn4	0	3	0	4	1
ZmAnn5	0	3	1	1	0
ZmAnn6	5	4	3	3	0
ZmAnn7	1	0	2	7	0
ZmAnn8	1	4	5	2	2
ZmAnn9	8	0	1	1	2
ZmAnn10	3	5	6	1	1
ZmAnn11	2	7	2	1	2
ZmAnn12	2	0	0	5	0

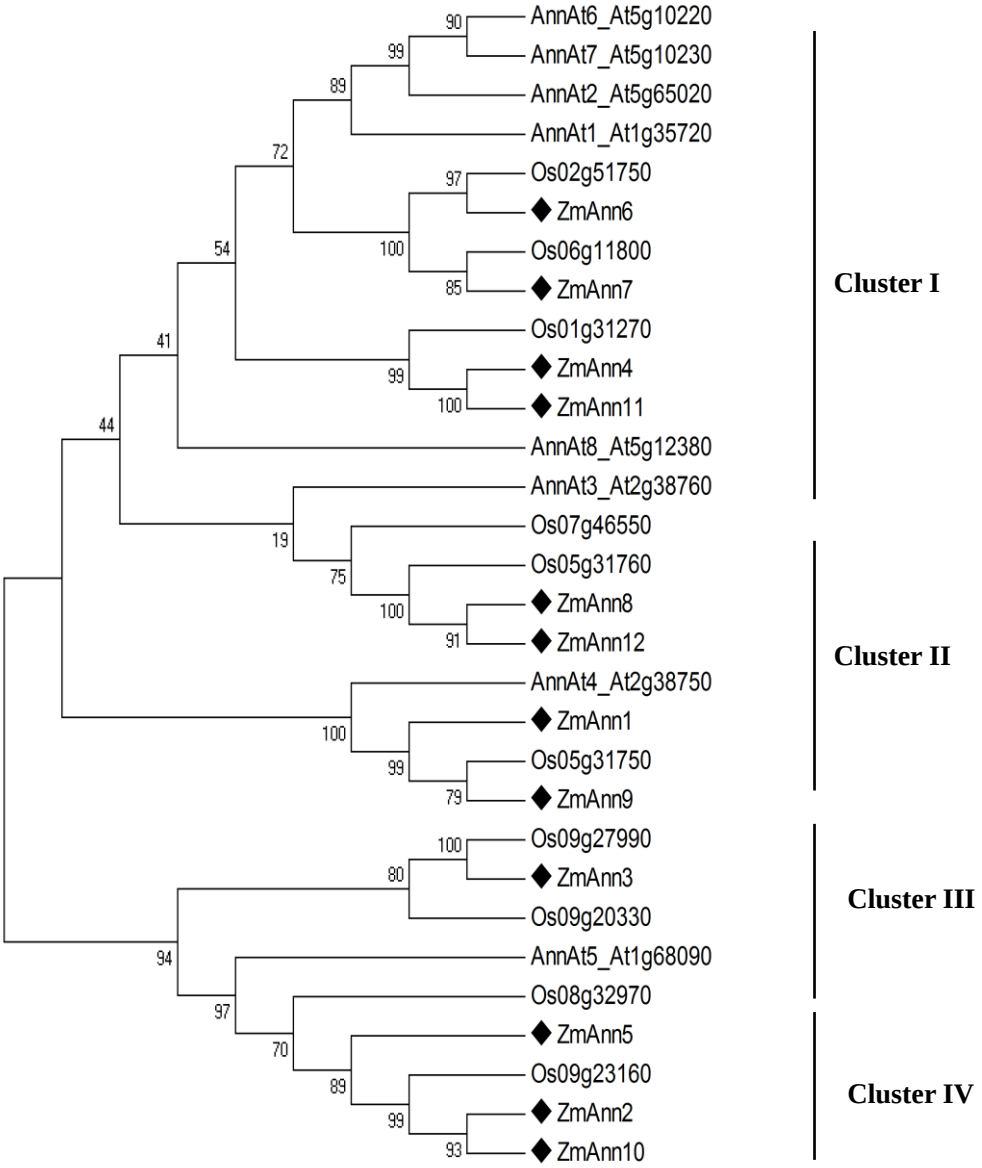

Fig 2. The phylogenetic tree for *Zea mays*, *Arabidopsis* and *Oryza sativa annexins*. The joint unrooted tree was generated using MEGA4 by the NJ method. Bootstrap values from 1,000 replicates are indicated at each branch.

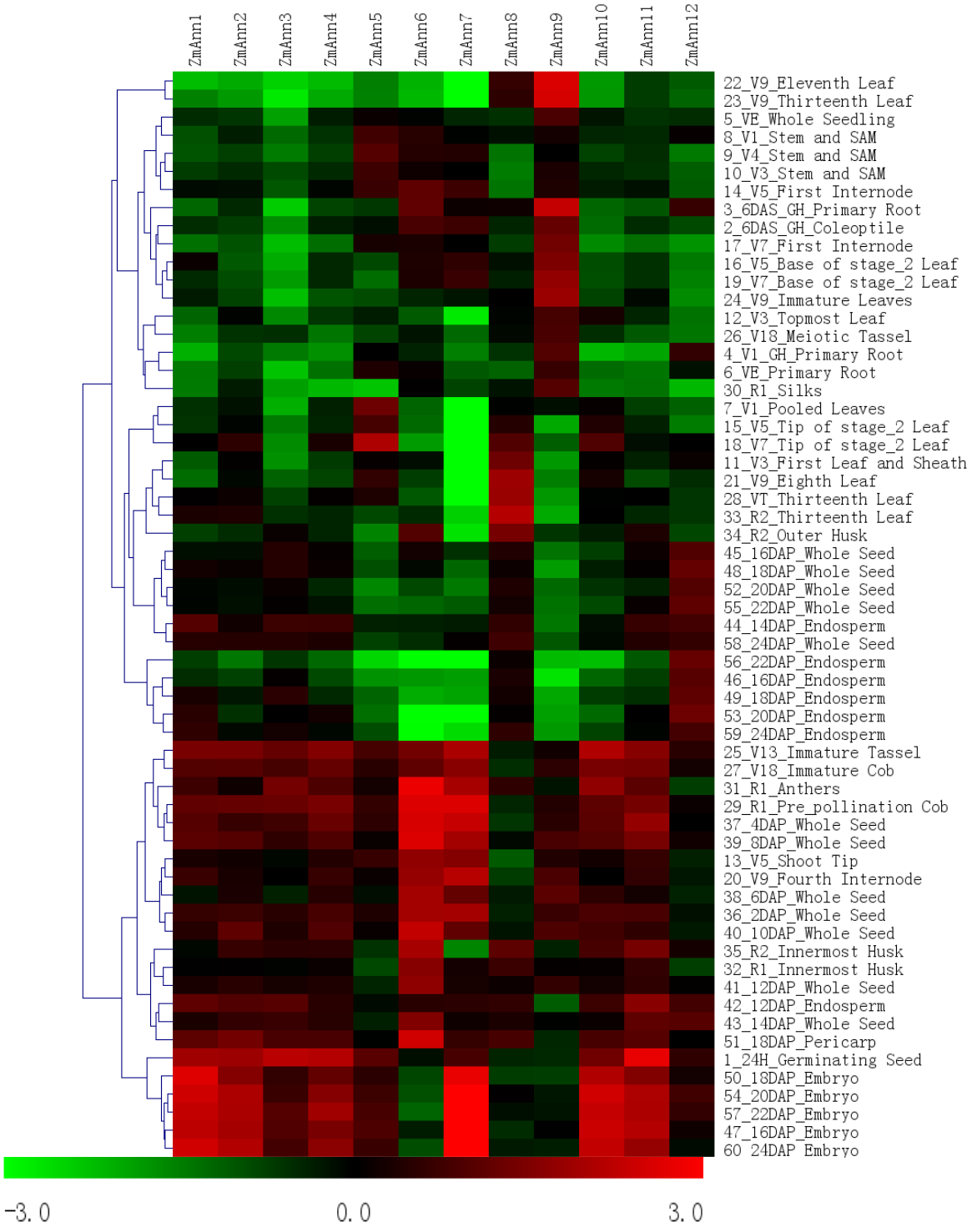

Fig 3. Organ-specific expression patterns of maize *annexin* genes detected in the microarray data. Log2 ratios of expression were used to make this heat map. A red color indicates a higher expression, whereas a green color signifies a lower expression in 60 different tissues.

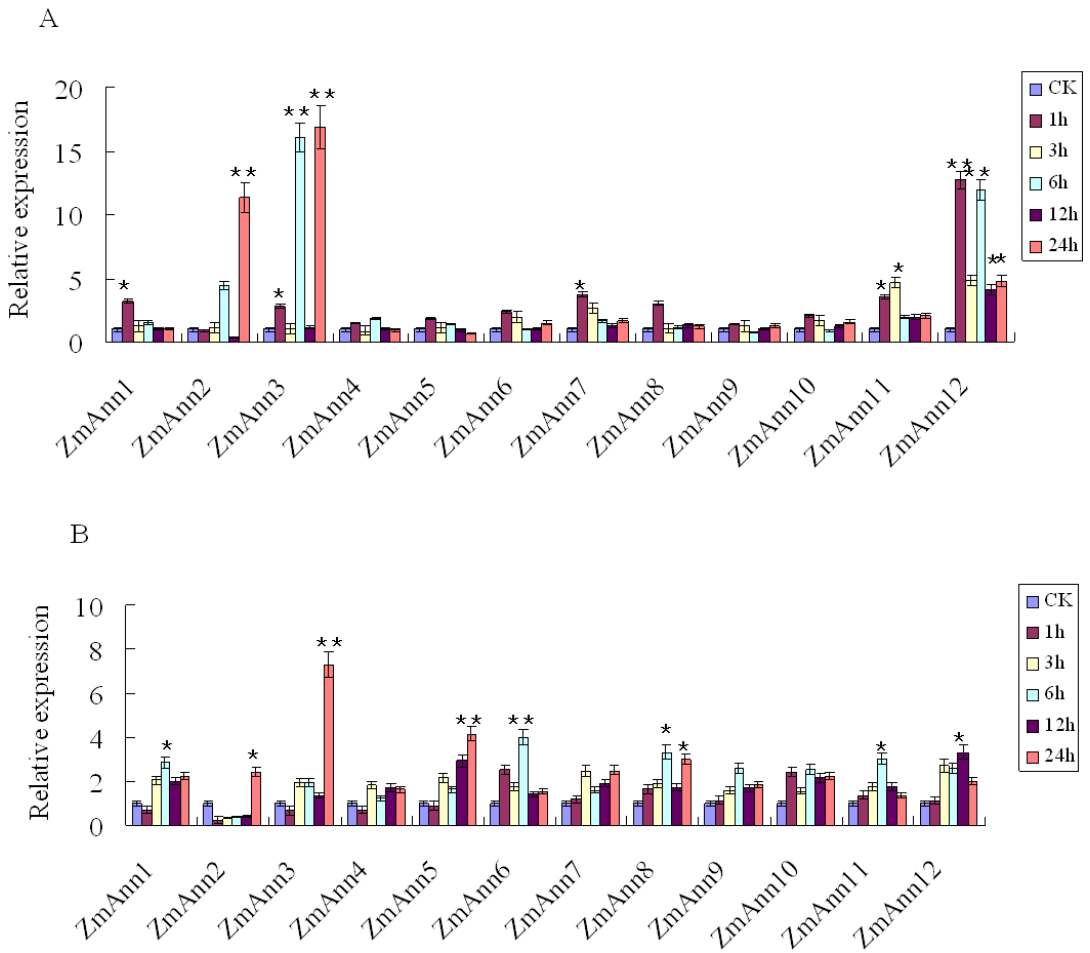

Fig 4. Expression levels of maize *annexin* genes under PEG (A) and NaCl (B) stress treatment, based on real-time quantitative PCR. The X axes are treatment time points and the Y axes are scales of relative expression level. The transcript level at time 0 h (untreated) was used as the calibrator and was given as 1. Error bars indicate ±SE (n=3). * and ** indicate significant differences at the 0.05 level and 0.01 level using the student's t-test, respectively.

In maize and *Arabidopsis*, annexins *AnnAt4–AnnAt8* (Konopka-Postupolska et al., 2009), tobacco annexin *NtAnn12* (Vandeputte et al., 2007) and Brassica annexins, *AnnBj3* and *AnnBj7* (Jami et al., 2009) are up-regulated by exposure to NaCl.

Materials and Methods

Plant materials and stress treatments

Seeds of the maize (*Zea mays*) B73 were surface-sterilized, germinated and hydroponically grown to the three-leaf stage in pots filled with vermiculite, with four seeds per pot. The pots were placed in a greenhouse at 28°C under 16 h light and 20°C under 8 h dark and watered once every three days. For NaCl and PEG treatments, the seedlings were carefully removed from the vermiculite, washed-clean with tap water and dried using filter paper. The roots of the seedlings were then submerged in 200 mM NaCl or 20% PEG (molecular weight 6,000) solution, air conditioned by a pump and sampled at 0, 1, 3, 6, 12 and 24 h, respectively (Zhang et al., 2012). All fresh samples were stored at -80°C after liquid nitrogen treatment, from which RNA was extracted for a differential expression analysis. All the experiments were repeated three times independently.

Isolation and gene structure analysis of Annexin genes in maize

The information on the maize *annexin* genes, including DNA and full length cDNA sequences, ORF length, chromosomal location and protein amino acids were obtained from the B73 maize sequencing database (http://www.gramene.org/Multi/blastview) (Jami et al., 2012; Zhang et al., 2014). Exon and intron structures of maize *annexin* genes were investigated by using the online software GSDS (http://gsds.cbi.pku.edu.cn/) (Guo et al., 2007).

Motif display and phylogenetic analysis of annexin proteins

The MEME (Multiple Expectation Maximization for Motif Elicitation) utility program (Bailey et al., 2009) was used to detect motifs of maize *annexin* proteins. The matrix for phylogenetic analysis included the 8, 10 and 12 *annexin* genes from *Arabidopsis*, rice and maize, respectively. The amino acid sequences of all of the proteins were aligned by using ClustalX2.0 (Larkin et al., 2007) and the unrooted phylogenetic tree was constructed by the neighbor-joining (NJ) method using MEGA4 software (Tamura et al., 2007).

Promoter regions analysis of maize annexin genes

To investigate the *cis*-elements in promoter sequences of maize *annexin* genes, 2000 bp of B73 genomic DNA sequences upstream of the ATG (initiation codon) were downloaded from the NCBI HTGS (high throughput genomic sequences) database. Then, the PLACE (http://www.dna.affrc.go.jp/PLACE/) was used to analyze the *cis*-elements in the promoters (Higo et al., 1999).

Analysis of maize annexin genes expansion patterns

Tandem and segmental duplication have an impact on gene family amplification (Wang et al., 2010). We considered paralogs segmental duplications if they resided within a region of conserved protein-coding genes (Yang et al., 2009). To investigate tandem and segmental duplications, we used Yang's (2009) and Maher's (2006) method (Maher et al., 2006; Yang et al., 2009). To classify apparent expansions of the maize *annexin* gene family, we detected the physical locations of all members of this family.

Microarray data collection and analyses of expression profiles

The expression behaviors of *annexin* genes in maize were analyzed in a set of maize transcriptome data at PLEX database (http://www.plexdb.org). The microarray data of genome-wide gene expression data of the maize inbred line B73 (GSE27004) and the data of transcriptomic analysis of induced senescence in maize were provided by Kaeppler from the University of Wisconsin (Sekhon et al., 2011, 2012). A heat map was used to present the number of maize *annexin* genes, and the map was generated using MultiExperiment Viewer (MeV, version 4.8.1) software. The data were adjusted by the median centering of genes and clustered by the complete linkage clustering method (Zhang et al., 2014).

Transcript level analysis maize annexin genes

Total RNA was extracted using TRIZOL reagent (Invitrogen, Karlsruhe, Germany) and then purified using the DNase I (TaKaRa, Dalian) according to the protocol. Real-time PCR was performed in an optical 96-well plate with an ABI StepOnePlus Real-Time PCR System. Gene-specific primers were designed for all 12 maize *annexin* genes (Supplementary Table 1) and *ZmGAPDH* transcript served as the control (Kozak et al., 1999). Each reaction contained 10 μl of SYBR Premix Ex Taq (TaKaRa, Dalian), 1.0 μl of cDNA samples, 0.4 μl ROX Reference Dye II and 10 μM gene specific primers in a final volume of 20 μl. The thermal cycle used was as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 60 s. For the melt curve, the thermal cycle was 95°C for 15 s, 60°C for 60 s and 95°C 15 s. The analysis of real-time PCR data used the $2^{-\Delta\Delta C}{}_{T}$ method (Livak et al., 2001).

Statistical analysis

Statistical analysis was analyzed following the Student's t-test using SPSS (Chicago). Differences were considered significant at a probability level of $P \leq 0.05$, one asterisk （*）indicated significant difference ($P \leq 0.05$), and double asterisks （**）indicated significant difference ($P \leq 0.01$).

Conclusion

In this study, we have systematically investigated the putative annexin gene family and revealed that the maize genome contained 12 gene members encoding annexin transcription factors. The detailed information on the genomic structures, chromosomal locations, protein architecture, promoter component and phylogenetic analysis among annexin genes in maize, *Arabidopsis* and rice were presented. In addition, the expression profiles of the genes were measured in various maize tissues and seedling leaves under various abiotic stresses. The data presented in this work provide vital clues for further investigating the functions of the genes in the maize annexin family.

Acknowledgments

We are grateful to our editors and reviewers for their helpful comments and the groups who submitted the microarray data to the public expression databases, which can be applied freely. This work was supported partly by the Beijing Nova Program (No. Z121105002512031), the National Special Program of Transgenic Research (2014ZX08003-003B), the Youth Foundation of Beijing Academy of Agriculture and Forestry Sciences (No. QNJJ201303) and the Beijing Academy of Agriculture and Forestry Sciences (No. KJCX20140202).

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