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ORIGINAL PAPER

Genome-wide identification and characterization of maize expansin genes expressed in endosperm

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Abstract By promoting cell wall loosening, expansins contribute to cell enlargement during various developmental processes. Nevertheless, the role of expansins in the expansion and development of endosperm—a major seed component whose cell size is significantly associated with grain yield—is poorly understood. To explore associated biological processes and the evolution of expansins in maize, we performed a systematic analysis of the expansin gene family encompassing gene structure, phylogeny, chromosomal location, gene duplication, and gene ontology. A total of 88 maize expansin genes (*ZmEXPs*) were identified and categorized into three subfamilies according to their phylogenetic relationships. Expression patterns of *ZmEXPs* were also investigated in nine different tissues by semi-quantitative RT-PCR. The expression of eight *ZmEXPs* was detected in endosperm, with five showing endosperm-specific expression. Quantitative RT-PCR was used to analyze expression patterns of the eight *ZmEXPs* in endosperm (10 days after pollination) under abscisic acid (ABA) and gibberellic acid (GA_3) treatments. All eight *ZmEXPs* were found to be significantly regulated by ABA and GA_3 in endosperm, suggesting important roles for these hormones in the regulation of *ZmEXPs* during endosperm development. Our results provide essential information for *ZmEXPs* cloning and functional exploration, which will assist research on expansin-related mechanisms and contribute to future enhancement of maize grain yield.

Keywords Maize · Endosperm · Expansin proteins · Abscisic acid · Gibberellin

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Introduction

Maize is one of the most important cereal plant crops, providing food for humans and animals as well as raw material for industry. A better understanding of endosperm development is vital for increased maize production, as endosperm is an important seed component (Guo et al. 2013) occupying 85 % of seed mass or volume (Lopes and Larkins 1993). Numerous studies have shown that expansin proteins have essential functions in plant developmental processes such as root growth, pollen tube elongation, and leaf expansion. Interestingly, several investigations have revealed that expression of expansins is associated with critical periods of seed (perisperm, endosperm and pericarp) development (Budzinski et al. 2011; Chen et al. 2001). For example, two expansins (*CaEXPA1* and *CaEXP3*) in different isoforms (IAPAR-59 and IAPAR-59 Graúdo) participate in grain size regulation in *Coffea arabica* (Budzinski et al. 2011). Expansins may thus be important factors in the final determination of grain size (Lizana et al. 2010). To date, the roles and expression patterns of expansins in maize endosperm remain unclear. A clearer understanding of maize expansin gene family systematics and the roles of expansins in endosperm

development would provide potentially beneficial insights for maize breeding.

Expansins are involved in plant cell enlargement and other developmental processes, such as endosperm expansion (Lizana et al. 2010), fruit softening, pollen tube growth, and root hair growth (Lee et al. 2001). The regulatory role of expansins in plant cell enlargement has been investigated in a number of plant species. In wheat, RNA *in situ* hybridization revealed that the *TaExpA6* transcript was principally found in the endosperm and pericarp during early grain development. Expansin gene expression profiles were well correlated with the critical period of early grain expansion, suggesting that expansins might be a factor in the final determination of grain size in wheat (Lizana et al. 2010). Transgenic plants overexpressing *OsEXPA8*, a root-specific α-expansin gene in rice, showed pleiotropic phenotypes: improved root system architecture, increased plant height, and increased leaf number and size. Expansins were thus proposed to be required for cell enlargement and cell wall loosening in rice (Ma et al. 2013). In another study, ectopic expression of the expansin gene *NtEXPA5* (Kuluev et al. 2013) increased leaf and stem sizes of transgenic tobacco plants. Further analysis revealed that the phenotypic changes were induced by cell enlargement (Kuluev et al. 2013). An increasing number of studies have demonstrated the involvement of expansin genes in cell growth of various plant species, including *Arabidopsis* (Goh et al. 2014), tobacco (Kuluev et al. 2013), rice (Ma et al. 2013), wheat (Zhao et al. 2012), and maize (Kapu and Cosgrove 2010; Valdivia et al. 2009).

Expansins were first identified through their involvement in the pH-associated mechanism of cell wall enlargement (Cosgrove 2000a; McQueen-Mason et al. 1992). These proteins are ubiquitous in cells throughout the plant world, ranging from green algae to land plants (Cosgrove 2000b). Expansin proteins include signal sequences and comprise two conserved domains (DPPB_1 and Pollen_allerg_1) (Yennawar et al. 2006) that cooperate during cell wall loosening (Sampedro and Cosgrove 2005; Yennawar et al. 2006). DPPB_1 domains share high homology with glycoside hydrolase family 45 (GH45) proteins (Sampedro and Cosgrove 2005), but do not possess enzymatic activity (Lee et al. 2001; Sampedro and Cosgrove 2005), presumably because of the highly specific and transient interaction between expansin and its substrate (McQueen-Mason and Cosgrove 1995). Pollen_allerg_1 domains share homology with group 2 grass pollen allergens, whose physiological roles remain unknown (Cosgrove 2005). No significant sequence similarity exists with any other proteins currently in GenBank. On the basis of conserved aromatic and polar residues on the protein surface, Pollen_allerg_1 domains are thought to bind polysaccharides (Cosgrove et al. 1997).

Low extracellular pH (4.5–6) (Cosgrove 2005) has long been known to activate expansin proteins, which then regulate plant cell enlargement by irreversibly loosening the cell wall (Sampedro and Cosgrove 2005). A hypothesized mechanism for this cell wall loosening under acidic conditions involves transient disruption of the non-covalent bond between hemi-cellulose and cellulose microfibrils by the activated expansins (Cosgrove 2005).

In the cell wall loosening process, expression of expansin genes is regulated by different plant hormones (Lee et al. 2001). These plant hormones exert their influence by stimulating the cell to modify the cell wall pH through control of the plasma membrane proton pump (H^+-ATPase) (Cosgrove 2005) or by other mechanisms (Zhao et al. 2012). In *Arabidopsis*, *AtEXPA2* is exclusively expressed in germinating seeds, suggesting its involvement in control of seed germination (Yan et al. 2014). During seed germination, *AtEXP2* expression increases when seeds are treated with exogenous gibberellin (GA) (Yan et al. 2014). In wheat, treatment of coleoptiles with exogenous abscisic acid (ABA) enhances expansin expression levels, as ABA induces expansin activity (Zhao et al. 2012). Expansin genes have also been found to be responsive to auxin and ethylene (Cho and Cosgrove 2002; Ding et al. 2008; Kwon et al. 2008). These plant hormones (GA and ABA) have been shown to regulate key processes during seed development (Chen et al. 2014).

By suggesting a probable role for expansins in seed development, previous studies of expansin gene expression have set the stage for further research (Wu et al. 2001). The B73 maize genome sequencing project was completed in 2009 (Schnable et al. 2009), thereby providing an important resource for whole-genome annotation and classification and comparative genomics research. Although genome-wide studies have been reported on expansins in *Arabidopsis*, rice (Lee et al. 2001; Lee and Kende 2002), and some other species (Dal Santo et al. 2013), a systematic analysis of gene family members in maize has not been published to date. Furthermore, the function and expression pattern of expansins in maize endosperm development remains unclear. To detect expressed expansin genes, including those specifically expressed in endosperm, we consequently explored the expression pattern of maize expansin genes in different tissues. Expansin genes whose expression was affected by ABA and gibberellic acid (GA_3) during endosperm development were identified, and their spatial expression patterns in endosperm were characterized. Our results should lead to further studies on expansin function in endosperm and should also be applicable to future efforts to improve maize yield.

Materials and methods

Identification and sequence analysis of expansin genes and proteins

Maize, rice, and *Arabidopsis* genome sequences were downloaded from the following databases: http://ftp.maizesequence.org/current/ (maize), http://rapdb.dna.affrc.go.jp/download/irgsp1.html (rice), and http://www.arabidopsis.org/browse/genefamily/index.jsp (*Arabidopsis*). Local genome and protein databases were constructed from these databases using the DNATOOLS program (Zhao et al. 2011b). To identify expansin proteins in these databases, a hidden Markov model (HMM) of the DPBB_1 domain (CL0199), based on the *Arabidopsis* AtEXP1 sequence, was used as a query in the Pfam database (http://pfam.sanger.ac.uk/). The maize genome was searched for sequences containing the DPBB_1 HMM using the BLASTp program ($p = 0.001$) (Lin et al. 2011). Pfam was then used to find additional genes encoding DPBB_1 and Pollen_allerg_1 domains, and the SMART program (http://smart.embl-heidelberg.de/) (Schultz et al. 2000) was used to confirm the presence of DPBB_1 domains. At this stage, all expansin gene family members were compiled for further analyses. Existing names of *ZmEXPs* were used (Table S4), and newly identified members were named according to Kende et al. (2004) (Kende et al. 2004).

Molecular weight and isoelectric point (PI) were calculated for all ZmEXPs using ExPASy (http://web.expasy.org/compute_pi/) (Gasteiger et al. 2005). The SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al. 2011) was used to predict the presence and location of signal peptide cleavage sites (Gan et al. 2011). Cellular roles and gene ontology (GO) categories were predicted using the ProtFun2.2 server (http://www.cbs.dtu.dk/services/ProtFun/) (Jensen et al. 2002, 2003), which relies solely on the protein sequence as input (Jain et al. 2008). A phylogenetic tree of the expansin family was constructed using the neighbor-joining method in MEGA 4.0 software (Tamura et al. 2007) with 1,000 bootstrap replicates. Subgroups were divided into classes based on bootstrap values ≥50 % (Peng et al. 2012). ClustalW was used to align gene and protein sequences (Thompson et al. 1994).

Using gene and cDNA sequences from the B73 genome database as inputs, GSDS (http://gsds.cbi.pku.edu.cn/) (Guo et al. 2007) was employed to predict gene structure. To analyze chromosomal locations, the start and end positions of each open reading frame were obtained from the genome database, with the MapInspect program (http://mapinspect.software.informer.com/) used to visualize this information (Cheng et al. 2012; Peng et al. 2012; Zhao et al. 2011b). Based on chromosomal location, gene clusters were identified that met specific clustering criteria, namely chromosome regions containing two or more homologous genes within 200 kb (Cheng et al. 2012; Holub 2001).

Phylogenetic and chromosomal location analyses were used to identify duplicated genes. For detection of tandem and segmental duplications, paralogs were regarded as tandemly duplicated genes separated by five or fewer gene loci according to the maize B73 genome annotation. Paralogs were designated as segmental duplicated genes if they were placed on duplicated chromosomal blocks as previously proposed by Wei et al. (2007) (Zhang et al. 2011; Zhao et al. 2011b). The number of nonsynonymous substitutions per nonsynonymous site (*Ka*) and synonymous substitutions per synonymous site (*Ks*) were calculated by DnaSPv5.0 (Librado and Rozas 2009; Rozas et al. 2003). To explore different selective constraints, *Ks* and *Ka*/*Ks* ratios for each pair of duplicated *ZmEXP* genes were calculated (Peng et al. 2012). Ratios of nonsynonymous to synonymous nucleotide substitutions (*Ka*/*Ks*) between paralogs were analyzed to determine the mode of selection (Peng et al. 2012).

To identify conserved motifs, all expansin protein sequences were submitted to the MEME program (http://meme.nbcr.net/meme/cgi-bin/meme.cgi) (Bailey et al. 2009) using the following parameters: optimum motif width ≥6 and ≤200, and maximum number of motifs = 20 (Lin et al. 2011). To predict hormone-responsive elements in promoter regions, 2-kb genomic sequences upstream of the transcription start site (ATG) were submitted to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002; Rombauts et al. 1999) and PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) (Higo et al. 1999; Peng et al. 2012). Four putative stress-related *cis*-elements were identified: ABA-responsive, GA-responsive, auxin-responsive, and ethylene-responsive elements.

Plant materials and treatments

The B73 maize line was used to investigate expression profiles. B73 plants were grown in the greenhouse at 28 ± 2 °C under a 14-h light/10-h dark cycle (Zhao et al. 2011b), and nine types of tissues were harvested at the following ages or stages: shoot (7 days), root (10 days), leaf (3 weeks), stem (6 weeks), ear (5–8 cm), silk (6–10 cm), tassel (8–12 cm), endosperm, and embryo (3 weeks after pollination). Tissues were frozen immediately in liquid nitrogen for at least 10 min and stored at −80 °C to preserve RNA for semi-quantitative reverse-transcription PCR (semi-RT-PCR). Plants were treated by spraying 100 nM ABA or GA_3 (Fu et al. 2012; Guan et al. 1996; Kaya et al. 2006) on the leaf surfaces 10 days after pollination (Jin et al. 2013; Lu et al. 2013b), and

endosperm tissue was harvested 0, 3, 6, 12, 24, and 48 h after treatment for quantitative real-time PCR (qRT-PCR).

RNA isolation and semi- and qRT-PCR

Total RNA was isolated from the stored samples using Trizol reagent (Invitrogen) and treated with DNase I (Takara). The first cDNA strand was synthesized using AMV reverse transcriptase (Promega), and the rTaq PCR system (Takara) was used for semi-RT-PCR. Primers were analyzed by Oligo 6 (Table S3A). Expression was normalized against the maize *GAPDH* housekeeping gene for semi-RT-PCR (Chen et al. 2012). Three biological and technical replicates were used for detection of expression patterns by semi-RT-PCR and subsequent qRT-PCR.

An ABI 7300 Real-Time System (Applied Biosystems) was used for qRT-PCR experiments. Primers were analyzed by Primer Express 3.0 (Applied Biosystems) (Table S3B). The first cDNA strand was synthesized using an M-MLV First Strand kit (Applied Biosystems). Reactions contained 2 µl of cDNA sample, 400 nM forward and reverse primers, 10 µl SYBR Select master mix, and sterile water to 20 µl. Thermal cycling was as follows: 50 °C for 2 min, 95 °C for 2 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. To analyze the specificity of each gene, melting curves were constructed by increasing the final temperature from 60 to 95 °C. The maize 18S rRNA gene was used as an internal control in the qRT-PCR experiments. Relative expression levels were calculated as $2_{\mathrm{T}}^{-\Delta\Delta C}$ [where $\Delta C_{\mathrm{T}} = C_{\mathrm{T,\ target}} - C_{\mathrm{T,\ 18S}}$ and $\Delta\Delta C_{\mathrm{T}} = \Delta C_{\mathrm{T,\ treatment}} - \Delta C_{\mathrm{T,\ CK\ (0\ h)}}$]. Relative expression levels ($2_{\mathrm{T}}^{-\Delta\Delta C,\ \mathrm{CK\ (0\ h)}}$) in untreated control plants were normalized to 1 as described previously (Livak and Schmittgen 2001; Peng et al. 2012). Statistical analyses were performed using SDS 1.3.1 (Applied Biosystems).

Results

Identification of expansin genes in maize

Using the conserved amino acid sequences of DPBB_1 HMM (Pfam: CL0199) and Pollen_allerg_1 domains as queries in the BLASTp program, we identified 121 candidate expansin family members in maize. Pfam and SMART analyses were used to confirm the presence of the two domains in all putative expansins, as both domains are required for expansin activity (Yennawar et al. 2006). As a result, 88 expansin genes were confirmed (Table 1). The maize genome was also found to contain a greater number of expansin genes than those present in rice and *Arabidopsis* genomes (Table 1). Maize expansin genes were named consistently and compiled, and their sequence characteristics were analyzed (including sequence ID, number of residues, molecular weight, PI, signal sequence position, and cleavage site) (Table 2). Lengths of the 88 *ZmEXPs* ranged from 175 to 717 bp, with 61 ranging from 250 to 290 bp (Fig. S1a). Lengths of their signal sequences varied between 20 and 30 bp (Fig. S1b).

Table 1 Summary of expansin gene subfamilies in three plant genomes

Category	Maize	Rice	*Arabidopsis*
EXPA	36	33	26
EXPB	48	18	6
EXPLA	4	4	3
EXPLB		1	1
Total	88	56	36

The total numbers of genes in each subfamily are shown

Phylogenetic analysis and evaluation of gene structure and conserved motifs

Based on the above analysis, full-length expansins sequences from maize and *Arabidopsis* were aligned to generate a phylogenetic tree (Fig. S2) for classification of maize expansin genes (Zhao et al. 2011a). According to the evolutionary relationships in this unrooted tree, the 88 ZmEXPs were divided into three subfamilies (Table 1): α-expansin (EXPA), β-expansin (EXPB), and expansin-like A (EXLA). To investigate evolutionary relationships between maize expansin protein family members, an unrooted phylogenetic tree was generated from aligned full-length sequences of all 88 ZmEXPs (Fig. 1a). In the resulting tree, EXPA and EXPB subfamilies contained 11 and 7 subgroups, respectively, with bootstrap support values >50 %. Eleven gene pairs were supported by bootstrap values >95 % (Fig. 1a). Comparative phylogenetic and exon–intron structural analysis (Fig. 1b) revealed that *ZmEXP* genes within each subgroup possessed similar gene structures. Most genes containing at least one intron were relatively short. Of the 88 *ZmEXPs*, 85 contained fewer than four introns (Fig. S3).

Besides containing two conserved domains (DPPB_1 and Pollen_allerg_1), expansin proteins carry several other functional domains. Twenty conserved motifs were identified in the identified ZmEXPs using the MEME program (Fig. S4, Table S1). All 88 ZmEXPs necessarily included some conserved motifs, although their types and locations varied. All proteins contained five motifs (motifs 1–5). Pfam analysis revealed that the DPPB_1 domain included motifs 1 and 3, while the Pollen_allerg_1 domain contained motifs 4 and 5. An additional motif, motif 2, was sited between the two domains DPPB_1 and Pollen_allerg_1.

Table 2 Characteristic properties of *ZmEXP*-encoded proteins

Gene code	Name	Size (aa)	Mw (Da)	PI	Chromosome	Signal position	Cleavage site
GRMZM2G339122_P01	*ZmEXPA1*	253	26,423.44	6.85	3	1–25	AAG-VD
GRMZM2G074585_P02	*ZmEXPA3*	262	27,775.19	8.62	10	1–29	AAC-FS
GRMZM2G106899_P01	*ZmEXPA3-a*	295	31,846.61	9.48	1	1–51	ANA-GH
GRMZM2G368886_P01	*ZmEXPA4*	268	28,753.8	9.38	1	1–27	AAA-RI
GRMZM2G361064_P01	*ZmEXPA5*	245	25,874.98	8.44	6	1–20	AAA-GG
GRMZM2G445169_P01	*ZmEXPA6*	265	28,450.34	9.36	5	1–25	AAA-RI
GRMZM2G066862_P01	*ZmEXPA7*	272	28,752.45	8.05	9	1–24	ASG-SG
GRMZM2G127029_P01	*ZmEXPA8*	259	27,765.62	9.17	9	1–24	ANA-RF
GRMZM5G871013_P01	*ZmEXPA9*	259	28,002.73	9.01	2	1–24	AVG-SE
GRMZM2G126196_P01	*ZmEXPA10*	277	29,590.01	5.71	6	1–35	SSG-GD
GRMZM2G149605_P01	*ZmEXPA11*	247	25,257.2	6.79	3	1–22	TAA-DS
AC219190.3_FGP002	*ZmEXPA12*	265	28,378.25	6.88	9	1–25	GGG-VR
GRMZM2G120724_P01	*ZmEXPA13*	258	27,993.26	8.69	2	1–19	AAG-HQ
GRMZM2G030531_P01	*ZmEXPA14*	274	29,371.61	9.2	1	1–24	AVA-AD
GRMZM2G105844_P01	*ZmEXPA15*	290	30,242.98	9.3	5	1–22	ALS-RG
GRMZM2G139695_P01	*ZmEXPA16*	281	29,853.94	9.14	1	1–24	AVA-AD
GRMZM2G072121_P01	*ZmEXPA17*	260	28,230.47	8.34	10	1–21	ADG-HQ
GRMZM2G173826_P01	*ZmEXPA18*	250	26,421.57	9.12	1	1–22	VRA-GW
GRMZM2G035189_P01	*ZmEXPA19*	252	26,497.78	8.57	1	1–23	AAA-GW
GRMZM2G332412_P01	*ZmEXPA20*	293	30,744.88	7.48	1	1–38	
GRMZM2G115909_P01	*ZmEXPA21*	211	22,469.14	8.42	1	1–36	
GRMZM2G414779_P01	*ZmEXPA22*	252	26,317.57	8.8	1	1–22	VVA-GW
AC234190.1_FGP001	*ZmEXPA23*	252	26,689.83	4.98	3	1–24	AVA-QQ
GRMZM2G094523_P01	*ZmEXPA24*	252	26,715.25	9.26	3	1–22	VAA-AQ
AC233901.1_FGP008	*ZmEXPA25*	254	26,868.27	8.9	9	1–26	AQG-WN
GRMZM2G073373_P01	*ZmEXPA26*	263	27,746.34	8.11	1	1–28	AGA-DD
GRMZM2G069332_P01	*ZmEXPA27*	217	23,282.61	8.44	5	1–23	VAA-DG
GRMZM2G359799_P01	*ZmEXPA28*	240	25,210.6	9.17	5	1–28	VVA-AN
GRMZM2G088791_P01	*ZmEXPA29*	269	28,734.78	9.07	5	1–25	SDA-AV
GRMZM2G146540_P01	*ZmEXPA30*	210	22,032.26	8.97	5	1–21	TMA-AS
GRMZM2G019398_P01	*ZmEXPA31*	254	27,558.09	6.02	6	1–23	ARS-DD
GRMZM2G303937_P01	*ZmEXPA32*	254	27,565.08	6.01	6	1–23	TRS-DD
GRMZM2G374248_P01	*ZmEXPA33*	254	27,531.12	6.02	6	1–23	ARS-DD
GRMZM2G064174_P01	*ZmEXPA34*	254	27,683.3	6.02	6	1–23	ARS-DD
GRMZM2G160314_P01	*ZmEXPA35*	256	27,848.49	5.7	6	1–25	TRS-DD
GRMZM2G450546_P01	*ZmEXPA36*	254	27,512.02	5.88	6	1–23	TRS-DD
GRMZM2G114322_P01	*ZmEXPL1*	272	29,125.22	8.99	2	1–19	ASA-GS
GRMZM2G095968_P01	*ZmEXPL2*	278	30,051.26	8.82	1	1–29	ASA-CD
GRMZM2G026147_P01	*ZmEXPL3*	275	29,492.52	8.76	1	1–29	ADG-CD
GRMZM2G095968_P02	*ZmEXPL4*	282	30,542.83	8.64	1	1–29	ASA-CD
GRMZM2G146551_P02	*ZmEXPB1*	269	29,112.69	8.99	9	1–24	GSC-GP
GRMZM2G146551_P01	*ZmEXPB1-a*	207	22,114.63	9.12	9	1–24	GSC-GP
GRMZM2G094990_P01	*ZmEXPB1a-a*	282	30,542.31	6.36	9	1–28	ARA-QQ
GRMZM2G021621_P02	*ZmEXPB1a-b*	282	31,180.17	8.07	1	1–24	AQC-RE
GRMZM2G082520_P01	*ZmEXPB1a-c*	270	29,127.72	5.19	9	1–29	GSC-AR
GRMZM2G177391_P01	*ZmEXPB1a-d*	281	29,970.77	5.34	1	1–29	
GRMZM2G100931_P02	*ZmEXPB2*	717	75,585.47	10.07	10	1–25	ASS-AS
GRMZM2G100931_P01	*ZmEXPB2-a*	292	30,631.29	9.54	10	1–25	ASS-AS

Table 2 continued

Gene code	Name	Size (aa)	Mw (Da)	PI	Chromosome	Signal position	Cleavage site
GRMZM2G169967_P01	*ZmEXPB3*	268	28,124.88	8.01	5	1–21	CAA-CL
GRMZM2G078279_P02	*ZmEXPB3-a*	274	29,252.58	9.06	2	1–25	VSA-TL
GRMZM2G154178_P01	*ZmEXPB4*	308	32,661.52	9.54	7	1–24	IAC-TR
GRMZM2G103672_P01	*ZmEXPB4-a*	307	32,216.3	5.1	1	1–25	CVS-TE
GRMZM5G859316_P01	*ZmEXPB4-b*	330	34,822.23	4.79	1	1–51	
GRMZM2G013671_P01	*ZmEXPB5*	267	29,263.96	6.2	9	1–26	ASC-AR
GRMZM2G176595_P01	*ZmEXPB6*	276	30,264.35	8.8	1	1–26	ARA-EQ
GRMZM2G342246_P01	*ZmEXPB7*	322	34,946.67	6.23	1	1–16	
GRMZM2G013002_P01	*ZmEXPB8*	266	31,626.6	8.07	9	1–26	GQC-RE
GRMZM2G072886_P02	*ZmEXPB9*	269	29,094.71	9.01	9	1–24	GSC-GP
GRMZM2G007685_P01	*ZmEXPB10*	269	29,109.44	8.25	3	1–30	AWC-GP
GRMZM2G164785_P01	*ZmEXPB10-a*	268	28,953.27	7.97	3	1–30	AWC-GP
GRMZM2G127106_P02	*ZmEXPB10-b*	270	29,341.81	8.82	5	1–31	AWC-GP
GRMZM2G089699_P01	*ZmEXPB11*	316	34,325.59	8.85	9	1–23	
GRMZM2G026956_P02	*ZmEXPB12*	267	27,715.1	5.53	5	1–25	
GRMZM2G148485_P01	*ZmEXPB13*	269	28,008.61	6.28	4	1–17	VAA-LA
GRMZM2G118873_P02	*ZmEXPB14*	265	27,546.99	8.02	9	1–26	ASA-VP
GRMZM2G121308_P01	*ZmEXPB15*	288	30,563.11	9.14	9	1–26	ASA-VP
GRMZM2G056236_P01	*ZmEXPB16*	294	30,659	8.86	5	1–24	IAC-SR
GRMZM2G327266_P01	*ZmEXPB17*	296	31,026.33	8.81	5	1–25	IAC-SR
GRMZM2G095204_P01	*ZmEXPB18*	295	31,640.06	8.92	7	1–24	IAC-SR
GRMZM2G401975_P01	*ZmEXPB19*	265	29,810.96	9.67	10	1–27	VSC-YG
GRMZM2G401983_P01	*ZmEXPB20*	262	26,892.33	5.85	10	1–25	CAS-VE
GRMZM2G405622_P01	*ZmEXPB21*	273	29,351.25	9.48	1	1–18	AVA-HP
GRMZM2G059785_P01	*ZmEXPB22*	264	28,746.8	9.2	9	1–28	AEA-GS
GRMZM2G073260_P01	*ZmEXPB23*	274	30,064.14	9.12	9	1–27	ARA-QP
GRMZM2G097229_P01	*ZmEXPB24*	273	30,200.22	9.06	1	1–29	ASG-GV
GRMZM2G021427_P01	*ZmEXPB25*	267	29,205.83	5.95	1	1–26	GSC-AR
GRMZM2G104013_P01	*ZmEXPB26*	313	34,164.86	6.43	2	1–22	GSC-TR
GRMZM2G474194_P01	*ZmEXPB27*	318	33,908.52	5.2	1	1–32	SDG-TR
GRMZM2G181202_P01	*ZmEXPB28*	269	29,110.67	8.99	9	1–24	GSC-GP
GRMZM2G020852_P01	*ZmEXPB29*	269	29,138.72	8.99	9	1–24	GSC-GP
GRMZM2G181202_P02	*ZmEXPB30*	207	22,112.61	9.12	9	1–24	GSC-GP
GRMZM2G409286_P01	*ZmEXPB31*	175	19,300.42	8.02	5	1–10	
GRMZM2G109973_P01	*ZmEXPB32*	270	29,159.58	8.59	5	1–31	AWC-GP
GRMZM2G110013_P01	*ZmEXPB33*	193	20,871.87	8.04	5	1–53	
AC233881.1_FGP006	*ZmEXPB34*	234	25,271	8.93	5	1–14	AWC-GP
GRMZM2G144856_P01	*ZmEXPB35*	270	29,253.66	8.71	5	1–31	AWC-GP
GRMZM2G110025_P01	*ZmEXPB36*	270	29,316.71	8.59	5	1–31	AWC-GP
GRMZM2G144898_P01	*ZmEXPB37*	274	29,943.28	8.26	5	1–24	AWC-GP

Chromosomal locations and gene duplications

On the basis of gene start positions on each chromosome, the 88 maize expansin genes were found to be unevenly distributed on all 10 chromosomes except for chromosome 8 (Fig. 2). Chromosome 1 contained the largest number of maize expansin genes (23), whereas chromosome 4 contained the fewest (1). In addition, 18 gene clusters including 63 genes were detected on these chromosomes. We searched for evidence of past gene duplication events (tandem and segmental) to elucidate the mechanism(s) responsible for the *ZmEXP* gene family expansion that is believed to have occurred during the process of evolution. According to the phylogenetic analysis and the chromosomal distribution of the *ZmEXP* genes, five gene pairs involved in segmental duplication

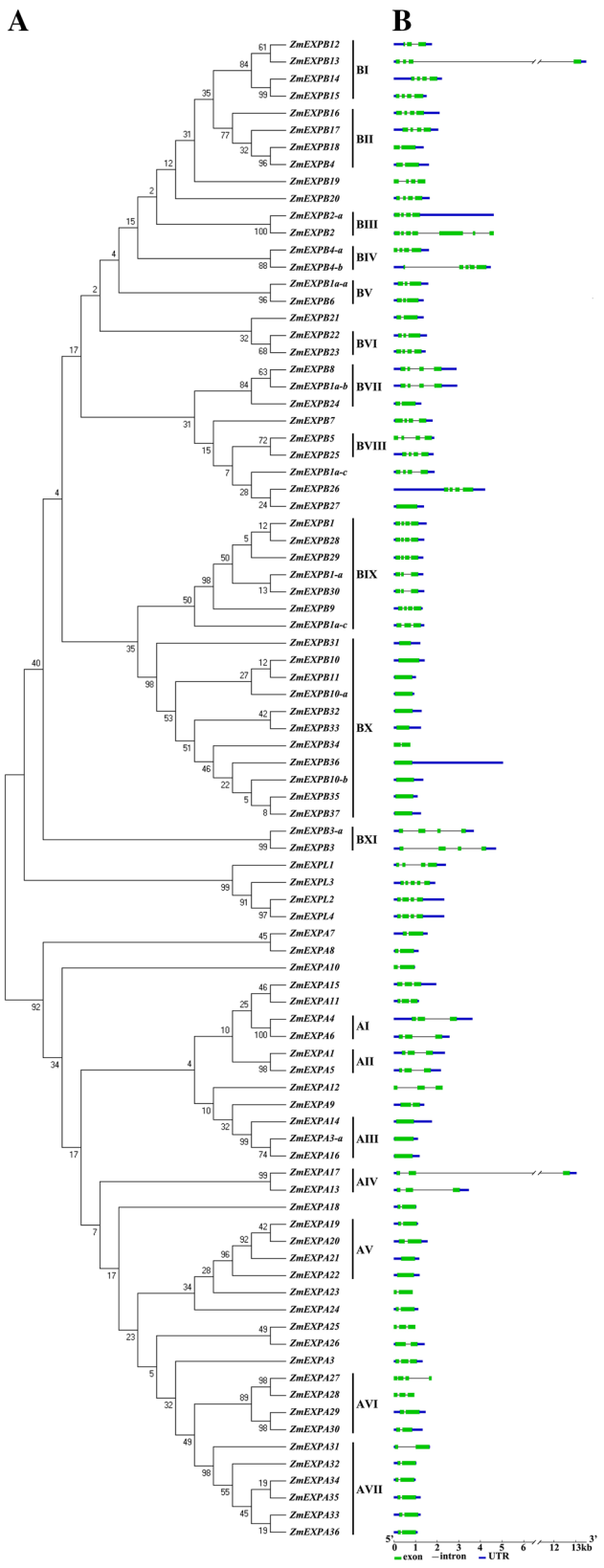

Fig. 1 **a** Phylogenetic relationships of *ZmEXPs*. **b** Exon–intron structures. Exons and introns are indicated by *green rectangles* and *thin lines*, respectively. Untranslated regions (UTRs) are indicated by *thick blue lines* (color figure online)

and five pairs involved in tandem duplication were identified (Table 3).

To explore different selective constraints on the duplicated *ZmEXP* genes, we calculated *Ks* and *Ka*/*Ks* ratios for each duplicated pair. A *Ka*/*Ks* ratio less than 1 generally indicates accelerated evolution with positive selection, a ratio equal to 1 corresponds to neutral selection, and a ratio less than 1 indicates negative or purifying selection. *Ka*/*Ks* ratios of all 10 duplicated pairs were less than 1. Except for the duplicated pair *ZmEXPL2–ZmEXPL4*, ratios were less than 0.4, implying strong purifying selection (Table 3). These results suggest that functions of the duplicated genes have not diverged drastically over the course of genome evolution following the duplication events.

GO analysis

To predict ZmEXP biological functions, cellular roles and GO category annotations were explored using the ProtFun server. The majority of genes fell into the cell envelope functional category and GO categories of stress response and immune response (Fig. 3, Table S2), indicating their possible roles in these processes. In particular, 86 % of ZmEXPs were classified into the cell envelope functional category, and more than 43 % of ZmEXPs belonged to the stress response category.

Detected *ZmEXPs* expressed in endosperm

Expression patterns of the 88 *ZmEXPs* in nine different tissues were studied using semi-RT-PCR (Fig. 4). Of the 88 *ZmEXPs*, only 33 were expressed in any of the nine tissues; eight were expressed in endosperm. We observed that 25 genes were expressed in specific tissues (16 in tassels, 5 in endosperm, 2 in shoots, 1 in ears, and 1 in embryos), but no single gene was expressed in all nine tissues. Some genes were expressed in more than one tissue, such as *ZmEXPA6* expressed in endosperm and embryos and *ZmEXPA4* and *ZmEXPA9* expressed in endosperm, embryos, and roots.

To explore the roles of *ZmEXPs* in endosperm development, qRT-PCR was used to further analyze expression patterns of the eight endosperm-expressed genes (*ZmEXPA4*, *A5*, *A6*, *A9*, *A23*, *A26*, *ZmEXPB12*, and *ZmEXPB13*) (Fig. 4). Many studies have revealed that expansin gene expression is regulated by GA_3 and ABA treatment (Vogler et al. 2003; Wu et al. 2001). For example, increased expression of five α-expansin genes has been observed in rice internodes under GA treatment (Lee and Kende 2002). Expansin expression increases in plants under various abiotic stresses, as exemplified by the induction of *RhEXPA4* promoter activity by ABA (Lu et al. 2013a). We accordingly harvested different stages of endosperm tissue 0, 3, 6, 12, 24, and 48 h after treatment (10 days post-pollination)

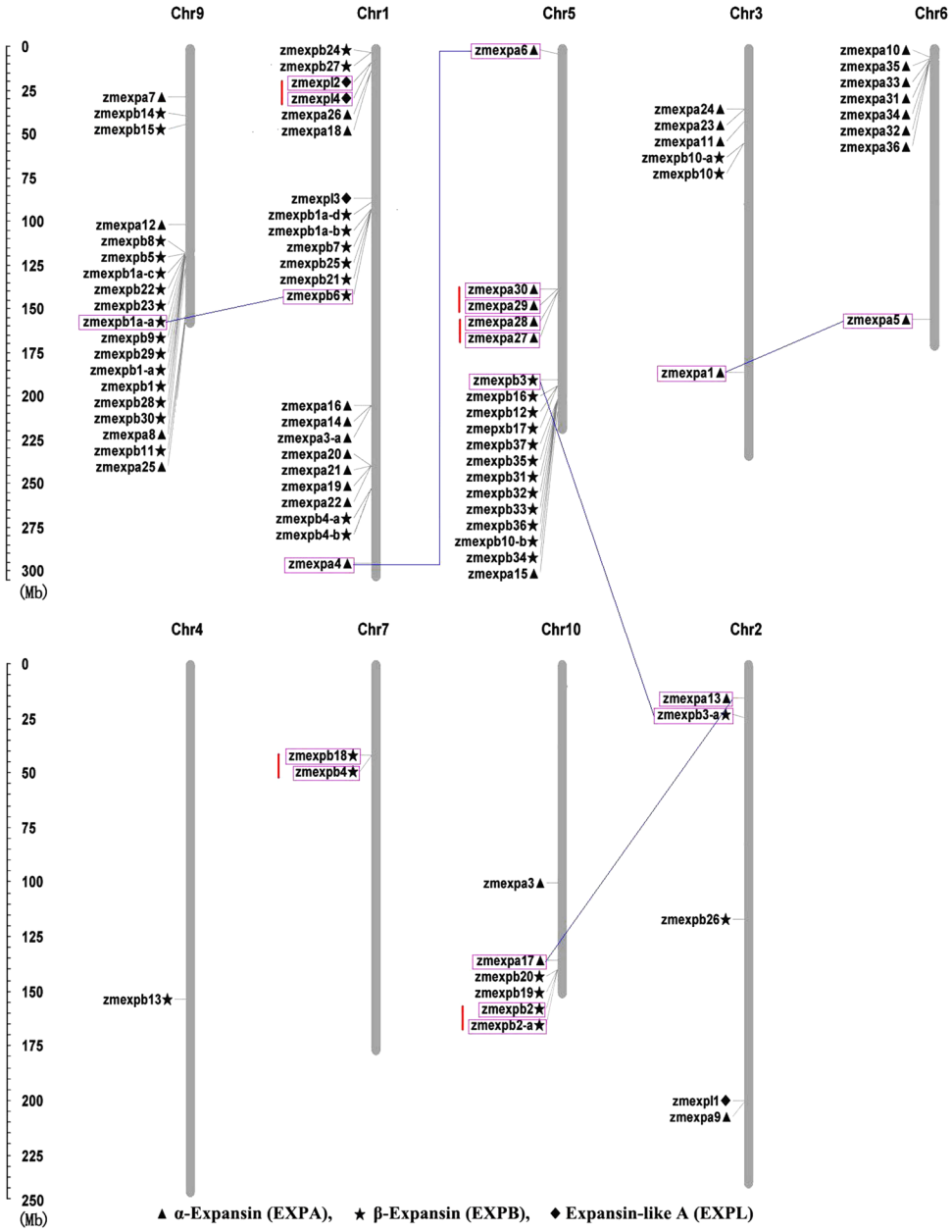

Fig. 2 Chromosomal locations of *ZmEXPs* on nine chromosomes. *Different markers* represent different subfamilies. Chromosome numbers are shown on top of each *vertical bar*. *Names* on the *left side* of each chromosome correspond to the approximate location of each expansin gene. Genes involved in segmental duplication are joined by *dashed lines*, and the *red rectangle indicates* the gene cluster on each chromosome. The scale is in megabases (Mb) (color figure online)

Table 3 *Ka*/*Ks* analysis and estimated divergence times for duplicated *ZmEXP* paralogs

Duplicated pairs	*Ka*	*Ks*	*Ka*/*Ks*	Purifying selection	Duplicate type
ZmEXPA1–ZmEXPA5	0.0679	0.2302	0.295	Yes	Tandem
ZmEXPA4–ZmEXPA6	0.0467	0.1101	0.424	Yes	Tandem
ZmEXPA13–ZmEXPA17	0.0173	0.1751	0.099	Yes	Tandem
ZmEXPA27–ZmEXPA28	0.4295	0.4402	0.976	Yes	Segmental
ZmEXPA29–ZmEXPA30	0.0172	0.0252	0.682	Yes	Segmental
ZmEXPB1a-a–ZmEXPB6	0.0692	0.1577	0.439	Yes	Tandem
ZmEXPB2–ZmEXPB2-a	0.0432	0.0496	0.871	Yes	Segmental
ZmEXPB3–ZmEXPB3-a	0.153	0.397	0.385	Yes	Tandem
ZmEXPB4–ZmEXPB18	0.1484	0.3954	0.375	Yes	Segmental
ZmEXPL2–ZmEXPL4	0	0	0		Segmental

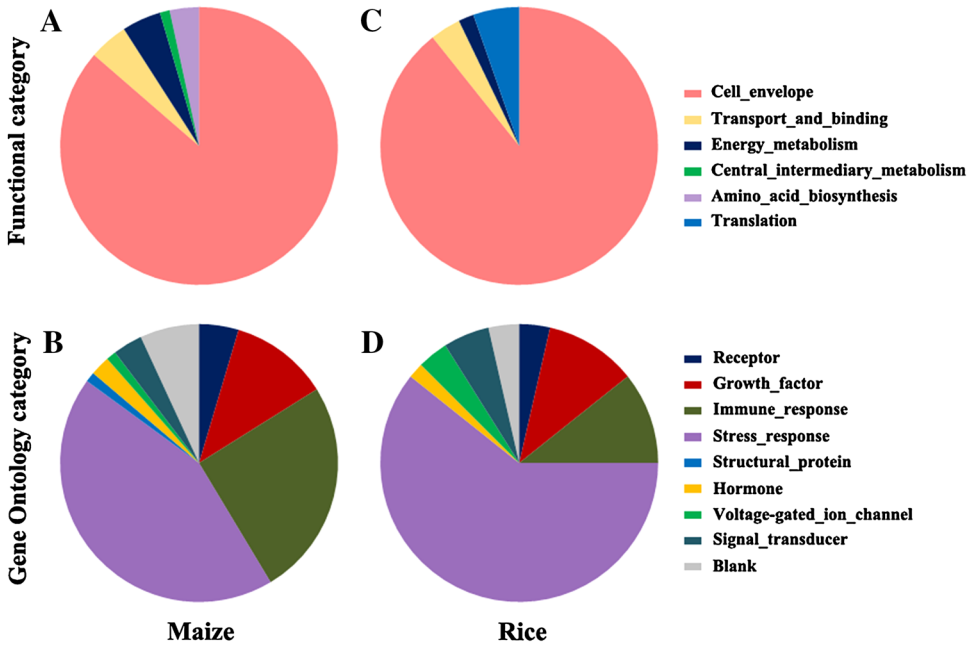

Fig. 3 Functional categorization of maize and rice expansins. Functional category (**a**, **c**) and GO category (**b**, **d**) were determined for maize (**a**, **b**), and rice (**c**, **d**) using ProtFun

with ABA or GA_3. Although *ZmEXPA9*, *ZmEXPA23*, and *ZmEXPA26* were not expressed in the untreated (0 h) endosperm sample, expression levels of all eight genes increased strongly after ABA or GA_3 treatment (Fig. 5). Three hours after treatment with ABA or GA_3, expression levels of all eight genes were noticeably higher than in the control. Expressions were much higher following treatment with ABA than with GA_3, except that the 24-h expression level of *ZmEXPB13* was higher after GA_3 exposure. Most notably, *ZmEXPA4* and *ZmEXPA6* were strongly up-regulated (almost 100- and 200-fold, respectively) 3 h after ABA or GA_3 treatment. These results demonstrate that expression levels of all eight *ZmEXPs* were modified by ABA or GA_3 treatment.

Discussion

Maize is one of the world's most important crop plants, and an increasing body of research has focused on improvement of maize yield. Because endosperm is a major seed component (Guo et al. 2013), regulation of endosperm cell size and volume is a key method for increasing crop yield. On the basis of previous studies, we conclude that expansins play crucial roles in endosperm development by stimulating the loosening of plant cell walls and thereby promoting plant cell enlargement (Cosgrove et al. 2002). The association of expansin expression with grain size dynamics has been reported in wheat (Lizana et al. 2010). In contrast to *Arabidopsis* and other species, however, a genome-wide analysis of the maize expansin gene family has not been reported, and the sequence characteristics and function of maize expansins remain unknown. A search for *ZmEXPs* possibly involved in endosperm development would provide essential information for cloning and functional research of candidate *ZmEXPs* that can be adopted in maize breeding programs to increase yield. In this study, we identified 88 expansins (*ZmEXPs*) in the maize genome using bioinformatics methods and investigated their sequence characteristics and evolutionary relationships. Importantly, expression profiles of *ZmEXPs* in nine different tissues were detected by semi-RT-PCR, allowing us to explore their functions in endosperm development. Previous research has shown that the expression of expansins is regulated by plant hormones such as ABA and GA_3 (Nakaune et al. 2012; Oka et al. 2001; Yan et al. 2014). We therefore used qRT-PCR to investigate expression patterns of *ZmEXPs* showing endosperm-specific expression under ABA or GA_3 treatment at 10 days after pollination (Xin et al. 2013). The qRT-PCR analysis not only confirmed some previously described features of this gene family but also identified some novel characteristics not reported in earlier studies.

To reconstruct the evolutionary history of maize expansins, we generated an unrooted phylogenetic tree of maize, *Arabidopsis*, and rice expansins (Fig. S5). These expansins were divided into four groups (EXPA, EXPB, EXPLA, and EXPLB) sharing a common origin in ancient organisms (Lee et al. 2001; Liu et al. 2011). None of the maize expansins were placed in the well-supported (>90 % bootstrap value) EXPLB subfamily in the phylogenetic tree, indicating that expansin-like A subfamily proteins have evolved more slowly in maize than in the other species. Although genetic relationships between proteins of the

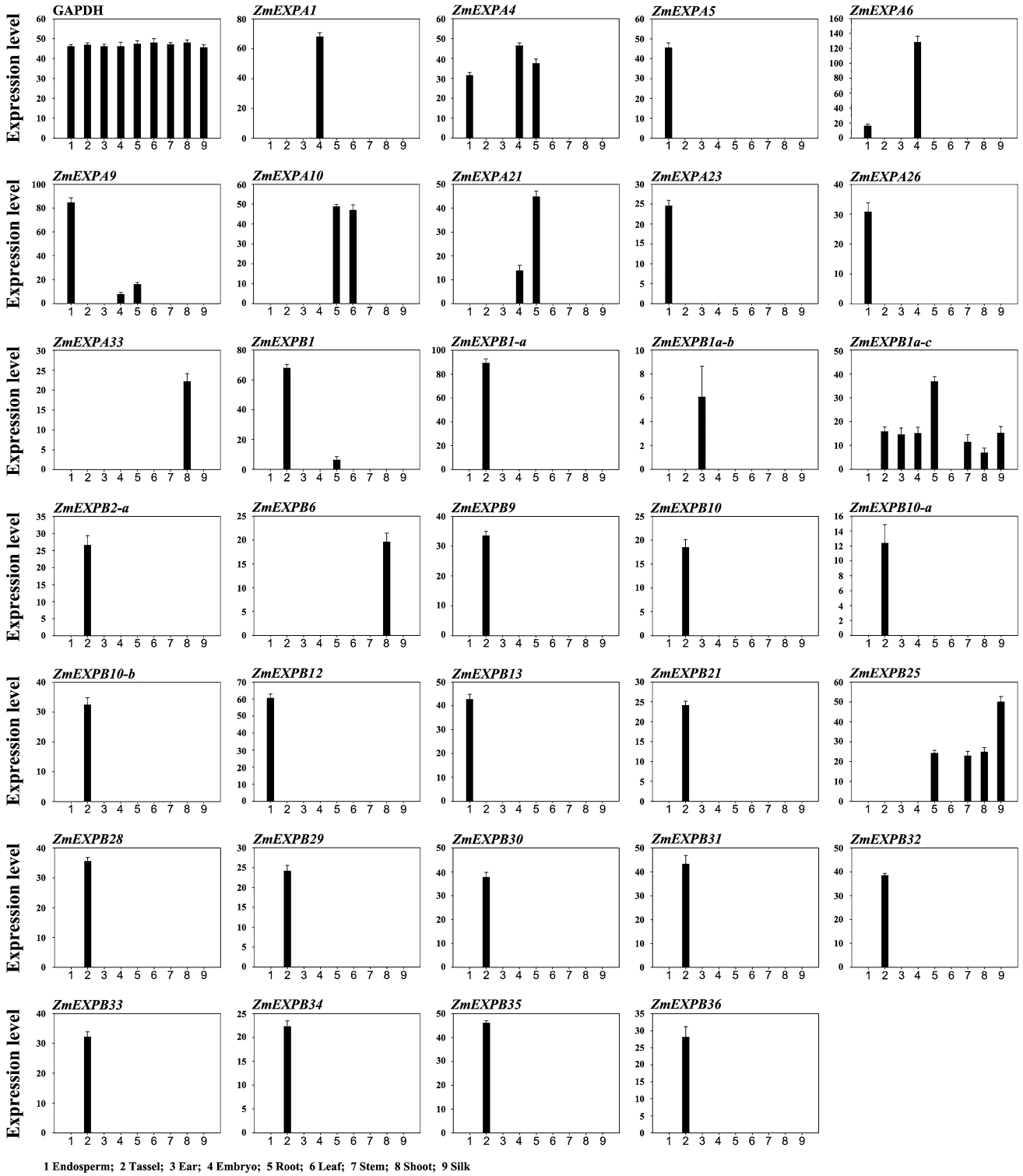

Fig. 4 Relative expression of *ZmEXPs* in nine different tissues (1 = endosperm, 2 = tassel, 3 = ear, 4 = embryo, 5 = root, 6 = leaf, 7 = stem, 8 = shoot, and 9 = silk). Data were normalized against *GAPDH*. Mean ± SE relative expression values were calculated from triplicate measurements

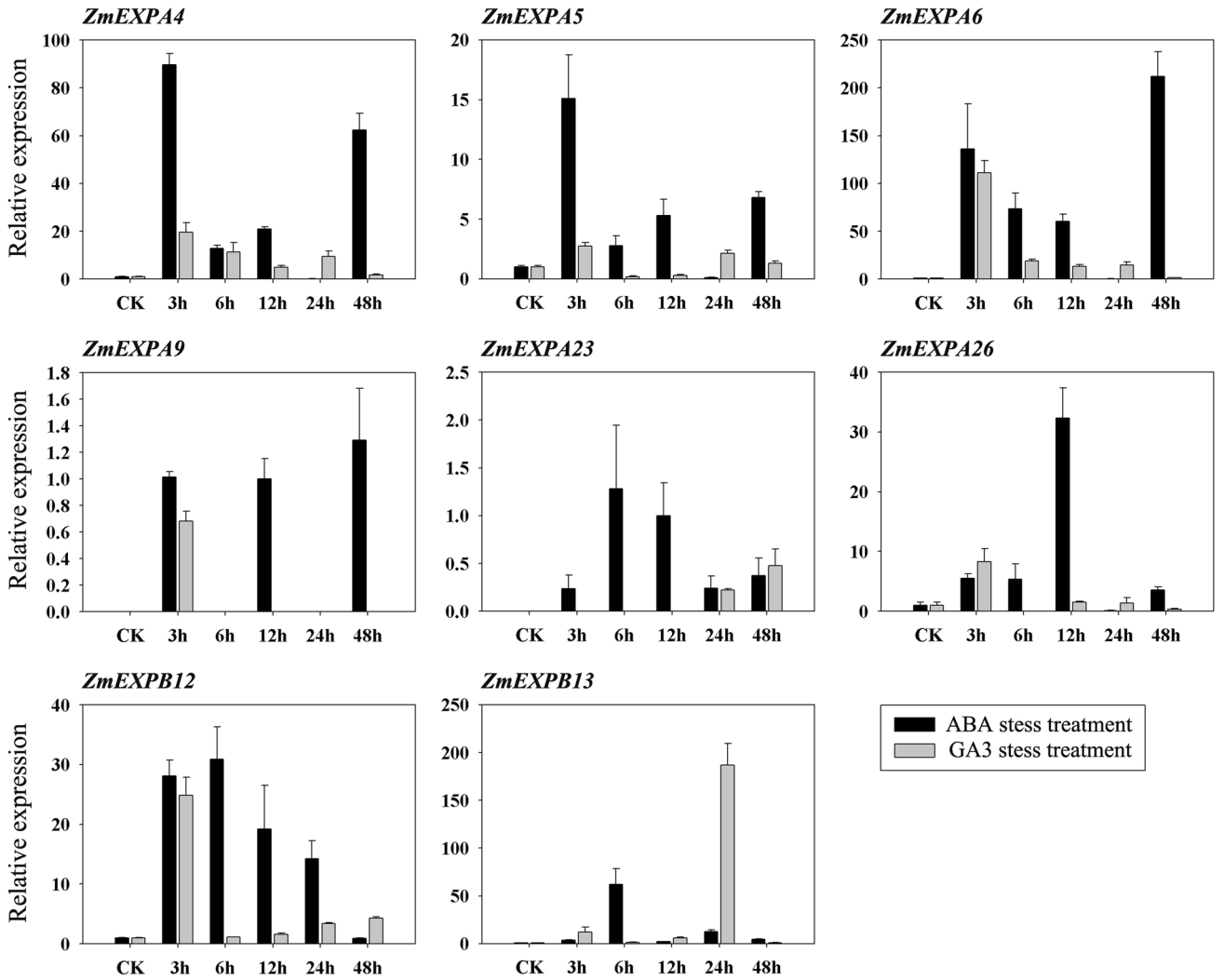

Fig. 5 Expression pattern analysis of eight endosperm-expressed *ZmEXPs* by qRT-PCR following ABA and GA_3 treatments (normalized against maize 18S rRNA). Endosperm was sampled at 0 (no treatment), 3, 6, 12, 24, and 48 h following ABA or GA_3 treatments 10 days after pollination. *Black bar* ABA treatment; *gray bar* GA_3 treatment. *Error bars* show ±SE

three species are very close, maize expansin genes are more closely related to those of rice than of *Arabidopsis* based on the prevalence of ZmEXP ortholog pairs in the phylogenetic tree. For example, ZmEXPs were more closely grouped with OsEXPs than with AtEXPs. This situation is likely due to the diversification of some clades of expansins that accompanied genome evolution following the monocot-dicot split, leading to sequence variation between their members (Peng et al. 2012; Zhao et al. 2011b).

Previous studies have demonstrated that gene duplication has been largely responsible for the expansion of gene families such as CCCH and HD-zip (Peng et al. 2012; Zhao et al. 2011b). In our study, five sister gene pairs of *ZmEXPs* were determined to be involved in segmental duplications, as deduced by shared phylogenetic clade combinations within the same groups and by their locations within segmentally duplicated blocks. An additional five sister gene pairs on the 10 chromosomes were determined to be involved in tandem duplications. We therefore conclude that segmental and tandem duplications are the main contributors to the diversification of the maize expansin gene family. *Ka*/*Ks* ratios calculated for the duplicated gene pairs were all less than 1, indicating that purifying selection may have played an important role in maize expansin gene family evolution (Peng et al. 2012).

Expansin-associated regulation of cell enlargement has been linked to a variety of developmental processes (Cosgrove 2005; Lee et al. 2001; Sampedro and Cosgrove 2005). Although many studies have addressed the function of expansins in numerous plant species, the involvement of ZmEXPs in endosperm development has not been reported. We therefore initiated this study to investigate the

functions of maize expansins in future study. Information regarding their functions was gleaned from known functions of rice proteins (Jain et al. 2008) and by comparative analyses using the ProtFun program (Table S2). The majority of maize and rice expansins fell into the cell envelope functional category, although the exact percentages varied between species and proteins.

To further confirm the involvement of expansin genes in seed development (Wu et al. 2001), we analyzed their expression patterns. Fifty-five of the 88 *ZmEXPs* were not expressed in any of the nine tissues studied. The low proportion of expressed genes may indicate that the expression of some *ZmEXPs* is highly specific and limited to a single organ or cell type (Wu et al. 2001). Alternatively, expression levels of some genes may have been lower than the detection limit of this study. Nevertheless, eight *ZmEXPs* were observed to be expressed in endosperm, with five of them exhibiting endosperm-specific expression.

The above results provide important information for future research on the roles of expansins in endosperm. Although regulation of expansin expression by auxin, GA_3, ABA, ethylene, and cytokine has been reported (Lee et al. 2001), no analyses have been conducted on hormone-responsive *cis*-elements of expansin gene families of any plant species. Our analysis of promoter regions revealed the presence of known ABA-, auxin-, GA-, and ethylene-responsive elements. Even more exciting, almost all of the 2-kb promoter sequences (Peng et al. 2012) of the 88 *ZmEXPs* contained GA_3- or ABA-responsive elements (Fig. 6, Table S5). This result indicates that ABA and GA_3 are the main hormone-responsive elements involved in *ZmEXP* expression. The eight *ZmEXP*s expressed in endosperm contained at least one GA_3- or ABA-responsive *cis*-element in their promoter regions, strongly suggesting their involvement in two hormone-responsive regulatory pathways (Lee et al. 2001) and the two signaling pathways that regulate development. On the basis on these analyses, we postulated that the two hormones upregulate the genes via pH modification (Cosgrove 2005; Zhao et al. 2012). Both ABA and GA_3 have been previously linked to pH regulation of seed development (Sampedro and Cosgrove 2005). To test this hypothesis, expressions of the eight *ZmEXPs* in endosperm under ABA and GA_3 treatment were studied using qRT-PCR. All eight *ZmEXPs* were sharply upregulated after ABA or GA_3 treatment. The sensitivity of these *ZmEXP* genes to ABA and GA_3 treatment suggests important roles for ABA and GA_3 in the regulation of the eight *ZmEXPs* during endosperm development and also indicates a potential role in the control of adaptive cell wall modification under treatment (Dal Santo et al. 2013). We plan to experimentally examine the biological functions of *ZmEXPs* in future studies (Fig. 6).

In conclusion, we identified and characterized expansin genes in maize by analyzing their structural diversity, chromosomal distribution, GO annotations, and phylogenetic relationships, and subsequently investigated their expression patterns. Eight *ZmEXPs* were observed to be expressed in endosperm, with five exhibiting endosperm-specific expression. Notably, almost all promoter regions of the eight *ZmEXPs* contained one or more GA_3- or ABA-responsive elements. This finding, which was subsequently verified by qRT-PCR expression analysis of these genes under ABA or GA_3 treatment in endosperm, suggests important roles for ABA and GA_3 in the regulation of *ZmEXPs* during endosperm development. Our comprehensive and systematic analysis of the maize expansin gene family and expression patterns of *ZmEXPs* in endosperm development has provided essential information for further

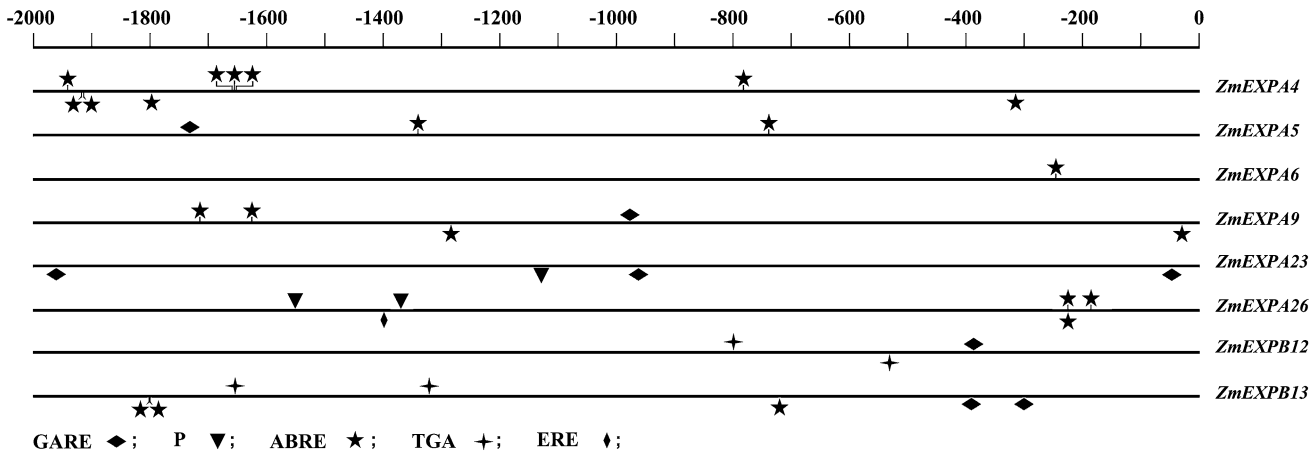

Fig. 6 ZmEXP promoter region hormone-responsive elements. *Cis*-elements on sense and reverse strands are shown above and below black lines, respectively. ABA-responsive elements (ABRE), GA-responsive elements (GARE, P), auxin-responsive elements (TGA), and ethylene-responsive elements (ERE) are indicated by *different markers*

research on the functions of *ZmEXPs* in the endosperm development process. These findings should assist research into expansin-related mechanisms and aid efforts to improve maize yield.

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