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# Maize Membrane-Bound Transcription Factor Zmbzip17 is a Key Regulator in the Cross-Talk of ER Quality Control and ABA Signaling

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**Abiotic stresses disrupt protein folding and induce endoplasmic reticulum (ER) stress, which in turn activates the unfolded protein response (UPR) to aid in the refolding or degradation of misfolded proteins. The phytohormone ABA regulates many aspects of plant development and plays a central role in the stress response; however, the role of ABA in transducing stress signals to activate the UPR has not been recognized. In this study, a gene encoding the maize ortholog of AtbZIP17, a transmembrane transcription factor functioning as an ER stress transducer, was identified from the MaizeGDB database, and designated *ZmbZIP17*. *ZmbZIP17* was induced by both ABA and ER stress-eliciting agents such as dithiotreitol (DTT) and tunicamycin (TM). Transiently expressed yellow fluorescent protein (YFP)–ZmbZIP17 co-localized with the ER marker HDEL–mCherry under control conditions, but partially translocated into the nucleus upon TM treatment or removal of the transmembrane domain. TM-induced processing of ZmbZIP17 was confirmed by Western blot analysis. When overexpressed in Arabidopsis, *ZmbZIP17* triggered ER stress response gene expression and tolerance to DTT and TM, elevated ABA-responsive gene expression and ABA sensitivity both pre- and post-germination. Additionally, ABA was found to induce ER stress response gene expression, alone or synergistically with ZmbZIP17, in the absence of DTT or TM; while ZmbZIP17 was capable of interacting with ABA-responsive *cis*-elements (ABREs) that exist in promoters of known ABA-responsive genes. Together, our results reveal a direct link between ER stress and ABA signaling pathways involving the ZmbZIP17 transcription factor.**

**Keywords:** ABA • bZIP • ER • Maize • Membrane-associated transcription factor • Signaling.

**Abbreviations:** ABRE, ABA-responsive element; 3-AT, 3-aminotriazole; ATF6, activating transcription factor 6; BiP, luminal binding protein; bZIP, basic domain/leucine zipper; CDS, coding DNA sequence; CNX, calnexin; CRT, calreticulin; DAPI, 4′,6-diamidino-2-phenylindole; DTT, dithiotreitol; ER, endoplasmic reticulum; ERdj3b, stromal-derived factor 2 (SDF2)-heat shock protein 40/ER lumen-localized Dnaj protein 3b; ER-QC, ER quality control; GAL-AD, GAL4 activation domain; GFP, green fluorescent protein; IRE1, inositol requiring enzyme 1; MS medium, Murashige and Skoog medium; NLS, nuclear localization signal; PDI, protein disulfide isomerase; PERK, double-stranded RNA-activated protein kinase (PKR)-like ER kinase; PPT, phosphinothricin; RT–PCR, reverse transciption–PCR; TM, tunicamycin; TMD, transmembrane domain; UPR, unfolded protein response; WT, wild type; YFP, yellow fluorescent protein.

## Introduction

The endoplasmic reticulum (ER) is a major site of synthesis and processing of all secretory proteins and membrane proteins in eukaryotic cells. The mechanisms termed ER quality control (ER-QC) safeguard the correct folding and assembly of secretory proteins to maintain ER homeostasis by at least three systems: the STROMAL-DERIVED FACTOR2 (SDF2)-HEAT SHOCK PROTEIN40/ER LUMEN-LOCALIZED DnaJ PROTEIN3b (ERdj3b)–LUMINAL BINDING PROTEIN (BIP) complex, the CALRETICULIN/CALNEXIN (CRT/CNX) cycle and the protein disulfide isomerase (PDI) system (Anelli and Sitia 2008). When normal protein folding or secretory processes are disturbed and unfolded or misfolded proteins accumulate in the ER, ER stress occurs, which activates a signaling network called the unfolded protein response (UPR) to aid in protein folding or in degradation of misfolded secretory proteins by producing protein folding factors and other factors (Ron and Walter 2007, Vitale and Boston 2008). The regulatory mechanism of UPR has been extensively explored in yeasts and metazoans (Walter and Ron 2011). Three principal components have been identified, namely inositol requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase (PKR)-

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like ER kinase (PERK) and activating transcription factor 6 (ATF6). In eukaryotic cells, ATF6 plays a predominant role. ATF6 is a type II transmembrane protein normally retained in the ER through association with the binding protein BiP/GRP78, and translocated into the nucleus for targeting stress response genes through proteolytic cleavage upon ER stress (Shen et al. 2002). In addition, the signaling transduction of IRE1 involves a non-conventional RNA splicing event and the PERK pathway regulates translation (Walter and Ron 2011).

In plants, bZIP (basic domain/leucine zipper) transcription factors that are involved in the UPR have been identified, including AtbZIP60 (Nagashima et al. 2011), OsbZIP50 (Hayashi et al. 2012), ZmbZIP60 (Lu et al. 2012, Wang et al. 2012), AtbZIP17 (Liu et al. 2007b), AtbZIP28 (Liu et al. 2007a, Gao et al. 2008, Tajima et al. 2008), OsbZIP39 (Takahashi et al. 2012) and OsbZIP60 (Hayashi et al. 2013). It has been reported that *AtbZIP60*, *OsbZIP50* and *ZmbZIP60* are activated by IRE1-catalyzed mRNA splicing (Nagashima et al. 2011, Hayashi et al. 2012, Lu et al. 2012). The others have a transmembrane domain (TMD), as does ATF6, and are activated by proteolysis mediated by the proteases S1P and S2P during the UPR, and thus transduce stress signals from the ER to the Golgi and then to the nucleus (Liu et al. 2007a, Tajima et al. 2008, Takahashi et al. 2012). In contrast to *AtbZIP28* and *OsbZIP39*, *AtbZIP17* is not induced by tunicamycin (TM) and dithiothreitol (DTT) (Tajima et al. 2008). However, in response to salt stress, the membrane-bound AtbZIP17 was activated by S1P-dependent proteolysis, and translocated to the nucleus to activate salt-responsive gene expression (Liu et al. 2007b). The constitutive expression of the TMD-truncated *AtbZIP17* activated the expression of stress response genes under unstressed conditions (Liu et al. 2008b). The involvement of AtbZIP17 in salt signaling was further supported by the salt-sensitive phenotype of an *AtbZIP17* loss-of-function mutant and the salt-tolerant phenotype of *RD29A::AtbZIP17ΔC* transgenic Arabidopsis seedlings (Liu et al. 2007b, Liu et al. 2008a).

Abiotic stresses have been shown to disturb protein folding and ER homeostasis. The phytohormone ABA regulates many aspects of plant development, including induction of seed dormancy and inhibition of the developmental transition from the mature embryo towards a young seedling, and plays a central role in plant adaptation to environmental challenges, such as drought, salt, hypoxic and cold stresses (Rock 2000, Shinozaki and Yamaguchi-Shinozaki 2000, Hauser et al. 2011). Members of bZIP transcription factors, such as ABI5, ABF2/AREB1, ABF4/AREB2, AREB3, GBF4, ABF1 and ABF3, are key regulators of ABA-dependent gene expression, via binding to ABA-responsive elements (ABREs) (Fujita et al. 2005, Yoshida et al. 2010, Liu et al. 2012). Recently, the core signaling pathway of the PYR/PYL/RCAR–ABA–PP2C module has been discovered, which activates a complex signal network in response to various developmental and environmental factors (Santiago et al. 2012). However, the role of ABA in regulating the ER stress response has not been addressed.

Maize is one of the most important crops for livestock and humans worldwide. Its growth and development are seriously affected by adverse environmental conditions. Abiotic stresses including drought, heat stress and salinity disturb protein folding and trigger ER stress (Ron and Walter 2007). So far, the characterization of ER stress regulators in maize has been limited to ZmbZIP60 (Li et al. 2012, Wang et al. 2012). In this study, we identified a maize homologous protein of AtbZIP17, which was designated ZmbZIP17. We found that *ZmbZIP17* is also regulated by ABA. Phenotypic characteriztion and downstream gene expression assays of the transgenic seedlings ectopically expressing *ZmbZIP17* suggest a role for ZmbZIP17 in the cross-talk of ER-QC control and ABA signaling.

## Results

### Cloning and sequence analysis of *ZmbZIP17*

In an attempt to investigate ER stress regulation in maize, we performed a search for homologs of bZIP factors that were known to function as ER stress transducers by querying the maize genome database MaizeGDB. A gene (accession No. BT040011) encoding an AtbZIP17 homolog was identified and was thus designated as *ZmbZIP17*. An ABRE (5′-TGCGCG TACG-3′) was found in the promoter of *ZmZip17*, suggesting that this gene might be regulated by an ABA-dependent signal pathway. A schematic diagram of the *cis*-elements located in the promoter region of *ZmbZIP17* is shown in **Supplementary Fig. S1a**. *ZmbZIP17* cDNA was amplified and cloned from leaves of the maize inbred line B73 and subsequently sequenced. The cloned cDNA is 1,914 bp in length, with a coding DNA aequence (CDS) located in the region from 120 bp to 1,811 bp. Compared with the sequence deposited in GenBank, the amplified cDNA contained 12 nucleotide differences, resulting in eight amino acid differences between the encoded proteins. None of these amino acids was distributed in the bZIP domain or TMD; however, one difference was found to be located in the predicted S1P site (**Supplementary Fig. S1b**). ZmbZIP17 shared the typical bZIP domain, TMD, nuclear localization signal (NLS) and the canonical S1P site with the other ATF6- counterpart bZIPs in Arabidopsis and rice, including OsbZIP60, OsbZIP39, AtbZIP17, AtbZIP49 and AtbZIP28 (**Fig. 1a**, **b**). An unrooted phylogenetic tree was constructed, which indicated that ZmbZIP17 was more closely related to AtbZIP17 in Arabidopsis and OsbZIP60 in rice (**Fig. 1c**).

### Subcellular localization of ZmbZIP17

ZmbZIP17 was predicted to have a TMD, implying that the transcriptional factor is associated with the ER membrane. To determine the subcellular localization, *ZmbZIP17* was fused in-frame to the C-terminus of yellow fluorescent protein (YFP), and the resulting construct (YFP–ZmbZIP17) was transiently co-expressed with the known ER marker HDEL–mCherry in epidermal cells of *Nicotiana benthamiana* leaves. Confocal laser scanning microscopy of living cells revealed that the YFP–ZmbZIP17 signal overlapped with HDEL–mCherry, suggesting that ZmbZIP17 is ER localized (**Fig. 2a**). Upon TM

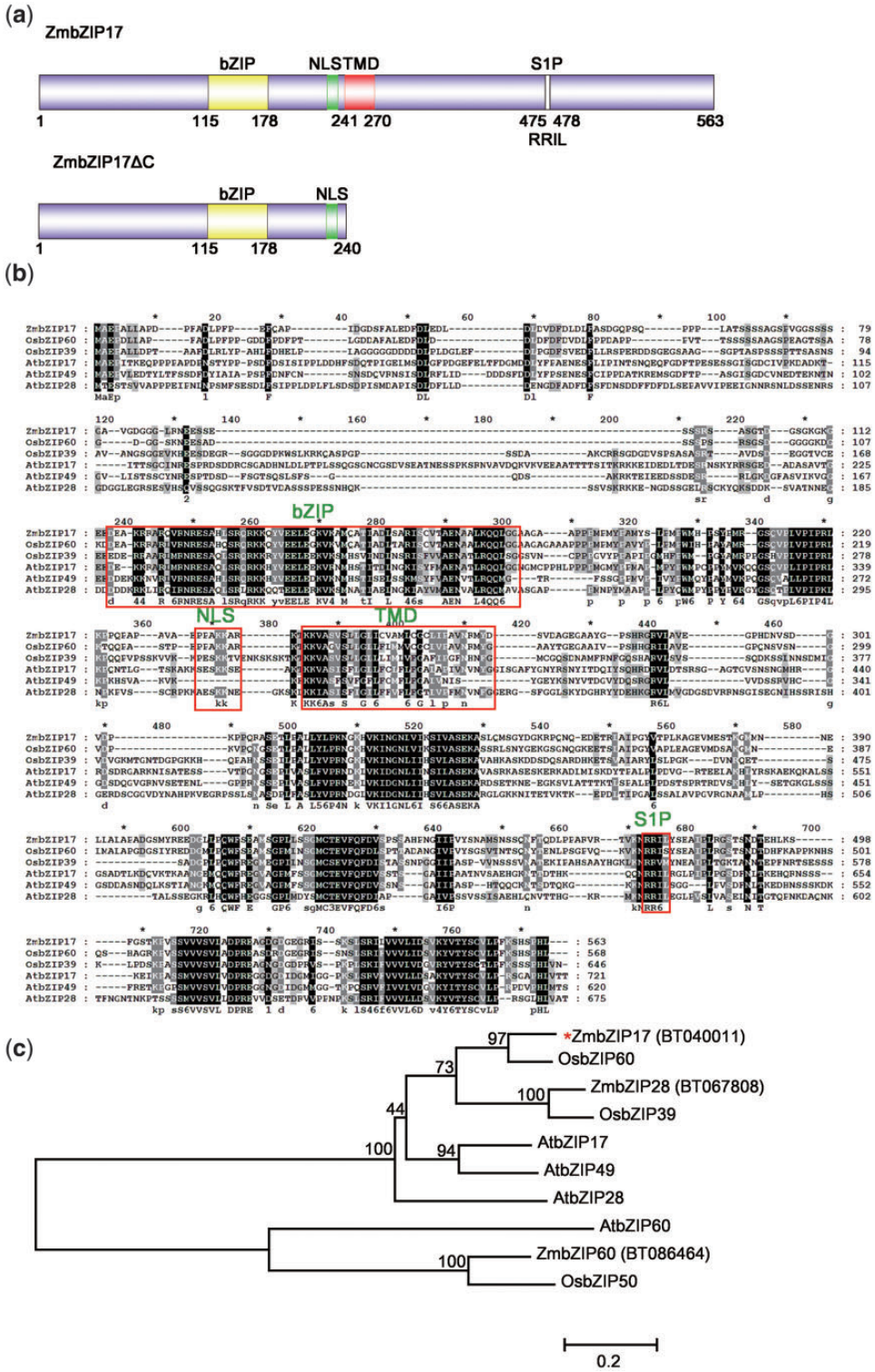


**Fig. 1** Structure of ZmbZIP17, and the alignment and phylogenetic tree generated with ER stress-associated membrane-associated bZIP factors from Arabidopsis, rice and maize. (a) Structure of ZmbZIP17 and ZmbZIP17ΔC. The map of the full-length ZmbZIP17 indicates the location of the putative bZIP, nuclear localization signal (NLS), transmembrane domains (TMDs) and the canonical S1P site. The map of the truncated protein ZmbZIP17ΔC indicates the deletion of TMDs and S1P domains. (b) Alignment of protein sequences for ZmbZIP17 and the known membrane-associated bZIP transcription factors. (c) The unrooted phylogenetic tree of ER stress-associated bZIP factors from Arabidopsis, rice and maize. The bZIP factors in (b) and (c) were AtbZIP17 (At2g40950), AtbZIP49 (At3g56660), AtbZIP28 (At2g17770), AtbZIP60 (AT1G42990), OsbZIP39 (Os05g34050), OsbZIP50 (Os06g0622700), OsbZIP60 (Os07g44950), ZmbZIP17 (BT040011), ZmbZIP28 (BT067808) and ZmbZIP60 (BT086464).

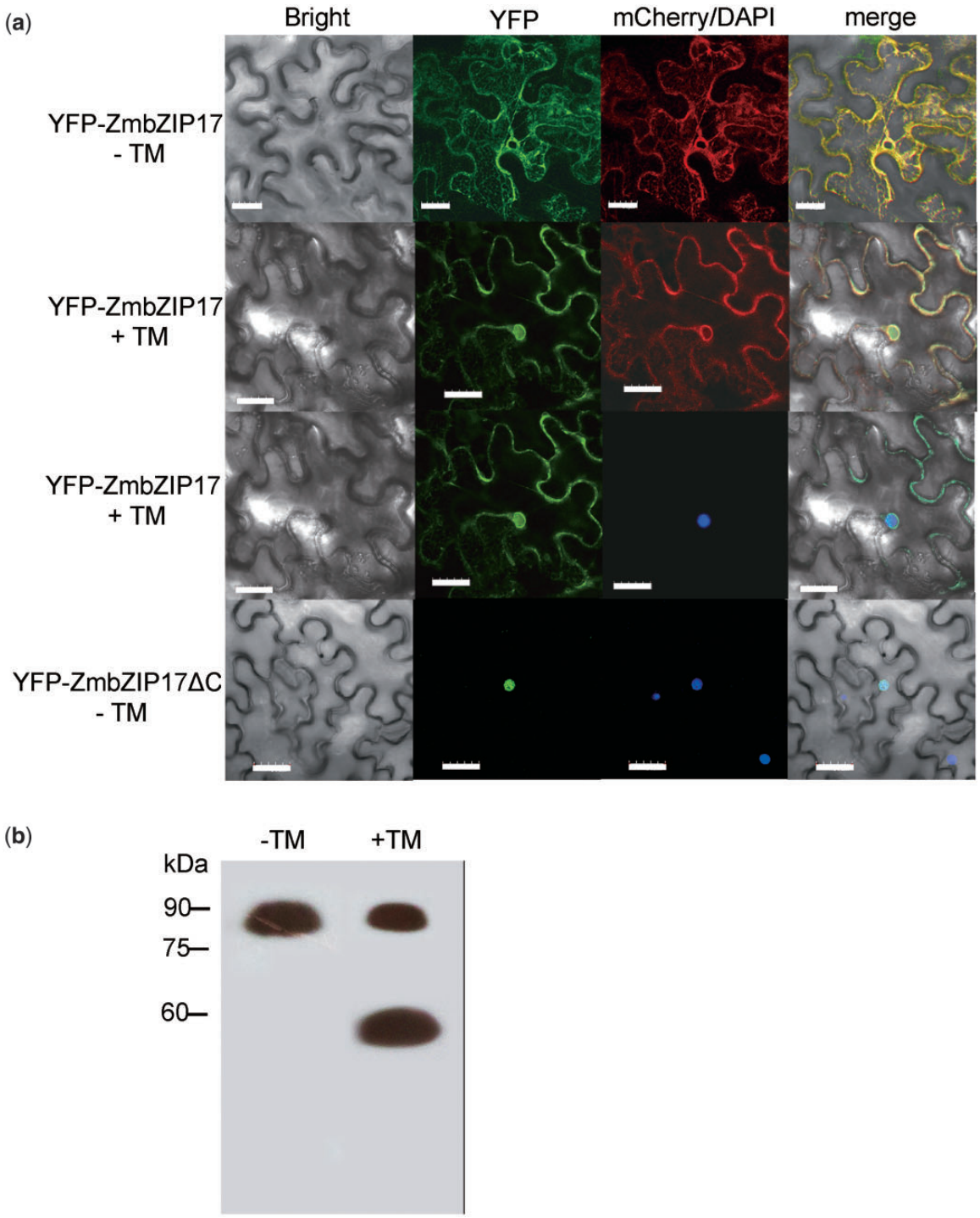


**Fig. 2** Subcellular localization of ZmbZIP17 and ZmbZIP17ΔC, and the processing of ZmbZIP17. (a) Subcellular localization of YFP–ZmbZIP17 and YFP–ZmbZIP17ΔC in a transient expression system of leaf epidermal cells in *Nicotina benthamiana* with or without treatment with 2 μg ml$^{-1}$ TM for 4 h. Green, red and blue fluorescence represent the signal of YFP, mCherry and DAPI, respectively. The YFP signals in the top two panels were merged with mCherry, and the YFP signal in the lower panels were merged with DAPI. For YFP–ZmbZIP17, photographs show the YFP signals merged with mCherry and DAPI, respectively, in the same cell. Bar = 30 μm. (b) Processing of ZmbZIP17 in Arabidopsis. Ten-day-old seedlings were treated with 5 μg ml$^{-1}$ TM for 4 h, and then Western blot was performed using anti-GFP antiserum.

treatment, YFP signals were also detected in the nucleus, as shown by the merge with 4′,6-diamidino-2-phenylindole (DAPI) staining (Fig. 2a), indicating that ZmbZIP17 could at least partially translocate from the ER membrane to the nucleus in response to ER stress. This observation was consistent with the presence of a typical S1P cleavage site in ZmbZIP17 between the 475th and 478th amino acid, which is involved in the processing of AtbZIP17 and AtbZIP28 (Liu et al. 2007b, Srivastava et al. 2012). S1P-dependent proteolysis of AtbZIP17 predicts its localization in the nucleus. To test this hypothesis, we constructed a truncated form (YFP–ZmbZIP17ΔC) where the TMD was removed. As expected, the YFP signal was detected exclusively in the nucleus under unstressed conditions (Fig. 2a). These results indicated that ZmbZIP17 was likely to translocate from the ER to the nucleus upon proteolysis of the C-terminus, similar to other ATF6-counterpart ER membrane-associated bZIP factors.

To confirm the processing of ZmbZIP17 protein, transgenic plants containing *YFP-ZmbZIP17* were generated and a Western blot analysis was performed using anti-green fluorescent protein (GFP) antibody. The result showed that a band of 90 kDa corresponding to the full-length YFP–ZmbZIP17 was detected in plants without TM treatment; however, the band was weaker and an additional 52 kDa band corresponding to the processed YFP–ZmbZIP17 appeared in TM-treated plants (Fig. 2b). Thus we concluded that ZmbZIP17 was partially processed by proteolysis by S1P upon TM treatment.

## Expression pattern of *ZmbZIP17* in maize

To investigate the expression pattern of *ZmbZIP17*, real-time PCR was performed to quantify the transcription levels of this gene under treatment by ABA and ER stress agents. As shown in Fig. 3, the transcript level of *ZmbZIP17* was increased during the first 2–6 h incubation in TM or DTT, but transcript levels were reduced afterwards. In contrast, the expression of *ZmbZIP17* increased gradually to the highest peak level after ABA treatment for 12 h. These results indicate that *ZmbZIP17* expression is regulated by both ER stress and ABA treatment.

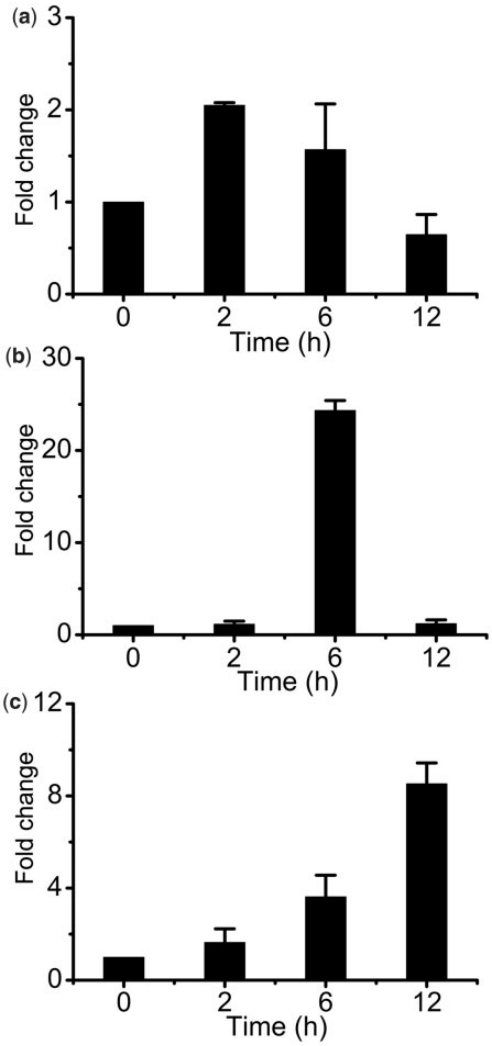


Fig. 3 Expression pattern of *ZmbZIP17* in maize under different treatments. (a–c) Maize seedlings at the three-leaf stage were treated with 2 mM DTT (a), 2 μg ml$^{-1}$ TM (b) and 100 μM ABA (c) for indicated periods, respectively. Transcription of *ZmbZIP17* was evaluated by real-time PCR analysis. Error bars indicate the SD ($n = 4$). Maize *β-tubulin* was used as the internal control to normalize the data, and fold change was calculated with the $2^{-\Delta\Delta T}$ method. Data were expressed as the mean ± SE of four independent experiments.

## Expression of *ZmbZIP17* affects the ER stress response in Arabidopsis

In order to investigate the role of ZmbZIP17 in the ER stress response, transgenic Arabidopsis plants overexpressing *ZmbZIP17* under the control of the 35*S* promoter were generated (Supplementary Fig. S2a). Fifteen independent transgenic lines were obtained, and three lines were confirmed to be homozygous (OE-2, OE-10 and OE-12). Transgene expression in these lines was confirmed by reverse transcription–PCR (RT–PCR), which revealed the highest and lowest expression of ZmbZIP17 in OE-10 and OE-2, respectively (Supplementary Fig. S2b).

When the transgenic plants were grown in parallel with wild-type (WT) control plants under unstressed conditions, the *ZmbZIP17*-expressing lines showed normal growth. In contrast, when grown under TM or DTT treatments, the transgenic plants exhibited a stress-tolerant phenotype in comparison with the WT, as shown by the improved seedling growth, in terms of increased size and vigor, indicating that ZmbZIP17 functions in regulating seeding morphogenesis under ER stress (Fig. 4).

## Expression of *ZmbZIP17* activated the expression of ER stress response genes

To gain insights into the mechanisms underlying ZmbZIP17-induced ER stress tolerance, the genes known to be critical components in the three branches of ER-QC, *BiP1*, *BiP2*, *BiP3*,

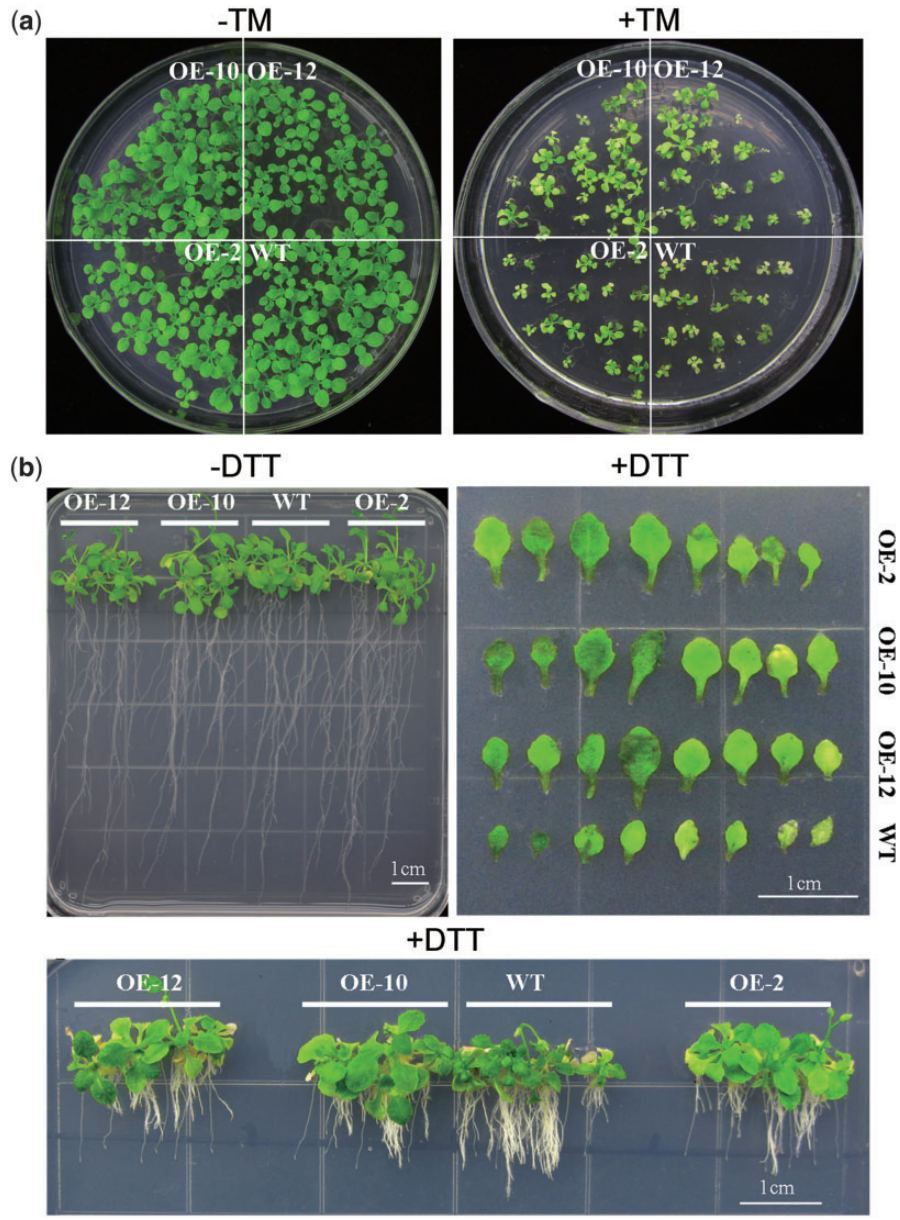


**Fig. 4** Phenotype of transgenic plants under TM-triggered ER stress in comparison with the wild type (WT). (a) Seeds were grown on MS medium plus 100 ng ml$^{-1}$ TM. After 10 d, the photographs were taken. (b) Three-day-old seedlings were transferred to MS medium with 2 mM DTT. Photographs were taken after 4 weeks. Transgenic lines showed larger leaves compared with the WT (b).

*CNX1*, *CRT1*, *ERdj3A* and *GRP94*, were selected to characterize the changes in expression between unstressed and ER stress conditions. As shown in **Fig. 5**, the transcript levels of all these genes were higher in transgenic lines compared with the WT under both unstressed and DTT-stressed conditions. Transcript levels of ER stress genes were higher in OE-10 than in OE-2, which was correlated with the higher transcript level of *ZmbZIP17* in OE-10. DTT further stimulated the expression of these genes up to higher levels in both the transgenic lines and the WT (**Fig. 5**).

AtbZIP17 is a known ER stress transducer and leads to repression of ER stress response genes such as *Bip1*, *Bip2*, *Bip3*, *CNX1* and *CRT1* (Liu et al. 2007b, Liu et al. 2008b). When *ZmbZIP17* was ectopically expressed in the *atbzip17* background, the expression of these genes (with the exception of *Bip3*) was activated. Thus the reduction of the transcripts of ER response genes in *atbzip17* can be rescued, at least partially, by the overexpression of *ZmbZIP17* (**Supplementary Fig. S3**).

## *ZmbZIP17* expression increases plant sensitivity to ABA

Members of the bZIP family function in ABA-dependent pathways (Jakoby et al. 2002). The regulatory machinery in the ABA

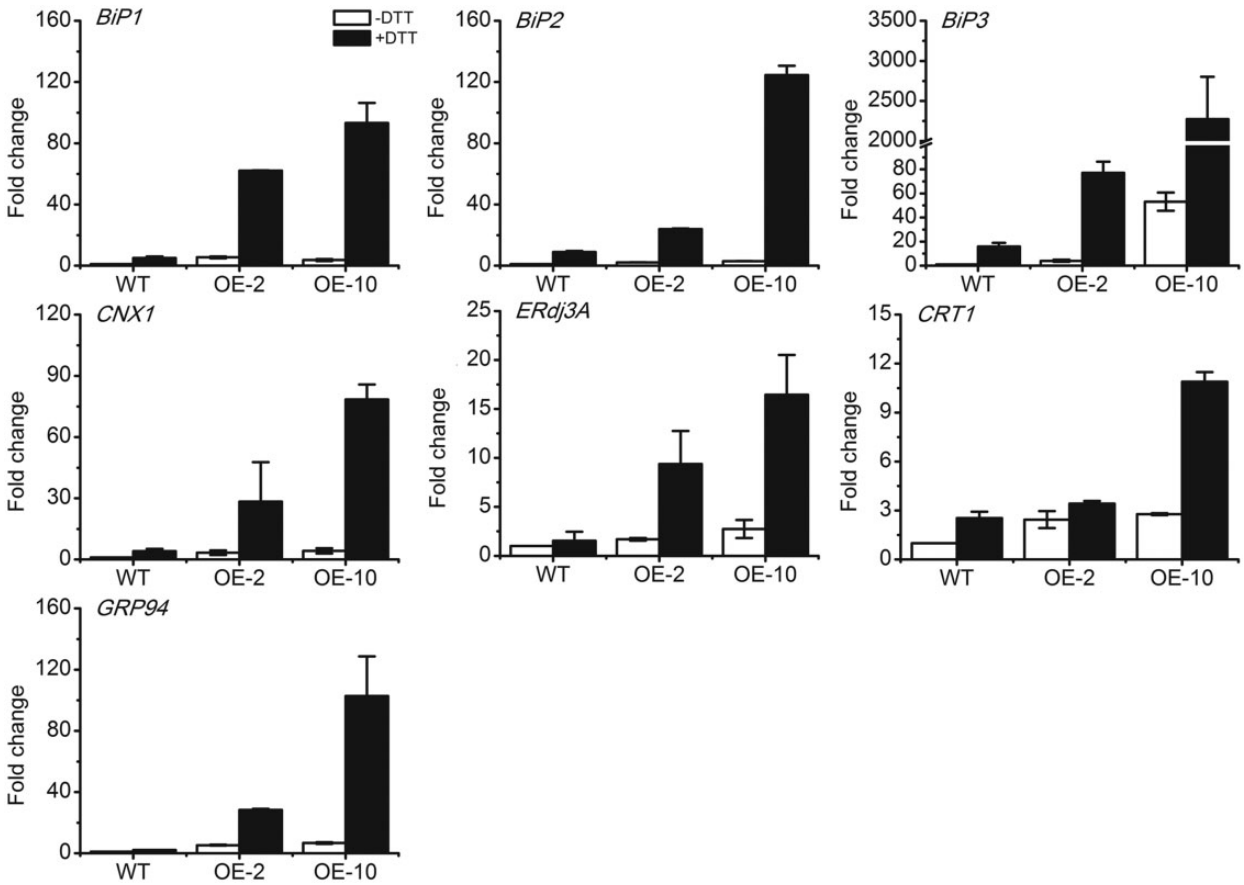


**Fig. 5** Expression of ER stress response genes in transgenic Arabidopsis overexpressing *ZmbZIP17*. Three-week-old seedlings of the wild type (WT) and *ZmbZIP17* overexpression lines (OE-2 and OE-10) were treated with 2 mM DTT for 3 h. Fold changes of expression levels of the ER response genes *BiP1*, *BiP2*, *BiP3*, *CNX1*, *ERdj3A*, *CRT1* and *GRP94* were detected by real-time PCR. The raw data (cycle threshold values) were normalized using *Actin1* as an internal reference gene. Data are the means ± SE of at least three independent experiments.

response varies with different developmental stages and tissues (Finkelstein et al. 2002, Pritchard et al. 2002, Garcia et al. 2008). To assess whether ZmbZIP17 participates in the ABA signaling pathway, ABA sensitivity of transgenic plants was examined during seed germination and post-germination growth. The results showed that ABA inhibited seed germination in all of the transgenic lines, and at each concentration more severely than that in the WT plants, when the plates contained 1 or 5 μM ABA (**Fig. 6a**). In contrast, germination of transgenic lines was similar to that of WT plants in the absence of ABA (**Fig. 6a**). Furthermore, post-germination growth of both transgenic and WT plants was arrested visibly in the presence of 1 μM ABA, but transgenic plants were affected more severely than the WT, as shown by the severe inhibition of greening/expansion of the cotyledons, the emergence of the true leaves and the elongation of the roots (**Fig. 6b**, **c**). The severity of the growth inhibition was correlated with the expression level of the *ZmbZIP17* transgene in each line. These data implied that the overexpression of *ZmbZIP17* enhanced plants hypersensitive to exogenous ABA treatment.

## Expression of *ZmbZIP17* activated the expression of ABA-responsive genes

To elucidate further the involvement of ZmbZIP17 in the ABA response, the expression of ABA/stress-responsive genes including *LEA* genes, *RD29A* and *Rab18*, and the alcohol dehydrogenase gene *ADH1* was assessed. As shown in **Fig. 7a**, the expression levels of *ADH1* and *RD29A* were much higher in the *ZmbZIP17* transgenic line OE-10 than in WT plants under control conditions. Expression levels were further increased in the presence of ABA. Modest expression levels of *Rab18* were observed in OE-10 in control conditions, but a significant increase of *Rab18* expression was induced upon ABA treatment (**Fig. 7a**). These data demonstrated that overexpression of *ZmbZIP17* resulted in the modulation of ABA/stress-responsive gene expression.

It is known that the promoters of *RD29A*, *Rab18* and *ADH1* contain ABREs, which are critical for the activation of ABA-inducible gene expression (Kang et al. 2002). A series of bZIP transcription factors that modulate ABA and the stress response such as ABF/AREB/ABI5 were found to bind ABREs

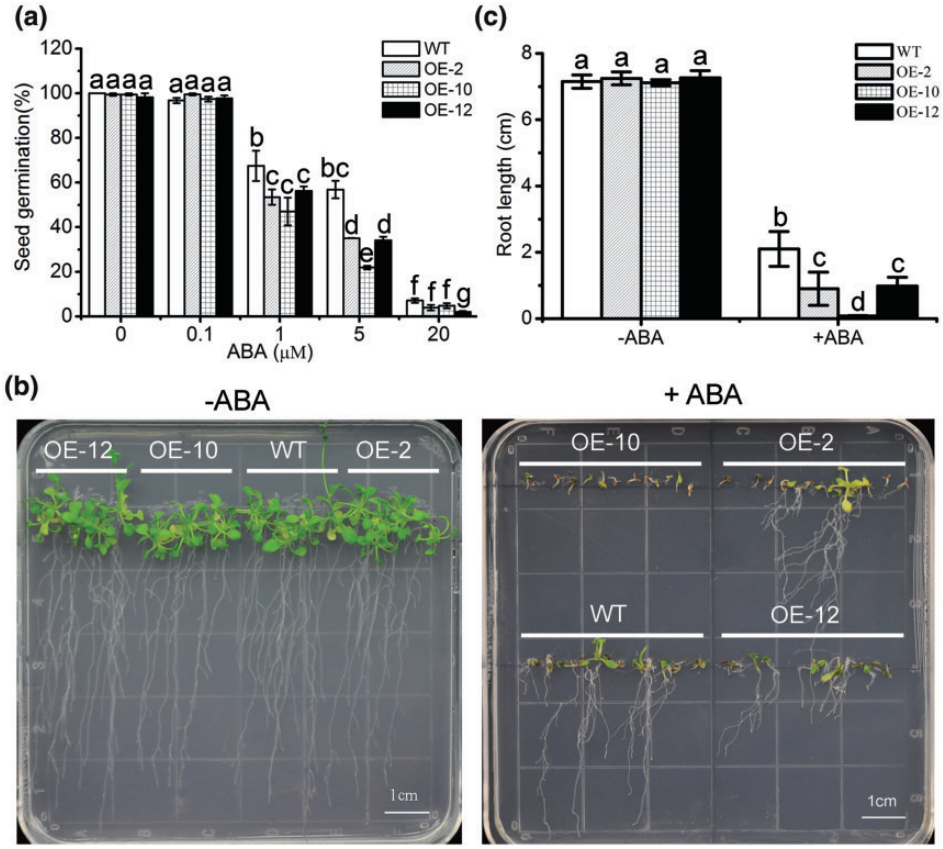


**Fig. 6** *ZmbZIP17* overexpression conferred plant hypersensitivity to ABA. (a) Seed germination of *ZmbZIP17*-overexpressing plants and the WT in the presence of ABA. Seeds were sown on MS medium containing 0–20 μM ABA. The average germination percentage was calculated after 6 d. (b) Post-germination growth of *ZmbZIP17*-overexpressing plants and the WT in the presence of ABA. Seedlings were grown in MS medium with or without 1 μM ABA, and photographs were taken after 3 weeks. (c) Root lengths of the seedlings in (b).

(Fujita et al. 2005, Yoshida et al. 2010, Liu et al. 2012). To examine the ABRE-binding affinity of ZmbZIP17, a yeast one-hybrid assay was performed. The interaction between ZmbZIP17 and *ABRE* genes was evidenced by the growth of yeast on media lacking leucine and histone but containing 3-aminotriazole (3-AT) (**Fig. 7b**). In contrast, *ZmbZIP17* was not able to bind to the mutated forms of *ABRE* genes, and the GAL4 activation domain (GAL-AD) alone was not able to bind ABREs as negative controls.

## Synergistic regulation of ABA and ER stress responsive genes mediated by ZmbZIP17

To evaluate the possible role of ABA in ER stress regulation, the ABA responsiveness of ER stress response genes was investigated using the Genevestigator database (https://www.genevestigator.com/gv/directlink.jsp). The results showed that the majority of the ER stress response genes presented in the database, including *BiP2*, *BiP3*, *CNX1*, *CRT1 PDIL* and *GRP94*, were up-regulated by ABA treatment in germinating seeds (**Supplementary Table S2**). In addition, the ABA-induced expression of these genes was further enhanced in *ahg1* (*ABA-hypersensitive germination 1*) and *ahg3* (*ABA-hypersensitive germination 3*) mutants (**Supplementary Table S2**). These data indicate that ER-QC may influence seed germination and early seedling growth via ABA signaling pathways.

The expression changes of ER stress genes *BiP1*, *BiP2*, *BiP3*, *GRP94* and *CNX1* in response to ABA were further monitored by real-time PCR in WT and *ZmbZIP17* transgenic Arabidopsis. The results revealed that these genes were highly induced by ABA in both WT and transgenic plants (**Fig. 8**). Furthermore, compared with the WT, ABA-induced expression of these ER marker genes was higher in the *ZmbZIP17* transgenic plants, indicating that ABA and ZmbZIP17 regulated ER response gene expression synergistically.

# Discussion

## *ZmbZIP17* functions as a regulator of plant responses to ER stress

There are four bZIP factors that are candidates for transducers of ER stress signals in Arabidopsis (Jakoby et al. 2002, Liu et al. 2007a, Liu et al. 2007b, Nagashima et al. 2011). AtbZIP17,

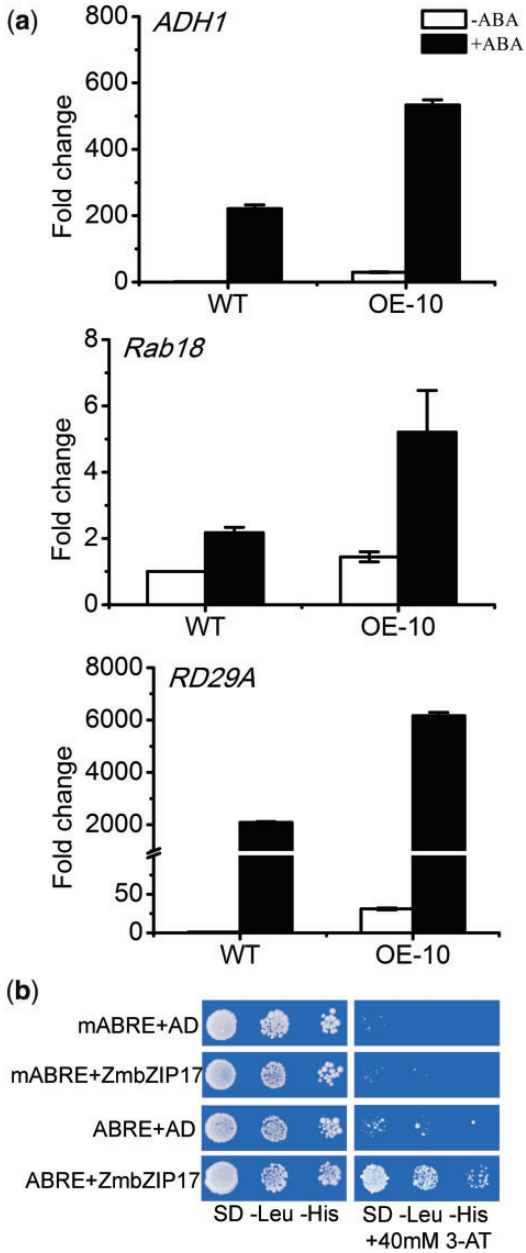


**Fig. 7** *ZmbZIP17* enhanced expression of ABA-responsive genes and showed positive interaction with ABREs in the yeast one-hybrid system. (a) The expression of the ABA-responsive genes including *ADH1*, *Rab18* and *RD29A*. Three-week-old seedlings were treated with 100 μM ABA for 3 h. Gene expression levels were determined in the WT and OE-10 by quantitative RT–PCR. *Actin1* was used as an internal reference gene. Error bars indicate the SD. The experiments were performed at least three times with similar results. (b) Yeast one-hybrid binding analysis of *ABRE* and ZmbZIP17. Yeast cells were co-transformed with a bait vector, containing tetramer *ABRE* sequence fused to a *HIS3* reported gene, and a prey vector, containing the *ZmbZIP17* coding sequence fused to the *GAL-AD*. The tetramer mutant *ABRE* (*mABRE*) was used as a negative control. Cells were grown in liquid medium to an $OD_{600}$ of 0.1 ($10^{-1}$) and diluted in a 10 × dilution series ($10^{-2}$–$10^{-3}$). Of each dilution, 5 μl was spotted on SD medium lacking leucine and histone, supplemented with 50 mM 3-AT to suppress background growth.

AtbZIP28 and AtbZIP49 belong to type II transmembrane proteins that are activated by proteolysis and translocate to the nucleus to trigger ER stress response gene expression, similar to ATF6, whereas AtbZIP60 was activated by IRE1-catalyzed mRNA splicing in response to ER stress (Ron and Walter 2007, Vitale and Boston 2008). In maize, only one bZIP factor, ZmbZIP60, has been reported to be involved in the ER stress response so far (Li et al. 2012, Wang et al. 2012). ZmbZIP60 belongs to the IRE1 pathway. Here we described ZmbZIP17, a type II transmembrane bZIP transcription factor, with an N-terminus facing the cytosol and C-terminus facing the ER lumen. Like its homologs from Arabidopsis and rice, ZmbZIP17 resides in the ER membrane under unstressed conditions and translocates to the nucleus after TM treatment or deletion of the C-terminal tail.

Full-length type II transmembrane proteins contain both an NLS and a TMD. Upon ER stress, the TMD domain is removed to allow the truncated form of the proteins to relocate into the nucleus, where those proteins function as transcription factors (Ron and Walter 2007, Tajima et al. 2008, Takahashi et al. 2012). It was described that AtbZIP28 is cleaved via intramembrane proteolysis by two distinct proteases, S1P and S2P, in the Golgi apparatus (Liu et al. 2007a, Che et al. 2010). The RxxL motif in AtbZIP28 was probably a S1P recognition sequence (Ye et al. 2000). AtbZIP17 processing also requires S1P which is located in the Golgi (Liu et al. 2008a, Tajima et al. 2008). Our data demonstrated that ZmbZIP17 was processed by the deletion of the TMD after TM treatment to translocate to the nucleus (**Fig. 2**). Thus ZmbZIP17 is likely to be activated by proteolysis in response to ER stress in a way similar to AtbZIP17 and AtbZIP28.

However, unlike *AtbZIP17*, *ZmbZIP17* expression was induced in maize by TM and DTT treatment. Expression of *ZmbZIP17* in Arabidopsis led to increased tolerance to ER stress and activated the expression of ER stress response genes, such as *BiP1*, *BiP2*, *BiP3*, *CNX1*, *CRT1* and *ERdj3A*. These genes act as chaperones or folding enzymes in the ER lumen, and were previously shown to be up-regulated by ER stress agents (Anelli and Sitia 2008). These data further support that ZmbZIP17 is an early sensor and positive regulator in the ER stress signaling pathway.

## ZmbZIP17 plays a key role in the cross-talk of ABA and ER stress signaling pathways

It has been established that many abiotic stresses, such as salt stress and drought stress, disrupt protein folding and assembly, resulting in cellular damage (Hampton 2000). Cells activate the UPR to protect the ER from being damaged by misfolded or unfolded proteins (Liu et al. 2007b, Liu et al. 2008b). Many genes associated with ER-QC and the UPR were reported to be responsive to environmental stress. For example, AtbZIP17 was activated by salt stress and heat stress, and enhanced salt tolerance was found in transgenic seedlings of the active form of *AtbZIP17* (Liu et al. 2008b,

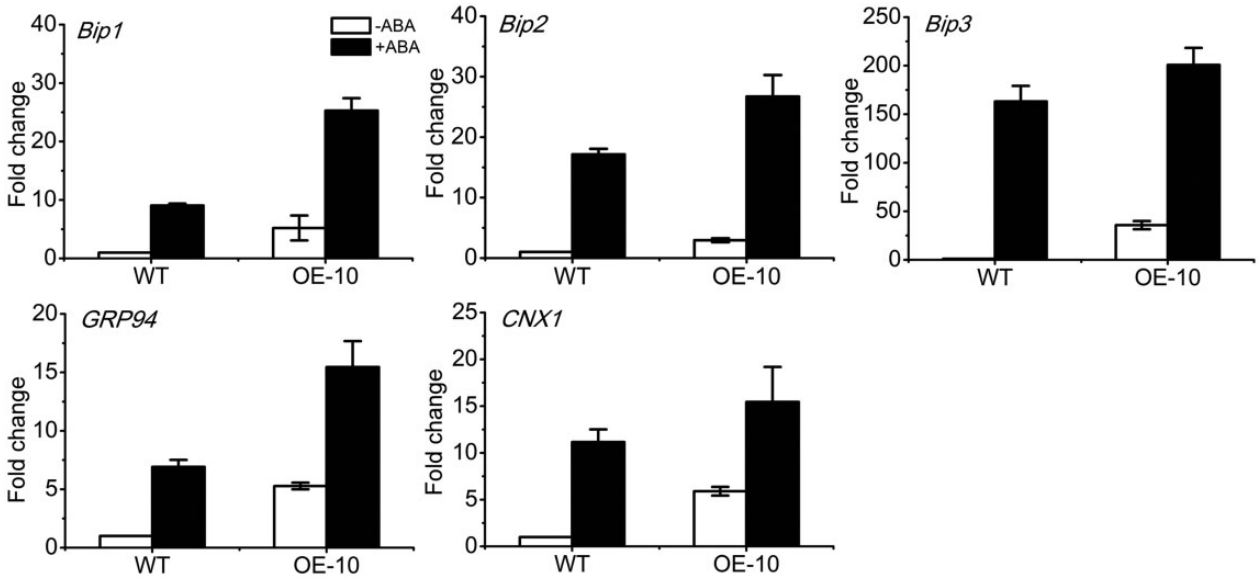


**Fig. 8** ABA regulated ER stress response gene expression in wild-type (WT) and transgenic Arabidopsis overexpressing *ZmbZIP17*. Three-week-old seedlings of WT and a *ZmbZIP17* overexpression line (OE-10) were treated with 100 μM ABA for 3 h. Fold changes of expression levels of genes (*BiP1*, *BiP2*, *BiP3*, *CNX1* and *GRP94*) were detected by quantitative real-time PCR. The raw data (cycle threshold values) were normalized using *Actin1* as an internal reference gene. Data are the means ± SE of at least three independent experiments.

Che et al. 2010). The knock-out mutant *atbzip28* showed a striking heat-sensitive phenotype (Gao et al. 2008). Correspondingly, these two transcription factors up-regulate different sets of genes that mitigate the corresponding stresses (Liu et al. 2007a, Liu et al. 2007b, Gao et al. 2008, Tajima et al. 2008). Plant *BiP* gene expression has been shown to be induced by drought, and overexpression of *BiP* resulted in more tolerance to drought in soybean and tobacco (Alvim et al. 2001, Valente et al. 2009). In contrast, *OsbZIP39* expression was shown to be induced by TM and DTT, but not by NaCl (Takahashi et al. 2012). In addition, the expression of *AtbZIP60* conferred an enhanced tolerance to salt stress (Fujita et al. 2007). These studies led to speculation on the involvement of ER-QC regulators in abiotic stress responses. Recent studies have revealed that AtbZIP17 is activated by proteolysis in salt stress, subsequently relocates to the nucleus and then activates salt stress response genes. However, it is not known how much this system contributes to ER-QC during salt stress, considering that *AtbZIP17* is not activated by TM and DTT, and so far none of the typical UPR downstream target genes was found to be up-regulated by salt stress (Liu et al. 2008a). The responses and functions of the ER-QC regulators under various abiotic stresses are plant species dependent, and, at least for AtbZIP17, it remains unclear as to whether abiotic stress activates the UPR or if ER-QC contributes to stress tolerance.

The phytohormone ABA serves as the central regulator of abiotic stress and development in plants, especially during seed germination and seedling establishment. It coordinates a complex network enabling plant gene expression to cope with stress and developmental signals (Himmelbach et al. 1998). To date, the involvement of ABA in ER stress response regulation has remained largely unknown. In this study, we have found that the expression of *ZmbZIP17* was increased after ABA treatment in maize, ZmbZIP17 was capable of binding ABREs and the overexpression of *ZmbZIP17* in Arabidopsis led to ABA-responsive gene expression and significant hypersensitivity to exogenous ABA in seed germination and seedling growth during the post-germination stage, thus providing the evidence for a positive and direct role for ZmbZIP17 in ABA signaling, especially during germination and post-germination seedling establishment. Furthermore, we also found that ABA could induce the expression of ER stress response genes alone or synergistically with ZmbZIP17. According to the Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), *AtbZIP17*, *AtbZIP28* and *AtbZIP60* are induced 1.18-, 1.19- and 2.2-fold, respectively, after 10 μM ABA treatment for 1 h. Gene expression data from the Genevestigator database also revealed that the majority of the ER stress response genes were up-regulated by ABA in germinating seeds and further induced in *ahg1* and *ahg3* mutants. It was reported that AHG1 functioned upstream of ABI3 and ABI5 in the ABA signaling pathway controlling seed germination and post-germination growth (Nishimura et al. 2007). The post-germination growth was remarkably reduced in the *ahg1 ahg3* double mutant compared with that of parental monogenetic mutants in the presence of ABA (Nishimura et al. 2007). Therefore, these data support that ER-QC is to some extent regulated by the ABA signaling pathway during seed germination and early growth.

Considering that ER agents also induced *ZmbZIP17* expression and ZmbZIP17 translocation to the nucleus, and *ZmbZIP17* overexpression up-regulated ER stress response

genes, it is likely that *ZmbZIP17* transcription is under the control of both ER stress agents and ABA, and the protein functions in both ER stress and ABA signaling to activate the expression of both sets of genes, thereby coordinating the UPR and ABA-triggered responses. Taken together, our study revealed a novel mechanism that directly links the ER-QC and ABA signaling by a type II transmembrane protein from maize which represents a critical node in the cross-talk signaling network.

## Materials and Methods

### Plant materials and stress treatments

Maize (*Zea mays L.*) inbred line B73 seeds were imbibed and incubated on moist filter paper at 28°C in the dark for 48 h, then sown in soil and grown in a growth chamber (28°C, 12 h light/12 h dark photoperiod). Seedlings with three fully expanded leaves were harvested for RNA extraction. For stress treatments, roots of three-leaf stage maize were submerged in 2 mM DTT, 2 μg ml$^{-1}$ TM or 100 μM ABA solutions for 0, 2, 6 and 12 h, respectively. The plant materials were harvested at each time point, frozen immediately in liquid nitrogen, and stored at −80°C for RNA extraction.

*Arabidopsis thaliana* (ecotype Col-0) seeds were surface-sterilized and sown on Murashige and Skoog (MS) medium plates, and vernalized at 4°C for 3 d in the dark. Seven-day-old seedlings were transferred to soil, and grown in a chamber (22°C under a 16 h light/8 h dark photoperiod). For phenotypic identification, WT and transgenic lines seeds were prepared and grown on MS medium plus 100 ng ml$^{-1}$ TM, 2 mM DTT or 1 μM ABA, horizontally or vertically, the growth was evaluated and photographs were taken at the appointed time. For germination test, seeds were sown on MS medium with or without 1 μM ABA. The average germination rate was determined after 6 d of cultivation. Seeds for germination assays were collected from plants of different genotypes and grown simultaneously, and stored under identical conditions. For RT–PCR, roots were submerged in 2 mM DTT or 100 μM ABA solutions for 3 h, respectively, and then the plant materials were harvested, frozen in liquid nitrogen, and stored at −80°C for RNA extraction.

### Molecular cloning and construction of plasmids

Based on sequence similarity, a gene (accession No. BT040011) coding for AtbZIP17 homologous protein was revealed from a screening against MaizeGDB (http://www.maizegdb.org, Soderlund et al. 2009), and designated *ZmbZIP17*. An ABRE was found in the promoter region of *ZmbZIP17*. Specific primers were designed to amplify the sequence of BT040011 from maize seedling using Phusion High-Fidelity DNA Polymerase (NEB). The PCR product was purified and cloned to pENTR vector. It was cloned into the binary vector pLeela through LR recombination reaction, using Gateway LR Clonase Enzyme Mix (Invitrogen) for constitutive expression in plants.

For determination of the subcellular location and Western blot analysis, *YFP-ZmbZIP17* and *YFP-ZmbZIP17ΔC* were generated by insertion of the full length or truncated form of *ZmbZIP17* without the sequence coding for TMD into an YFP-tag vector pENSG-YFP and pPLV17, respectively. The primers used in this study are listed in **Supplementary Table S1**. All constructs were confirmed by sequencing the entire inserted fragments.

### Sequence analysis and construction of phylogenetic tree

The gene and promoter sequences of *ZmbZIP17* were obtained from PlantGDB (http://www.plantgdb.org/). *ABRE* was identified through searching against the PLACE database (http://www.dna.affrc.go.jp/PLACE/index.html) and the Plant CARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) (Higo et al. 1999, Lescot et al. 2002). The amino acid sequence was predicted using DNAMAN program. The subcellular localization was predicted with the online software available at http://ipsort.hgc.jp and http://wolfpsort.org. The homology was analyzed against the TAIR database (http://www.Arabidopsis.org/index.jsp). The phylogenetic tree was constructed using the Neighbor–Joining (NJ) method in clustalx1.83 and viewed using the software MEGA4 according to the method of Kumar et al. (2004).

### Generation of Arabidopsis transgenic plants

*35SS::ZmbZIP17* and *35SS::YFP-ZmbZIP17* were transformed into the *Agrobacterium tumefaciens* strain GV3101 (pMP90RK) through electroporation and then transferred into *A. thaliana* ecotype Col-0 using a floral dip method (Bechtold et al. 1993). Transgenic plants were screened with phosphinothricin (PPT) and confirmed by PCR analysis. $T_3$ plants displaying 100% PPT resistance were considered homozygous and used for further experiments. Seeds of different genotypes were collected simultaneously and stored under identical conditions.

### RNA extraction and RT–PCR

Total RNA was isolated from leaves of Arabidopsis and maize using Trizol (TAKARA) according to the manufacturer's instructions, and cDNA was synthesized with random primers using SuperScript TM III Reverse Transcriptase (Invitrogen). Semi-quantitative RT–PCR was carried out according to the method described by Che et al. (2002). Quantitative real-time PCR was performed using SYBR Green Real-Time PCR Master Mix (Toyobo) on a Mastercycler ep realplex real-time PCR system (Eppendorf). The primers of ABA and ER stress response genes were synthesized according to Kang et al. (2002), Liu et al. (2007a), Qiang et al. (2012) and Wang et al. (2012). Expression levels were calculated with the $2^{-\Delta\Delta T}$ method (Livak and Schmittgen 2001) and statistically analyzed according to Liu et al. (2007a). The raw data (cycle threshold values) were normalized using maize *β-tubulin* (NCBI accession No.

X74654) and Arabidopsis *Actin1* as internal reference genes for maize and Arabidopsis gene expression, respectively. The relative expression of each gene was calculated as the mean fold change compared with the untreated WT sample in each experiment. Data are means ± SE of at least three independent experiments.

## Western blot analysis

Western blot analysis was performed as described by Liu et al. (2007a). Ten-day-old seedlings of transgenic plants overexpressing *YFP-ZmbZIP17* were treated or not with 5 μg ml$^{-1}$ TM for 4 h before being frozen and homogenized in liquid nitrogen. The extraction buffer contained 25 mM Tris–HCl (pH 6.8), 10% glycerol, 0.1% NP-40 and 150 mM NaCl. A 30 μg aliquot of protein was loaded per lane, and transferred onto a nitrocellulose membrane using a semi-dry transfer method (BIO-RAD). The membrane was incubated with rabbit anti-GFP antiserum (1 : 5,000, Abmart), which recognizes GFP and its YFP variant. Immunoblots were incubated with anti-rabbit IgG (1 : 10,000, Abmart).

## Yeast one-hybrid assays

Yeast one-hybrid assays were performed according to the manufacturer's instructions (Clontech). The bait plasmids were constructed by transferring fragments containing tetramer *ABRE/mABRE* genes, which were amplified from pRSA4 and pRSmA4 into pHISi, respectively (Wang et al. 2002), using primers 5′-CCCGGGCCGCGGTGGCGGCCGCT CTAG-3′ and 5′-ACGCGTGGCCAGGAATTCCCCGGATC-3′. The full-length CDS of *ZmbZIP17* was fused to the GAL-AD in the vector pACT2, resulting in plasmid pACT2-ZmbZIP17. Each pair of bait and prey plasmids was co-transformed into yeast YM4271 cells using a lithium acetate method (Clontech) and analyzed for yeast growth on selective medium containing 50 mM 3-AT but lacking histone and leucine.

## Confocal microscopy

The plasmids containing *YFP-ZmbZIP17* or *YFP-ZmbZIP17ΔC* were transiently co-transformed with HDEL–mCherry into epidermal cells of *N. benthamiana* leaves via an *Agrobacterium*-mediated method (Wydro et al. 2006). Leaves were incubated with 2 μg ml$^{-1}$ TM for 4 h separately or counterstained with 1 μg ml$^{-1}$ DAPI before observation. Fluorescent images were obtained by laser excitation at 488 nm for YFP, 591 nm for mCherry and 358 nm for DAPI, respectively. A confocal laser scanning microscope (FV1000MPE; Olympus) was used to visualize DAPI and GFP signals. Image Browser software was used for image acquisition.

## Supplementary data

**Supplementary data** are available at PCP online.

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## Disclosures

The authors have no conflicts of interest to declare.

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