Functional analysis of a transcription factor involved in the endoplasmic reticulum stress response in *Arabidopsis thaliana*

Yuji Iwata

2005

Abstract

The endoplasmic reticulum (ER) consists of a three-dimensional structure in eukaryotic cells where proteins for the secretory pathway are synthesized. Proper folding and assembly of proteins synthesized in the ER are necessary for transport to their final destinations. When folding or assembly of proteins in the ER is disordered, unfolded proteins accumulate in the ER and expression of genes for ER-resident chaperones such as BiP and folding enzymes are induced. This phenomenon is conserved among eukaryotic cells and is referred to the ER stress response or the unfolded protein response. The molecular mechanism of the ER stress response has been characterized in yeast and mammalian cells. Recent studies have shown that the ER stress response plays essential roles not only under specific stresses but also under normal growth conditions. In contrast, the molecular mechanism and physiological function of the ER stress response are poorly understood in plants. Therefore, I aimed to study signal transduction pathway of the ER stress response in a model plant *Arabidopsis thaliana*.

In this study, I focused on transcription factors (TFs) that function on the gene expression in the ER stress response. Since bZIP TFs are involved in the ER stress response of yeast and mammals, I analyzed putative bZIP genes found in the *Arabidopsis* genome assuming that bZIP TFs are also involved in the response of plants. Screening of transcripts of 75 putative bZIP genes identified *AtbZIP60* highly induced by tunicamycin, an inhibitor of asparagine-linked glycosylation that induces the ER stress response. Although the expression level of *AtbZIP60* is very low without stresses, analysis using GUS reporter gene indicated that promoter of *AtbZIP60* is activated in pollens and premature seeds. The deduced amino acid sequence suggested that AtbZIP60 contains a transmembrane domain (TMD) following a bZIP domain. This implies conversion of AtbZIP60 to a soluble protein by proteolysis in response to ER stress. Indeed, a truncated form of AtbZIP60 without TMD (AtbZIP60ΔC) fused with GFP localized to the nucleus,

suggesting cleavage and translocation to the nucleus of endogenous AtbZIP60.

In order to examine whether AtbZIP60 enhances the expression of genes induced in the ER stress response, effect of expression of AtbZIP60 on activation of three *BiP* and two *calnexin* (*CNX*) promoters was analyzed by transient assay in protoplasts using luciferase as a reporter. Interestingly, although expression of the intact form of AtbZIP60 did not activate any *BiP* or *CNX* promoter, AtbZIP60ΔC clearly activated all promoters. These results supported the hypothesis that cleavage of AtbZIP60 is necessary for its activation. This activation was considered through *cis*-elements such as P-UPRE and ERSE found in many chaperone promoters. Involvement of these *cis*-elements was proven by introduction of mutation in the promoters (loss of function) and by use of tandem repeats of *cis*-elements (gain of function). In addition, AtbZIP60ΔC also seemed to activate the expression of *AtbZIP60* itself through an ERSE-like element in its promoter.

Subsequently, I obtained a T-DNA insertion mutant of *AtbZIP60* and subjected to microarray analysis. When expression profile of ER stress-responsive genes was compared between wild type and the mutant, some genes such as *BiP3* were clearly repressed in the mutant, indicating AtbZIP60 functioning in the ER stress response. However, some genes including *BiP1* and *BiP2* were still induced in the mutant by tunicamycin, although their promoters were activated by expression of AtbZIP60ΔC in transient assay described above. Therefore, there would be additional pathways that do not need AtbZIP60.

In summary, I isolated AtbZIP60, a novel TF activating the expression of genes induced in the ER stress response. Activation of AtbZIP60 was considered to occur by release of the protein from the membrane after cleavage. AtbZIP60 also appeared to enhance its transcription, resulting in amplifying the signal. It was also shown that *Arabidopisis* has an additional signaling pathway enhancing the ER stress response.

Table of Contents

Abbreviations

bZIP	basic leucine zipper
CNX	calnexin
CRT	calreticulin
ER	endoplasmic reticulum
ERAD	ER-associated protein degradation
ERSE	ER stress-response element
GAL4 BD	GAL4 DNA binding domain
GAL4 BS	GAL4 binding site
GUS	β-glucuronidase
PDI	protein disulfide isomerase
P-UPRE	plant UPR element
TMD	transmembrane domain
UPR	unfolded protein response
UPRE	UPR element

Introduction

Proteins translated according to DNA sequence are major functional molecules catalyzing various reactions in cellular response. After translation in ribosome, nascent proteins are further modified, correctly folded and assembled for their proper functions. If such maturation processes are failed, resulting malfolded proteins may aggregate giving serious damage to cells. To avoid such situations, malfoded proteins are monitored, correctly folded by various chaperones, or eventually degraded if correct structure is not achieved. Importance of such quality control after translation has been recognized and molecular mechanism of regulating protein quality control needs to be clarified.

Proteins for the secretary pathway (approximately one third of total protein) are synthesized in the endoplasmic reticulum (ER). Their proper folding and assembly necessary for transport to their final destinations are ensured by the protein quality control in the ER. Perturbations that alter ER homeostasis often disrupt folding, and lead to the accumulation of unfolded proteins and protein aggregates, which is detrimental to cell survival. More specifically, disturbances in calcium homeostasis or redox status in the ER, increased demand for protein folding due to elevated synthesis of secretory proteins, lack of asparagine-linked glycans that facilitate protein folding, result in prevention of correct folding or assembly of proteins. Such perturbations

preventing protein maturation have been referred to as ER stress. The ER quality control process that alleviates ER stress is called the ER stress response. In mammals, the ER stress response consists of three major mechanisms; attenuation of protein synthesis to prevent supplying additional unfolded proteins, induction of chaperones and folding enzymes to facilitate protein folding (unfolded protein response; UPR), and degradation of unfolded proteins in proteasome after retrotranslocation to the cytoplasm (ER-associated protein degradation; ERAD) (Kaufman et al., 2002; Mori, 2000; Patil and Walter, 2001; Rutkowski and Kaufman, 2004). In yeast, attenuation of protein synthesis is not observed, however, the UPR and the ERAD are conserved. Upon the UPR, expression of genes for ER-resident chaperones such as BiP, calreticulin (CRT), calnexin (CNX), and protein disulfide isomerase (PDI) are highly induced. Especially, mRNA induction of BiP, an ER-resident Hsp70, has been considered as an indication of the UPR. Induction of these genes implies there is a signal transduction pathway from the ER where malfolded proteins generate to the nucleus where gene expression occurs.

Recent studies conducted in yeast and mammalian cells have shown that the ER stress response plays essential roles not only under specific stresses, but also under normal growth conditions (Harding et al., 2001; Iwakoshi et al., 2003; Reimold et al., 2001; Scheuner et al., 2001). For instance, the ER stress response is required for terminal differentiation of B lymphoid cells to plasma cells, where the ER compartment expands five fold to accommodate the large increase in immunoglobulin synthesis.

3

Pancreatic β-cell is also reported to require proper function of the ER stress response.

In plants, the ER stress response was observed in the *floury-2* endosperm mutant of maize (Boston et al., 1991; Fontes et al., 1991). This mutant produces an aberrant 24 kDa α-zein storage protein with a defective signal peptide-processing site. As a result, the defective storage proteins accumulate as a membrane-anchored protein in the ER and in ER-derived protein bodies. The seeds show the ER stress response with dramatically increased levels of BiP and other ER-resident chaperones (Coleman et al., 1995; Gillikin et al., 1997). Expression of BiP is also regulated during development and by the environment. Regulation of BiP expression was observed during seed development in soybean, rice, pumpkin and Douglas fir, in which high amounts of seed storage proteins are folded and assembled in the ER (Forward and Misra, 2000; Hatano et al., 1997; Kalinski et al., 1995; Muench et al., 1997). It was also observed that BiP expression is regulated under various environmental conditions, such as salt/osmotic stress (Koiwa et al., 2003). The induction of BiP and other ER chaperones has been also observed on application of artificial stress such as tunicamycin, a potent inducer of ER stress inhibiting asparagine-linked glycosylation (Vitale and Ceriotti, 2004). Treatment of tunicamycin stimulates expression of BiP and other ER-resident chaperones in several plant systems (Cascardo et al., 2000; Denecke et al., 1991; Koizumi, 1996; Okushima et al., 1999; Wrobel et al., 1997). However, it remains to be elucidated the significance of the ER stress response in cellular processes.

The mechanism of signal transduction for the ER stress response has been extensively characterized in yeast and mammalian cells (Fig. 1). In yeast cells, IRE1, an ER membrane-located protein kinase/ribonuclease, plays a pivotal role for perception of ER stress (Cox et al., 1993; Mori et al., 1993). Sensing ER stress, IRE1 dimerizes and trans-autophosphorylates, activating its ribonuclease activity (Bertolotti et al., 2000; Shamu and Walter, 1996). Activated IRE1 catalyzes the spliceosome-independent splicing of *Hac1* mRNA, encoding a basic leucine zipper (bZIP) transcription factor. Hac1 protein is efficiently synthesized from spliced *Hac1* mRNA and binds to a *cis*-element, UPRE (CAGCGTG), resulting in induction of downstream chaperone genes such as *BiP* (Kohno et al., 1993; Mori et al., 1996; Mori et al., 1992).

The ER stress response pathways of mammalian cells are multiple in contrast to that of yeast, which is explained by a linear pathway consisting of IRE1, Hac1, UPRE and induction of chaperone genes. In mammals, at least two bZIP transcription factors functioning in the ER stress response, XBP1 and ATF6, have been identified. The *XBP1* mRNA is spliced by IRE1α through unconventional splicing, similarly to yeast Hac1 (Yoshida et al., 2001). This splicing removes 26 nucleotides from authentic *XBP1* mRNA, resulting in a frameshift. XBP1 protein, with an activation domain at the C-terminus, is synthesized after splicing and enhances target gene expression through the *cis*-elements ERSE (CCAAT-N9-CCACG), ERSE-II (ATTGG-N-CCACG) or XBP1-BS (GA-TGACGT-G(T/G)) (Kokame et al., 2001; Shen et al., 2001; Wang et al.,

2000; Yamamoto et al., 2004; Yoshida et al., 1998). Another protein, ATF6, is a transmembrane protein located in the ER membrane with a bZIP domain on the cytoplasmic side. In response to ER stress, ATF6 protein is translocated to the Golgi and processed by S1P and S2P proteases in the transmembrane domain (Haze et al., 1999; Lee et al., 2002). The processing localizes the cytoplasmic bZIP domain to the nucleus that activates downstream genes through ERSE or ERSE-II cooperating with NF-Y transcription factor complex (Yoshida et al., 2000; Yoshida et al., 2001). The active form of ATF6 is produced prior to that of XBP1 in response to ER stress, since the former is derived from preexisting precursor protein, whereas the latter must be newly translated from transcriptionally induced mRNA and then processed by IRE1-dependent splicing (Yoshida et al., 2003; Yoshida et al., 2001). Since *XBP1* contains ERSE in its promoter, ER stress signaling can be amplified through the transcription of *XBP1* as long as IRE1 is activated.

Plants also show a clear ER stress response (Boston et al., 1991; Jelitto-Van Dooren et al., 1999; Koizumi, 1996; Koizumi et al., 1999; Leborgne-Castel et al., 1999; Martinez and Chrispeels, 2003), while knowledge of the molecular mechanism for the response is limited. To date, IRE1 homologs have been isolated in *Arabidopsis thaliana* and rice (*Oryza sativa*) (Koizumi et al., 2001; Okushima et al., 2002). A *cis*-element P-UPRE (for plant UPR element) responsible for the ER stress response was identified in *BiP2* (AGI code; At5g42020) promoter of *Arabidopsis* (Oh et al., 2003). Interestingly,

P-UPRE consisted of two *cis*-elements identified in the mammalian ER stress response, ERSE-II and XBP1-BS. In addition to the *BiP2* promoter, P-UPRE was found in the promoters of other ER-chaperone genes including *BiP1* (AGI code; At5g28540). A transcriptomic approach using microarrays showed that ERSEs were also found in promoters of several genes induced by ER stress (Martinez and Chrispeels, 2003; Noh et al., 2003). Further, the third BiP, *BiP-L* (AGI code; At1g09080, *BiP3* in the present study), also contains two functional ERSEs, since mutation of ERSE in the *BiP3* promoter abolishes induction in response to ER stress (Noh et al., 2003). Namely, *cis*-elements are conserved between mammals and plants, however, a database search for *Arabidopsis* genomic information did not succeed in finding possible homologs of XBP1 or ATF6. In the present study, I aimed to isolate a transcription factor involved in the ER stress response in plants using *Arabidopsis*.

Materials and Methods

Analysis of bZIP transcripts

Total RNA was extracted from *Arabidopsis* (Col-0 ecotype) leaves treated with or without tunicamycin (5 µg/mL) for 12 h. From each RNA sample, cDNA synthesis and subsequent PCR was conducted using the RNA PCR kit (AMV) ver. 2.1 (Takara, Otsu, Japan) according to the manufacturer's instructions. The size and signal intensity of PCR products using specific primers for 75 bZIP genes were examined by gel electrophoresis.

RNA blot analysis

Arabidopsis seedlings were grown in one-half strength MS medium supplemented with 2% (w/v) sucrose in a 16 h light and 8 h dark cycle. Total RNA was extracted using the aurintricarboxylic acid method (Gonzalez et al., 1980) from two-week-old seedlings treated with tunicamycin (5 µg/mL), dithiothreitol (2 mM) or azetidine-2-carboxylate (5 mM). 5 µg of RNA per lane was fractionated on a 1.2% agarose gel containing 2% formaldehyde, capillary-blotted onto a nylon membrane (Hybond N, Amersham Biosciences, Piscataway, NJ) in 20 x SSC, and fixed by UV irradiation. Hybridization probes of *BiP*, *AtbZIP60*, *Sec61γ*, *Sar1* and *Hap5b* cDNAs were labeled with [α-^{32}P] dCTP using a DNA labeling kit (BcaBEST labeling kit, Takara, Otsu, Japan). To

8

distinguish between *BiP1* and *BiP2*, and *BiP3*, 200 bp fragments of 5' regions of *BiP1* and *BiP3* were PCR amplified by primers (CAAAAAGAGAGATCGTACGCAAAAG and ACTGATCCTAACTTCGTAGCCTCTT for *BiP1* and *BiP2*, and ACAAACGAGATCGAAGAAGAGTTCTC and ACCGTCCCCAGTTTCTGCTCTTCGC for *BiP3*) and used for labeling reaction. The membrane was washed with 0.2 x SSC, 0.1% SDS at 65 ˚C three times, then exposed to X-ray film.

DNA constructs for protoplast transformation

For observation of subcellular localization of truncated AtbZIP60, a cDNA fragment corresponding to amino acids 1-216 in AtbZIP60 was PCR amplified using primers GTCGACATGGCGGAGGAATTTGGAAGCATAG and CCATGGTAGACTCCTGCTTCGACATCATGG, then fused to the N-terminus of the sGFP in the CaMV35S-sGFP(S65T)-NOS3' vector, a gift of Dr. Niwa (Chiu et al., 1996).

For transactivation assay, plasmids yy64 and yy96 were used as an effector and a reporter, respectively (Yamamoto and Deng, 1998). Each cDNA fragment of AtbZIP60 was amplified by PCR using primers (GGATCCATGGCGGAGGAATTTGGAAGC and GTCGACTCAAGACTCCTGCTTCGACATCATGGTAG for AtbZIP60(1-216), GGATCCATGGCGGAGGAATTTGGAAGC and

GTCGACTCACTCCTTCCCGGAATCATCAGCGGCG   for   AtbZIP60(1-110),

GGATCCATGAATTCGGATTTGGTTGTTGAGAAGAAG   and

GTCGACTCAAGACTCCTGCTTCGACATCATGGTAG   for   AtbZIP60(111-216),

GGATCCATGGCGGAGGAATTTGGAAGC   and

GTCGACTCAATCCGGTGAAGACTGAAGAAAATC   for   AtbZIP60(1-40),

GGATCCATGGCGGAGGAATTTGGAAGC   and

GTCGACTCAATAATCAACGAGTAGATCCGCTAT   for   AtbZIP60(1-80),

GGATCCTCATGGATCGGAGAAATCGAGAAT   and

GTCGACTCAATAATCAACGAGTAGATCCGCTAT   for   AtbZIP60(41-80),   and

GGATCCTCATGGATCGGAGAAATCGAGAAT   and

GTCGACTCACTCCTTCCCGGAATCATCAGCGGCG for AtbZIP60(41-110)). PCR

products were fused to C-terminus of GAL4-BS by insertion into the BamHI-SalI site of

the multi cloning site in yy64, and then used as effectors.

For transient luciferase assays, the β-glucuronidase (GUS) gene in pBI221

(Clontech) was replaced with the firefly luciferase gene derived from pGL3-Basic

(Promega), obtaining the plasmid pBI221-Luc. Approximately 1.2 kb of *BiP*, *calnexin*

(*CNX*) and *Hsp70* promoters was amplified by PCR using primers

(CTCGAGAGAGGAGGTTGAGAGAGAAGATAGAC   and

ACTAGTAGCCATATCGGAAACTTTTGCGTACG   for   *BiP1*,

CTCGAGTGTATTGTAAAGCCCTTAGCGTTACCGG   and

GGATCCAGCCATATCGGAAACTTTTGCGTACG   for   *BiP2*,

CTCGAGCAAACATAGCACCGAACGACTTACTAC   and

CGCATGGATCCAATCATTTTTCGTTGTTGAGAACTCTTCTTCG   for   *BiP3*,

CTCGAGGACGAGATGGTTGCTTTGGGTCTA   and

GGATCCTCTCATTCTCGGAATCTCTAAAAT   for   *CNX1*,

CTCGAGCGTCGTTTCTCTATGATTCATTTG   and

GGATCCTCTCATTATCGCAATCTCAAGAGA   for   *CNX2*,   and

CTCGAGCGAACATTTTGCTGAACTGATTAG   and

GGATCCCGCCATTATTAGAGATCAGAATTG for *Hsp70*). PCR products were

translationally fused to the firefly luciferase gene by replacing the CaMV 35S promoter

of pBI221-Luc, and were designated BiP1pro-Luc to BiP3pro-Luc, CNX1pro-Luc,

CNX2pro-Luc and Hsp70pro-Luc, respectively. To obtain mutations in *BiP1* and *BiP3*

promoters, two mutated PCR fragments for each mutation were amplified. Subsequent

PCR was performed to obtain full-length mutated promoters, and designated m1 to m3.

These were substituted for the CaMV 35S promoter of pBI221-Luc. A P-UPRE

hexamer fused with the CaMV35S -46 minimal promoter (min) and firefly luciferase,

designated as P-UPREx6-min-Luc, was used as previously described (Oh et al., 2003).

For ERSE, a TTACCAATCACTTCTTGACACGAGA hexamer was synthesized and

used to replace that of P-UPREx6-min-Luc to generate ERSEx6-min-Luc. For

overexpression of intact and truncated AtbZIP60, cDNA sequences encoding each

polypeptide were substituted with the *GUS* gene of pBI221. Resulting constructs were designated 35S-AtbZIP60 and 35S-AtbZIP60ΔC. For overexpression of HY5, a cDNA fragment amplified with primers GGATCCATGCAGGAACAAGCGACTAGCTCT and GAGCTCTCAAAGGCTTGCATCAGCATTAGA was substituted with *GUS* gene of pBI221. The resulting construct was designated 35S-HY5. For promoter analysis of *AtbZIP60*, an approximately 1.2 kb region of promoter amplified by PCR with primers AAGCTTCGTAAAACAATTTAATAGATGTTAATG and GGATCCCATGGTCAAAAAAAAAAAAATATACAAAGAAGAAAAAAAAAAGC was translationally fused to the firefly luciferase gene by replacing the CaMV 35S promoter of pBI221-Luc (AtbZIP60pro-Luc). To obtain mutations in the promoter, two mutated PCR fragments were amplified using a combination of AAGCTTCGTAAAACAATTTAATAGATGTTAATG and AGATGAGAGAAGGCTTAGTTCTGGAAGAATAGGATCACAG, and GAACTAAGCCTTCTCTCATCTTGTGTGACGGCACATAAAA and GGATCCCATGGTCAAAAAAAAAAAAATATACAAAGAAGAAAAAAAAAAGC, respectively. Subsequent PCR was performed using AAGCTTCGTAAAACAATTTAATAGATGTTAATG and GGATCCCATGGTCAAAAAAAAAAAAATATACAAAGAAGAAAAAAAAAG to obtain full-length mutated promoter, which was substituted for the CaMV 35S promoter of pBI221-Luc (AtbZIP60mpro-Luc).

Stable transformation with a chimeric gene consisting of AtbZIP60 promoter and the GUS gene

The promoter region used to construct AtbZIP60pro-Luc was fused with the *GUS* gene by replacing the CaMV 35S promoter of pBI121 to generate AtbZIP60pro-GUS. Stable transformation of *Arabidopsis* was carried out according to Clough et al. (Clough and Bent, 1998). GUS activity of T1 plants was measured using 4-methylumbelliferyl-β-D-glucuronide (4-MUG) as described previously (Oh et al., 2003). For GUS histochemical staining, whole transgenic seedlings and organs were incubated with GUS buffer (25 mM sodium phosphate buffer, pH 7.2, 1mM $KFe(CN)_2$, 1mM $KFe(CN)_3$, 10mM EDTA, and 1mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide) at 37 ˚C. The GUS buffer was removed and the tissues were dehydrated by increasing the ethanol concentrations gradually from 70% (v/v) to absolute ethanol. Samples were visualized in the light microscope.

Transient expression analysis by GFP and dual luciferase assays

Protoplasts were isolated from *Arabidopsis* suspension cells and transiently transformed using polyethylene glycol according to Ueda et al. (Ueda et al., 2001). In brief, 3g of cultured cells was incubated in 25 ml of enzyme solution (0.4 M mannitol, 5 mM EGTA, 1% cellulose R-10 and 0.05% pectolyase Y-23) for 2 hr at 30 ˚C and filtered

with two layers of Miracloth (Calbiochem, #475855). Protoplasts were washed twice with 25 ml of solution A (0.4 M mannitol, 70 mM $CaCl_2$, and 5 mM MES-KOH, pH 5.7) and resuspended in 1 ml of MaMg solution (0.4 M mannitol, 15 mM $MgCl_2$, and 5 mM MES-KOH, pH 5.7). After the addition of 20 μg of plasmid and 50 μg of carrier DNA to 100 μl of protoplast solution, 400 μl of DNA uptake solution (0.4 M mannitol, 40% polyethylene glycol 6000 and 0.1 M $Ca(NO_3)_2$) was added. The protoplasts were incubated on ice for 20 min and then diluted with 10 ml of dilution solution (0.4 M mannitol, 126 mM $CaCl_2$, 5 mM KCl, 5 mM glucose and 1.5 mM MES-KOH, pH 5.7). Collected protoplasts were resuspended in 4 ml of MS medium containing 0.4 M mannitol. Fluorescence of GFP was observed by an LSM510 confocal laser scanning microscope (Carl Zeiss) after incubation at 23 ˚C for 16 h. For the dual luciferase assay, transformed protoplasts were incubated at 23 ˚C for 16 h in the dark and luciferase activities were measured using the dual luciferase assay system (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Isolation of atbzip60 mutant

A T-DNA insertion mutant having a Columbia background, *atbzip60* (SALK_050204), was obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Insertion mutant information was obtained from the Salk Institute

Genomic Analysis Laboratory Web site (http://signal.salk.edu/).

Microarray analysis

Arabidopsis seedlings were grown in one-half strength MS medium supplemented with 2% (w/v) sucrose in a 16 h light and 8 h dark cycle. Total RNA was extracted from 10-day-old seedlings treated with 5 μg/ml of tunicamycin for 5 h as a tunicamycin-treated sample, and from seedlings before tunicamycin treatment as nontreated sample. Two independent experiments were performed to obtain samples. Total RNA was extracted using RNeasy Plant Mini Kit (QIAGEN, Valencia, CA) according to the manufacture's instructions. Total RNA samples were processed to cDNA, labeled, and hybridized to Arabidopsis 2 Oligo Microarray (Agilent Technologies, California, USA), and the fluorescence was scanned by Agilent Technologies Microarray Scanner. The data were analyzed using GeneSpring (Silicon Genetics, San Carios, CA). Each RNA sample labeled by Cy5 was hybridized competitively with common reference (CR) labeled by Cy3; an equal mixture of each total RNA was used for CR. Hybridization and scanning were performed by Hokkaido System Science Co.,Ltd. (Hokkaido, Japan).

Results

Identification of AtbZIP60

I assumed that bZIP transcription factors play roles in the ER stress response of plants, since bZIPs are involved in the response of yeast (Hac1) and mammals (XBP1 and ATF6). Thus, according to the prediction of 75 bZIP genes in the *Arabidopsis* genome (Jakoby et al., 2002), they were screened one by one by RT-PCR using RNA prepared from *Arabidopsis* leaves treated with and without tunicamycin. Among the transcripts detected, only transcripts of *AtbZIP60* (AGI code; At1g42990) were highly induced by tunicamycin and the induction was confirmed by RNA gel blotting analysis using *BiP* as a positive control (Fig. 2A). *AtbZIP60* encoded an open reading frame consisting of 295 amino acids (Fig. 2B) having a bZIP DNA binding domain followed by a putative transmembrane domain (TMD) (Fig. 2C). This implies conversion of AtbZIP60 to a soluble protein by proteolysis in response to ER stress in analogy to ATF6 in mammals. Indeed, a truncated AtbZIP60 containing amino acids 1-216 (AtbZIP60ΔC) fused to GFP localized to the nucleus when transiently expressed in *Arabidopsis* protoplasts (Fig. 2D).

Expression profile of AtbZIP60

In order to examine whether other agents inducing ER stress affect the expression

16

of *AtbZIP60*, *Arabidopsis* seedlings treated with tunicamycin, dithiothreitol (a reducing agent inhibiting disulfide bond formation), or azetidine-2-carboxylate (a proline analog that perturbs protein structure) were subjected to RNA blot analysis. These agents also induced *AtbZIP60* as well as *BiP* (Fig. 3A). As shown in Fig. 3B, the time course of *AtbZIP60* induction in response to tunicamycin treatment was similar to that of *BiP* induction.

In order to investigate expression profile of *AtbZIP60* in organ or tissue level, a transgenic plant carrying chimeric gene consisting of *AtbZIP60* promoter (approximately 1.2 kb) and *GUS* gene was generated. When plants were treated with tunicamycin, GUS activity was clearly (approximately 7 fold) induced, indicating that the promoter contains a sequence necessary for the ER stress response (Fig. 4A). When seedlings were treated with tunicamycin, GUS staining was obviously enhanced (Fig. 4B, a), being consistent with the result observed in Fig. 4A. Under normal growth condition, clear GUS staining was observed only in anthers and immature seeds (Fig. 4B, b-d). However, mature seeds were not stained (Fig. 4B, e).

Transactivation activity of AtbZIP60

In order to examine whether AtbZIP60 has transactivation activity, transient assay was conducted. In this experiment, a reporter plasmid with luciferase gene driven by a synthetic promoter with GAL4 binding site (GAL4 BS) and an effector plasmid for a

17

fusion protein with the GAL4 DNA binding domain (GAL4 BD) were co-introduced

into protoplasts prepared from *Arabidopsis* suspension cells. As shown in Fig. 5, fusion

of AtbZIP60ΔC (amino acid 1-216) at C-terminus of GAL4 BD clearly showed

luciferase activity. When AtbZIP60ΔC was devided into two parts, only N-terminal half

(amino acid 1-110) revealed luciferase activity. In contrast, C-terminal half containing

the bZIP domain (amino acids 111-216) did not show activity. Detailed analysis of

N-terminal half showed that the region corresponding to amino acids 1-80 was

responsible for transcriptional activation.

Effect of AtbZIP60 on activation of BiP *and* CNX *promoters*

Since AtbZIP60 was proven to be a transcriptional activator, next question was

what the target genes are. In general, induction of *BiP* is considered as an indication of

the ER stress response. Thus, I examined effect of AtbZIP60 on activation of *BiP*

promoters by using transient assay. The *Arabidopsis* genome has been known to contain

three *BiP* genes (Fig. 6A). *BiP1* and *BiP2* are closely related including promoter and

intron sequences. Homology of amino acid sequence between BiP1 and BiP2 are 98%

(Koizumi and Sano, 1997), and both genes have one copy of P-UPRE on their

promoters. *BiP3* has different characteristics from *BiP1* and *BiP2* (Fig. 6B) (Noh et al.,

2003). Its amino acid sequence is 80% homologous with BiP1 and BiP2, and it does not

have P-UPRE on the promoter. Instead, the *BiP3* promoter contains two copies of ERSE,

which is identical to a *cis*-element responsible for the ER stress response in mammals and has been assumed to function also in plants. Expression profile of *BiP* genes also differs as shown in Fig. 6C; transcripts for *BiP1* and *BiP2* are detected under non-stressed condition, whereas basal level of *BiP3* is very low. In addition to *BiP* genes, two *CNX* genes (*CNX1* and *CNX2*) that are also induced by ER stress were included in the experiments.

First, each of the three *BiP* promoters and two *CNX* promoters (approximately 1.2 kb) was fused with the firefly luciferase reporter gene and introduced into protoplasts as described above. When transformed protoplasts were treated with tunicamycin, luciferase activity driven by three *BiP* and two *CNX* promoters was clearly enhanced, indicating these promoters are responsible for ER stress (Fig. 7A). Induction of endogenous *BiP* was also confirmed by RNA blots (data not shown). As a negative control promoter, the promoter of cytosolic heat shock-inducible *Hsp70* (AGI code; At3g12580) (Sung et al., 2001) was tested. Although luciferase activity was enhanced by heat shock (data not shown), it was not affected by tunicamycin treatment (Fig. 7A).

Next, either intact AtbZIP60 or the truncated form, AtbZIP60ΔC (amino acids 1-216), was co-expressed under the CaMV35S promoter as effectors. As control effectors, GUS and HY5, a bZIP transcription factor involved in signal transduction of photomorphogenesis (Chattopadhyay et al., 1998) were used, since HY5 showed the highest similarity with XBP1 in a database search of the *Arabidopsis* genome. Although

GUS, HY5 and the intact AtbZIP60 did not affect induction of luciferase activity, co-expression of AtbZIP60ΔC significantly enhanced luciferase activity driven by all *BiP* and *CNX* promoters (Fig. 7B). Production of full-length or truncated AtbZIP60 protein was confirmed by using antibodies for AtbZIP60 (data not shown). The level of induction was higher for the *BiP3* promoter than for *BiP1* and *BiP2*. No effect was observed on the *Hsp70* promoter.

Importance of two cis-*elements in chaperone promoters for induction*

Two *cis*-elements, P-UPRE and ERSE, found in many ER chaperone genes have been considered responsible for the ER stress response. Thus, it was likely that activation of *BiP* and *CNX* promoters by AtbZIP60ΔC depends on these *cis*-elements. Following transient assay using dual luciferase reporter and effector plasmids was conducted to examine whether this is the case or not.

First, *cis*-elements on *BiP* promoters were mutated. Mutation was introduced into P-UPRE of the *BiP1* and *BiP2* promoters and ERSE of the *BiP3* promoter (Fig. 8A). Since P-UPRE consists of two *cis*-elements responsible for the ER stress response, ERSE-II and XBP1-BS, three mutated promoters, m1 (mutation in XBP1-BS), m2 (mutation in ERSE-II), and m3 (mutation in both ERSE-II and XBP1-BS) were generated from the *BiP1* and *BiP2* promoters. Three mutated promoters were also generated for *BiP3* that contains two copies of ERSE; m1, m2 (mutation in each ERSE),

20

and m3 (mutation in both ERSEs). As shown in Fig. 8B, treatment with tunicamycin enhanced wild type *BiP* promoters. Disruption of one element (m1 and m2) in *BiP* promoters also responded to tunicamycin. In contrast, when mutation was introduced into both *cis*-elements (m3), induction was abolished, indicating that ERSE-II, XBP1-BS and ERSE are necessary for activation of *BiP* promoters by tunicamycin.

Subsequently, the effect of mutation in *cis*-elements on transcriptional activation by AtbZIP60ΔC was examined. As shown in Fig. 8C, co-expression of AtbZIP60ΔC activated wild type, m1 and m2 promoters of *BiP1*. Although these results were similar with tunicamycin treatment, AtbZIP60ΔC unexpectedly enhanced the m3 promoter with complete disruption of P-UPRE in *BiP1* even though the basal level and induction rate was low. In the case of *BiP3*, wild type, m1 and m2 promoters were activated by AtbZIP60ΔC, while m3 promoter abolished activation of the *BiP3* promoter by AtbZIP60ΔC.

Since loss of function of the *cis*-elements was confirmed, I next examined whether P-UPRE or ERSE are sufficient for the ER stress response. A hexamer of either P-UPRE or ERSE fused to the CaMV 35S -46 minimal promoter and the luciferase gene was served as a reporter plasmid. As shown in Fig. 9A, P-UPRE responded to tunicamycin as previously reported (Oh et al., 2003), and ERSE also responded to tunicamycin. When AtbZIP60ΔC was co-expressed under the CaMV 35S promoter, luciferase activities driven by both P-UPRE and ERSE were clearly enhanced, in

contrast to no effect of co-expression of GUS (Fig. 9B). Intact AtbZIP60 had little effect on induction (data not shown). It should be noted that induction through ERSE was higher than through P-UPRE in both tunicamycin treatment and co-expression of AtbZIP60ΔC.

Autoregulation of AtbZIP60 transcripts

Since *AtbZIP60* was induced by ER stress similarly to *BiP*, AtbZIP60 may induce its own transcription. Indeed, the promoter of *AtbZIP60* contains a sequence, CCAAT-N9-TCAAG, similar to the general ERSE sequence CCAAT-N9-CCACG. As indicated in Fig. 10A, CCAAT is conserved exactly, and TCAAG has two base mismatches with the CCACG. Then I predicted that this ERSE-like sequence functions in the induction of *AtbZIP60*. In order to test this idea, mutation was introduced into the ERSE-like sequence (Fig. 10A) and subjected to transient luciferase assay. As shown in Fig. 10B, authentic *AtbZIP60* promoter responded to tunicamycin; however, the mutated promoter responded little to tunicamycin. This result indicates that the induction of *AtbZIP60* is dependent on the ERSE-like sequence.

Subsequently, the effect of AtbZIP60ΔC on the *AtbZIP60* promoter was examined. As shown in Fig. 10C, co-expression of AtbZIP60ΔC clearly activated the authentic *AtbZIP60* promoter. However, this activation was almost completely abolished by mutation of the ERSE-like sequence, suggesting that AtbZIP60ΔC activates

transcription through the ERSE-like sequence.

Isolation of T-DNA insertion mutant of AtbZIP60

Since experiments described above were mostly conducted by transient assay, I considered that more direct evidence was necessary to show involvement of AtbZIP60 in the ER stress response. Therefore, I obtained a T-DNA insertion mutant of *AtbZIP60* gene from the collection generated in Salk Insitute. In this mutant, the first exon was disrupted by insertion of T-DNA (Fig. 11A). After self crossing, homozygote (*atbzip60*) was isolated and disruption was confirmed by PCR and southern blot analysis (data not shown). RNA blot analysis further confirmed that transcript was not detected in *atbzip60* by tunicamycin treatment (Fig. 11B). When wild type and *atbzip60* plants were grown on soil in normal condition (16 h light and 8 h dark cycle at 22 ˚C), apparent difference on plant growth was not observed.

Microarray analysis

It is likely that induction of ER stress-inducible genes is affected in *atbzip60*, since AtbZIP60ΔC activated *BiP* and *CNX* promoters. Thus, difference of expression profile of genes by ER stress was analyzed between wild type and *atbzip60* using microarray. I used an oligonucleotide array (Agilent Arabidopsis 2 Oligo Microarray), which contains 60-mer oligonucleotides for each 21,500 gene derived from ATH1 ver. 3 database of

23

The Institute for Genomic Research (TIGR). For the analysis, RNA was isolated from 10-day-old seedlings of wild type and *atbzip60* before or after tunicamycin treatment, and subjected to microarray analysis.

Among 21,500 genes, 19,583 genes showed considerable signal intensity and 133 genes were activated more than 3-fold by tunicamycin treatment in wild type. These were categorized according to the annotations in the database with normalized signal intensities and fold inductions (Table 1). The results for *atbzip60* were also shown. Dominant components of genes responding to tunicamycin were for protein folding and for secretory pathway. Namely, 21 genes were chaperones (BiP, GRP94, DnaJ, CRT, and CNX), and enzymes for disulfide bond formation (PDI and ERO1) and prolyl isomerization (ROC7). 19 genes were identified as genes for protein transport through the secretory pathway such as protein import into the ER (Sec61 translocon complexes and signal peptidase) and small GTPases involved in vesicle transport (Sar1 and Arf). Genes for each step of ERAD pathway were also induced. They are a Der1-like transmembrane protein for retrotranslocation, proteins related to ubiguitin/proteasome system such as ubiquitin ligases or F-box protein, and AAA-type ATPases for degradation. Genes for signal transduction such as transcription factors including *AtbZIP60*, for protein kinase and for two-component system were also up-regulated. In addition, 22 genes for various metabolism including protein glycosylation and 40 genes without defined function were identified.

24

Among 133 genes induced in wild type, a considerable number of genes showed reduced induction in the *atbzip60*. In table 1, 34 genes of which fold induction was 2-fold lower in *atbzip60* than in wild type were indicated by shadow. Regarding the *BiP* genes, mRNA induction of *BiP3* was clearly repressed in the mutant, while that of *BiP1* and *BiP2* showed similar activation rate between wild type and mutant plants. In order to confirm this repression, several genes (three *BiP* genes, *AtbZIP60*, *Sec61γ* (AGI code; At4g24920), *Sar1* (AGI code; At1g09180) and *Hap5b* (AGI code; At1g56170)) were selected and analyzed by RNA blot. As shown in Fig. 12, induction of *AtbZIP60*, *BiP3*, *Sar1* and *Hap5b* was apparently suppressed in *atbzip60*.

Relationship between cis-*elements and expression profile*

As described above, the ER stress response is considered to be dependent on *cis*-elements ERSE and P-UPRE (combination of ERSEII and XBP1-BS). Thus, these *cis*-elements were searched in promoter sequences of genes up-regulated by tunicamycin. Since the first cytosine residue of CCACG of ERSE (CCAAT-N9-CCACG) and ERSE-II (ATTGG-N-CCACG) has been reported to have a minor effect, consensus sequences of ERSE and ERSE-II used in this search were defined CCAAT-N10-CACG (for ERSE) and ATTGG-N2-CACG (for ERSE-II). A consensus sequence of XBP1-BS (GA-TGACGT-GR) was used without modification. I also defined an ERSE-like sequence with one mismatch as ERSE-L, and an XBP1-BS

core sequence (TGACGT-GR) as UPRE. With these consensus sequences, 500 bp of promoter sequences from defined or predicted 5' end of each transcription start site were surveyed. These c*is*-elements were found in 71 out of 133 up-regulated genes (Table 1). It is noteworthy that *cis*-elements are more abundant in promoters of genes for protein folding than others. In 34 genes whose induction was clearly repressed in *atbzip60,* ERSE or ERSE-L was found in 12 genes, and XBP1-BS or UPRE in 4 genes. Although typical *cis*-elements were not identified in other 19 genes, it does not mean they do not contain *cis*-elements since they may locate outside of 500 bp region.

Discussion

The purpose of this study was identification of a transcription factor functioning in the ER stress response in plants. Since previous studies indicated that *cis*-elements for the ER stress response were conserved between mammals and plants, I assumed that transcription factors also have common characteristics with those of other organisms. That is, a bZIP transcription factor may play roles in the ER stress response of plants, since all transcription factors known to date are bZIPs (Hac1 in yeast, XBP1 and ATF6 in mammals).

According to this assumption, *Arabidopsis* genes encoding putative bZIP were analyzed by RT-PCR and *AtbZIP60* was identified to be induced by tunicamycin treatment. In addition to tunicamycin, other reagents activating the ER stress response also induced transcripts of *AtbZIP60*. Thus, I predicted that AtbZIP60 plays a role in the ER stress response. However, since the expression profile of *AtbZIP60* was close to that of *BiP*, induction of *AtbZIP60* transcript was not considered to be the first trigger of activation of *BiP* expression. Instead, it is likely that a conformational change of AtbZIP60 is the initial trigger for induction of chaperone genes. This prediction was based on the fact that AtbZIP60 contains a putative TMD similar to ATF6. From analogy to ATF6, I hypothesized that AtbZIP60 is synthesized as a precursor protein anchored in the ER membrane, and converted to a soluble form, localizing to the

27

nucleus. This soluble form may function to induce expression of chaperone genes. Indeed, a truncated form of AtbZIP60 without C-teminal domain including TMD (AtbZIP60ΔC) fused with GFP localized to the nucleus. In addition, N-terminal region of AtbZIP60 (amino acids 1-80) has considerable transactivation activity.

Furthermore, transient assay using luciferase reporter showed that co-expression of AtbZIP60ΔC clearly activated three *BiP* and two *CNX* promoters responding to tunicamycin, in contrast to no effect of the intact AtbZIP60. This result strongly supported the hypothesis that cleavage of AtbZIP60 in protein level is necessary for activation. In fact, preliminary result using antibody for AtbZIP60 and transgenic plants expressing AtbZIP60 under CaMV 35S promoter indicated cleavage of AtbZIP60 upon tunicamycin treatment (data not shown). In mammals, ATF6 is translocated to the Golgi apparatus where it is cleaved by S1P and S2P proteases. However, conserved sequences necessary for cleavage by proteases (Ye et al., 2000) was not found in AtbZIP60. Thus, the mechanism of cleavage remains to be clarified.

Activation of *BiP* and *CNX* promoters by AtbZIP60ΔC is considered to be through the *cis*-elements P-UPRE and ERSE, since disruption of these *cis*-elements in *BiP* promoters abolished the activation. It was exception however that mutation into P-UPRE in the *BiP1* promoter still revealed activation by AtbZIP60ΔC even though induction by tunicamycin was almost abolished with this mutation. This unexpected induction may be due to the incomplete disruption of P-UPRE. In fact, the mutated

BiP1 and *BiP2* promoters with disruption of P-UPRE (m3) still slightly responded to tunicamycin. It is likely that excess production of AtbZIP60ΔC has stronger effect on the incomplete disruption of P-UPRE than endogenous truncated AtbZIP60 that is generated by tunicamycin. In fact, large amount of AtbZIP60ΔC protein was detected in protoplasts when expressed by CaMV 35S promoter, although endogenous protein could not be detected in protoplasts treated with tunicamycin (data not shown).

It should be mentioned that AtbZIP60ΔC activated both ERSE and P-UPRE that have been shown to be responsible for the ER stress response in plants. P-UPRE consists of ERSE-II and XBP1-BS. However, ERSE (CCAAT-N9-CCACG) and ERSE-II (ATTGG-N-CCACG) seems different in the primary sequence level. How does AtbZIP60ΔC recognize both ERSE and ERSE-II? Most probable interpretation is that the secondary structures of DNA for ERSE and ERSE-II are similar as reported in mammalian cells. That is, CCAAT in ERSE functions as ATTGG (complementary to CCAAT) in ERSE-II (Kokame et al., 2001; Yamamoto et al., 2004). It is likely that AtbZIP60ΔC has a higher binding affinity for ERSE than for ERSEII (or P-UPRE), since higher induction was observed in assays with ERSE. This result was consistent with the observation that the induction rate of *BiP3* is higher than that of *BiP1* and *BiP2*.

The AtbZIP60 promoter was activated by co-expression of AtbZIP60ΔC (Fig. 10C), indicating an interesting characteristic that AtbZIP60 may amplify the signal of the ER

stress response by activating its own transcription. This amplification of signal by transcriptional induction is similar to that of XBP1 in mammalian cells, while XBP1 is activated by IRE1-dependent mRNA splicing and ATF6 is responsible for transcriptional activation of XBP1 (Yoshida et al., 2001). Thus, AtbZIP60 has characteristics similar to both ATF6 and XBP1, namely activation by proteolysis and transcriptional activation of itself.

Genes up-regulated by tunicamycin were identified using microarray containing *Arabidopsis* 21,500 genes. Being basically consistent with the previous results using an array with 8,000 genes (Martinez and Chrispeels, 2003), various genes were regulated by tunicamycin. Here, I mention about 133 genes that were up-regulated more than three fold. Many of them were genes for protein folding in the ER and for secretory pathway including translocon of the ER. This result is similar to yeast rather than mammals. That is, the ER stress response in yeast activates genes for secretory pathway as well as ER protein folding, while in mammals induction of genes is rather restricted to protein folding in the ER (Okada et al., 2002; Travers et al., 2000). This difference of transcriptional regulation between yeast and mammals has been considered to be due to the presence of PERK, a transmembrane ER stress-sensing protein in mammalian cells, but not in yeast cells. The PERK pathway induces transient attenuation of protein translation to decrease the load of newly synthesized proteins into the ER, reducing the amount of unfolded proteins. The yeast cells may have evolved a system triggering

dynamic remodeling through the secretory pathway in response to ER stress instead of translational attenuation. In this respect, plants may be similar to yeast, being consistent with the fact that PERK homolog is not found in the *Arabidopsis* genome.

The 133 genes also contain genes for protein degradation that are probably related to ERAD and protein glycosylation. These were already reported by other researchers and seemed interpretable. A considerable number of genes for signal transduction was also detected. They were protein kinases and transcription factors including *AtbZIP60*, which is highly induced by tunicamycin treatment. In addition, a number of genes for putative proteins were also detected. Although their functions were not known, they may be involved in the ER stress response. Some genes such as chlorophyll binding protein seemed not to be directly related to the ER stress response.

It was not easy to detect correlation between expression profile and *cis*-clements, namely some genes contained apparent ERSE or P-UPRE in their promoters while some do not even though they were clearly induced. Of course it is possible that some variations of nucleotide sequence are allowed for the functional *cis*-elements. Another possibility is that *cis*-elements locate outside of 500 bp regions from the transcription start sites, even though a considerable number of genes, especially for protein folding, contains *cis*-elements on their promoters.

When expression profile of these 133 genes was compared between wild type and T-DNA insertion mutant *atbzip60*, induction of at least 34 genes was clearly repressed

in the *atbzip60*. Reduction or abolishment of induction of *BiP3*, *Sar1B* and *Hap5b* was confirmed by northern blot. However, those genes do not necessarily have apparent *cis*-elements responsible for the ER stress response in their promoters. In contrast, genes that contain ERSE are not necessarily repressed in *atbzip60*. This interpretation is similar to no strict relationship between gene expression and *cis*-elements described above. However, it could be mentioned that AtbZIP60 is one of the transcription factors mediating ER stress since induction of some genes such as *BiP3* and *Sar1B* were clearly diminished by deletion of *AtbZIP60*.

It would be also true that AtbZIP60 is not only the factor functioning in the ER stress response. In other words, *Arabidopsis* is considered to have other pathways for the ER stress response in addition to the AtbZIP60 pathway. Regarding the *BiP* genes, although co-expression of AtbZIP60ΔC activated all three *BiP* promoters, induction of *BiP1* and *BiP2* was not affected in *atbzip60*. Transcriptional induction of *BiP3* was clearly but not completely repressed in the *atbzip60* mutant. This observation indicated an additional signaling pathway of the ER stress response.

Taken together, current model was summarized in Fig. 13. Without stress *AtbZIP60* is weakly transcribed and AtbZIP60 locates in the membrane with its TMD, possibly in the ER membrane. Sensing stress or malfolded proteins, AtbZIP60 is processed to a soluble form and located to the nucleus where it functions as a transcription factor. Namely, the initial trigger of activation of this pathway is considered as conversion to a

soluble form. This idea is supported by the observation that overexpression of truncated form (AtbZIP60ΔC) but not intact protein enhanced the reporter genes driven by *BiP* and *CNX* promoters in transient assay. However, mechanisms for sensing ER stress and for protein processing are unclear. ATF6 in mammals is also processed at protein level. In this case, ATF6 is translocated to the Golgi and then processed by proteases (Shen and Prywes, 2004; Ye et al., 2000). However, AtbZIP60 does not contain consensus sequences for those proteases. In addition, the C-terminal region of AtbZIP60 is much shorter than that of ATF6, which has been considered to function as a sensor for ER stress that interacts with BiP (Shen et al., 2002; Shen et al., 2005). Thus, the mechanism of conformational change of AtbZIP60 to the active form is considered to differ from that of ATF6. Thus, further analysis to clarify the mechanism of signal perception and conversion to the active form is necessary.

After conversion to a soluble form, possibly similar to AtbZIP60ΔC, AtbZIP60 is supposed to activate promoters of chaperone genes such as *BiP* and *CNX* through ERSE or P-UPRE. This transcriptional activation was confirmed by transient assay from aspects of 'loss of function' and 'gain of function'. However, it was not determined whether soluble form of AtbZIP60 directly binds to those sequences. Analysis of T-DNA insertion mutant of *AtbZIP60* also proved the involvement of AtbZIP60 in the ER stress response. That is, induction of some genes such as *BiP3* is clearly repressed in the mutant. Interestingly, AtbZIP60 contains a *cis*-element similar to ERSE in its own

promoter and activates own transcription through this sequence. In this way, AtbZIP60 is considered to amplify the signal. This characteristic is similar to that of XBP1.

It should be also pointed out that an alternative pathway for the ER stress response exists in *Arabidopsis*, since many genes such as *BiP1* and *BiP2* are still inducible in the T-DNA insertion mutant. Currently it is not known which molecules are involved in the response. One candidate is IRE1 homologs since they play roles in the ER stress response of other organisms. In addition, microarray analysis detected several transcription factors highly induced by tunicamycin treatment. Thus, such transcription factors may also function in signal transduction of the ER stress response. Analysis of these candidates will provide new insight into the ER stress response in plants.

Physiological function of AtbZIP60 *in planta* remains unclear. Although a T-DNA insertion mutant showed a different response of gene expression to tunicamycin, the mutant grow normally under unstressed condition. Tunicamycin treatment did not give obvious difference of growth between the wild type and the mutant (data not shown). This is considered to be due to redundant pathways for the ER stress response. Genes for chaperones and folding enzymes are also redundant. For example, *Arabidopsis* has three *BiP* genes in contrast to mammals that have single *BiP* gene. Although those *BiP* genes similarly respond to ER stress, there may be multiple pathways for induction. Such redundancy of genes and pathways for the ER stress response seems to be unique for plants and also important to survive under various stress conditions. Indeed, it has

been reported that transcription of *AtbZIP60* is activated by several environmental cues.

AtbZIP60 was induced by cadmium stress (Suzuki et al., 2001). Accumulation of

AtbZIP60 transcript was also observed in response to oxidative stress (i.e. exposure to

ozone) (Tamaoki, personal communication), being consistent with the report that the ER

stress response has been implicated in response to oxidative stress (Harding et al., 2003).

In a microarray database in the TAIR web site, *AtbZIP60* transcript was found to

respond to salt stress and some pathogen elicitors. The transcriptional induction in

response to salt stress is consistent with the previous observation that *BiP* expression

was activated in response to salt stress (Koiwa et al., 2003). It was also reported that a

set of ER stress-responsive genes including secretory pathway genes can be induced by

NPR1, an essential positive regulator of salicylic acid-induced PR gene expression and

systemic acquired resistance (Wang et al., 2005). In addition to response to stress,

AtbZIP60 expression was observed in pollens and premature seeds from histochemical

analysis of *GUS* reporter gene (Fig. 4B) This observation indicates that the ER stress

response occurs in these tissues where high amounts of secretory protein are

synthesized. Being consistent with this result, transgenic plants with *GUS* gene under

the *BiP2* promoter also showed signals in these tissues (Koizumi et al, unpublished

data).

Although T-DNA insertion mutant did not show clear phenotype, identification of

AtbZIP60 will serve a clue to clarify the physiological importance of the ER stress

response in plants. Generation of transgenic plants expressing a truncated form of AtbZIP60 driven by the CaMV 35S promoter was not succeeded so far. Transgenic plants are barely obtained or easily die after germination (data not shown). It suggests that constitutive activation of the ER stress response is toxic to plants. Thus, transgenic plants expressing AtbZIP60ΔC under the inducible promoter have been generated. Also plants suppressing targets of AtbZIP60ΔC by using transcriptional repressor peptides (Hiratsu et al., 2004; Hiratsu et al., 2002) have been generated. If these plants show any visible phenotype, they will help to understand the physiological significance of the ER stress response in plants.
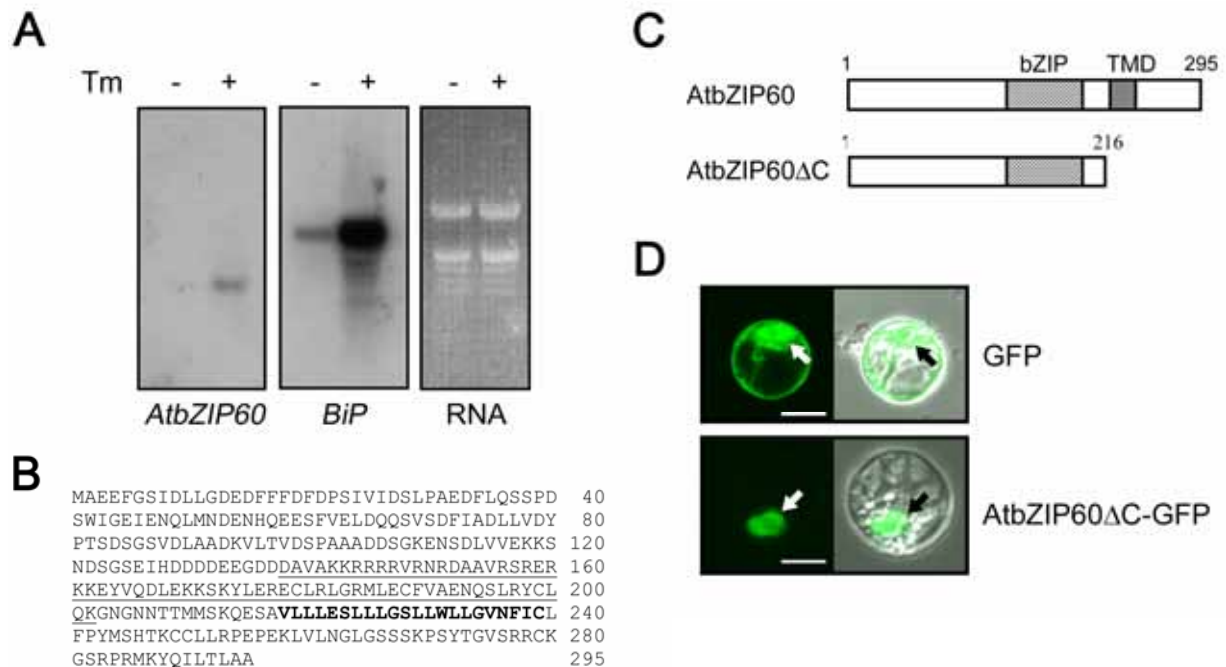
Figures and Tables
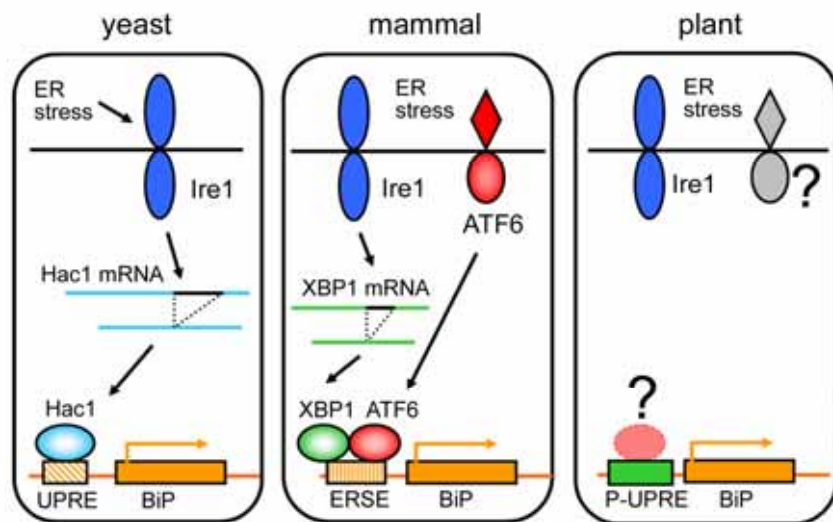
Fig. 1 The molecular mechanism of the ER stress response.
After sensing ER stress, Ire1 catalyzes splicing of mRNA for Hac1 (yeast) and XBP1 (mammals) to produce active transcription factors. In mammals, ATF6 anchored in the ER membrane is cleaved in response to ER stress converting to a soluble and active transcription factor. It should be noted that Hac1, XBP1 and ATF6 are all bZIP transcription factors.

A

Tm   − +   − +   − +

AtbZIP60   *BiP*   RNA

B

```
MAEEFGSIDLLGDEDFFFDFDPSIVIDSLPAEDFLQSSPD    40
SWIGEIENQLMNDENHQEESFVELDQQSVSDFIADLLVDY    80
PTSDSGSVDLAADKVLTVDSPAAADDSGKENSDLVVEKKS   120
NDSGSEIHDDDDEEGDDDAVAKKRRRRVRNRDAAVRSRER   160
KKEYVQDLEKKSKYLERECLRLGRMLECFVAENQSLRYCL   200
QKGNGNNTTMMSKQESAVLLLESLLLGSLLWLLGVNFICL   240
FPYMSHTKCCLLRPEPEKLVLNGLGSSSKPSYTGVSRRCK   280
GSRPRMKYQILTLAA                           295
```

C

AtbZIP60

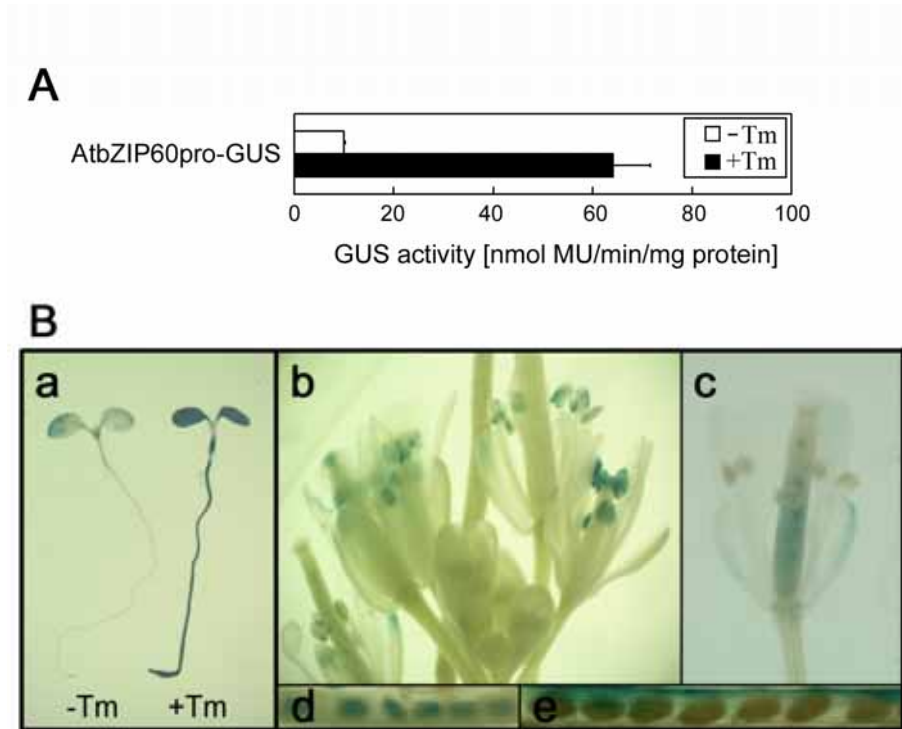AtbZIP60ΔC

D

GFP

AtbZIP60ΔC-GFP

Fig. 2 Identification and characterization of AtbZIP60.
(A) RNA blot analysis of *AtbZIP60* and *BiP*. Total RNA was extracted from 2-week-old *Arabidopsis* seedlings that had been placed in water with DMSO (as a solvent control; -) or 5 μg/ml tunicamycin (+) for 12 h and used for RNA blot analysis. *AtbZIP60* or *BiP* cDNA was used as a probe. (B) Deduced amino acid sequence of AtbZIP60. The bZIP domain is underlined, and a putative TMD is indicated in bold. (C) A schematic structure of AtbZIP60 protein. The locations of the bZIP domain and the TMD are indicated. AtbZIP60ΔC represents the truncated form used in later experiments. (D) Observation of fluorescence of GFP alone and of the AtbZIP60ΔC-GFP fusion protein expressed transiently in protoplasts. Confocal and brightfield images were captured from the same cells. Arrows indicate position of the nucleus (Bar, 10 μm).

38
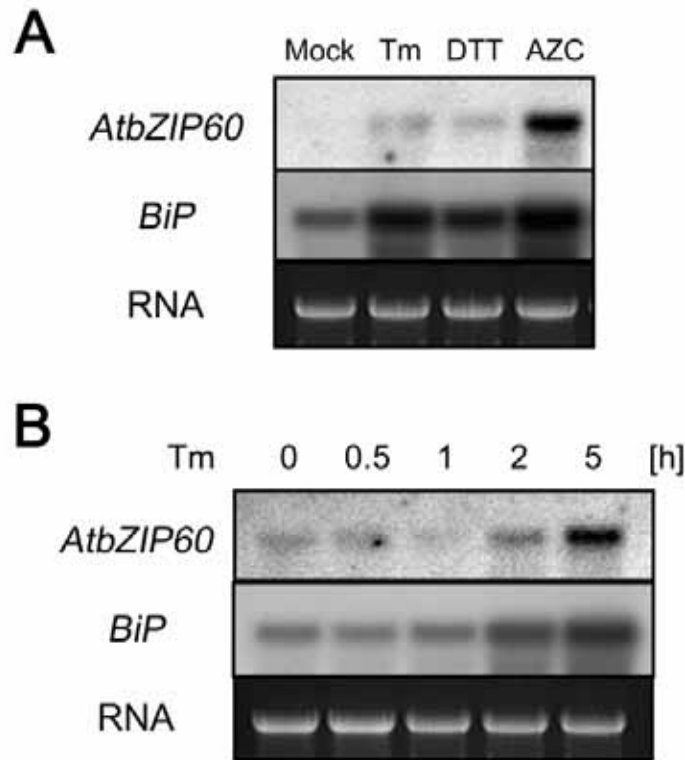
Fig. 3 Expression profiles of *AtbZIP60* and *BiP* transcripts.
(A) Effects of various reagents inducing the ER stress response. Total RNA was extracted from *Arabidopsis* seedlings treated with DMSO (Mock), 5 μg/ml tunicamycin (Tm), 2 mM DTT, or 5 mM azetidine-2-carboxylate (AZC) for 5 h and analyzed by RNA blotting. (B) Induction of time course after tunicamycin treatment. *Arabidopsis* seedlings were treated with 5 μg/ml tunicamycin, and RNA was extracted and analyzed at the indicated time periods. The exposure for *AtbZIP60* in B was conducted for five times longer than that in A.

Fig. 4 Analysis of *AtbZIP60* promoter::*GUS* plants.
(A) GUS activity of *AtbZIP60* promoter::*GUS* plants. Extracts from leaves treated with or without 5 μg/ml tunicamycin (Tm) for 12 h were subjected to quantitative GUS assay. (B) Histochemical staining of *AtbZIP60* promoter::*GUS* plants. a, 5-day-old seedlings were treated with 5 mg/ml tunicamycin (+Tm), or with DMSO (as a solvent control, -Tm) for 10 h, and subjected to GUS histochemical staining. b-e, GUS histochemical analysis under normal growth condition. b, flowers with blue-stained anthers. c, flower after fertilization. d, immature seeds. e, mature seeds.

40
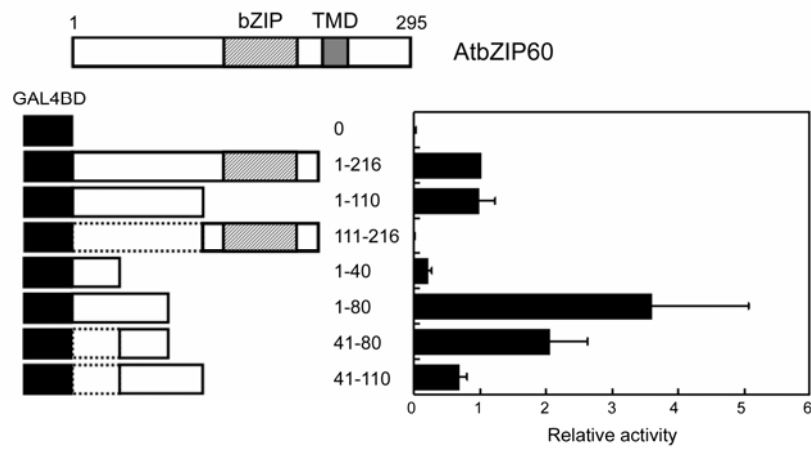
Fig. 5 Transactivation assay of AtbZIP60.

Protoplasts were transiently transformed with effector plasmids carrying each AtbZIP60 fragment fused with GAL4 BD and reporter plasmids expressing a luciferase gene driven by a synthetic promoter with GAL4 BS. A plasmid carrying *Renilla* luciferase gene driven by CaMV 35S promoter was co-transformed for normalization. Relative activity represents activities relative to basal activity obtained from the plasmid expressing AtbZIP60(1-216) fused with GAL4 BD.

41
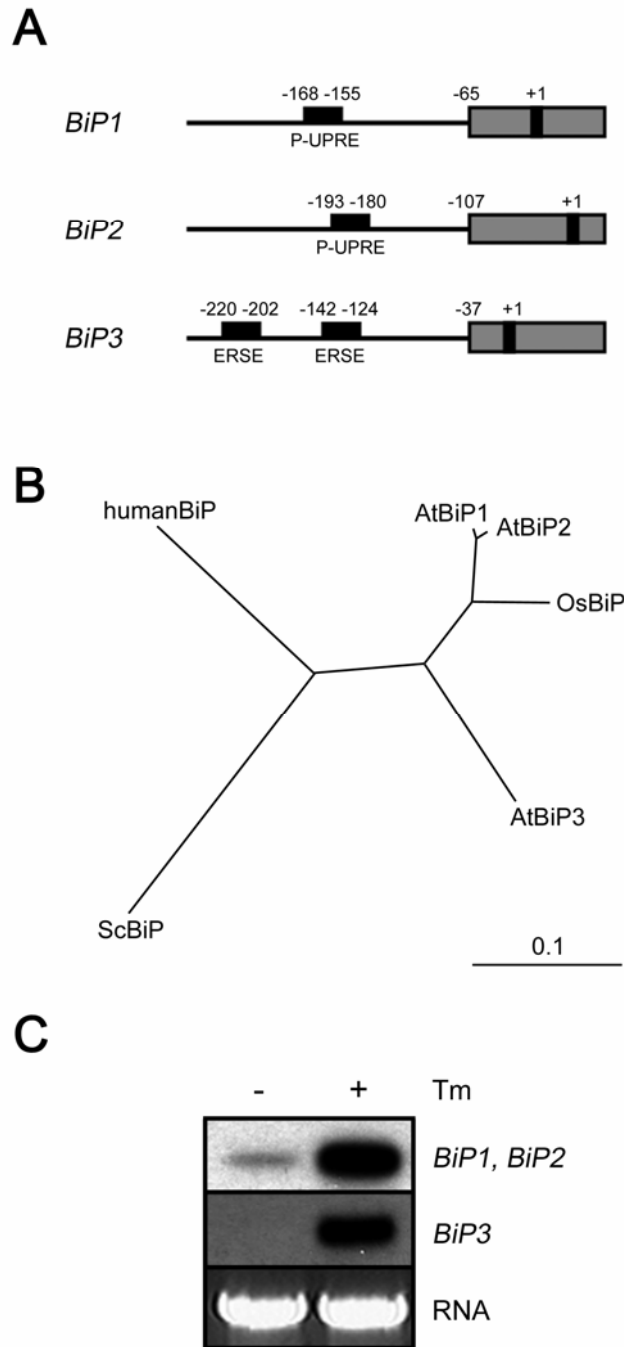
Fig. 6 *Arabidopsis* three *BiP* genes.

(A) Schematic representation of *Arabidopsis* three *BiP* genes. Shaded boxes and black boxes indicate transcribed regions and ATG start codons, respectively. The locations of P-UPRE and ERSE are also indicated. The nucleotide sequences of these *cis*-elements are shown in Fig. 8A. (B) A phylogenetic tree of BiP proteins from yeast (*Saccharomyces cerevisiae*), human, *Arabidopsis* and rice (*Oryza sativa*). (C) RNA blot analysis of *Arabidopsis BiP* genes. Probes for *BiP1* and *BiP2* are considered to cross-hybridize due to high homology.
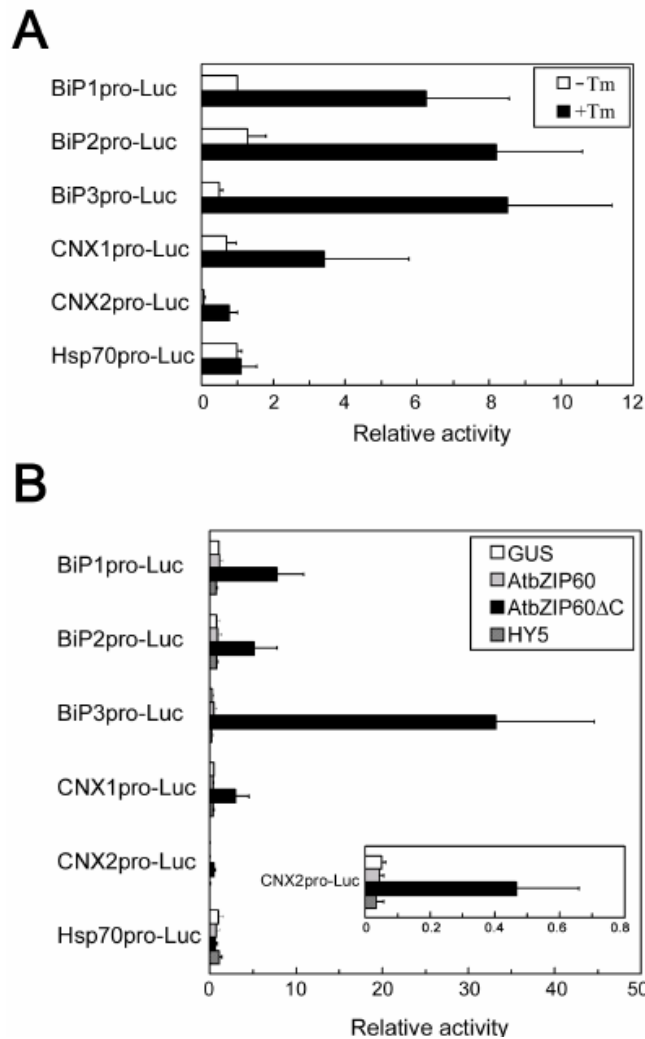
Fig. 7 Effect of AtbZIP60 on gene expression in the ER stress response.

(A) Activation of *BiP* and *CNX* promoters with tunicamycin (Tm) treatment. Protoplasts were transiently transformed with plasmids carrying either the firefly luciferase gene under the control of each *BiP* and *CNX* promoter or *Renilla* luciferase driven by the CaMV 35S promoter. After transformation, protoplasts were incubated with or without 5 μg/ml tunicamycin for 16 h. Luciferase activities were normalized by the ratio of firefly and *Renilla* luciferase activities. Relative activity represents activities relative to basal activity obtained from the construct with the *BiP* promoter. (B) Effects of AtbZIP60 and AtbZIP60ΔC on *BiP* and *CNX* promoters. Transient assays were carried out as described above. Instead of tunicamycin treatment, effector plasmids carrying GUS, AtbZIP60, AtbZIP60ΔC, or HY5 driven by the CaMV 35S promoter were cotransformed. (Inset) An enlarged view of the activity of the *CNX2* promoter. Relative activity represents activity relative to basal activity obtained from constructs with the *BiP1* promoter and *GUS*.
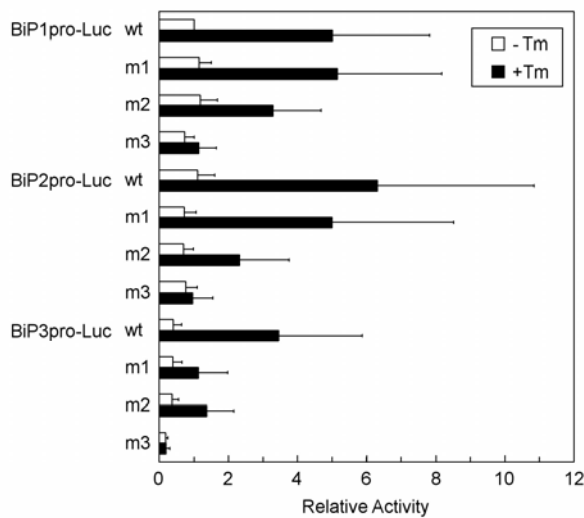
43

Fig. 8 Disruption of *cis*-elements in *BiP* promoters.
(A) Nucleotide sequence of P-UPRE in *BiP1* promoter and ERSEs in *BiP3* promoter. ERSE-II and XBP1-BS in P-UPRE. Authentic (wt) and mutated (m1 to m3) sequences used in later experiments are indicated in bold Mutated sequences are indicated as lower-case characters. P-UPRE in *BiP2* promoter is not shown, since it is the same as that in *BiP1* promoter. (B) Effect of tunicamycin treatment on induction of mutated *BiP* promoters. Transient transformation, including tunicamycin treatment and dual luciferase assay, was carried out as in Fig. 7A. Relative activity represents activities relative to basal activity obtained from the construct with the wild type *BiP1* promoter. (C) Effect of AtbZIP60ΔC on mutated *BiP* promoters. A transient assay was carried out as described in Fig. 7B. Relative activity represents activity relative to that of constructs with the wild type *BiP1* promoter and *GUS*.

44
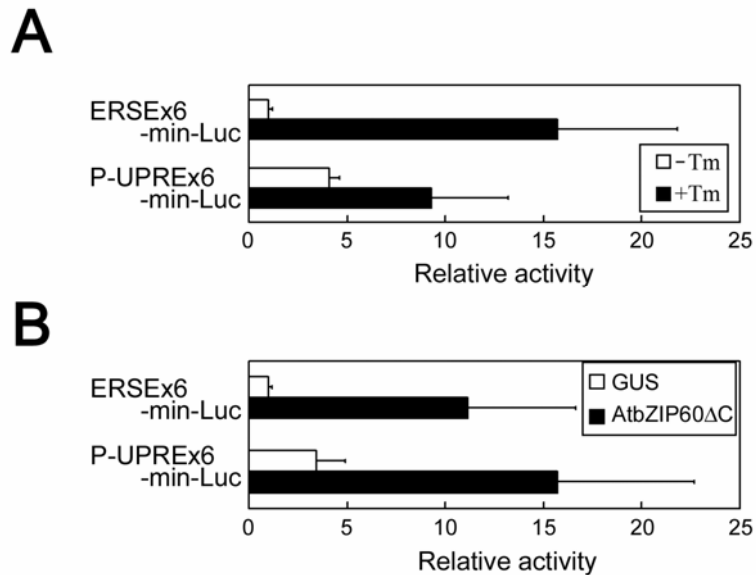
Fig. 9 Effect of AtbZIP60 on ERSE and P-UPRE.

(A) Activation of ERSE and P-UPRE by tunicamycin. Transient transformation, including tunicamycin treatment and dual luciferase assay, was carried out as in Fig. 7A. Plasmids consisting of hexamers of ERSE or P-UPRE, the minimal promoter of CaMV 35S (min) and firefly luciferase (Luc), were introduced. Relative activity represents activities relative to basal activity obtained from the construct with the ERSE hexamer. (B) Effects of AtbZIP60ΔC on ERSE and P-UPRE. Transient assays were carried out as described in Fig. 7B. Relative activity represents activity relative to that of constructs with the ERSE hexamer and *GUS*.
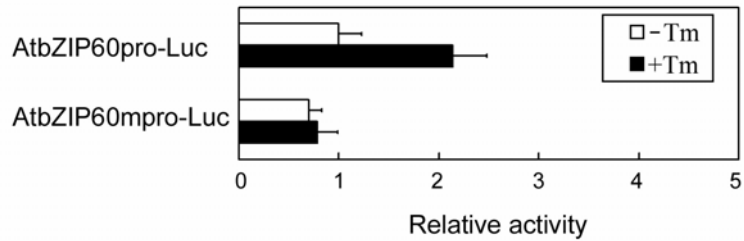
45

A

```
AtbZIP60pro    -263 ACAACCAATGAGAAGGCTTCAAGCTG -288
AtbZIP60mpro   -263 ACAAgatgaGAGAAGGCTTagttCTG -288
```

B

C
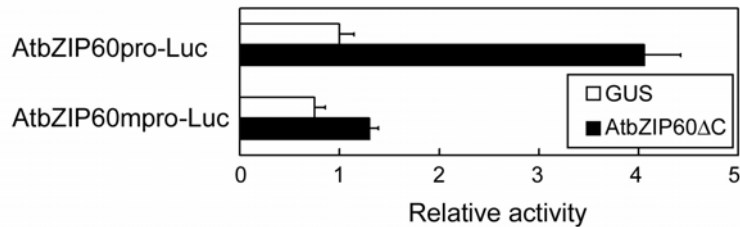
Fig. 10 Regulation of *AtbZIP60* promoter through an ERSE-like sequence.

(A) Nucleotide sequence from -288 to -263 of the *AtbZIP60* promoter. The ERSE-like sequence and mutated sequence (AtbZIP60mpro) used in later experiments are indicated in bold. (B) Activation of the *AtbZIP60* promoter by tunicamycin in a transient assay. Transient transformation of protoplasts was carried out as described in the legend for Fig. 7A. Reporter plasmids consisting of authentic or mutated promoter and the firefly luciferase gene were used for transfection. Tunicamycin (5µg/ml) treatment was conducted for 16 h. Relative activity represents activity relative to basal activity obtained from constructs with the intact *AtbZIP60* promoter. (C) Effect of AtbZIP60ΔC on the *AtbZIP60* promoter. Protoplasts were transfected with reporter plasmids carrying authentic and mutated promoters fused to the firefly luciferase gene and effector plasmid carrying *GUS* and *AtbZIP60ΔC* genes driven by the CaMV 35S promoter. Relative activity represents activity relative to that obtained from constructs with intact *AtbZIP60* promoter and *GUS*.
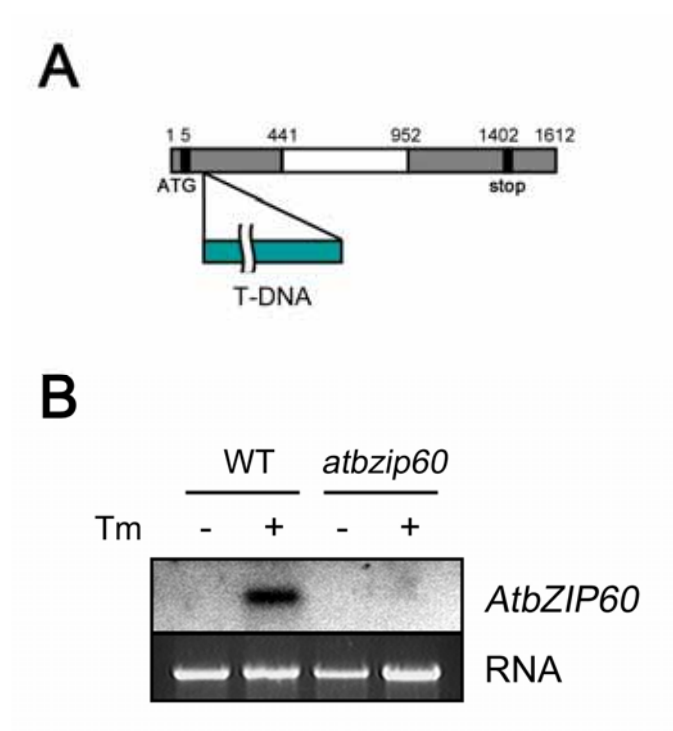
Fig. 11 Isolation of T-DNA insertion mutant.

(A) Schematic representation of the *AtbZIP60* gene and the position of the T-DNA insertion in *atbzip60* mutant. Shaded boxes indicate exons and a white box indicates intron. The positions of a start codon and stop codon are also indicated as black boxes. (B) RNA blot analysis of rosette leaves from the wild type and *atbzip60* mutant with or without tunicamycin (5 μg/ml) for 12 h.
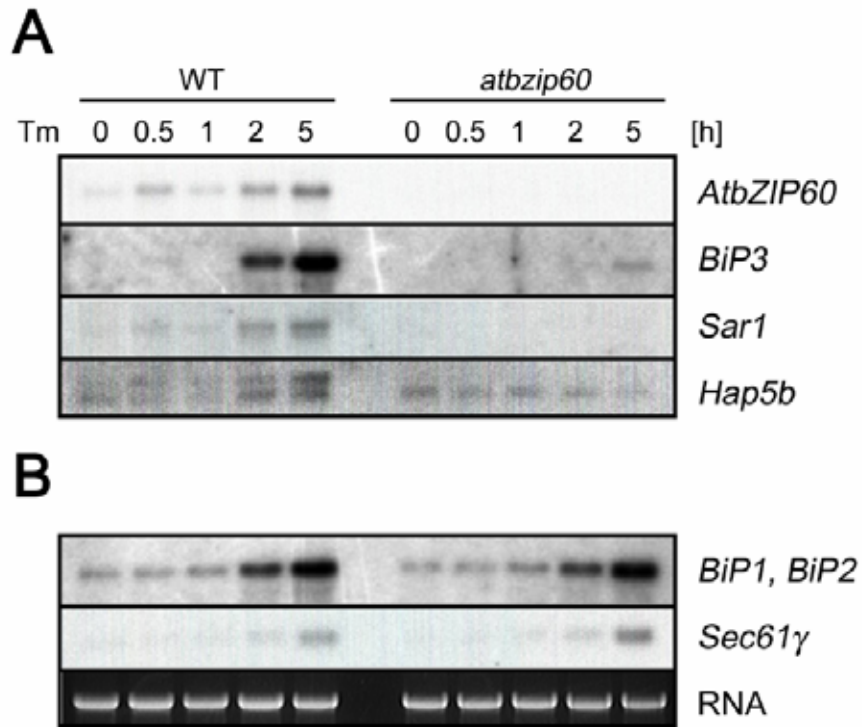
Fig. 12 RNA blot analysis of wild type and *atbzip60* mutant.
Arabidopsis seedlings of wild type and *atbzip60* mutant were treated with 5 μg/ml tunicamycin, and RNA was extracted and analyzed at the indicated time periods. (A) Genes that show repressed induction in *atbzip60* mutant compared to wild type. (B) Genes that show similar expression profile between wild type and *atbzip60* mutant.
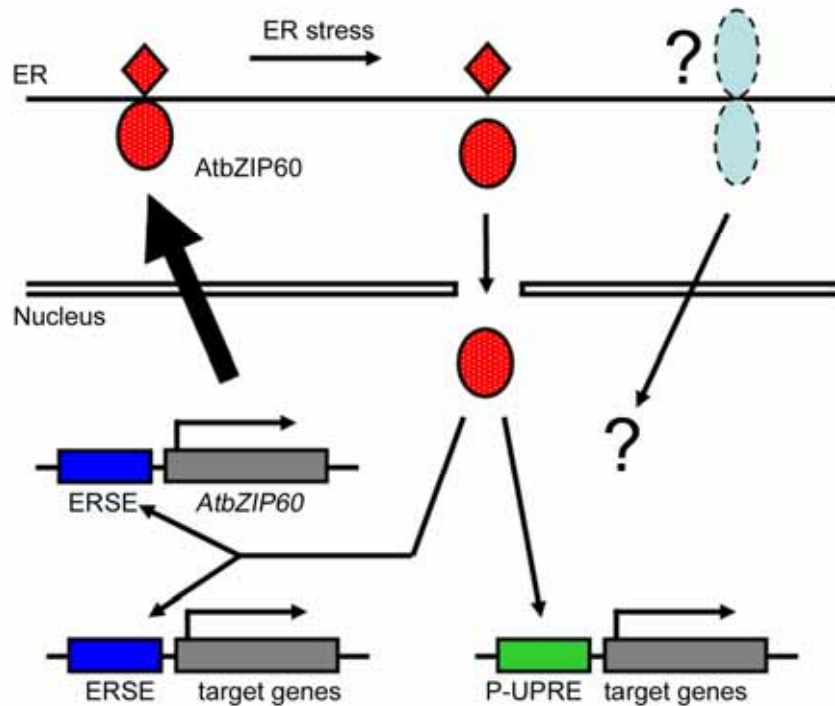
Fig. 13 A proposed model for the function of AtbZIP60 in the ER stress signaling pathway.

AtbZIP60 is synthesized at a low level as a precursor protein that may be anchored in the ER membrane under unstressed conditions. Sensing ER stress by an unknown mechanism, the N-terminal domain of AtbZIP60, which is similar to AtbZIP60ΔC, is cleaved and translocated to the nucleus. Truncated AtbZIP60 activates transcription of target genes, such as *BiP* genes, through either P-UPRE or ERSE. Transcription of *AtbZIP60* is also activated through an ERSE-like sequence to amplify the signal. Definitely, other pathways for the ER stress response exist.

Table 1 Genes induced more than three fold by tunicamycin

AGI code	Description	WT 0 h	WT 5 h	WT FI	*atbzip60* 0 h	*atbzip60* 5 h	*atbzip60* FI	*cis*-element
protein folding (21 genes)								
AT1G09080	BiP3	1.3	151.8	119.5	0.7	17.5	25.8	ERSEx2
AT3G08970	putative DnaJ protein	1.0	24.0	24.0	1.0	15.8	15.9	
AT1G72280	ER oxidoreductin (ERO1)	1.0	13.7	13.5	1.0	6.2	6.4	
AT1G77510	PDI	1.1	13.8	12.1	0.8	6.9	8.2	ERSE
AT4G24190	GRP94	1.1	12.3	11.4	0.9	6.8	7.5	ERSE, ERSE-L, UPRE
AT5G42020	BiP2	1.1	10.3	9.5	0.9	7.2	8.1	ERSE-II, XBP1BS
AT1G04980	PDI-related	1.1	9.9	9.0	0.9	7.2	8.3	ERSE
AT5G28540	BiP1	1.1	9.8	8.9	0.9	7.1	8.0	ERSE-II, XBP1BS
AT2G32920	PDI, putative	1.1	9.5	8.7	0.9	3.6	4.0	ERSE-L
AT3G62600	DnaJ homolog	1.1	8.0	7.5	0.9	5.5	5.9	ERSE
AT1G09210	CRT2	1.1	7.8	7.1	0.9	5.2	5.7	ERSE, ERSE-L
AT5G61790	CNX1	1.1	7.5	7.0	0.9	6.3	6.8	ERSE, UPRE
AT1G56300	DnaJ protein, putative	1.0	5.9	6.2	1.0	5.2	5.0	
AT4G16660	HSP like protein (containing HDEL)	1.1	6.4	6.1	0.9	4.0	4.3	ERSE, UPRE
AT1G21750	putative PDI	1.1	6.4	6.0	0.9	4.1	4.4	ERSEx2, UPRE
AT5G07340	CNX2	1.1	4.8	4.5	0.9	3.7	4.0	
AT2G47470	putative PDI precursor	1.1	4.6	4.3	0.9	3.2	3.5	UPRE
AT1G56340	CRT1	1.1	4.4	4.1	0.9	2.7	2.9	ERSE, ERSE-L, UPREx2
AT3G54960	PDI-like	1.1	4.4	3.9	0.8	1.9	2.2	ERSE-L
AT4G21180	DnaJ-like (containing Sec63 domain)	1.0	4.0	3.9	1.0	2.5	2.6	ERSE-L
AT5G58710	ROC7	1.0	3.4	3.4	1.0	2.5	2.5	ERSE, ERSE-L, UPRE
secretory pathway (19 genes)								
AT1G09180	SAR1B	1.1	10.5	9.7	0.9	1.9	2.2	ERSE-Lx2
AT1G70490	putative ADP-ribosylation factor 1 (Arf)	1.0	9.0	8.9	1.0	1.8	1.8	ERSE
AT1G29310	Sec61 alpha	1.1	8.0	7.2	0.9	4.6	5.2	ERSE
AT3G15980	putative coatomer complex subunit (COPI-vesicle coat)	1.0	6.9	6.7	1.0	1.2	1.2	
AT5G14670	ADP-ribosylation factor (Arf)– like	0.9	5.9	6.3	1.1	1.5	1.5	
AT3G60540	Sec61beta	1.1	6.1	5.8	0.9	4.5	4.8	ERSEx2
AT2G03290	emp24/gp25L/p24 family protein, similar to Transmembrane protein Tmp21	0.8	4.2	5.2	0.9	1.1	1.2	ERSE
AT3G05230	signal peptidase	1.0	5.1	5.1	1.0	3.5	3.5	
AT5G50460	Sec61 gamma	1.0	4.7	4.5	1.0	4.0	4.2	
AT4G24920	SEC61 Gamma –like	1.1	4.4	4.2	0.9	3.3	3.4	

AT2G45070	putative SEC61 beta	1.0	4.0	4.0	1.0	3.2	3.2	ERSE-L
AT1G10630	ADP-ribosylation factor (Arf), putative	1.0	3.9	3.9	1.0	1.4	1.4	
AT3G44340	Sec24 like	1.0	3.9	3.8	1.0	1.7	1.7	
AT2G03120	signal peptide peptidase	1.0	3.8	3.8	1.0	3.0	3.0	
AT1G21900	emp24/gp25L/p24 family protein, similar to Transmembrane protein Tmp21	1.0	3.5	3.5	1.0	3.6	3.7	
AT3G07680	putative coated vesicle membrane protein	1.0	3.4	3.4	1.0	2.4	2.4	ERSE, ERSE-L, UPRE
AT1G52600	signal peptidase subunit, putative	1.0	3.2	3.2	1.0	2.8	2.7	XBP1BS, UPREx2
AT1G62020	coatomer alpha subunit, putative	1.0	3.3	3.2	1.0	1.2	1.2	
AT2G34250	SEC61 alpha	1.0	3.1	3.1	1.0	2.9	2.9	ERSE-L

protein degradation (10 genes)

AT5G40010	AAA-type ATPase	1.0	7.5	7.6	1.0	4.0	3.9	
AT2G02230	phloem-specific lectin, F-box family	1.0	5.6	5.6	1.0	6.1	6.1	
AT4G05010	F-box family protein	0.9	4.7	5.1	1.1	1.8	1.7	ERSE, ERSE-L
AT5G17760	AAA-type ATPase (BCS1-like)	1.0	4.6	4.5	1.0	3.9	4.0	
AT4G05380	AAA-type ATPase (BCS1-like)	0.9	3.9	4.5	1.1	1.1	1.0	
AT1G64470	ubiquitin, putative	1.0	4.1	4.0	0.9	2.4	2.5	ERSE-L
AT3G17000	E2, ubiquitin-conjugating enzyme, putative	0.9	3.8	4.0	1.0	2.1	2.0	UPREx2
AT4G21810	Derlin-like	0.9	3.1	3.3	1.1	2.3	2.2	ERSE-L
AT1G80110	expressed protein, similar to SKP1 interacting partner 3	1.0	3.2	3.3	1.0	3.1	3.0	ERSE-L
AT3G23280	zinc finger (C3HC4-type RING finger) family protein	1.0	3.2	3.2	1.0	2.9	2.9	

transcription factor (14 genes)

AT1G01380	myb family TF (CPC, ETC1)	1.0	8.4	8.5	1.0	1.0	1.0	
AT2G40340	AP2 domain TF (DREB subfamily)	0.9	7.3	8.4	1.1	6.5	6.0	XBP1BS
AT1G42990	AtbZIP60	1.6	13.6	8.3	0.4	0.4	1.1	ERSE-L
AT5G22290	NAM (no apical meristem)-like protein	1.0	6.5	6.7	1.0	3.6	3.5	ERSE-L, UPRE
AT5G64060	NAC1-like	1.2	7.8	6.6	0.8	1.1	1.4	
AT3G28210	zinc finger protein (PMZ), putative	0.9	3.8	4.3	1.1	2.1	1.9	
AT3G49530	NAC2-like protein	0.9	3.5	3.8	1.1	1.8	1.7	ERSE-L
AT1G67970	putative heat shock transcription factor	1.0	3.5	3.7	1.0	2.8	2.7	
AT1G26780	myb-related protein, putative	0.9	3.3	3.6	1.1	1.0	0.9	UPRE
AT3G46080	zinc finger (C2H2-type)-like protein	0.8	2.9	3.5	1.2	3.4	2.9	
AT5G03720	heat shock transcription factor -like protein	1.0	3.6	3.5	1.0	3.7	3.8	XBP1BS
AT4G28790	bHLH TF	0.8	2.6	3.2	1.2	2.0	1.7	
AT1G56170	Hap5b	1.0	3.0	3.0	1.0	1.2	1.1	ERSE-L
AT3G48360	speckle-type POZ protein-related	0.8	2.8	3.6	1.2	3.8	3.3	

protein kinase (4 genes)

AT2G19190	putative receptor-like protein kinase	0.8	6.3	7.5	1.1	3.7	3.3	

AT3G51990	putative serine/threonine protein kinase	1.1	4.6	4.1	0.8	3.6	4.3	ERSE
AT1G78290	serine-threonine protein kinase, putative	1.0	4.1	4.0	1.0	5.1	5.2	
AT3G18750	mitogen activated protein kinase kinase, putative	1.1	3.6	3.4	0.9	1.2	1.2	

two-component (3 genes)

AT5G60100	pseudo-response regulator 3 (APRR3)	1.0	3.6	3.7	1.0	4.0	4.0	ERSE-L
AT5G24470	putative protein (APRR5)	1.0	3.4	3.5	1.0	3.9	3.9	
AT2G40670	ARR16, two-component response regulator protein	1.0	3.3	3.2	1.0	2.4	2.5	

glycosylation (2 genes)

AT2G02810	UDP-galactose/UDP-glucose transporter, contains TMD	1.0	10.7	10.4	1.0	9.5	9.9	ERSE-L
AT1G14360	UDP-galactose/UDP-glucose transporter	1.0	7.3	6.9	1.0	6.6	7.0	ERSE

others (20 genes)

AT1G64460	phosphatidylinositol 3- and 4-kinase family protein	1.0	5.6	5.4	1.0	3.4	3.6	
AT3G24090	glutamine:fructose-6-phosphate amidotransferase, putative	1.0	7.1	7.1	1.0	6.4	6.4	ERSE, UPRE
AT3G03640	glycosyl hydrolase family 1 (beta-glucosidase)	0.9	4.4	4.7	1.1	3.5	3.3	
AT2G27690	cytochrome p450, putative	0.8	3.3	4.0	1.1	4.5	3.9	
AT2G30750	cytochrome p450 family (CYP71A12)	1.1	4.1	3.8	0.8	2.4	3.0	
AT5G47120	Bax inhibitor-1 like (AtBI-1)	1.0	4.5	4.6	1.0	2.8	2.8	ERSE
AT4G14420	elicitor like protein, lesion inducing protein	1.0	5.6	5.5	1.0	4.0	4.2	
AT5G43860	AtCLH2 (chlorophyllase)	1.0	3.9	4.0	1.0	4.4	4.3	
AT2G40880	putative cysteine proteinase inhibitor B (cystatin B)	1.0	3.7	3.9	1.0	3.3	3.2	
AT5G43440	1-aminocyclopropane-1-carboxylate oxidase	0.9	3.1	3.3	1.0	3.2	3.1	
AT2G28630	beta-ketoacyl-CoA synthase family	1.0	4.3	4.5	1.0	4.6	4.6	
AT2G38240	oxidoreductase, similar to flavonol synthase	0.6	1.8	3.1	1.3	3.8	2.9	
AT2G45790	putative phosphomannomutase	1.0	3.5	3.6	1.0	2.5	2.4	
AT5G52760	heavy-metal-associated domain-containing protein	0.9	4.2	4.5	1.0	2.0	1.9	
AT1G14540	anionic peroxidase, similar to lignin forming anionic peroxidase	1.0	2.9	3.0	1.0	3.0	3.0	
AT1G07050	expressed protein (CONSTANS-like)	1.0	13.8	14.1	1.0	14.8	14.5	ERSE-L
AT2G34430	photosystem II type I chlorophyll a /b binding protein	1.0	6.5	6.4	1.0	3.7	3.8	
AT4G21820	putative protein, calmodulin binding	1.1	7.1	6.7	0.9	2.9	3.0	
AT1G78340	glutathione transferase, putative (GST7-like)	0.9	6.0	6.6	1.1	6.2	5.9	
AT1G17960	threonyl-tRNA synthetase, putative	1.1	4.6	4.3	0.9	0.5	0.6	

unknown (40 genes)								
AT5G64510	putative protein	1.0	26.7	26.8	1.0	10.7	10.7	XBP1BS, UPREx2
AT5G23575	transmembrane protein, putative	1.1	10.1	9.4	0.9	2.6	2.8	
AT1G56580	expressed protein	1.0	9.0	8.8	1.0	1.5	1.5	
AT2G25460	expressed protein	0.9	8.1	8.6	1.1	2.0	1.9	ERSE
AT3G51980	hypothetical protein	1.0	8.2	8.3	1.0	5.1	5.1	ERSE
AT3G55700	UDP-glucoronosyl/UDP-glucosyl transferase family protein, glucuronosyl transferase	1.1	8.1	7.6	0.9	2.1	2.2	
AT4G34630	putative protein	1.0	7.7	7.4	1.0	6.9	7.2	ERSE-L
AT4G29520	putative protein	1.1	7.3	6.8	0.9	6.2	6.7	ERSE-L
AT5G42050	putative protein, similar to gda-1	1.0	5.9	6.1	1.0	2.6	2.5	UPRE
AT2G25110	MIR domain-containing protein	1.0	6.2	6.0	1.0	5.2	5.4	ERSE-Lx2
AT5G02220	Expressed protein	1.0	5.6	5.9	1.0	2.9	2.8	ERSE-L, UPRE
AT5G35080	putative protein	1.0	5.7	5.6	1.0	3.8	3.9	ERSE
AT5G15190	putative protein	1.0	5.1	5.3	1.0	3.7	3.5	ERSE-L
AT3G51400	putative protein	1.0	5.1	5.3	1.0	6.5	6.4	
AT1G27350	unknown protein with TMD, ribosome-associated	1.0	5.1	5.0	1.0	4.3	4.4	ERSE
AT1G27330	unknown protein	1.0	5.2	5.0	1.0	4.4	4.6	ERSE, ERSE-L
AT1G11210	expressed protein	0.9	4.5	5.0	1.1	5.9	5.5	
AT4G32670	putative protein	1.1	5.3	5.0	0.9	1.2	1.3	
AT2G42530	cold-regulated protein cor15b precursor	0.9	4.1	4.8	1.1	3.6	3.4	
AT5G42900	putative protein	0.8	3.9	4.6	1.1	4.4	3.9	XBP1BS
AT1G29060	expressed protein	0.8	3.7	4.5	1.1	1.6	1.5	ERSE
AT4G08230	glycine rich protein	1.0	4.5	4.4	1.0	4.1	4.2	ERSE, ERSE-L
AT4G04330	expressed protein	0.9	3.9	4.3	1.0	4.9	4.7	
AT4G32340	putative protein	0.9	3.7	4.3	1.1	3.9	3.6	
AT5G47420	putative protein	1.0	4.3	4.3	1.0	4.0	4.0	ERSE
AT1G18260	suppressor of lin-12-like protein-related / sel-1 protein-related	1.0	4.2	4.0	1.0	2.9	2.9	ERSE
AT2G19460	expressed protein	1.0	3.9	4.0	1.0	3.6	3.5	
AT4G33980	putative protein	0.8	3.3	3.9	1.1	3.6	3.2	XBP1BS
AT1G13340	hypothetical protein	0.8	3.2	3.8	1.1	3.6	3.2	
AT4G30500	putative protein	1.0	3.5	3.7	1.0	3.0	2.9	ERSE-L
AT1G52590	expressed protein	0.8	3.1	3.7	1.2	2.3	2.0	XBP1BS, UPREx2
AT1G67960	unknown protein	1.0	3.7	3.6	1.0	2.2	2.2	ERSE
AT3G15630	expressed protein	0.7	2.6	3.6	1.3	3.6	2.9	
AT4G29960	expressed protein	1.0	3.5	3.5	1.0	3.0	3.1	ERSE
AT1G42480	expressed protein	1.0	3.6	3.5	1.0	2.6	2.8	XBP1BS, UPRE
AT3G53670	putative protein	1.0	3.5	3.4	1.0	3.2	3.2	ERSE-L
AT3G26740	light regulated protein, putative	0.8	2.8	3.4	1.1	4.4	3.8	

AT4G14270	Expressed protein	0.8	2.6	3.2	1.2	2.6	2.2	
AT5G10695	Expressed protein	0.8	2.3	3.0	1.2	2.3	1.9	UPRE
AT3G44860	methyltransferase-related	0.5	1.5	3.0	1.3	3.2	2.5	ERSE-Lx2

Genes whose induction was repressed in the *atbzip60* mutant more than 2-fold compared to the wild type were shaded. FI, fold induction.

Concluding remarks

The ER stress response is a mechanism monitoring malfolded proteins, correcting their structure and degrading proteins that could not be correctly folded in the ER. This quality control mechanism after protein synthesis has been considered to play important roles not only under specific stresses but also under normal growth conditions in eukaryotic cells. In contrast to yeast and mammals, the signaling mechanism and physiological role of the ER stress response in plants have been largely unknown although plants also reveal clear response. Thus I aimed to clarify the signaling mechanism of the ER stress response using a model plant *Arabidopsis thaliana*.

First, according to the assumption that bZIP transcription factors are involved in the ER stress response in plants as well as in yeast and mammals, I screened 75 putative bZIP genes in *Arabidopsis* and identified *AtbZIP60* transcriptionally induced under ER stress. AtbZIP60 contains a transmembrane domain following a bZIP domain, suggesting that conversion to a soluble form and translocation to the nucleus are necessary for activation. This hypothesis was confirmed by transient assay using luciferase reporter genes driven by promoters of ER chaperone genes such as *BiP* or tandem repeats of *cis*-elements ERSE and P-UPRE. This is the first identification of a transcription factor involved in the ER stress response in plants. Analysis of T-DNA insertion mutant of AtbZIP60 clearly indicated that AtbZIP60 plays roles in the ER

stress response since induction of a number of genes were repressed in the mutant. It was also shown that additional pathways that do not need AtbZIP60 function for the induction of the ER stress-responsive genes.

With data obtained in the present study I propose a following model for the molecular mechanism of the ER stress response. Without stress AtbZIP60 is synthesized at a low level as a precursor protein that may be anchored in the ER membrane. Sensing ER stress by unknown mechanism, N-terminal domain of AtbZIP60 is cleaved and translocated to the nucleus. This soluble form activates ER-resident chaperone genes and secretory pathway genes through the *cis*-elements ERSE and P-UPRE. AtbZIP60 also activates its own promoter to amplify the signal. I would like to emphasize that the characteristics of AtbZIP60 and the current model for signaling in the ER stress response in plants proposed in the present study is different from those in yeast and mammals. The present study serves as a new clue in dissecting the molecular mechanism. It will be possible in the near future to regulate expression of truncated form of AtbZIP60 that will help understanding of physiological function of the ER stress response in plants.

References

Bertolotti, A., Zhang, Y., Hendershot, L.M., Harding, H.P. and Ron, D. (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol*, **2**, 326-332.

Boston, R.S., Fontes, E.B., Shank, B.B. and Wrobel, R.L. (1991) Increased expression of the maize immunoglobulin binding protein homolog b-70 in three zein regulatory mutants. *Plant Cell*, **3**, 497-505.

Cascardo, J.C., Almeida, R.S., Buzeli, R.A., Carolino, S.M., Otoni, W.C. and Fontes, E.P. (2000) The phosphorylation state and expression of soybean BiP isoforms are differentially regulated following abiotic stresses. *J Biol Chem*, **275**, 14494-14500.

Chattopadhyay, S., Ang, L.H., Puente, P., Deng, X.W. and Wei, N. (1998) Arabidopsis bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. *Plant Cell*, **10**, 673-683.

Chiu, W., Niwa, Y., Zeng, W., Hirano, T., Kobayashi, H. and Sheen, J. (1996) Engineered GFP as a vital reporter in plants. *Curr Biol*, **6**, 325-330.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J*, **16**, 735-743.

Coleman, C.E., Lopes, M.A., Gillikin, J.W., Boston, R.S. and Larkins, B.A. (1995) A defective signal peptide in the maize high-lysine mutant floury 2. *Proc Natl Acad Sci U S A*, **92**, 6828-6831.

Cox, J.S., Shamu, C.E. and Walter, P. (1993) Transcriptional induction of genes encoding endoplasmic reticulum resident proteins requires a transmembrane protein kinase. *Cell*, **73**, 1197-1206.

Denecke, J., Goldman, M.H., Demolder, J., Seurinck, J. and Botterman, J. (1991) The

tobacco luminal binding protein is encoded by a multigene family. *Plant Cell*, **3**, 1025-1035.

Fontes, E.B., Shank, B.B., Wrobel, R.L., Moose, S.P., GR, O.B., Wurtzel, E.T. and Boston, R.S. (1991) Characterization of an immunoglobulin binding protein homolog in the maize floury-2 endosperm mutant. *Plant Cell*, **3**, 483-496.

Forward, B.S. and Misra, S. (2000) Characterization and expression of the Douglas-fir luminal binding protein (PmBiP). *Planta*, **212**, 41-51.

Gillikin, J.W., Zhang, F., Coleman, C.E., Bass, H.W., Larkins, B.A. and Boston, R.S. (1997) A defective signal peptide tethers the floury-2 zein to the endoplasmic reticulum membrane. *Plant Physiol*, **114**, 345-352.

Gonzalez, R.G., Haxo, R.S. and Schleich, T. (1980) Mechanism of action of polymeric aurintricarboxylic acid, a potent inhibitor of protein--nucleic acid interactions. *Biochemistry*, **19**, 4299-4303.

Harding, H.P., Zeng, H., Zhang, Y., Jungries, R., Chung, P., Plesken, H., Sabatini, D.D. and Ron, D. (2001) Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. *Mol Cell*, **7**, 1153-1163.

Harding, H.P., Zhang, Y., Zeng, H., Novoa, I., Lu, P.D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D.F., Bell, J.C., Hettmann, T., Leiden, J.M. and Ron, D. (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell*, **11**, 619-633.

Hatano, K., Shimada, T., Hiraiwa, N., Nishimura, M. and Hara-Nishimura, I. (1997) A rapid increase in the level of binding protein (BiP) is accompanied by synthesis and degradation of storage proteins in pumpkin cotyledons. *Plant Cell Physiol*, **38**, 344-351.

Haze, K., Yoshida, H., Yanagi, H., Yura, T. and Mori, K. (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and

activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell*, **10**, 3787-3799.

Hiratsu, K., Mitsuda, N., Matsui, K. and Ohme-Takagi, M. (2004) Identification of the minimal repression domain of SUPERMAN shows that the DLELRL hexapeptide is both necessary and sufficient for repression of transcription in Arabidopsis. *Biochem Biophys Res Commun*, **321**, 172-178.

Hiratsu, K., Ohta, M., Matsui, K. and Ohme-Takagi, M. (2002) The SUPERMAN protein is an active repressor whose carboxy-terminal repression domain is required for the development of normal flowers. *FEBS Lett*, **514**, 351-354.

Iwakoshi, N.N., Lee, A.H., Vallabhajosyula, P., Otipoby, K.L., Rajewsky, K. and Glimcher, L.H. (2003) Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. *Nat Immunol*, **4**, 321-329.

Jakoby, M., Weisshaar, B., Droge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T. and Parcy, F. (2002) bZIP transcription factors in Arabidopsis. *Trends Plant Sci*, **7**, 106-111.

Jelitto-Van Dooren, E.P., Vidal, S. and Denecke, J. (1999) Anticipating endoplasmic reticulum stress. A novel early response before pathogenesis-related gene induction. *Plant Cell*, **11**, 1935-1944.

Kalinski, A., Rowley, D.L., Loer, D.S., Foley, C., Buta, G. and Herman, E.M. (1995) Binding-protein expression is subject to temporal, developmental and stress-induced regulation in terminally differentiated soybean organs. *Planta*, **195**, 611-621.

Kaufman, R.J., Scheuner, D., Schroder, M., Shen, X., Lee, K., Liu, C.Y. and Arnold, S.M. (2002) The unfolded protein response in nutrient sensing and differentiation. *Nat Rev Mol Cell Biol*, **3**, 411-421.

Kohno, K., Normington, K., Sambrook, J., Gething, M.J. and Mori, K. (1993) The promoter region of the yeast KAR2 (BiP) gene contains a regulatory domain that

responds to the presence of unfolded proteins in the endoplasmic reticulum. *Mol Cell Biol*, **13**, 877-890.

Koiwa, H., Li, F., McCully, M.G., Mendoza, I., Koizumi, N., Manabe, Y., Nakagawa, Y., Zhu, J., Rus, A., Pardo, J.M., Bressan, R.A. and Hasegawa, P.M. (2003) The STT3a subunit isoform of the Arabidopsis oligosaccharyltransferase controls adaptive responses to salt/osmotic stress. *Plant Cell*, **15**, 2273-2284.

Koizumi, N. (1996) Isolation and responses to stress of a gene that encodes a luminal binding protein in Arabidopsis thaliana. *Plant Cell Physiol*, **37**, 862-865.

Koizumi, N., Martinez, I.M., Kimata, Y., Kohno, K., Sano, H. and Chrispeels, M.J. (2001) Molecular characterization of two Arabidopsis Ire1 homologs, endoplasmic reticulum-located transmembrane protein kinases. *Plant Physiol*, **127**, 949-962.

Koizumi, N. and Sano, H. (1997) Isolation of two genes (Accession nos. D89341 and D89342) encoding luminal binding protein from Arabidopsis thaliana. *Plant Physiol*, **113**, 664.

Koizumi, N., Ujino, T., Sano, H. and Chrispeels, M.J. (1999) Overexpression of a gene that encodes the first enzyme in the biosynthesis of asparagine-linked glycans makes plants resistant to tunicamycin and obviates the tunicamycin-induced unfolded protein response. *Plant Physiol*, **121**, 353-361.

Kokame, K., Kato, H. and Miyata, T. (2001) Identification of ERSE-II, a new cis-acting element responsible for the ATF6-dependent mammalian unfolded protein response. *J Biol Chem*, **276**, 9199-9205.

Leborgne-Castel, N., Jelitto-Van Dooren, E.P., Crofts, A.J. and Denecke, J. (1999) Overexpression of BiP in tobacco alleviates endoplasmic reticulum stress. *Plant Cell*, **11**, 459-470.

Lee, K., Tirasophon, W., Shen, X., Michalak, M., Prywes, R., Okada, T., Yoshida, H., Mori, K. and Kaufman, R.J. (2002) IRE1-mediated unconventional mRNA

splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev*, **16**, 452-466.

Martinez, I.M. and Chrispeels, M.J. (2003) Genomic analysis of the unfolded protein response in Arabidopsis shows its connection to important cellular processes. *Plant Cell*, **15**, 561-576.

Mori, K. (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell*, **101**, 451-454.

Mori, K., Kawahara, T., Yoshida, H., Yanagi, H. and Yura, T. (1996) Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. *Genes Cells*, **1**, 803-817.

Mori, K., Ma, W., Gething, M.J. and Sambrook, J. (1993) A transmembrane protein with a cdc2+/CDC28-related kinase activity is required for signaling from the ER to the nucleus. *Cell*, **74**, 743-756.

Mori, K., Sant, A., Kohno, K., Normington, K., Gething, M.J. and Sambrook, J.F. (1992) A 22 bp cis-acting element is necessary and sufficient for the induction of the yeast KAR2 (BiP) gene by unfolded proteins. *EMBO J*, **11**, 2583-2593.

Muench, D.G., Wu, Y., Zhang, Y., Li, X., Boston, R.S. and Okita, T.W. (1997) Molecular cloning, expression and subcellular localization of a BiP homolog from rice endosperm tissue. *Plant Cell Physiol*, **38**, 404-412.

Noh, S.J., Kwon, C.S., Oh, D.H., Moon, J.S. and Chung, W.I. (2003) Expression of an evolutionarily distinct novel BiP gene during the unfolded protein response in Arabidopsis thaliana. *Gene*, **311**, 81-91.

Oh, D.H., Kwon, C.S., Sano, H., Chung, W.I. and Koizumi, N. (2003) Conservation between animals and plants of the cis-acting element involved in the unfolded protein response. *Biochem Biophys Res Commun*, **301**, 225-230.

Okada, T., Yoshida, H., Akazawa, R., Negishi, M. and Mori, K. (2002) Distinct roles of

activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response. *Biochem J*, **366**, 585-594.

Okushima, Y., Koizumi, N. and Sano, H. (1999) Glycosylation and its adequate processing is critical for protein secretion in tobacco BY2 cells. *J Plant Physiol*, **154**, 623-627.

Okushima, Y., Koizumi, N., Yamaguchi, Y., Kimata, Y., Kohno, K. and Sano, H. (2002) Isolation and characterization of a putative transducer of endoplasmic reticulum stress in Oryza sativa. *Plant Cell Physiol*, **43**, 532-539.

Patil, C. and Walter, P. (2001) Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. *Curr Opin Cell Biol*, **13**, 349-355.

Reimold, A.M., Iwakoshi, N.N., Manis, J., Vallabhajosyula, P., Szomolanyi-Tsuda, E., Gravallese, E.M., Friend, D., Grusby, M.J., Alt, F. and Glimcher, L.H. (2001) Plasma cell differentiation requires the transcription factor XBP-1. *Nature*, **412**, 300-307.

Rutkowski, D.T. and Kaufman, R.J. (2004) A trip to the ER: coping with stress. *Trends Cell Biol*, **14**, 20-28.

Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S. and Kaufman, R.J. (2001) Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell*, **7**, 1165-1176.

Shamu, C.E. and Walter, P. (1996) Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. *EMBO J*, **15**, 3028-3039.

Shen, J., Chen, X., Hendershot, L. and Prywes, R. (2002) ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi

localization signals. *Dev Cell*, **3**, 99-111.

Shen, J. and Prywes, R. (2004) Dependence of site-2 protease cleavage of ATF6 on prior site-1 protease digestion is determined by the size of the luminal domain of ATF6. *J Biol Chem*, **279**, 43046-43051.

Shen, J., Snapp, E.L., Lippincott-Schwartz, J. and Prywes, R. (2005) Stable binding of ATF6 to BiP in the endoplasmic reticulum stress response. *Mol Cell Biol*, **25**, 921-932.

Shen, X., Ellis, R.E., Lee, K., Liu, C.Y., Yang, K., Solomon, A., Yoshida, H., Morimoto, R., Kurnit, D.M., Mori, K. and Kaufman, R.J. (2001) Complementary signaling pathways regulate the unfolded protein response and are required for C. elegans development. *Cell*, **107**, 893-903.

Sung, D.Y., Vierling, E. and Guy, C.L. (2001) Comprehensive expression profile analysis of the Arabidopsis Hsp70 gene family. *Plant Physiol*, **126**, 789-800.

Suzuki, N., Koizumi, N. and Sano, H. (2001) Screening of cadmium-responsive genes in Arabidopsis thaliana. *Plant Cell Environment*, **24**, 1177-1188.

Travers, K.J., Patil, C.K., Wodicka, L., Lockhart, D.J., Weissman, J.S. and Walter, P. (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell*, **101**, 249-258.

Ueda, T., Yamaguchi, M., Uchimiya, H. and Nakano, A. (2001) Ara6, a plant-unique novel type Rab GTPase, functions in the endocytic pathway of Arabidopsis thaliana. *EMBO J*, **20**, 4730-4741.

Vitale, A. and Ceriotti, A. (2004) Protein quality control mechanisms and protein storage in the endoplasmic reticulum. A conflict of interests? *Plant Physiol*, **136**, 3420-3426.

Wang, D., Weaver, N.D., Kesarwani, M. and Dong, X. (2005) Induction of protein secretory pathway is required for systemic acquired resistance. *Science*, **308**,

1036-1040.

Wang, Y., Shen, J., Arenzana, N., Tirasophon, W., Kaufman, R.J. and Prywes, R. (2000) Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. *J Biol Chem*, **275**, 27013-27020.

Wrobel, R.L., GR, O.B. and Boston, R.S. (1997) Comparative analysis of BiP gene expression in maize endosperm. *Gene*, **204**, 105-113.

Yamamoto, K., Yoshida, H., Kokame, K., Kaufman, R.J. and Mori, K. (2004) Differential Contributions of ATF6 and XBP1 to the Activation of Endoplasmic Reticulum Stress-Responsive cis-Acting Elements ERSE, UPRE and ERSE-II. *J Biochem (Tokyo)*, **136**, 343-350.

Yamamoto, Y.Y. and Deng, X.W. (1998) A new vector set for GAL4-dependent transactivation assay in plants. *Plant Biotechnol*, **15**, 217-220.

Ye, J., Rawson, R.B., Komuro, R., Chen, X., Dave, U.P., Prywes, R., Brown, M.S. and Goldstein, J.L. (2000) ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol Cell*, **6**, 1355-1364.

Yoshida, H., Haze, K., Yanagi, H., Yura, T. and Mori, K. (1998) Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *J Biol Chem*, **273**, 33741-33749.

Yoshida, H., Matsui, T., Hosokawa, N., Kaufman, R.J., Nagata, K. and Mori, K. (2003) A time-dependent phase shift in the mammalian unfolded protein response. *Dev Cell*, **4**, 265-271.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T. and Mori, K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell*, **107**, 881-891.

Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M. and Mori, K. (2000) ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to

the cis-acting element responsible for the mammalian unfolded protein response. *Mol Cell Biol*, **20**, 6755-6767.

Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M. and Mori, K. (2001) Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6alpha and 6beta that activates the mammalian unfolded protein response. *Mol Cell Biol*, **21**, 1239-1248.

List of Publications

Yuji Iwata and Nozomu Koizumi (2005) An *Arabidopsis* transcription factor, AtbZIP60, regulates the endoplasmic reticulum stress response in a manner unique to plants. *Proc Natl Acad Sci USA*, **102**, 5280-5285.

Yuji Iwata and Nozomu Koizumi (2005) Unfolded protein response followed by induction of cell death in cultured tobacco cells treated with tunicamycin. *Planta*, **220**, 804-807.

Acknowledgments