Arabidopsis bZIP60 Is a Proteolysis-Activated Transcription Factor Involved in the Endoplasmic Reticulum Stress Response

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Proteins synthesized in the endoplasmic reticulum (ER) of eukaryotic cells must be folded correctly before translocation out of the ER. Disruption of protein folding results in the induction of genes for ER-resident chaperones, for example, BiP. This phenomenon is known as the ER stress response. We report here that bZIP60, an Arabidopsis thaliana basic leucine zipper (bZIP) transcription factor with a transmembrane domain, is involved in the ER stress response. When compared with wild-type Arabidopsis plants, homozygous bzip60 mutant plants show a markedly weaker induction of many ER stress-responsive genes. The bZIP60 protein resides in the ER membrane under unstressed condition and is cleaved in response to ER stress caused by either tunicamycin or DTT. The N-terminal fragment containing the bZIP domain is then translocated into the nucleus. Cleavage of bZIP60 is independent of the function of Arabidopsis homologs of mammalian S1P and S2P proteases, which mediate the proteolytic cleavage of the mammalian transcription factor ATF6. In Arabidopsis, expression of the bZIP60 gene and cleavage of the bZIP60 protein are observed in anthers in the absence of stress treatment, suggesting that the ER stress response functions in the normal development of active secretory cells.

INTRODUCTION

Secretory proteins are synthesized and folded in the endoplasmic reticulum (ER) of eukaryotic cells. Proper folding of proteins is necessary for transport to their final destinations. When this process is perturbed, unfolded proteins accumulate in the ER, inducing expression of many genes. Among them are genes encoding ER-resident chaperones, such as BiP, and enzymes involved in protein folding, such as protein disulfide isomerase. This phenomenon is conserved among eukaryotic cells and is referred to as the ER stress response or the unfolded protein response (Mori, 2000; Kaufman et al., 2002; Rutkowski and Kaufman, 2004).

There is growing recognition that the ER stress response also plays a role in a wide variety of normal cellular processes (Harding et al., 2001; Reimold et al., 2001; Scheuner et al., 2001; Iwakoshi et al., 2003; Lipson et al., 2006). Mammalian cells specialized for secretion, including plasma cells, pancreatic β-cells, hepatocytes, and osteoblasts, require an intact ER stress response. For instance, an intact ER stress response is required for terminal differentiation of B lymphoid cells to plasma cells, during which the ER compartment expands fivefold to accommodate the large increase in immunoglobulin synthesis (Iwakoshi et al., 2003).

In plants, the ER stress response was initially described in the floury-2 endosperm mutant of maize (Zea mays), which produces an aberrant 24-kD α-zein storage protein with a defective signal peptide processing site (Boston et al., 1991; Fontes et al., 1991). The defective storage protein accumulates as a membrane-anchored protein in the ER and in ER-derived protein bodies. As a result, seeds exhibit the ER stress response, with dramatically elevated levels of BiP and other ER-resident chaperones (Coleman et al., 1995; Gillikin et al., 1997). BiP expression is upregulated during seed development in soybean (Glycine max), rice (Oryza sativa), pumpkin (Cucurbita maxima), and Douglas fir (Pseudotsuga menziesii) when large amounts of seed storage proteins are synthesized, folded, and assembled in the ER (Forward and Misra, 2000; Kalinski et al., 1995; Hatano et al., 1997; Muench et al., 1997). BiP expression is induced under stress conditions, such as salt stress and high sugar levels, and in response to pathogens (Koiwa et al., 2003; Wang et al., 2005; Tajima and Koizumi, 2006). The function of the ER stress response in these cellular processes has yet to be elucidated.

The signal transduction mechanism that triggers the ER stress response has been characterized extensively in yeast and mammalian cells. In mammalian cells, two transcription factors, XBP1 and ATF6, activate ER stress-responsive genes. XBP1 is activated by IRE1, an ER membrane–localized protein kinase/ribonuclease. Upon perception of ER stress, IRE1’s ribonuclease domain is activated to catalyze the spliceosome-independent splicing of XBP1 mRNA, which encodes a basic leucine zipper (bZIP) transcription factor (Yoshida et al., 2001a). The splice removes 26 nucleotides from the XBP1 mRNA, resulting in a frame shift that causes production of a nonfunctional protein. The XBP1 protein, which has a transcription-activation domain at the
C terminus, is synthesized from the spliced mRNA and enhances target gene expression through cis-elements designated the ER stress response element (ERSE), ERSE-II, or the unfolded protein response element (UPRE) (Yoshida et al., 1998; Wang et al., 2000; Kokame et al., 2001; Shen et al., 2001; Yamamoto et al., 2004).

ATF6 is a transmembrane protein located in the ER membrane and has a bZIP domain on its cytoplasmic side. In response to ER stress, ATF6 protein is transported to the Golgi apparatus, where it is sequentially cleaved by the site-1 and site-2 proteases (S1P and S2P) (Haze et al., 1999; Lee et al., 2002). S1P cleaves ATF6 on the luminal side to generate ATF6 with a shorter luminal domain. The first cleavage by S1P allows S2P to recognize ATF6 and cleave its transmembrane segment. This two-step processing liberates the cytoplasmic fragment containing the bZIP domain from the membrane. It then translocates into the nucleus, where it activates downstream genes through ERSE or ERSE-II elements, cooperating with the NF-Y transcription factor complex (Yoshida et al., 2000, 2001b). Knockouts of both of the two ATF6 genes have been reported to cause embryonic lethality in mice, further suggesting that the ER stress response plays a critical role in normal development (Yamamoto et al., 2007).

The molecular mechanism of the ER stress response is much less well understood in plants than in yeast or mammalian cells. Tunicamycin, an inhibitor of N-glycosylation commonly used to induce ER stress in yeast and mammals, also induces the ER stress response in plants (Koizumi et al., 1999; Iwata and Koizumi, 2005b; Udade, 2007). Although IRE1 homologs have been identified in Arabidopsis thaliana and rice (Koizumi et al., 2001; Okushima et al., 2002), their involvement in the ER stress response has not been analyzed. We previously reported that the Arabidopsis bZIP60 gene is transcriptionally activated by tunicamycin (Iwata and Koizumi, 2005a). bZIP60 is a protein of 295 amino acids that has a bZIP domain and an adjacent putative transmembrane domain (Figure 1A). Expression of bZIP60ΔC, a truncated form of bZIP60 lacking the transmembrane domain (Figure 1A), activated BiP and calnexin (CNX) promoters through ERSE and P-UPRE cis-elements in a transient expression assay, suggesting that bZIP60 functions in a manner analogous to ATF6 during the ER stress response. bZIP28 has also been reported to be involved in the ER stress response (Liu et al., 2007a; Tajima et al., 2008). bZIP28 activates expression of BiP genes in response to ER stress through cis-elements P-UPRE and ERSE, and T-DNA insertion mutants of bZIP28 show reduced induction of all BiP genes.

Here, we present evidence that bZIP60 is both involved in the plant ER stress response and upregulated and activated during normal development in anther cells specialized for secretion. We show that many of the ER stress-responsive genes are less strongly induced in homozygous bzip60 mutant plants than in wild-type plants. We present evidence that the bZIP60 protein resides in the ER membrane in the unstressed condition and that it is cleaved in response to ER stress, allowing the N-terminal fragment containing the bZIP domain to translocate into the nucleus to function as a transcription factor. We report that cleavage of bZIP60 does not depend on the Arabidopsis homologs of the mammalian S1P and S2P proteases that cleave the ATF6 transcription factor during ER stress. Moreover, cleavage of the bZIP60 protein is observed in actively secreting anther cells in unstressed plants, suggesting a possible function for the ER stress response during normal anther development.

RESULTS

Isolation of a T-DNA Insertion Mutant of bZIP60

We isolated a T-DNA insertion mutant of bZIP60 from the SALK collection. The first exon was disrupted by insertion of the T-DNA in this mutant (Figure 1B). A homozygous bzip60 mutant plant was isolated and disruption was confirmed by PCR. Sequencing of the T-DNA flanking region showed that the T-DNA had inserted 16 nucleotides downstream from the ATG start codon. RNA gel blot analysis further showed that bZIP60 transcripts were undetectable in bzip60 plants treated with tunicamycin (Figure 1C). When wild-type and bzip60 plants were grown in soil under normal growth conditions (16-h-light and 8-h-dark cycle at 22°C), there was no apparent difference in plant growth or morphology.

Microarray Analysis of Wild-Type and bzip60 Mutant Seedlings

Our previous observation that bZIP60 lacking its transmembrane and C-terminal domains (bZIP60ΔC, Figure 1A) can activate BiP and CNX promoters (Iwata and Koizumi, 2005a) prompted us to ask whether induction of ER stress-inducible genes is affected in bzip60 mutant plants. We therefore compared the expression profile of genes induced by ER stress in wild-type and bzip60 mutant plants using Agilent Arabidopsis 2 Oligo Microarrays containing 60-mer oligonucleotides for each of 21,500 genes derived from the ATH1 version 3 database of The Institute for Genomic Research. RNA for the microarray analysis was isolated from 10-d-old wild-type and bzip60 tunicamycin-treated and control seedlings.

Of the 21,500 genes represented on the microarray, 19,583 genes showed significant signal intensity, and of these, 129 were activated more than threefold by tunicamycin treatment in wild-type seedlings (Figure 1D, Table 1). The relative changes in gene expression for these 129 genes in wild-type and bzip60 seedling are shown in Supplemental Table 1 online. The numerically dominant component of the tunicamycin response comprises 25 genes encoding ER chaperones and folding enzymes, including BiP, GRP94 (Klein et al., 2006), J protein (Yamamoto et al., 2008), calreticulin (Christensen et al., 2008), calnexin, protein disulfide isomerase (Houston et al., 2005), ERO1, and ROC7, and enzymes involved in modification, such as glycosylation. An additional 21 of the tunicamycin-induced genes code for proteins involved in protein transport into or through the secretory pathway. These include the Sec61 translocon complexes and signal peptidases as well as small GTPases involved in vesicular transport (Sec1 and Arf). Genes coding for proteins of the ER-associated degradation pathway were also induced. These include a Der1-like transmembrane protein for retrotranslocation of unfolded proteins from the ER to the cytosol (Kirst et al., 2005) and proteins related to ubiquitin/proteasome system, such as...
ubiquitin ligases and F-box proteins. In addition, genes coding for proteins involved in signal transduction, including the transcription factors bZIP60, were also upregulated. An additional 25 genes involved in various other metabolic processes and 34 genes of unknown function were upregulated as well.

Among the 129 genes induced by tunicamycin treatment in wild-type seedlings, 54 showed a significantly lower level of induction in \textit{bzip60} than in wild-type seedlings (Welch’s \(t\) test, \(P < 0.05\)). These are identified in boldface type in Supplemental Table 1 online. Among the chaperone genes, the \textit{BiP} gene was clearly less strongly induced in \textit{bzip60} than in wild-type seedlings, whereas induction of \textit{BiP1} and \textit{BiP2} was similar in both. Several genes were subjected to RNA gel blot analysis to confirm the microarray results. These included the three \textit{BIP} genes, \textit{bZIP60}, \textit{Sar1} (At1g09180), and \textit{Sec61\(\gamma\)} (At4g24920). As shown in Figure 1E, induction was much less marked for \textit{BiP3} and undetectable for \textit{Sar1} in \textit{bzip60} compared with wild-type seedlings, while induction of others was unaffected, as indicated by the microarray data. The lower level of \textit{BiP3} gene induction was reflected at the protein level as well, with little or no increase in \textit{BiP3} protein in response to tunicamycin treatment in \textit{bzip60} mutant seedlings compared with wild-type seedlings (Figure 1F).

The ER stress response is considered to be mediated by the cis-elements designated ERSE and P-UPRE (a combination of ERSE-II and XBPI-BS or UPRE) (Oh et al., 2003). We therefore searched the promoter sequences of genes upregulated by tunicamycin for such motifs (ERSE, ERSE-II, ERSE-L, XBP1-BS, and UPRE; see Methods) and found them in 68 of the 129 upregulated genes (see Supplemental Table 1 online). Among them, ERSE or ERSE-L elements were found in 54 genes, and XBP1-BS or UPRE elements were found in 24 genes. We found

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Characterization of ER Stress-Inducible Genes in Wild-Type Plants and Plants with a T-DNA Insertion in the \textit{bZIP60} Gene.}
\end{figure}
that these elements were overrepresented (P = 1.28E-22 for ERSE or ERSE-L elements and P = 7.65E-11 for XBP1-BS or UPRE elements; see Methods), showing that these motifs were enriched among ER stress-responsive genes. Among the 54 genes whose induction was clearly lower in bzip60 mutants than in wild-type seedlings, ERSE or ERSE-L elements were found in 12 genes and XBP1-BS or UPRE elements were found in 12 genes. Thus, only about half of the bZIP60-responsive genes had known ER stress-responsive motifs within 0.5 kb of the transcription start site.

To confirm that the reduced induction of the 54 putative bZIP60-responsive genes was due to disruption of the bZIP60 gene, we performed a complementation experiment by introducing a genomic fragment containing the bZIP60 gene into bzip60 mutant plants and then analyzed the response of the transformed plants to tunicamycin. As shown in Supplemental Figure 1 online, induction of the BiP3 gene was restored by introduction of the genomic fragment containing the bZIP60 gene. This confirms that the reduced induction of ER stress-responsive genes was due to disruption of the bZIP60 gene.

Evidence That bZIP60 Is a Transcription Factor in Vivo

We previously reported that the N-terminal fragment of bZIP60 activates several ER stress-responsive promoters in a protoplast assay (Iwata and Koizumi, 2005a). To provide further evidence that bZIP60 activates ER stress-responsive genes in vivo, we asked whether the minimal repression domain (RD) from SUPERMAN, an Arabidopsis transcriptional repressor regulating flowering time, could override transcription induction by bZIP60. The RD domain consists of the six amino acids DLELRL, and fusion of RD has been reported to convert a transcriptional activator to a strong transcriptional repressor (Hiratsu et al., 2002, 2004). We fused the C terminus of the bZIP60ΔC coding sequence to RD coding sequence to generate the bZIP60ΔC-RD construct and expressed it from a dexamethasone (DEX)-inducible pro-
from a cauliflower mosaic virus 35S promoter (bZIP60-OX) and used qRT-PCR to investigate transcriptional induction of ER chaperone genes in response to ER stress. We confirmed that tunicamycin induces the bZIP60 gene in wild-type cells, and we established that the bZIP60 gene is constitutively expressed at a high level in bZIP60-OX cells (Figure 3A). Overexpression of bZIP60 in bZIP60-OX cells was also confirmed at the protein level (see below; Figure 4A). Thus, both the gene and the protein are overexpressed in bZIP60-OX cells. However, BiP1 and BiP3 transcripts were detected at similar levels in unstressed wild-type and bZIP60-OX cells (Figure 3B). These results demonstrate that overexpression of bZIP60 gene does not increase the abundance of BiP transcripts.

The abundance of both BiP1 and BiP3 transcripts increased in response to ER stress in both wild-type and bZIP60-OX cells (Figures 3B and 3C). Both BiP1 and BiP3 transcripts were induced in both wild-type and bZIP60-OX cells upon treatment with either tunicamycin (Figure 3B) or DTT, a reducing agent that inhibits disulfide bond formation and induces ER stress (Figure 3C). Thus, despite overexpression of the bZIP60 gene and bZIP60 protein, ER stress-responsive target genes still require the ER stressor for induction. These observations imply the existence of a posttranslational activation step.

Consistent with the greater abundance of bZIP60 protein in bZIP60-OX cells than in wild-type cells, both BiP1 and BiP3 genes were more strongly induced by both tunicamycin and DTT in the bZIP60-OX cells than in the wild-type cells (Figures 3B and 3C). This implies that the overproduced protein contributes to the ER stress response, once activated. The BiP3 gene was more strongly induced by both tunicamycin and DTT treatment in bZIP60-OX cells than in wild-type cells than the BiP1 gene (Figures 3B and 3C). This finding is consistent with the earlier observations that bZIP60 has a more marked effect on the transcription of the BiP3 gene than the BiP1 gene.

Proteolysis and Subcellular Localization of bZIP60 Protein in Response to ER Stress

The results of the foregoing experiments, combined with our earlier report that the N-terminal fragment of bZIP60 lacking the transmembrane domain shows nuclear localization (Iwata and Koizumi, 2005a), suggest that bZIP60 is activated by proteolytic

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(A) qRT-PCR analysis of bZIP60 transcripts in wild-type and bZIP60-OX cells in response to tunicamycin. RNA was extracted from wild-type and bZIP60-OX Arabidopsis suspension cells treated with 5 μg/mL tunicamycin at each time point and subjected to qRT-PCR analysis. The abundance of bZIP60 transcripts was normalized to that of Act8 transcripts. Inset: An enlarged view of the result obtained with wild-type cells. Data represent means with SD of three independent experiments. (B) qRT-PCR analysis of BiP transcripts in wild-type and bZIP60-OX in response to tunicamycin. RNA was extracted as in (A). The abundance of BiP transcripts was normalized to that of Act8 transcripts. Data represent means with SD of three independent experiments. (C) qRT-PCR analysis of BiP transcripts in the wild type and bZIP60-OX in response to DTT. qRT-PCR was performed as in (B), except that the ER stress treatment was 2 mM DTT instead of tunicamycin.
and intracellular localization of the bZIP60 protein.

and Figure 1A) to investigate the effect of ER stress on the size
unstressed cells and a new, faster-migrating band expected for
single band corresponding to full-length bZIP60 was detected in
and subjected to immunoblot analysis. As shown in Figure 4A, a

A

\[ \text{WT} \quad \text{bZIP60-OX} \]

\[ \begin{array}{c|c|c|c}
\text{bZIP60} & \text{\textit{kD}} & \text{\textit{+}} & \text{\textit{+}} \\
\hline
\text{WT} & 40 & + \\
\text{bZIP60} & 33 & + \\
\text{\textit{α-Tub}} & 33 & + \\
\end{array} \]


B

\[ \text{Tm} \quad \text{0.5} \quad 1.5 \quad 2 \\
\text{bZIP60} \\
\text{α-Tub} \\
\end{array} \]

\[ \text{DTT} \quad \text{0} \quad 0.5 \quad 1.5 \quad 2 \\
\text{bZIP60} \\
\text{α-Tub} \\
\end{array} \]

\[ \text{anti-FLAG} \quad \text{anti-HA} \]

\[ \begin{array}{c|c|c|c}
\text{DTT} & \text{0} & 0.5 & 1.5 & 2 \\
\hline
\text{anti-FLAG} & - & - & - & - \\
\text{anti-HA} & - & - & - & - \\
\end{array} \]


Figure 4. Proteolytic Processing of bZIP60 in Response to ER Stress.

(A) Detection of bZIP60 protein. Total protein was extracted from wild-
type and bZIP60-OX cells treated with 5 μg/mL tunicamycin (+) or 0.1% DMSO (−) for 2 h, and 20 μg of protein was subjected to immunoblot analysis using an anti-bZIP60 antibody as well as an anti-α-tubulin antibody as a loading control. Closed and open triangles in (A) and (B) indicate the positions of the full-length and faster-migrating putative cleaved forms of bZIP60, respectively. Positions of protein marker bands were indicated at the left side.

(B) Time-course analysis of bZIP60 protein by ER stress. Arabidopsis suspension cells were treated with 5 μg/mL tunicamycin (Tm), 2 mM DTT, or 0.1% DMSO (Control) at the indicated times, and total protein extracts (20 μg) were gel-fractionated and subjected to immunoblot analysis using an anti-bZIP60 antibody as well as an anti-α-tubulin antibody as a loading control.

(C) Identification of cleaved bZIP60. Arabidopsis suspension cells expressing either bZIP60 tagged with 3xFLAG at the N terminus (3xFLAG-bZIP60) or bZIP60 tagged with 3xFLAG and HA at the N and C termini, respectively (3xFLAG-bZIP60-HA) were treated with 2 mM DTT for 1 h (+) or maintained as controls (−). Total protein was extracted, and 20 μg of protein was gel-fractionated and subjected to immunoblot analysis using anti-FLAG and anti-HA antibodies. The positions of bZIP60 tagged with both 3xFLAG and HA, and bZIP60 tagged with only 3xFLAG are indicated by closed and gray triangles, and the position of the cleaved form of bZIP60, tagged with 3xFLAG at the N terminus, is indicated by the open triangle.

cleavage in or near the transmembrane domain, freeing the
N-terminal fragment to translocate to the nucleus to function as a
transcriptional activator. To test this hypothesis directly, we used
an antibody that recognizes the bZIP60JC protein (see Methods
and Figure 1A) to investigate the effect of ER stress on the size
and intracellular localization of the bZIP60 protein.

Arabidopsis suspension cells were treated with tunicamycin and
subjected to immunoblot analysis. As shown in Figure 4A, a
single band corresponding to full-length bZIP60 was detected in
unstressed cells and a new, faster-migrating band expected for
the cleaved form was detected in wild-type cells treated with
tunicamycin (Figure 4A, left lanes). Similar results were obtained
with bZIP60-OX cells that overexpress bZIP60 (Figure 4A, right
lanes). A temporal analysis of bZIP60 protein during tunicamycin
treatment revealed that the putative cleaved bZIP60 form first
becomes detectable at 1.5 h of tunicamycin treatment (Figure
4B), consistent with the observation that the abundance of BiP1/
BiP2 and BiP3 transcripts increases at 2 h after the onset of
tunicamycin treatment (Figure 3B).

To determine whether the faster-migrating band is the
N-terminal fragment of the bZIP60 protein, rather than a modified
form of the intact protein, we incorporated different epitope tags
at the N and C termini of bZIP60 and followed their intracellular
fate after DTT treatment. A 3xFLAG epitope was added to the N
terminus, and an HA epitope was added to the C terminus of the
bZIP60 coding sequence. Cauliflower mosaic virus 3S promoter–
driven constructs coding for just the FLAG-tagged protein or one
tagged with both FLAG and HA epitopes were transformed into
wild-type Arabidopsis cells. As shown in Figure 4C, only the
slower-migrating band was detected with anti-FLAG antibody in
unstressed cells, while either just the faster-migrating band or
both were detected with anti-FLAG antibody in DTT-treated cells
(Figure 4C, lanes 1 to 4). Neither band was detectable with anti-
HA antibody in cells expressing only the FLAG-tagged bZIP60
construct (Figure 4C, lanes 5 and 6), while only the more slowly
migrating band was detectable with anti-HA antibody in cells
expressing a construct tagged with both FLAG and HA epitopes
(Figure 4C, lanes 7 and 8). This shows that the C terminus of
bZIP60 is missing from the faster-migrating species, providing
direct evidence that the faster-migrating band is a cleaved form
of bZIP60 lacking the C terminus.

To investigate the intracellular location of the cleaved bZIP60
fragment, nuclear proteins were isolated and subjected to im-
munoblot analysis. Consistent with previous observations, only
full-length bZIP60 was detected in total extracts of unstressed
cells, while both the full-length and cleaved forms were detected
in extracts of tunicamycin-treated cells (Figure 5A). Neither form
was detected in nuclei of untreated cells, while only the cleaved
form was detected in the nuclei of cells treated with tunicamycin
(Figure 5A). This observation supports the hypothesis that pro-
eteolytic cleavage of bZIP60 in response to ER stress liberates the
cytosolic N-terminal fragment to translocate into the nucleus.

To investigate the intracellular localization of full-length
bZIP60, we first determined whether bZIP60 associates with
the membranes. We prepared the microsomal and soluble
fractions from untreated Arabidopsis cells by ultracentrifugation
and subjected them to immunoblot analysis. As shown in Figure
5B, bZIP60 protein was detected in the microsomal fraction,
indicating membrane localization. A microsomal preparation
from unstressed cells was further fractionated on a sucrose
density gradient, followed by immunoblot analysis for bZIP60
and various subcellular marker antibodies. We used two kinds of
buffers throughout this analysis, one containing MgCl2 and
the other containing MgCl2 + MgCl2. MgCl2+ keeps the ER membrane–ribosome
interaction intact so that ER-localized proteins sediment more
rapidly in a buffer containing MgCl2 than in buffer containing
EDTA. As shown in Figure 5C, bZIP60 cofractionates with
BiP1 in both buffers. bZIP60 sedimented more rapidly in the
Mg^2+-containing buffer than in the buffer containing EDTA, as did the ER marker BiP1. The sedimentation rates of the other subcellular markers used were unaffected by the presence of Mg^{2+} (Figure 5C). The cofractionation of bZIP60 with the ER marker BiP1 establishes its presence in the ER. The foregoing experiments show that the bZIP60 protein resides in the ER membrane under unstressed condition and that it is cleaved and translocates to the nucleus in ER-stressed cells.

Golgi-localized proteases S1P and S2P are known to mediate the proteolytic cleavage of the mammalian membrane-bound transcription factor ATF6 (Ye et al., 2000). A survey of the genomic database revealed that Arabidopsis has one gene each with significant homology to mammalian S1P and S2P genes. The S1P homolog is encoded by At5g19660, which has been identified previously (Liu et al., 2007b), and S2P is encoded by At4g20310. To determine whether these proteases cleave bZIP60, T-DNA insertion mutants of S1P and S2P were isolated from the SALK and GABI-Kat collections (Figure 6A). Plants homozygous for each of two independent insertion alleles of each gene were isolated and confirmed to be null mutations by RT-PCR (Figure 6B). Seedlings of these mutants were treated with tunicamycin and subjected to immunoblot analysis. As shown in Figure 6C, bZIP60 cleavage was observed in both s1p and both s2p mutants as well as in wild-type plants. Thus, neither the Arabidopsis S1P nor the S2P homolog appears to be involved in bZIP60 cleavage. Since both S1P and S2P must cleave mammalian ATF6 in order for it to be released to translocate to the nucleus, we conclude that bZIP60 cleavage is either performed by different proteases or by a different mechanism than that reported in mammalian cells.

**Activation of bZIP60 without Stress Treatment**

To gain insight into the physiological function of the ER stress response, we analyzed untreated organs and tissues for the presence of the cleaved and uncleaved forms of bZIP60. We first performed RT-PCR to detect expression of the bZIP60 gene. As shown in Figure 7A, the bZIP60 transcripts were detected at similar levels in all tissues examined. Transgenic plants carrying a chimeric gene consisting of the bZIP60 promoter (1.2 kb) and a β-glucuronidase (GUS) gene were used to further investigate the expression pattern of the bZIP60 promoter. When seedlings were treated with tunicamycin, GUS staining was obviously enhanced (Figure 7B), indicating that the promoter contains a sequence necessary for the ER stress response. Under normal growth condition, marked GUS staining was observed in anthers and in immature seeds (Figures 7C to 7E). We observed GUS staining of both pollen grains and tapetal cells in sectioned anthers (Figure 7F).

We used immunoblot analysis to detect endogenous bZIP60 without stress treatment. As shown in Figure 8A, cleaved bZIP60

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**Figure 5.** Translocation of bZIP60 from the ER to the Nucleus in Response to ER Stress.  
(A) Nuclear localization of the cleaved form of bZIP60. Arabidopsis suspension cells were treated with 5 μg/mL tunicamycin (+) or 0.1% DMSO (−) for 2 h, and total protein and nuclear extracts were gel-fractionated and subjected to immunoblot analysis using anti-bZIP60 antibody. Anti-Histone H2B and anti-cFBPase antibodies were used as nuclear and cytosolic markers, respectively. Closed and open triangles indicate the positions of the full-length and cleaved forms of bZIP60, respectively.  
(B) Membrane localization of full-length bZIP60 protein. Total protein extract (Total) from untreated Arabidopsis suspension cells was ultracentrifuged at 100,000g for 1 h to obtain pelleted microsomal membrane fraction (M) and soluble fraction (S). Each fraction was analyzed by SDS-PAGE and immunoblotting using anti-bZIP60 antibody. Anti-VHA-a and anti-cFBPase antibodies were used as markers for membrane and soluble fractions, respectively.  
(C) Sucrose gradient centrifugation. A microsomal fraction from untreated Arabidopsis suspension cells was fractionated through a 15 to 55% sucrose gradient. Aliquots of fractions were analyzed by SDS-PAGE and immunoblotting using anti-bZIP60 antibody as well as antibodies for various subcellular marker proteins; anti-BiP1 (ER), anti-RGP (Golgi), anti-VHA-a (vacuolar membrane), anti-AHA (plasma membrane), and anti-COX-II (mitochondria).
was detected in buds and flowers. Immunoblot analysis after dissection of buds showed that the cleaved bZIP60 was in anthers (Figure 8B). The tapetum is a highly secretory tissue that produces pollen surface proteins, and pollen grains are poised for the high secretory activity that supports rapid pollen tube growth (Hepler et al., 2001; Hsieh and Huang, 2007). The observation that these tissues contain the transcriptionally active N-terminal fragment of bZIP60 suggests that the genes activated by this transcription factor are upregulated in cells with high secretory activity.

**DISCUSSION**

We have presented evidence that bZIP60 is a posttranslationally activated transcription factor involved in the Arabidopsis ER stress response. The results of microarray analysis of wild-type plants identified 129 genes that showed increases in transcript abundance of threefold or more in response to tunicamycin treatment. A majority of the proteins encoded by the induced genes are involved in protein folding, transport, secretion, or degradation. These observations are consistent with those reported in earlier studies using an 8000-gene microarray (Martinez and Chrispeels, 2003; Noh et al., 2003) and a fluid microarray (Kamauchi et al., 2005). ERSE and UPRE cis-elements were identified in the 500-bp upstream sequences of 68 of the 129 upregulated genes. Although these characteristic cis-elements were not identified in the other genes, it does not mean they do not contain such elements since they may be located outside of the 500-bp region examined.
We were unable to establish a simple correlation between the expression profile, currently known ER stress-responsive cis-elements, and the bZIP60 transcription factor. Thus, although 54 of the 129 genes observed to be upregulated by ER stress in wild-type plants showed a reduced transcriptional response to ER stress in homozygous bzip60 mutant plants, not all of these contain either an ERSE or UPRE element within 0.5 kb of the transcription start site. Furthermore, not all genes that contain an ERSE or UPRE element showed a reduced transcriptional response to ER stress in bzip60 mutant seedlings. However, it is clear that bZIP60 is at least one of the transcription factors that mediate gene activation in the ER stress response, since induction of many ER stress-responsive genes, including BiP3 and Sar1B is substantially reduced in bzip60 mutant plants lacking the bZIP60 protein. It appears likely that bZIP60 is the main regulator of BiP3 gene expression but makes only a minor contribution to BiP1/BiP2 regulation. This inference is supported by the observation that expression of the transcriptional repressor bZIP60ΔC-RD, derived by adding a repressor domain to the C-terminal sequence of bZIP60, more strongly interfered with tunicamycin induction of BiP3 than that of BiP1. It is also supported by the observation that overexpression of bZIP60 enhances induction of the BiP3 gene more than that of the BiP1 gene.

Our observations also imply that induction of the ER stress response is complex in Arabidopsis, since many genes, including BiP1/BiP2, are still inducible in bzip60 mutant plants. Even the transcriptional induction of BiP3, although significantly reduced in bzip60 mutants, was not completely abolished. It was recently reported that bZIP28 is also involved in induction of BiP genes during the Arabidopsis ER stress response (Liu et al., 2007a). Thus, it appears that bZIP60 and bZIP28 perform complementary and partially overlapping regulatory functions in the Arabidopsis ER stress response. IRE1 homologs are candidate molecules mediating the Arabidopsis ER stress response in view of their roles in the ER stress responses of other organisms. However, plant homologs of IRE1 have not, to date, been shown to function in the plant ER stress response. Other possible candidates are the transcription factors highly induced by tunicamycin treatment identified by our microarray analysis (see Supplemental Table 1 online).

We have presented evidence that the bZIP60 protein resides in the ER membrane under unstressed condition and is cleaved in response to ER stress, translocating into the nucleus to function as a transcription factor. This is consistent with the observation that overexpressing the bZIP60 gene increased the abundance of the full-length bZIP60 protein but did not activate BiP genes in the absence of ER stress. We observed a further increase, rather than a decrease, in the abundance of full-length bZIP60 protein following ER stress, although the cleaved form of bZIP60 is derived from the full-length form. Similar observations have been reported for other membrane-bound transcription factors, such as the mammalian ER stress transducer BBF2H7 (Kondo et al., 2007) and the Arabidopsis transcription factor bZIP17 activated in response to salt stress (Liu et al., 2007b). The most likely explanation is that the full-length form of bZIP60 turns over during unstressed conditions and that both the full-length and cleaved forms are stabilized under ER stress conditions.

The mechanism by which a membrane-bound transcription factor is activated by proteolytic cleavage is called regulated intramembrane proteolysis (RIP), and we have presented evidence here that bZIP60 is activated by RIP. Indeed, the transmembrane domains of bZIP60 and its orthologs contain a Pro residue (see Supplemental Figure 2 online), which has been considered to unwind the α-helix to be efficiently cleaved by RIP proteases (Akiyama et al., 2004), as do other RIP-regulated transcription factors, including ATF6. However, the RIP mechanism by which bZIP60 is activated in response to ER stress in Arabidopsis differs from that known to be involved in activation of ATF6. In the case of ATF6, the Golgi localization signal in the luminal domain of ATF6 is masked by BiP binding, and dissociation of BiP in response to ER stress allows translocation of ATF6 to the Golgi, where it is cleaved by S1P and S2P proteases (Shen et al., 2002). Recent studies have identified OASIS and CREBH as additional bZIP transcription factors with a transmembrane domain involved in the mammalian ER stress response, both of which are targets for S1P and S2P cleavage (Kondo et al., 2005; Zhang et al., 2006).

We showed here that proteolytic cleavage of bZIP60 is not affected in T-DNA insertion mutants of the genes coding for the Arabidopsis S1P and S2P homologs. This observation is consistent with the observation that bZIP60 does not contain the
stress (Liu et al., 2007b). Moreover, ATAF1,2/CUC2) transcription factor family. It was also reported (Kim et al., 2006), which belongs to a plant-specific NAC (NAM/
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RNA Gel Blot Analysis

Arabidopsis seedlings were grown in half-strength MS medium supplemented with 2% (w/v) sucrose in a 16-h-light/8-h-dark cycle. Total RNA was extracted using the aurintricarboxylic acid method (Gonzalez et al., 1980) from 2-week-old seedlings treated with tunicamycin (5 μg/mL) for the indicated time periods. Five micrograms of RNA was applied per lane and fractionated on a 1.2% agarose gel containing 2% formaldehyde. The RNA was capillary blotted onto a nylon membrane (Hybond N; Amersham Biosciences) in 20× SSC and fixed by UV irradiation. To detect BiP transcripts, ~200-bp fragments of the 5′ regions of BiP1 and BiP3 were PCR amplified using primers 5′-CAAAAAGGAGATCGTACAGCAAAAG-3′ and 5′-ACTGATCCTAATCTGGACTTCTT-3′ for BiP1 and 5′-ACAAGAGGAGAGGTGATTTCC-3′ and 5′-ACCGTCCCGAGCTTCCGTCGC-3′ for BiP3 and used for the labeling reaction. The BiP1 fragment also recognizes BiP2 because the homology between BiP1 and BiP2 is very high. To detect bZIP60, Sar1B, and Sec61γ transcripts, their cDNAs were PCR amplified using primers 5′-AGGGGAATTTGGAAGGTTAAG-3′, 5′-GAGCTCTACGACGCGAACG-3′, and 5′-CTACTTGATATACTGAGATAGCCA-3′ for Sar1B, and 5′-AGGGCAATGGCGGAGGAATTTGGAAGGTTAAG-3′ and 5′-TCAGATCACCGAAGAAAAAACGTCGC-3′ for Sec61γ. These cDNA hybridization probes were labeled with [α-32P]dCTP using a DNA labeling kit (BcaBest labeling kit; Takara). The membrane was washed with 0.2× SSC, 0.1% SDS at 55°C three times for BiP1 and BiP3 or with 0.2× SSC, 0.1% SDS at 65°C three times for others, and then exposed to x-ray film.

Stable Transformation of Arabidopsis

For complementation of bZIP60, a genomic fragment containing ~2.1 kb of the 5′ sequence upstream from the start codon and 0.6 kb of the 3′ sequence downstream from the stop codon of bZIP60 coding sequence was PCR amplified using primers 5′-CTCTGGAGGCTGATGATAATTAA-GACTAGACGAC-3′ and 5′-CGATACCCGGTAGACTTGCTGAGGGGACAC-3′. The PCR product was digested by XhoI and BamHI and inserted into pBIKS and was used as a gift from Keiji Nakajima (Nara Institute of Science and Technology); pBIB-BS was generated by inserting ori and LacZ DNA fragments of pBluescript II KS (Atting-Meers and Short, 1989) into pBIB (Becker, 1990).

To generate bZIP60-OX, bZIP60 cDNA was amplified using primers 5′-AGGGGAATTTGGAAGGTTAAG-3′ and 5′-GAGCTCTACGACGCGAACGACGAGTGAATTGGAAGGTTAAG-3′. The PCR product was digested by XhoI and BamHI and inserted into pBIKS and used to replace the Gus gene of pBI121 (Clontech Laboratories). To generate DEX-bZIP60-OC-RD plants, bZIP60-OC-RD was amplified by PCR using primers 5′-CTCTGGAGGCTGATGATAATTAA-GACTAGACGAC-3′ and 5′-ACTGATCCTACACTTCACATGACATGAGAGTGAATTGGAAGGTTAAG-3′, digested with XhoI and Spel, and inserted into pTAT7002 (Aoyama and Chua, 1997). These constructs were transferred to Agrobacterium tumefaciens C58 by the freeze-thaw method (Holsters et al., 1978). Stable transformation of Arabidopsis plants was performed using the floral dip method (Clough and Bent, 1998). Stable transformation of MM2d cells was performed by coculturing with Agrobacterium (Menges and Murray, 2004).

RT-PCR

Wild-type and DEX-bZIP60-OC-RD seedlings grown in half-strength MS medium for 10 d were treated with 5 μg/mL tunicamycin, 10 μM DEX (Sigma-Aldrich), or both for 5 h. Wild-type and bZIP60-OC MM2d cells were treated with 5 μg/mL tunicamycin or 2 mM DTT for indicated times. Total RNA was extracted using the RNeasy plant mini kit. RNA (100 ng) was reverse transcribed using the PrimeScript RT reagent kit (Takara) according to the manufacturer’s instructions with oligo(dT) primers. Real-time qRT-PCR measurements were performed using a Roche LightCycler 480 (Roche Diagnostics). bZIP60, BiP1, BiP3, and Act8 transcripts were amplified using SYBR Premix Ex Taq (TakaRa) with primers 5′-CGATGATGCGTGTCGAAA-3′ and 5′-TCTAAAAAGGTTCTTCTGAGAT-3′ for bZIP60, 5′-TCATGCTTGGAGGATTGATTG-3′ and 5′-TGCCCTTGAGCATCATTGAA-3′ for BiP1, 5′-CTAGGAACTCTGATGAGAA-3′ and 5′-GCTGCCCACTCTCTGATGAC-3′ for BiP3, and 5′-TCAGCACCTTCTCCAGAGTGTG-3′ and 5′-ATGCCTGGACCCGTCTTTG-3′ for Act8. Expression values of BiP1 and BiP3 were normalized to those of Act8.

For detection Act8, S1P, S2P, and bZIP60 transcripts by PCR, cDNAs were amplified for 25 cycles using the primers 5′-GCTGGAGACATGGCTTGACC-3′ for Act8, 5′-GATCATCACCCTCTGATGTTCT-3′ and for 28 cycles using the primers 5′-CAAAAGGCCCAAGAACCGAAGGCTTCTC-3′ and 5′-ATGGAATTTCAACGACGCGAATGAG-3′ and 5′-TCAGATCACCACAGAAAAACGTCGC-3′ for S2P and for 30 cycles using the primers 5′-ATGCGGAGAAATTTGGAAGAC-3′ and 5′-GAGCTCTACGGCGAAGTTGGTAAGTGG-3′ for bZIP60. PCR products were separated by electrophoresis and visualized by ethidium bromide staining.

Generation of Antibodies

bZIP60ΔC protein was used as an antigen for preparation of an antibody recognizing the bZIP60 protein (Figure 1A). The bZIP60ΔC fragment was PCR amplified using primers 5′-CATCGTGGGAGGAATTTGGAAGGTTAAG-3′ and 5′-TCAGATCACCACAGAAAAACGTCGC-3′ and cloned into pBAD102 vector (Invitrogen) according to the manufacturer’s instruction. The recombinant bZIP60ΔC fused with thioredoxin and hexahistidine Trx-bZIP60ΔC-His was expressed in Escherichia coli Top10 cells (Invitrogen), purified by Ni-NTA agarose (Qiagen), and used to immunize rabbits. The resulting serum was passed through a column containing hexahistidine-tagged thioredoxin to remove antibodies recognizing thioredoxin and hexahistidine, and purified using Trx-bZIP60ΔC-His, resulting in an antibody specifically recognizing bZIP60ΔC. Peptides corresponding to 645 to 658 amino acids of bZIP1 or 657 to 670 amino acids of bZIP3 were used as antigens for preparation of an antibody recognizing BiP1 and BiP3, respectively. Each antibody raised in a rabbit was affinity purified using the peptide antigens.

Protein Extraction and Subcellular Fractionation

For total protein extracts, Arabidopsis seedlings or MM2d cells were treated with 5 μg/mL tunicamycin or 2 mM DTT for the indicated times and homogenized in an extraction buffer (50 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.05% Tween 20, 1 mM EDTA, 1 mM PMSF, and 5 μg/mL leupeptin). The homogenate was centrifuged at 12,000g for 10 min. Soluble proteins in the supernatant were used as total proteins. The protein concentration was quantified using a Bio-Rad protein assay kit (Bio-Rad) with BSA as a standard. Nuclear protein was extracted from MM2d cells treated with or without 5 μg/mL tunicamycin for 2 h using a plant nucleus isolation/extraction kit (Sigma-Aldrich) according to the manufacturer’s instruction. To determine membrane localization of bZIP60, MM2d cells were collected, homogenized in LE buffer (80 mM Tris-HCl, pH 7.5, 12% sucrose, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 5 μg/mL leupeptin), and centrifuged at 100,000g for 1 h. The resulting pellet was resuspended with the same amount of LE buffer as the supernatant and used for immunoblot analysis. For sucrose gradient centrifugation, MM2d cells were collected and homogenized in LE buffer, which contains EDTA, or LM buffer, which contains Mg2+ (80 mM Tris-HCl, pH 7.5, 12% sucrose, 2 mM MgCl2, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 5 μg/mL leupeptin). The homogenate was centrifuged at 10,000g for 10 min. The supernatant was further centrifuged at 100,000g for 60 min to collect the microsomal fraction. The pellet was resuspended in the same
buffer (0.5 mL), subjected to sucrose gradient (15 to 55%, 11.5 mL), centrifugation at 100,000 g for 16 h, and then fractionated into 13 fractions.

**Immunoblot Analysis**

Protein extracts were loaded on an SDS-PAGE gel (Mini Electrophoresis System; Bio-Rad). After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, and immunoreactive proteins were detected with ECL Plus protein gel blotting detection reagent (Amersham Biosciences) according to the manufacturer’s instructions. The antibodies used were anti-BiP1, anti-BiP3, anti-bZIP60, anti-RGP (Dhugga et al., 1997), anti-VHA-a (Kobae et al., 2004), anti-AHA (Kobae et al., 2004), anti-cFBPase (Strand et al., 2000), anti-COX-II (Agrisera), anti-Histone H2B (Abcam), anti-α-tubulin (Santa Cruz), anti-HA 3F10 (Roche), anti-FLAG M2-HRP (Sigma-Aldrich), and anti-rabbit IgG-HRP (Bio-Rad), and anti-rat IgG-HRP (Bio-Rad). The following materials are available in the online version of this article. Supplemental Data

**Histochemical Staining for GUS Activity**

Generation of a transgenic plant carrying the bZIP60 promoter:GUS fusion was previously described (Iwata and Koizumi, 2005a). Seedlings treated with 5 μg/mL tunicamycin for 10 h and untreated seedlings were incubated with GUS staining solution (0.1 M sodium phosphate buffer, 10 mM EDTA, pH 8.0, 0.5 mM K3[Fe(CN)6], 0.5 mM K4[Fe(CN)6], 1 mM X-glucuronidase, and 0.1% Triton X-100) at 37°C for 16 h. Buds, open flowers, and siliques were immersed in ice-cold 90% acetone for 15 min and then stained with GUS staining solution. Tissues were dehydrated by increasing the ethanol concentration gradually from 70% to absolute ethanol and then subjected to microscopy observation. Dehydrated buds were incubated at 60°C in melted Paraplast Plus (Sherwood Medical). After five changes of Paraplast over 72 h, the tissues were embedded in Paraplast Plus blocks, sectioned into 8-μm slices, and observed under a light microscope.

**Accession Numbers**

Sequence data can be found in the Arabidopsis Genome Initiative database under accession numbers At1g24290 (bZIP60), At5g25850 (BiP1), At5g20200 (BiP2), At1g09980 (BiP3), At1g09180 (Sar1), At4g24920 (Sec61γ), At5g19660 (S1P), At4g20310 (S2P), and At1g49240 (Act8). Identifiers for T-DNA insertion mutants are Salk_050204 (bzip60), Salk_020530 (s1p-1), Salk_111474 (s1p-2), 459C12 (s2p-2), and 816A08 (s2p-3). Microarray data can be found in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under the accession number E-MEXP-1753 “bzip60 vs wild type in response to tunicamycin.”

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Complementation of bzip60.

**Supplemental Figure 2.** Amino Acid Alignment of bZIP60 Homologs.

**Supplemental Table 1.** Genes Induced More Than Threefold by Tunicamycin Treatment.

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