Heat induces the splicing by IRE1 of a mRNA encoding a transcription factor involved in the unfolded protein response in Arabidopsis

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Adverse environmental conditions produce endoplasmic reticulum (ER) stress in plants. In response to heat or stress agents, Arabidopsis seedlings mitigate stress damage by activating ER-associated transcription factors and a RNA splicing factor, IRE1. IRE1b splices the mRNA-encoding bZIP60, a basic leucine-zipper domain containing transcription factor associated with the unfolded protein response in plants. bZIP60 is required for the up-regulation of \textit{BIP3} in response to ER stress, and loss-of-function mutations in IRE1b or point mutations in the splicing site of bZIP60 mRNA are defective in \textit{BIP3} induction. These findings demonstrate that bZIP60 in plants is activated by RNA splicing and afford opportunities for monitoring and modulating stress responses in plants.

\textbf{Heat and drought tolerance are some of the most complex and important adaptive traits in plants. These stresses are foremost in placing limits on plant productivity worldwide, and tolerance to these stresses are among the most highly sought after traits in crops (1), particularly in the face of climate change.}

The unfolded protein response (UPR) in eukaryotes is an ER stress response that activates three different classes of membrane-associated sensor transducers in mammalian cells—activating transcription factor 6 (ATF6), inositol-requiring enzyme-1 (IRE1) and protein kinase RNA (PKR)-like ER kinase. Yeast has only one ER stress transducer, \textit{Ire1}, nonetheless, this factor sets off a massive UPR by triggering the expression of >5% of genes in the yeast genome. Many of these encode chaperones and ER-associated protein degradation components (2). IRE1 in yeast and mammalian cells acts by splicing a messenger RNA encoding a transcription factor that, then in turn, activates the expression of stress response genes (see recent reviews; refs. 3–5). Yeast cells splice an mRNA encoding a transcription factor called Hac1p (6, 7). The unsliced form of the \textit{Hac1} messenger RNA attenuates its own translation, and splicing relieves the translational repression (8).

IRE1-mediated splicing is unconventional because mRNA splicing normally occurs in the nucleus, not in the cytoplasm (9). IRE1 is a type I membrane-spanning protein situated in the ER with its N terminus facing the ER lumen and its C terminus, which possesses catalytic functions, facing the cytosol. IRE1 is regarded as a dual functional enzyme possessing both serine/threonine protein kinase and endoribonuclease activity (10). Upon activation, the IRE1 dimer undergoes autotransphosphorylation in which one monomer phosphorylates the other (11). Through the analysis of the structure of the cytosolic domain of IRE1, Lee et al. (12) found that dimerization brings together the kinase domains in a face-to-face manner that would seemingly facilitate autotransphosphorylation.

Autotransphosphorylation is then thought to open a nucleotide-binding site that, when occupied, produces a conformational change in the cytosolic domain so as to position the ribonuclease domains in a back-to-back dimer configuration. The crystal structure of the cytosolic domain shows two ribonuclease centers symmetrically arrayed. Lee et al. (12) reasoned that the configuration is meaningful to the operation of the enzyme because IRE1 cleaves the \textit{Hac1} mRNA in two places. They proposed that the predicted hairpin loops in the RNA substrate pair in a staggered, antiparallel manner so as to form “kissing” hairpin loops. In such a structure, the projected cleavage sites lie close to the ribonuclease catalytic sites in the cytosolic domain of IRE1.

Mammalian cells have two genes that encode IRE1 (\textit{IRE1a} and \textit{IRE1b}). \textit{IRE1a} is widely expressed in animal tissues, but \textit{IRE1b} is largely restricted to cells of the intestinal epithelium (13). Mammalian IRE1 appears to function in the same way as its yeast counterpart. However, mammalian IRE1 splices \textit{XPB1} mRNA (X-box binding protein), which is not related in sequence to \textit{Hac1} but, nonetheless, encodes a bZIP transcription factor that activates stress response genes (14). IRE1 splicing of \textit{XB1} mRNA produces a frameshift resulting in a protein with a different C terminus. When the unspliced \textit{pXB1(U)} (U) and spliced forms \textit{pXB1(S)} were overproduced in animal cells, the spliced form, \textit{pXB1(S)}, strongly transactivated a stress promoter-reporter gene (14). The authors also demonstrated that the C terminus of the spliced form is a potent transcriptional activator when fused to a Gal4 binding domain. Yoshida et al. (15) found that \textit{pXB1(U)} complexed with \textit{pXB1(S)}, and the complex was readily degraded because of a degradation domain in \textit{pXB1(U)}. Therefore, \textit{pXB1(S)} is transcriptionally active, whereas \textit{pXB1(U)} appears to be a negative regulator of \textit{pXB1(S)}. During the early phases of the stress response, ATF6 is activated and, in turn, it up-regulates \textit{XPB1}. IRE1 is also activated and presumably splices the \textit{XB1} message to produce an active transcription factor that up-regulates stress gene expression. In the later phase of the stress response, \textit{IRE1} expression is thought to fade, resulting in the production of \textit{pXB1(U)}, which shuts down \textit{XB1} action (16).

\textit{Arabidopsis} has two genes with \textit{IRE1}-related sequences: \textit{AtIRE1a} (At2g17520, formerly Atire1-2) and \textit{AtIRE1b} (At5g24360, formerly Atire1-1), which were first described by Koizumi et al. (17). They found that \textit{AtIRE1a} and \textit{b-GFP} fusions were located in the perinuclear ER. The genome also encodes a shorter \textit{IRE1}-related sequence, At3g11870, lacking the luminal and transmembrane domain. Koizumi et al. (17) demonstrated functional complementation of the \textit{Arabidopsis} luminal domain (sensor domain) in yeast and that the plant \textit{IRE1a} has autophosphorylation activity in vitro, but they were unable to demonstrate splicing. Noh et al. (18) attempted, but were unsuccessful, in showing that \textit{AtIRE1a} could splice yeast \textit{Hac1} RNA in \textit{Arabidopsis} protoplasts.


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UPR has been demonstrated in plants in response to ER stress agents such as dithiotreitol (DTT) and tunicamycin (TM) (19). ER stress activates bZIP transcription factors, AbtZIP17 and -28, members of a small family of membrane-associated bZIP factors in Arabidopsis (20, 21). AbtZIP17 and -28 are activated by mechanisms similar to the activation of ATF6 in mammalian cells (20–22). Mammalian ATF6 is activated by liberation from the ER and migration to the Golgi apparatus where its C-terminal, lumen-facing domain is cleaved by SIP and S2P proteases, releasing its N-terminal, cytosol-facing component, which enters the nucleus and up-regulates target genes.

bZIP60 also plays an important role in ER stress responses in Arabidopsis through the up-regulation of genes encoding factors that aid in protein folding and degradation (23, 24). In response to stress, bZIP60 appears in an active, truncated form seemingly to have undergone proteolytic processing similar to AtbZIP17 and AtbZIP28 (20–22). However, the mechanism for the presumed proteolysis event is not understood, because the C-terminal tail of bZIP60 does not have a canonical S1P cleavage site and its activation does not require SIP or S2P (23). For these reasons, we considered the possibility that activated forms of bZIP60 might arise by different means, by RNA splicing, in a manner similar to the activation of Hac1 in yeast or XBP1 in mammalian cells.

**Results**

*Arabidopsis bZIP60 mRNA is the Target of Splicing.* The target sites for XBP1 and Hac1 mRNA splicing are pairs of kissing hairpin loops with three conserved bases in each loop (14, 25). Using a RNA structure prediction program (M-fold; ref. 26), we found that the predicted lowest free energy forms for bZIP60 mRNA folded into twin kissing hairpin loops with three bases in each loop similar to the conserved bases in XBP1 and Hac1 mRNAs (Fig. S1 and Fig. S2A). Based on this structure and the known splice sites in the mammalian and yeast RNAs (14, 25), we predicted that if bZIP60 mRNA is spliced by an IRE1-like activity, then the splice should remove a 23b segment of RNA from that site.

![Fig. 1.](https://example.com) Twin hairpin loop structure at splicing site in bZIP60 mRNA. (A) Each of the two loops contains three conserved bases (highlighted). Solid block arrows indicate predicted cleavage sites. (Equivalent cleavage sites indicated by open block arrows.) Base substitution mutations are indicated with blue arrows. For constructs with a single point mutation (1PM), the G at nucleotide position 672 in loop 2 has been substituted by a C. This base substitution is not a coding change for bZIP60 mRNA. Constructs with two point mutations (2PM) involve the base substitution in loop 2 and another one in loop 1 in which the G at nucleotide position 672 has been substituted by a T. The second base substitution changes the coding capacity of bZIP60 mRNA; however, the change is a conservative one, exchanging a hydrophobic amino acid for another, a V to a L. (B) Splicing assay using primers immediately flanking splice site (Upper). Ethrophoretic gel band pattern of RT-PCR products from RNA sample taken from untreated seedlings or seedlings treated for 2 h with 2 μg/mL tunicamycin. Interpretation of the various RT-PCR products (labeled 1–2) based on reamplification of cDNA from individual bands (Lower). (C) Partial sequence of cDNA derived from unspliced and spliced forms of AbtZIP60 mRNA shown in B. Arrows indicate splice sites inferred from the sequence of the spliced mRNA. Underlined segment of the amino acid sequence derived from the spliced form is a predicted transmembrane domain. Amino acid sequence in red predicted from the spliced RNA form represents differences in the sequence predicted from the unspliced form.
HIS-cIRE1a and HIS-cIRE1b containing the putative kinase and ribonuclease domains were active in vitro producing a series of discrete RNA fragments (Fig. S3). The fragment patterns produced by the two enzymes, HIS-cIRE1a and HIS-cIRE1b, were similar although the 58/55 fragment was recovered less frequently among the HIS-cAtIRE1a products. Nonetheless, the fragment pattern for HIS-cAtIRE1b, in particular, was consistent with predictions for cuts in the two loops (loop 1 and 2) as shown in Fig. L4.

According to the Arabidopsis cFP browser (www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), both Arabidopsis IRE1 genes are expressed at low levels. IRE1b is more generally expressed throughout the plant, while IRE1a is more locally expressed in embryos and seeds (17, 18). The activity capable of splicing bZIP60 mRNA did not appear to be localized to a specific region of the seedling because splicing activity and BIP3 induction were observed following 2 h of DTT treatment in seedling shoots, hypocotyls and roots (Fig. S4). T-DNA insertion mutations are available for both IRE1 genes, so the splicing reactions in the mutants were tested. The IRE1a (i12-2-2) mutation is an insertion in the third exon and is a null mutant, in that full-length transcripts were not detectable in the mutant (Fig. S5A). Homozygous i12a mutant seedlings were subjected to DTT treatment, but bZIP60 mRNA splicing appeared unaffected (Fig. 2B). The ire1b mutation is a T-DNA insertion in the penultimate (fourth) intron, and no full-length IRE1b transcripts were detectable (Fig. S5B). When homozygous ire1b mutants were subjected to ER stress agents, splicing of bZIP60 mRNA was much reduced (Fig. 2B). Thus, Arabidopsis IRE1b is necessary for normal levels of bZIP60 mRNA splicing in stressed seedlings but IRE1a alone is not.

The excision of the 23b segment in the splicing of bZIP60 RNA creates a frame shift, which results in the expression of downstream sequences in a different reading frame (Fig. 1C). The unspliced bZIP60 RNA (bZIP60(u)) is predicted to encode a bZIP transcription factor with a transmembrane domain (TMD) in the C-terminal third of the protein (Fig. 2C). Splicing removes the TMD and alters the amino acid sequence further downstream.

to determine whether splicing alters the subcellular localization of bZIP60, cDNAs representing the spliced and unspliced forms of bZIP60 were linked to YFP and transiently expressed in tobacco BY-2 cell protoplasts (Fig. 2D). Unspliced bZIP60-YFP is colocalized in the cytoplasm with an ER marker, whereas the spliced form is located in the nucleus.

**bZIP60 Function Requires Ire1b-Catalyzed Splicing of bZIP60 mRNA.**

To show that splicing of mRNA is required for bZIP60 function, base substitution mutations were created in the conserved bases of the paired loops of bZIP60 mRNA (Fig. L4). In the single point mutant (1PM), the G at nucleotide position 672 in loop 2 was substituted for a C. This base substitution did not represent a coding change for bZIP60 mRNA. Constructs with two point mutations (3PM) involve the aforementioned base substitution in loop 2 and another one in loop 1 in which the G at nucleotide position 652 was substituted for a C. The second base substitution represented a coding change for bZIP60 mRNA, however, the change was a conservative one, exchanging a hydrophobic amino acid for another, a V to L.

The RNA splicing assay was carried out in the background of a T-DNA mutant (bzip60-1) with an insertion near the start codon (ATG) in bZIP60 mRNA. The mutation reduced the background of endogenous bZIP60 mRNA splicing in response to DTT treatment (Fig. 3A, compare lanes 1 and 2 with 3 and 4) to permit us to monitor the splicing of mRNA derived from the bZIP60 transe. Splicing was observed in the mRNA derived from a nonmutant (0PM) cDNA construct of bZIP60 (myc-bZIP60) in the bzip60-1 background (Fig. 3A, lanes 5–8). Splicing did not occur in RNA derived from a full-length cDNA construct of bZIP60 (myc-bZIP60) with one (1PM) or two point mutations (2PM, Fig. 3A, lanes 9–16). The level of RNA splicing in the mutants was comparable with the background of endogenous bZIP60 splicing in bzip60-1 (Fig. 3A, lanes 3 and 4).

To confirm that the lack of spliced bZIP60 mRNA derived from point mutants was not due to the absence of unspliced

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**Fig. 2.** Induction of bZIP60 splicing by treatment with DTT. (A) Seedlings were transferred to liquid LS medium containing 2 mM DTT and incubated for the times indicated. RNA samples were analyzed for the presence of unspliced (U) and spliced (S) bZIP60 mRNA by RT-PCR using the flanking primers (FP) assay or the specific primers assay using primers for unspliced bZIP60 mRNA (SPU) or for spliced mRNA (SPS). Amplification with primers for actin mRNA was used as a control. (B) Effects of T-DNA mutations in IRE1a and Ire1b on splicing of bZIP60 mRNA in seedlings. Seedlings were treated with 2 mM DTT and RNA was extracted 2 h later. bZIP60 mRNA splicing was analyzed by SPU and SPS assays for the presence of unspliced and spliced RNA forms, respectively. (C) Diagrams illustrating the predicted structure of proteins derived from the unspliced and the spliced forms of bZIP60 mRNAs. (D) Localization of bZIP60 proteins derived from unspliced (Top and Middle) and spliced mRNAs (Bottom). Constructs encoding GFP-tagged forms of bZIP60 were introduced by biolistics into tobacco BY-2 cells. RFP-HDEL was used as an ER marker, and RFP alone was used to mark both nuclei and cytoplasm.
mRNA, the transgenics were tested for presence of bZIP60 mRNA. Endogenous bZIP60 gene expression was up-regulated somewhat after DTT treatment (Fig. S6, lanes 1–2); however, bZIP60 expression was undetectable with or without DTT treatment in the bZIP60 T-DNA mutant (bzip60-1; Fig. S6, lanes 3 and 4). Full-length bZIP60 mRNA was detected in all of the transgenics, roughly at levels comparable with endogenous RNA levels in wild type (Fig. S6, lanes 5–16).Expression of the bZIP60 transgenes was relatively unaffected by stress treatment, although a slight upswing in expression after DTT treatment was noted in the 0PM sample #2 (Fig. S6, lanes 5 and 6). Hence, we can conclude that the lack of spliced forms in the point mutants is not due to the absence of mRNA derived from the transgenes and that the single or double point mutations in the conserved bases of the paired loops interfere with bZIP60 mRNA splicing.

Equipped with mutations with defects in RNA splicing, we could address whether mRNA splicing is required to confer functionality on bZIP60. We observed that BIP3 induction in response to DTT treatment was significantly reduced in the bzip60-1 and bzip60-2 mutant (a T-DNA line with an insertion near the splicing site in bZIP60) (Fig. 3B, lanes 3–6) compared with wild type (Fig. 3B, lanes 1 and 2). We also found that BIP3 induction in response to DTT treatment largely depended on IRE1b (Fig. 3B, lane 8). Thus, BIP3 up-regulation in response to ER stress substantially depends on bZIP60 and IRE1b.

To determine whether the function of bZIP60 specifically depends on the splicing of its mRNA, BIP3 expression supported by various bZIP60 constructs was monitored in a bzip60-1 background. As before with bzip60-2, bzip60-1 showed only modest up-regulation of BIP3 expression in comparison with wild type (Fig. 3C, lanes 1–4). It was found that the nonmutant bZIP60 construct complemented bzip60-1 by restoring full induction of BIP3 expression in response to DTT treatment (Fig. 3C, lanes 5–8). However, the bZIP60 constructs with point mutations in the conserved bases of the paired RNA loops did not support BIP3 induction (Fig. 3C, lanes 9–16). The lack of BIP3 induction is evident even in the case of the single point mutation (1PM), which does not represent a codon change in the mRNA. Thus, we conclude that bZIP60 mRNA splicing is necessary for the functionality of bZIP60.

### Conditions for Expression of AtIRE1 Activity.

Arabidopsis bZIP60 mRNA splicing can be induced in the laboratory by agents that interfere with protein folding, such as TM and DTT, but what natural environmental conditions elicit the response? We tested a variety of conditions and found using both the FP and SP assays that heat stress was most effective in eliciting bZIP60 mRNA splicing. Exposure of seedlings to 42 °C for 30 min was sufficient to elicit the splicing response (Fig. 4A). Other conditions tested such as salt stress, hypoxia, and nutrient stress (nitrogen deprivation) did not generate this response (Fig. S7).

Heat treatment at 42 °C did not immediately lead to BIP3 induction. However, if heat-treated seedlings were allowed to recover for 2 h at room temperature, then bZIP60-dependent BIP3 induction was observed (Fig. S8A). BIP3 expression was induced and modest amounts of bZIP60 mRNA were spliced when seedlings were heat treated at a lower temperature, 37 °C (Fig. S8B). We interpret the difference in response at the two different temperatures to mean that heat treatment at 42 °C may also inactivate processes necessary for the BIP3 up-regulation, but these processes recover at room temperature before the levels of spliced bZIP60 mRNA fall. Alternatively, BIP3 induction may be outcompeted by the induction of heat shock genes at the higher temperature. In either case, the lack of BIP3 induction immediately following heat treatment at 42 °C may explain why it has been reported that BIP3 is not induced by heat treatment in other plants (27, 28).

### Discussion

bZIP60 was identified by Iwata and Koizumi (24) as a gene induced by UPR stress agents. They also demonstrated that a truncated form of bZIP60 expressed as a transgene could activate other UPR genes in the absence of stress. Because AtbZIP60 has features in common with ATF6 in mammalian cells, they assumed that Arabidopsis TF might be activated in the same way. Like ATF6, bZIP60 is a membrane-associated bZIP TF. ATF6 is activated by relocating from the ER to the Golgi apparatus in response to ER stress, and in the Golgi apparatus, ATF6 is proteolytically processed by SIP and S2P and released to the nucleus (29, 30). Recently, Iwata et al. (23) demonstrated that, in fact, bZIP60 is present in a full-length form associated with microsomes in untreated seedlings, and in response to stress, bZIP60 appears in a shorter form without its TMD and a substantial portion of bZIP60 population is found in the nucleus. The authors conclude that bZIP60 undergoes proteolytic processing in response to ER stress. However, unlike ATF6, bZIP60 does not have a canonical SIP site and still undergoes apparent proteolytic processing in an AtSIP.
mutant. Iwata et al. (23) concluded that plants may have an un-described mechanism for processing this stress-activated TF.

Since the discovery of bZIP60, two other stress-induced, membrane-associated TFs have been described in Arabidopsis, AtbZIP17, which responds to UPR agents, heat, and salt stress (20, 22) and AtbZIP28 that is activated by UPR agents and heat (21, 22, 31). Both of these factors are activated conventionally in a manner similar to the activation of ATF6. Both are membrane associated, have canonical SIP sites in their ER-lumen-facing, C-terminal tails, and appear to rely on SIP and S2P for processing (29, 30). Thus, plants appear to have a conventional mechanism as described in mammalian cells for the processing of membrane-associated TFs.

The previous studies on bZIP60 (23, 24) present a dilemma for understanding how it is activated, and this study offers a solution. Activated forms of bZIP60 appear to be produced in a manner similar to Hac1 or XBP1 rather than like ATF6. bZIP60 mRNA is spliced in response to stress, and the nature of the splice predicts a form of bZIP60 that lacks a transmembrane domain, but has acquired a putative nuclear targeting signal.

It is of interest to know how IRE1 in Arabidopsis is activated by environmental conditions. Crede et al. (2) determined the structure of luminal domain of IRE1 in yeast with the aim of understanding how it senses stress. The prevailing view had been that BiP dissociation from the luminal domain activates IRE1. BiP is thought to be a negative regulator of IRE1, and the accumulation of unfolded proteins in the ER is presumed to recruit BiP away from IRE1 allowing it to dimerize and oligomerize (32).

Cred et al. (2) proposed that unfolded proteins directly activate IRE1. They found that the core luminal domain of IRE1 forms an antiparallel β-sheet linking two IRE1 monomers “through their zipper central strands.” Along either side of the β-sheet platform lies two α-helices forming the walls of a deep groove, which is thought to be able to bind the exposed loops of unfolded proteins leading to stabilize IRE1 dimers or to cross-link oligomers, thereby activating IRE1. In analyzing the structure of the human IRE1 luminal domain, Zhou et al. (13) also found that dimerization creates a similar groove, however, they concluded that the groove was too narrow to accommodate peptides and that peptide binding was not required for dimerization. Instead, they argued that BiP binding maintains IRE1 in an inactive monomeric state and that the imposition of ER stress promotes BiP release, leading to IRE1 dimerization (or oligomerization) and activation. Comparable studies on the structure and oligomerization of IRE1 will be needed to understand its activation in plants.

Materials and Methods

Lines and Growth Conditions. Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used in this study, and the mutants bzip60-1 (Col-0; SALK_050203), bzip60-2 (Col-0, SAIL_283_B03, ire1a (Col-0; SALK_018112), and ire1b (Col-0; SAIL_238_F07) were obtained from Arabidopsis Biological Resource Center. Seeds were stratified at 4 °C for 3 d before germination. Unless indicated otherwise, plants were grown under continuous white light at 23–25 °C in soil or on Linsmaier Skoog (LS) medium (1× LS salts, 1% sucrose, 0.8% agar). Agrobacterium-mediated transformation of Arabidopsis plants was carried out by the floral dip method (33). Agrobacterium strain GV3101 was used in all transformation experiments.

Stress Assays. To determine whether bZIP60 mRNA is spilled in response to various stress agents or treatments, 2-wk-old wild-type Col-0 seedlings were grown on LS agar medium and then treated with liquid LS medium containing 10 μM ABA, 1 mM ethephon, or an equivalent volume of DMSO and incubated for the indicated times. In testing for salt stress, 8-d-old seedlings were grown on LS as described above and treated with liquid LS medium containing 100 mM NaCl or LS medium alone for the indicated time. To test for the effects of nitrogen limitation, plants were grown on soil and fertilized by using limiting nitrogen (0.5 mM KNO₃) or optimal nitrogen (10 mM KNO₃) solutions as described in Peng et al. (34). Leaf tissue was harvested 3 wk after germination.

In Vitro Ribonuclease Assay. The double loop region of bZIP60 mRNA was synthesized and radiolabeled for use as a substrate in in vitro ribonuclease assays. A DNA template for in vitro RNA transcription was PCR-amplified by using primers T7bZIP-F and bZIP-8R and added to bZIP60 sequences. The reverse sequences, the primers respectively contain the T7 promoter used for in vitro transcription and a hairpin-forming sequence to prevent exonuclease degradation of the transcript (35). Using this template, the resulting RNA is expected to be 125 bases long and includes a 98-base sequence corresponding to the region surrounding the predicted splice sites in bZIP60 mRNA (bases 624–721 of the transcript). The PCR product was gel-extracted and precipitated by using sodium acetate and ethanol. In vitro transcription was performed by using the MEGAscript kit (Ambion AM1354) following the manufacturer’s instructions. Radioactive CTP was incorporated by using 4 μL of [α-32P]-CTP per reaction (Perkin-Elmer, 3,000 Ci/mmol, 10 μCi/mL). The reactions were incubated 4 h at 37 °C and treated with TURBO DNase. The product was denatured 5 min at 95 °C and run on a 0.4-mm-thick denaturing 10% polyacrylamide-urea gel for 2 h at 400 V. The gel was then exposed to a filter paper, wrapped in saran, and exposed to an X-ray film to localize the labeled RNA band, which was eluted overnight at 4 °C in equal volumes of [0.3 M NaOAc, 10 mM Mg(OAc)₂] and phenol:chloroform:isoamyl alcohol (25:24:1). The labeled RNA was purified two additional times, precipitated with ethanol, and resuspended in 20 μL of DEPC-treated H₂O before being used in the ribonuclease reaction.

Partial IRE1α and IRE1β sequences were amplified from wild-type Arabidopsis cDNA and cloned into pET28a by using EcoRI and NsiI sites. The ribonuclease reaction procedure was modified from Back et al. (36). The 50–μL reaction mixture contained 1 μg of recombinant His-tagged protein (or an equivalent volume of 10 mM Hepes at pH 7.6), 50 mM KOAc, 10 mM Mg(OAc)₂, 1 mM DTT, 2 mM ATP, 40 U RNAase, and 2 μL of radio-labeled RNA substrate. The reaction was incubated at 37 °C for 1 h and stopped by using 5 volumes of stop buffer (100 mM NaOAc at pH 5.2, 2 mM EDTA, 0.2% SDS). The RNA products were extracted and precipitated with ethanol and resolved on a 12% polyacrylamide-urea gel.

Transient Expression in Tobacco Protoplasts. Constructs used in transient expression assays were pRTL2/JRF-MCS, pRTL2/JRF-HDEL, pRTL2/N5/GFP-MCS, and puc18-NcoI/GFP were kind gifts from S. Gidda (University of Guelph, Guelph, Ontario, Canada) and have been described in Shockey et al. (2006) (37). The construct used to express GFP-bZIP60(U) was obtained by inserting the unspliced version of bZIP60 coding sequence in pRTL2/N5/GFP-MCS by using BamHI and XbaI sites. The construct used to express bZIP60(G)-GFP was obtained by inserting the spliced version of bZIP60 coding sequence in puc18-NcoI/GFP by using the NcoI site. In addition to missing the 23-base intron, this version of bZIP60 is truncated at its 3′ end (starting from base 802 in the unspliced bZIP60 mRNA transcript) and, thus, does not contain open reading frames. Constructs used in transient expression assays were pRTL2/JRF-MCS, pRTL2/JRF-HDEL, pRTL2/N5/GFP-MCS, and puc18-NcoI/GFP were kind gifts from S. Gidda (University of Guelph, Guelph, Ontario, Canada) and have been described in Shockey et al. (2006) (37). The construct used to express GFP-bZIP60(U) was obtained by inserting the unspliced version of bZIP60 coding sequence in pRTL2/JRF-MCS by using BamHI and XbaI sites. The construct used to express bZIP60(G)-GFP was obtained by inserting the spliced version of bZIP60 coding sequence in puc18-NcoI/GFP by using the NcoI site. In addition to missing the 23-base intron, this version of bZIP60 is truncated at its 3′ end (starting from base 802 in the unspliced bZIP60 mRNA transcript) and, thus, does not contain open reading frames.
the early stop codon that would prevent the translation of the C-terminal fusion with GFP.

Tobacco (Nicotiana tabacum cv BY-2) suspension cell cultures were prepared for biolistic bombardment as described in Banjoko and Trelease (38). Transient cotransformations were performed by using 2 μg of each plasmid DNA with a biolistic particle delivery system (Bio-Rad Laboratories). After bombardment, cells were incubated for 6–24 h, then fixed in 4% (wt/vol) formaldehyde. Confocal Laser Scanning Microscopy images were acquired with a Leica DM RE (Leica Microsystems) microscope connected to a Leica TCS SP2 system by using a Leica 63× Plan Apochromat oil-immersion objective.

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