BAX Inhibitor-1 Modulates Endoplasmic Reticulum Stress-mediated Programmed Cell Death in Arabidopsis\textsuperscript{*S}\textsuperscript{1}

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The components and pathways that regulate programmed cell death (PCD) in plants remain poorly understood. Here we describe the impact of drug-induced endoplasmic reticulum (ER) stress on Arabidopsis seedlings and present evidence for the role of Arabidopsis BAX inhibitor-1 (AtBI1) as a modulator of ER stress-mediated PCD. We found that treatment of Arabidopsis seedlings with tunicamycin (TM), an inhibitor of N-linked glycosylation and an inducer of ER stress by triggering accumulation of unfolded proteins in the ER, results in strong inhibition of root growth and loss of survival accompanied by typical hallmarks of PCD such as accumulation of H$_2$O$_2$, chromatin condensation, and oligonucleosomal fragmentation of nuclear DNA. These phenotypes are alleviated by co-treatment with either of two different chemical chaperones, sodium 4-phenylbutyrate, and tauroursodeoxycholic acid, both with chaperone properties that can reduce the load of misfolded protein in the ER. Expression of AtBI1 mRNA and its promoter activity are increased dramatically prior to initiation of TM-induced PCD. Compared with wild-type plants, two AtBI1 mutants (atbi1–1 and atbi1–2) exhibit hypersensitivity to TM with accelerated PCD progression. Conversely, overexpressing AtBI1 markedly reduces the sensitivity of Arabidopsis seedlings to TM. However, alterations in AtBI1 gene expression levels do not cause a significant effect on the expression patterns of typical ER stress-inducible genes (AtBip2, AtPDI, AtCRT1, and AtCNX1). We propose that AtBI1 plays a pivotal role as a highly conserved survival factor during ER stress that acts in parallel to the unfolded protein response pathway.

In eukaryotes, programmed cell death (PCD)\textsuperscript{2} occurs as a genetically controlled series of events, which is essential for maintenance of cellular homeostasis, development, aging, and removal of damaged or infected cells during environmental and pathogen insults (1, 2). The genes that control PCD are conserved across wide evolutionary distances in metazoans (3). In mammals, apoptosis is prominently controlled through functionally conserved proteins such as CED9/BCL-2 (anti-apoptotic protein) and BAX (pro-apoptotic protein). To date, no such genes have been identified in plants. Nonetheless, several studies have revealed that transgenic expression of mammalian anti- or pro-apoptotic proteins in plants can influence regulatory pathways of cell death activation or suppression (3, 4). Furthermore, a number of studies have suggested similarities in morphological changes with apoptotic features as well as in some biochemical events such as activation of nucleases and caspase-like proteases, and release of cytochrome c from the mitochondrion (3, 4). Plants may thus possess mediators that serve analogous functions in cell death signaling and execution pathways.

BAX inhibitor-1 (BI-1) was first identified as a suppressor of cell death activated by BAX in yeast or mammalian cells (5). BI-1 is evolutionarily conserved and predicted to be a transmembrane protein that localizes predominantly to the ER (6, 7). Expression of plant BI-1 mRNA has been detected in various tissues, and its expression level is enhanced during senescence and under several types of biotic and abiotic stresses (8–13). Overexpression of BI-1 from various plant species was shown to suppress BAX-, pathogen-, or abiotic stress-induced cell death in a variety of cells from yeast, plant, and mammalian origins (8–12, 14–16). These observations support the idea that BI-1 could have conserved function in diverse organisms. Recent genetic analysis of Arabidopsis BI-1 (AtBI1) demonstrated that AtBI1 is dispensable for normal plant growth and development, but plays a protective role against both phytotoxin- and heat stress-induced PCD (13). Plant BI-1 is thus likely to play an important role as a survival factor under multiple stress conditions that could trigger PCD.

The assembly and folding of secreted proteins in the ER is exquisitely regulated by a complex mechanism that maintains equilibrium between folded and unfolded proteins. Accumulation of misfolded or unfolded proteins in the ER triggers ER stress and may seriously affect the viability of cells. To cope with ER stress, ER-resident sensors (inositol-requiring 1, IRE1; PKR-
like ER kinase, PERK; and membrane-tethered activating transcription factor 6, ATF6) detect misfolded or unfolded proteins and elicit ER stress signaling in animal cells, which includes induction of the highly conserved unfolded protein response (UPR) (17). In metazoans, if cells cannot relieve the ER stress caused by excessive and prolonged inputs, apoptosis is activated concomitant with induction of caspase activation, cytochrome c release and DNA fragmentation (18). In mammals, members of the BCL-2 family (BCL-2, BAX, and BAK) localize not only to mitochondria but also to the ER and have been shown to influence ER homeostasis, apparently by influencing membrane permeability and Ca\(^{2+}\) levels of the ER (19). Recently, the function of mammalian BI-1 was shown to link to protection of cells from ER stress-induced apoptosis. Cells isolated from BI-1 knockout mice exhibited heightened apoptosis induction during ER stress triggered by pharmacological agents such as tunicamycin (TM), thapsigargin, and brefeldin A. Conversely, overexpression of BI-1 protected cells in vitro against apoptosis induced by those ER stress inducers (20, 21) or by reducing the calcium content of ER (20–22).

Unlike in mammals, the mechanisms of UPR and ER stress-mediated cell death in plants remain unclear. Structural homologues for an ER stress sensor protein, IRE1, have only been characterized from Arabidopsis and rice (23, 24). Although homologues of ATF6 and PERK have not been identified in plants, the TM-inducible bZip60 transcription factor was shown to function in the signal transduction pathway of the UPR unique to plants (25). Interestingly, gene expression studies have suggested that plants should possess the ability to induce a set of UPR-related genes in response to drug-induced ER stress in a similar manner as that found in mammalian systems (23, 26, 27). Furthermore, recent studies have shown that treatment of plant cell cultures with ER stress agents such as TM and cyclopiazonic acid (CPA) induces cell death with apoptotic morphology (28, 29) or causes growth arrest and cell death with induction of Hsr203J, a specific marker for hypersensitive response cell death in tobacco (30). At present, however, the molecular components and pathways that regulate ER stress-mediated cell death in plants remain obscure.

As plant BI-1 appears to localize predominantly to the ER, we hypothesized that plant BI-1 could also regulate cell death triggered by ER stress. Here, we report that Arabidopsis root cells exposed to TM, which has been extensively used as a pharmacological ER stress inducer in mammals and plants, die exhibiting typical hallmarks of PCD. We further show by reverse genetic approaches that AtBI1 level is a critical determinant for plant survival under ER stress. Together with the recent work in BI-1-deficient mice (20, 21), our results demonstrate that BI-1 is a highly conserved core component that plays a pivotal role as a cell survival factor that is required to delay the onset of PCD upon ER stress signaling.

**Experimental Procedures**

**Plant Materials, Growth Conditions, and Stress Treatments**—All wild-type and mutant Arabidopsis thaliana plants were in the ecotype Columbia (Col-0) background. The atbi-1 mutant was obtained from the Syngenta Arabidopsis T-DNA Insertion Library (SAIL_228_D08), and the atbi-1-2 mutant was obtained from the GABI-Kat collection (line# 117805) (13). Surface-sterilized seeds were first plated on one-half-strength Murashige and Skoog (0.5× MS) basal media (Sigma-Aldrich) supplemented with 1% (w/v) sucrose, at 4 °C in the dark for 2 days and transferred to a controlled growth room under continuous light (100 \( \mu \)mol photons m\(^{-2}\) s\(^{-1}\) of light intensity) at 22 °C. Unless stated otherwise, 5- to 7-day-old plants were subjected to treatment of an ER stress inducer TM (0 to 0.5 \( \mu \)M). TM was purchased from Sigma-Aldrich. Seedlings were treated with TM in a liquid culture containing 0.5× MS medium supplemented with 1.0% (w/v) sucrose for 6 h (temporal treatment) or for up to 72 h (continuous treatment). To evaluate the effect of chemical chaperones on TM-induced growth defect and cell death, 5- to 7-day-old plants were incubated in 0.5× MS medium supplemented with either 1 mM sodium 4-phenylbutyrate (PBA, Calbiochem) or 0.5 mM tauroursodeoxycholic acid (TUDCA, Calbiochem) in the presence of 0.5 \( \mu \)M TM. To observe the growth of Arabidopsis seedlings after chemical treatment, seedlings were washed five times with 0.5× MS medium and kept in the same well of a culture plate supplied with liquid 0.5× MS medium or transferred to normal 0.5× MS agar plates, and then grown for up to 5 days under constant illumination (100 \( \mu \)mol m\(^{-2}\) s\(^{-1}\) at 22 °C. Root growth was analyzed on 0.5× MS agar plates placed in a vertical position.

**RNA Extraction and Analysis**—Total RNA was isolated from 5- to 10-day-old Arabidopsis seedlings using Plant RNA Purification Reagent (Invitrogen) according to the manufacturer’s instruction. After electrophoresis in denaturing conditions, 10 \( \mu \)g of RNA was transferred and UV cross-linked to a membrane (Hybond N\(^{+}\), GE Healthcare) according to standard protocols. Full-length cDNA fragments of AtBI1 were labeled with \( ^{32}P \)dCTP using a random-primed labeling kit (Invitrogen) and used as hybridization probes. Partial cDNA fragments encoding AtBip2 (At5g42020), PR-1 (At2g14610), and AtAct2 (At3g18780) were amplified by PCR (see bellows) and used as hybridization probes. Hybridization was performed using ExpressHyb solution (Clontech), as suggested by the manufacturer.

For semi-quantitative RT-PCR analysis, total RNA was isolated from root tissues of 9- to 10-day-old seedlings using Plant RNA Purification Reagent (Invitrogen) according to the manufacturer’s instruction. Reverse transcription was performed in a reaction using iScript cDNA synthesis kit (Bio-Rad) in a 10-\( \mu \)l reaction (25 °C for 5 min, 42 °C for 30 min, and 80 °C for 3 min) containing 0.5 \( \mu \)g of DNase I-treated total RNA. cDNA was diluted 1:5 in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA buffer prior to use as a template in semi-quantitative RT-PCR analysis. PCR was performed for 25–30 cycles (94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s) in a 25-\( \mu \)l reaction containing 0.5 unit of Choice Tag Blue DNA polymerase (Denville Scientific), 200 \( \mu \)M dNTP, and 0.25 \( \mu \)M primers. The RT-PCR reactions (10 \( \mu \)l) were resolved by 1.5% (w/v) agarose gel electrophoresis, and ethidium bromide-stained gels were digitally photographed with a GelDoc 2000 photostation (Bio-Rad), and the invert image was analyzed using the software Quantify One (version 4.2.1).

Primers used for amplifications were as follows: EL1627 (5’-CAGAAGCTTGGACTATGATC-3’) and EL1629 (5’-ACG-
TCGTTGAGTGGACTATG-3′) for AtBII, EL1946 (5′-CACCAGTTCACATTGGAATGGA-3′) and EL1947 (5′-GCTCATGTCAGTTTCATT-3′) for AtBIIp2, EL682 (5′-GTAGGCTGCTTGGTCTCCCC-3′) and EL683 (5′-CATATAATCCCACTCAGGAGTAC-3′) for PR-1, EL2705 (5′-CACCCTGTGGAAAAAGAGAAACACCAG-3′) and EL2706 (5′-CAAGACACCAGTGTACTACTG-3′) for AtCNX1 (At5g61790), EL2707 (5′-CATAAGTATATCCCAAGTCTACC-3′) and EL2708 (5′-ATGGGGAT-AATTGTGGGTTCAG-3′) for AtPDI (At5g56340), and EL3031 (5′-ATCCAGCTGTCTCTCTGTTGTTCC-3′) and EL3033 (5′-AGAGCTTCTCCTTGATGTCTC-3′) for AtAct2.

Isolation and Analysis of Genomic DNA—Total genomic DNA was extracted from root tissues using DNAeasy Plant Kit (Qiagen) according to manufacturer’s instruction. For each sample, 4 μg of DNA was subjected to 2.0% (w/v) agarose gel electrophoresis, stained with 0.1 μg ml−1 ethidium bromide in Tris-EDTA buffer (10 mM Tris-HCl, pH 8.0, and 0.5 mM EDTA), and then washed with Tris-EDTA buffer. Fragmented DNA was visualized under UV light with a GelDoc 2000 photodocumentation (Bio-Rad), and the image was analyzed using the software Quantity One (Bio-Rad).

Analysis of AtBII Promoter Fused to GUS Reporter Lines—The DNA fragment containing a putative promoter region of AtBII (−753 to −1 bp from the translational initiation codon) was amplified by PCR using primers EL2368 (5′-AAGCTTTTGCAGGTTAGTTGGAATGTGTTG-3′) and EL2369 (5′-TCTAGATTTGTTTTTTGCTTGACCTGGA-3′) from genomic DNA isolated from WT and cloned into a pCR2.1-TOPO vector (Invitrogen). The BamHI and XhoI fragment from genomic DNA isolated from WT and cloned into a pBI101 (31). This construct, designated pNW166 (35S:AtBII-VSHis6), was used for A. tumefaciens-mediated transformation as described above. The resulting transgenic T1 seeds were selected by plating on 0.5× MS media containing 50 μg ml−1 kanamycin and 200 μg ml−1 carbenicillin, and then transferred to soil for seed production. Independent transformants were screened for expression levels of the AtBII transgene by RNA gel blot analysis and/or the protein expression level by Western blot analysis using anti-V5 antibody (Invitrogen). Rescue of atbi1-2 mutation by 35S:AtBII-VSHis6 was confirmed in multiple independent transgenic lines at the T2 generation.

Measurement of Chlorophyll Content—Total chlorophylls were extracted from individual seedlings with 80% (v/v) acetone at 4 °C overnight. Chlorophyll content was determined spectrophotometrically from the absorbance at 663 nm and 646 nm according to Lichtenthaler (33).

Ion Leakage Measurement—The progression of cell death was assayed by measuring ion leakage from shoots obtained at different time points following TM treatment. For each measurement, shoots from four seedlings were immersed in 4 ml of distilled water in a tissue-culture plate for 6 h at room temperature. After incubation with gentle shaking (100 rpm), the conductivity of the bathing solution was directly measured with a conductivity meter (model 604, VWR Scientific). Measurements for each time point were performed at least in triplicate.

Histochemistry and Microscopy—Fluorescein diacetate (FDA, Sigma-Aldrich) was used as a fluorescent indicator of cell viability via endogenous esterase activity (34). Seedlings were stained with 2.5 μg ml−1 FDA in phosphate-buffered saline (PBS, 20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 2.7 mM KCl) for 10 min at room temperature. After washing three times with PBS, root cells were immediately observed under a Leica MZ FLIII stereo fluorescence microscope equipped with an enhanced green fluorescence protein filter set (excitation 470/40 nm, emission 525/50 nm, dichroic 495 nm). For quantification of endogenous esterase activity, fluorometric assay using FDA was used. In brief, total soluble proteins were extracted using PBS buffer, and insoluble materials were removed by centrifugation (20,000 × g, 10 min, 4 °C). The resulting supernatants were used as samples for direct measurement of esterase activity in vitro.

H2O2 was detected by an endogenous peroxidase-dependent in situ histochemical staining procedure using 3,3′-diaminobenzidine (DAB) (35). Whole seedlings were placed in a solution containing 1 mg ml−1 DAB (pH 5.5, Sigma-Aldrich) for 2 h at room temperature. The seedlings were washed with water and cleared by boiling for 2 min in alcoholic lactophenol (95%
ethanol: lactophenol, 2:1), rinsed in 50% ethanol, followed by distilled water.

For whole-mount TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling) staining, roots were fixed overnight in 4% (v/v) paraformaldehyde in PBS at room temperature. Fixed root tissues were washed five times with PBS, treated with Proteinase K (20 μg ml⁻¹, Invitrogen) in 10 mM Tris-HCl (pH 7.5) at 37°C for 30 min, and then washed three times with PBS. TUNEL reaction was performed in a microcentrifuge tube (1.5 ml) using the In Situ cell death detection kit with fluorescein (Roche Applied Science) according to the manufacturer’s instructions. To visualize nuclei in root cells, samples were stained using 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) at 0.5 μg ml⁻¹ in 0.1% (v/v) Triton X-100 for 10 min, then washed twice with water. DAPI-stained and TUNEL-positive nuclei were observed under a Leica MZ FLIII stereo fluorescence microscope equipped with a BFP filter set (excitation 390/32 nm, emission 460/50 nm, dichroic 420 nm) and an enhanced green fluorescence protein filter set, respectively.

GUS staining was performed as described previously (31). In brief, materials were stained at 37°C overnight in 1 mM 5-bromo-4-chloro-3-indolylglucuronic acid, 1 mM potassium ferri-cyanide, 1 mM potassium ferrocyanide, 0.05% (v/v) Triton X-100, and 0.1 M sodium phosphate buffer (pH 7.0). After staining, seedlings were fixed in 10% (v/v) formamid/5% (v/v) acetic acid/45% (v/v) ethanol for 30 min and cleared with 95% ethanol followed by 50% (v/v) ethanol and 50% (v/v) glycerol.

All images were taken under an MZ FLIII stereo fluorescence microscope with a charge-coupled device camera (Leica, Wetzlar, Germany) and finally processed with Photoshop 5.5 (Adobe).

**RESULTS**

ER Stress Induces Programmed Cell Death in Arabidopsis Roots—

To assess the impact of drug-induced ER stress on the growth and survival of wild-type Arabidopsis (Col-0) plants, 5-day-old seedlings grown on MS solid medium were transferred to a new culture plate containing liquid MS media with or without 0.5 μg ml⁻¹ TM and then grown for up to 3 days. TM-treated seedlings showed strong growth retardation with chlorotic leaves at 3 days post-treatment (dpt) (Fig. 1A). The viability of Arabidopsis seedlings was assessed with FDA, which can assess cell viability in vitro or in vivo based on endogenous esterase activities (34). In vitro FDA assay revealed that TM treatment reduces endogenous esterase activities slightly at 2 dpt and dramatically at 3 dpt, whereas the activities in control seedlings remain relatively unchanged at 3 dpt (Fig. 1B). Similarly, in vivo FDA staining revealed that TM-treated seedlings at 3 dpt show a dramatic loss of cell viability in rosette leaves and roots (Fig. 1C). DAB staining demonstrated enhanced accumulation of H₂O₂ in TM-treated root cells (Fig. 1D) and shoot (data not shown), suggesting that the loss of cell viability is correlated with oxidative stress from enhanced H₂O₂ production, which is often associated with PCD in plants (36). In addition, phase-contrast imaging showed that TM-treated roots displayed irregular morphology with root tip swelling as well as alterations in the shape of cells on the root surfaces and distorted root growth (Fig. 1E).

We next addressed the question of whether TM kills root cells via necrosis or a programmed mechanism (i.e., PCD). A characteristic of PCD is the occurrence of morphological changes in the nucleus, which can be examined by DAPI staining. As shown in Fig. 1E, nuclei in the TM-treated roots appeared more brightly fluorescent due to chromatin condensation. In contrast, the nuclei of control cells are more rounded or oval in shape, and a uniform granular appearance was found in most root cells. Another specific feature of PCD is the cleavage of genomic DNA at internucleosomal sites by endogenous nucleases. To detect fragmented nuclear DNA in situ, a TUNEL procedure was applied to Arabidopsis roots (37). TM induced
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chemical chaperone, TUDCA, which can also relieve ER stress (38). This chemical chaperone was shown to alleviate the effects of TM by improving protein folding in the ER and thus result in the reduction of accumulation of misfolded proteins in the ER (49). Not types and the observed PCD shown in Fig. 1 could result from the accumulation of misfolded proteins in the ER (49). In the course of this study, we observed that TM treatment results in the inhibition of primary root elongation as well as the formation of root hair and lateral roots (Figs. 2, A and B). These morphological phenotypes and the observed PCD shown in Fig. 1 could result from the accumulation of misfolded proteins in the ER (i.e. ER stress) or may be direct consequences from inhibiting protein glycosylation that impairs critical protein functions. To distinguish between these possibilities, we tested the ability of 4-phenyl butyric acid (PBA), a low molecular weight chemical chaperone known to stabilize protein conformation, to alleviate the effects of TM by improving protein folding in the ER and thus relieve ER-stress (38). This chemical chaperone was shown to attenuate ER stress-induced cell death in mammalian cells (39).

When 5-day-old wild-type Arabidopsis (Col-0) seedlings were grown on MS liquid media supplemented with 0.5 μg ml−1 TM for 3 days, co-treatment of PBA (1 mM) significantly attenuated the retardation of root hair growth and lateral root formation (Figs. 2, A and B). PBA also increased survival of co-treated seedlings even at a lethal dose of TM (0.5 μg ml−1). Furthermore, DAPI staining revealed that PBA reduced the appearance of condensed chromatin in the nuclei of meristematic and elongation zones upon TM treatment (Fig. 2C). Similarly, another type of chemical chaperone, TUDCA, which can also relieve ER stress-mediated cell death in mammalian cells (39), dramatically alleviated the lethal effect of TM (see supplemental Fig. S1). These results provide supporting evidence that TM induces root growth defect and PCD via defective protein folding, which leads to ER stress.

### Chemical Chaperones PBA and TUDCA Alleviate TM-induced Growth Retardation and Cell Death

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### Induction of AtBI1 Expression by Tunicamycin Is Transcriptionally Controlled

To analyze the expression of AtBI1 transcript under TM-induced ER stress, total RNA was isolated from root cells of 9- to 10-day-old wild-type Arabidopsis (Col-0) after incubation with 0.5 μg ml−1 TM and then subjected to RNA gel blot analysis. As shown in Fig. 3, rapid increase of AtBI1 and AtBip2 transcripts can be detected at 2, 6, 12, and 24 hpt. AtBip2 is an ER-localized chaperone that belongs to the HSP70 family, which is required for the transport and secretion of proteins in the ER and has been used as a common marker for UPR activation in eukaryotes, including plants (23, 26). We also tested the expression of PR-1, which encodes a pathogenesis-related protein that is secreted via the ER-Golgi network, and its expression is induced by pathogen attack or abiotic stress stimuli (40). It was shown that Bip expression is induced before accumulation of PR-1 during plant-microbe interaction and overexpression of Bip triggers the constitutive expression of PR-1 in tobacco (41). As shown in Fig. 3, PR-1 expression was induced at 12 and 24 h after TM treatment, which was significantly delayed compared with Bip2 and AtBI1. In addition, co-treatment of TM roots with PBA resulted in the reduction of accumulation of AtBI1 and AtBip2 transcripts, whereas induction of PR-1 was almost completely suppressed. These results suggest that PR-1 induction is associated with AtBip2 accumulation under ER stress and is more downstream in the signaling process of the UPR. As a control, we show that AtAct2, a constitutively expressed Actin2 gene,
did not show any altered expression patterns in response to those chemical treatments. Similar results to PBA treatment were obtained with TUDCA. Suppression of both UPR induction in terms of AtBII and AtBip2 expressions and inducible PR-1 expression was found with this chemical chaperone as well (supplemental Fig. S2). These data thus provided evidence for the activation of the UPR in planta by TM treatment via accumulation of unfolded and/or misfolded proteins. Because the level of AtBII transcript is up-regulated by TM-induced ER stress (Fig. 3), its regulation may be transcriptional and conferred by cis elements in the promoter region of AtBII. Indeed, AtBII promoter has a sequence element between −298 to −280 (CGTGGatgattcttATTGG) that is conserved in other ER stress-inducible genes, including AtBip2 (26, 27). To examine the promoter activity of AtBII during ER stress at the cell- and tissue-specific levels, ProAtBII::GUS lines were analyzed (see “Experimental Procedures”). Under normal conditions, weak GUS expression driven by the AtBII promoter was observed in several tissues, including epidermal cells of the cotyledon, rosette leaves, and in the differentiation zone (root hair zone) of roots (supplemental Fig. S3A). Stronger GUS expression was observed in hydathodes and vascular bundles in the cotyledon and rosette leaves (supplemental Fig. S3A), in meristematic and elongation zones of primary and lateral roots (supplemental Fig. S3B), and in stipules and hypocotyls (data not shown). Under TM-induced ER stress conditions, AtBII promoter activity was dramatically increased by TM treatment at the whole seedling level, especially in roots where strong GUS staining was obtained after just 2 h of staining with X-Gluc (supplemental Fig. S3, C and D). We also quantified relative GUS enzyme activity of whole seedlings by in vitro biochemical assay. TM treatment resulted in GUS activity increases of ~4-fold at 6 hpt and 12-fold at 24 hpt as compared with the control (supplemental Fig. S3E). Thus, the highly responsive nature of AtBII expression to ER stress suggests that this gene may also play a role in the UPR and/or its related cell death process (i.e. PCD).

**AtBII Mutants Exhibit Enhanced Sensitivity to ER Stress**—To explore the role of AtBII during ER stress, the response of 5-day-old seedlings of the wild-type and two AtBII mutants (atbii-1 and atbii-2) to TM was examined. The two AtBII mutants are indistinguishable from wild-type Col-0 under normal growth conditions (13). However, at 3 days after a transient TM treatment of 6 h, the two mutants showed more severe growth defects (supplemental Fig. S4A) and chlorophyll loss (Fig. 4A) than wild-type seedlings when they were treated with either 0.3 or 0.5 μg ml⁻¹ TM. Because the TM sensitivity of the two AtBII mutants is quite similar, subsequent experiments were performed using only the atbii-2 mutant allele, a functional null. DAB staining revealed that accumulation of H₂O₂ in the shoot of atbii-1 mutant is slightly higher than wild-type plants at sub-lethal concentration of 0.3 μg ml⁻¹ TM, whereas there is no significant difference in the level of H₂O₂ accumulation between the wild-type and atbii-2 seedlings in the absence of TM treatment (supplemental Fig. S5A). In contrast to the shoot, root cells of atbii-2 mutant showed more intense accumulation of H₂O₂ than that of wild-type (supplemental Fig. S5B). To quantify the sensitivity of wild-type and atbii-2 seedlings to TM, primary root elongation, chlorophyll content, and ion leakage (an indicator of plasma membrane damage) were monitored in the presence or absence of chemical chaperone (PBA or TUDCA). Elongation of primary root as well as chlorophyll content of atbii-2 mutant were markedly reduced by TM treatment compared with wild-type, whereas those phenotypes were alleviated significantly by co-treatment with PBA (Fig. 4, B and C, and supplemental Fig. S4B) or TUDCA (supplemental Fig. S1, B and C). Moreover, elevated ion leakage of atbii-2 seedlings by TM was also alleviated by co-treatment either with PBA (Fig. 4D) or TUDCA (supplemental Fig. S1D). We further examined whether enhanced sensitivity of atbii-2 mutant seedlings to TM is due to accelerated...
PCD induction in root cells. At 3 dpt, viability of *atbi1-2* root cells was significantly decreased as determined by FDA assay (Fig. 5A), indicating that growth inhibition of *atbi1-2* root is due to enhanced cell death progression rather than growth arrest. Furthermore, consistent with this rapid loss of cell viability, *atbi1-2* mutant seedlings displayed more intense PCD phenotypes with enhanced H$_2$O$_2$ accumulation in roots (supplemental Fig. S5B) as well as increased chromatin condensation (revealed by DAPI staining) that coincides with TUNEL-positive nuclei in the elongation zones of the root (Fig. 5B). These results provide evidence that enhanced sensitivity of *atbi1-2* mutant seedlings to TM is caused by TM-induced ER stress followed by PCD activation.

Seedlings Overexpressing AtBI1 Are More Resistant to ER Stress—Because *atbi1* mutants displayed hypersensitivity to TM, which exhibits increased PCD phenotype, we next explored the effect of overexpression of AtBI1 protein for increased survival under ER stress. To this end, a 35S:AtBI1-V5His$_6$ transgene was constructed and then introduced into wild-type *Arabidopsis* and *atbi1-2* backgrounds (supplemental Fig. S6A). Multiple independent transgenic lines expressing AtBI1-V5His$_6$ at different levels were selected by Northern blot and/or Western blot. Western blot analysis using anti-V5 antibody confirmed the accumulation of AtBI1 protein with a fusion tag, which shows the predicted molecular mass of ~25 kDa (supplemental Fig. S6B).

To compare the sensitivity of the wild-type, *atbi1-2* and AtBI1 overexpressors to TM, 5-day-old seedlings were treated with different concentrations of TM (0, 0.1, 0.3, and 0.5 µg ml$^{-1}$) for 6 h, washed with 0.5× MS liquid media, and then grown for up to 5 days without TM. Compared with *atbi1-2* seedlings, transgenic *Arabidopsis* seedlings overexpressing AtBI1-V5His$_6$ in the *atbi1-2* mutant background (AtBI1-OE/*atbi1-2 #2) are markedly more resistant to TM treatment, even when these seedlings were treated with TM at the lethal concentration (0.3 and 0.5 µg ml$^{-1}$) for *atbi1-2* (supplemental Fig. S7). These results demonstrate that the AtBI1-V5His$_6$ construct can successfully rescue the *atbi1-2* phenotype. Similarly, the AtBI1 overexpressor in the wild-type background (AtBI1-OE/WT#5) exhibited reduced sensitivity to TM. Another transgenic line (AtBI1-OE/WT#9) with similar expression level to AtBI1-OE/WT#5 also exhibits reduced sensitivity to TM treatment (data not shown). In contrast, other transgenic lines with low expression of AtBI1-V5His$_6$ (AtBI1-OE/WT#3 and AtBI1-OE/atbi1-2#1) failed to improve survival under TM-induced ER stress (data not shown). These results suggest that the increased tolerance to TM observed with our transgenic lines is correlated with the accumulation of AtBI1-V5His$_6$ proteins. Therefore, it appears that a loss of AtBI1 function results in the hypersensitivity of *Arabidopsis* seedlings to ER stress in the *atbi1* mutant.

Cell death of *Arabidopsis* seedlings was monitored by ion leakage measurements at 3 days after exposure to TM for 6 h (Fig. 6A). TM-induced cell death exhibited dose dependence, and consistent with the results shown in Fig. 4A, increased cell death was observed in *atbi1-2* mutant seedlings even at a low concentration of TM (0.1 µg ml$^{-1}$), whereas cell death of wild type reached at comparable levels to *atbi1-2* mutant when they were treated with 0.5 µg ml$^{-1}$ of TM (Fig. 6A). Conversely, seedlings of AtBI1-overexpressing lines in either wild-type or *atbi1-2* mutant backgrounds exhibited much less cell death compared with their parental lines even at 0.5 µg ml$^{-1}$ TM.

In addition to quantifying cell death at the whole plant level, we also compared growth of primary roots of wild-type, *atbi1-2*, and two AtBI1 overexpressors after exposure to 0.3 µg ml$^{-1}$ TM for 6 h. As shown in Fig. 6B, all seedlings tested exhibited similar elongation rates without TM treatment. Upon TM
treatment, root elongation of atbi1-2 was severely impaired and further growth at between 2 to 3 dpt was arrested, whereas the primary root of the wild-type seedlings continued to elongate for at least up to 5 dpt. Conversely, root elongation of AtBI-1 overexpressors displayed increased resistance to TM treatment and dramatically rescues the growth arrest phenotype of atbi1-2. Because this root growth arrest phenotype in the atbi1-2 mutant can be alleviated by PBA (Fig. 4B) or TUDCA (supplemental Fig. S1B) co-treatment, ER stress due to misfolded proteins is likely a major contributor.

**Induction Patterns of Representative UPR Transcripts under TM-induced ER Stress in Root Cells**—The fact that atbi1 mutants exhibit hypersensitivity to TM suggests the possibility that loss-of-function of AtBI1 may cause an impairment of the UPR. Furthermore, it is possible that overexpression of AtBI1 protein might directly or indirectly interfere with the UPR leading to attenuated TM sensitivity. To examine these possibilities, we performed semi-quantitative RT-PCR analysis to examine the expression patterns of AtBip2, as well as other UPR-related genes identified in Arabidopsis (AtPDI, AtCRT1, and AtCNX1), in roots of wild-type, atbi1-2, and the AtBI1 overexpressor (AtBI1-OE/WT/#5). As seen in Fig. 7, accumulation of representative UPR transcripts as well as the AtBI1 transcript in wild-type roots started 2 h after TM treatment, whereas AtAct2 expression was essentially constitutive under these conditions, indicating induction of the UPR. Similar UPR induction in terms of gene expression was seen in atbi1-2 mutant and AtBI1 overexpressor backgrounds. These results suggest that alterations in AtBI1 expressions are unlikely to interfere with the UPR at the transcriptional level and that normal UPR signaling can occur.

**DISCUSSION**

**AtBI1 Belongs to the UPR/ER Stress Protein Family**—Transcriptional regulation of gene expression is a fundamental response to stress signals. Several independent lines of evidence support the rapid induction of AtBI1 mRNA as reflecting the activation of the UPR pathway during ER stress. First, several features of AtBI1 up-regulation correspond closely to those observed for transcripts of Bip2, a well studied ER chaperone measured in parallel in our system (Figs. 3 and 7). Second, the coordinated induction of AtBI1 with AtBip2 by TM-induced ER stress was further demonstrated by the administration of two different chemical chaperones PBA and TUDCA (Fig. 3 and supplemental Fig. S2), providing strong supportive evidence that rapid up-regulation of AtBI1 and AtBip2 transcripts in root cells are associated with the ER stress as the result of accumulation of misfolded proteins in the ER. Third, expression analyses of transgenic lines with an AtBI1 promoter fusion to the GUS reporter gene indicate that AtBI1 promoter activity is also rapidly induced in response to TM (supplemental Fig. S3). We also found the rapid induction of AtBI1 transcript as well as activation of its promoter in the presence of other types of ER stress inducers, CPA, and a proline analog 1-azetidine-2-carboxylic acid (AZC). Third, sequence analysis of the AtBI1 promoter region revealed a sequence element between −298 to −280 (CGTGGatgattctATTGG), called ER stress response element (ERSE), that is conserved in the promoter region of other ER stress-inducible genes that include AtBip2, AtPDI, AtCRT1, and AtCNX1 (26, 27). By RT-PCR, expression of these genes was shown to increase rapidly in roots at 2 h after TM treatment (Fig. 7), indicating that inductive expression of AtBI1 under ER stress is coordinated at the transcriptional level with other UPR-related genes. AtBI1 could be induced by the ATZ 60-dependent UPR pathway during ER stress, which is responsible for the induction of Arabidopsis UPR genes containing the ERSE element(s) (25). Lastly, the onset and severity of ER stress-induced PCD correlate inversely with AtBI1 expression levels, indicating that AtBI1 plays an important role in maintaining plant growth and survival under ER stress (Figs. 4–6). However, cell death acceleration and a more severe PCD phenotype resulting from the disruption of AtBI1 is likely to be independent of the UPR signaling pathway that serves to relieve ER stress as this response remains intact in the atbi1-2 mutant (Fig. 7). In addition, overexpressing AtBI1 did not affect significantly the UPR signaling pathway, suggesting that the mechanisms underlying PCD and UPR might thus be partially or temporally uncoupled in plants. Overall, we propose that AtBI1 is a critical survival factor for suppression of PCD induced by ER stress, thereby allowing the UPR sufficient time to re-establish proper homeostasis in the cell. In the absence of AtBI1, plant cells become hypersensitive to ER stress despite the induction of the UPR.

We show here that two different chemical chaperones, PBA and TUDCA, which have been clinically used for treatment of many human diseases and studied for their therapeutic potential to ER stress-related neurodegenerative diseases (38, 39, 42, 43), can attenuate TM-induced growth defect and cell death of Arabidopsis seedlings (Figs. 4, S1, and S4). Recent studies showed that PBA and TUDCA have different chemical properties in their ability to improve ER protein folding capacity. PBA was shown to suppress ER stress-mediated but not mitochondria-derived cell death by chemically enhancing ER protein folding capacity to stabilize proteins in their native conformation, thus contributing in some cases to rescuing the folding.

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defect of mutant proteins (42, 44, 45). However, unlike PBA, TUDCA has not been well characterized as a chaperone that chemically promotes protein folding. Its capacity to protect cells from ER stress appears to involve reduced UPR induction directly and suppression of calcium-mediated apoptosis pathway in animal cells (45, 46). These two chemical chaperones would be useful agents to dissect the mechanisms of ER stress response and PCD in plants, because proper regulation of ER protein-folding capacity is critical for growth and survival in Arabidopsis plants as we showed in this present work.

Consistent with TM induction of PCD via ER stress, we also found that treatment of Arabidopsis seedlings with two other agents, CPA and AZC, that can induce ER stress by different mechanisms resulted in growth defect and loss of survival. In 100 μM CPA- or 1 mM AZC-treated roots, severe PCD phenotypes were observed in the differentiation zone at much higher frequencies than in meristematic and elongation zone. Those phenotypes were also partially alleviated by cotreatment with PBA, indicating that CPA and AZC trigger ER stress response in a similar manner with TM. Compared with wild-type plants, atbi1-2 mutant plants also show increased sensitivity to CPA and AZC, whereas overexpression of AtBI1-V5His6 in the atbi1-2 background decreased sensitivity to these agents. These results support the model that AtBI1 is an important survival factor during drug-induced ER stress response. How these chemicals with their distinct modes of action contribute to quantitative differences in the observed phenotypes remains to be determined.

Role of AtBI1 in ER Stress and Its Related Cell Death Process—The ER is very sensitive to perturbation of its environment in eukaryotes in response to a variety of stress stimuli (17). It was shown that salt (NaCl) stress induces apoptosis-like cell death in barley and Arabidopsis roots (47–49). In Arabidopsis, PCD phenotypes (TUNEL-positive nuclei and DNA laddering) induced by a sublethal dose of salt treatment (0.16 M NaCl) were observed in the root meristematic and elongation/differentiation zones, whereas root cells of salt-hypersensitive mutant plants (sos1) exhibited more severe PCD phenotype under salt stress (49). It is postulated that localized PCD in root cells is important for the wild-type seedlings to adapt and cope with a saline condition, because salt-treated wild-type seedlings can form secondary and lateral roots after transfer to medium without salt (49). Subsequently, it was shown that salt stress induces the UPR in wild-type Arabidopsis seedlings while mutations in one of the subunits for an oligosaccharyltransferase (OST) complex (sst3a-1 and sst3a-2) involved in N-glycosylation in the ER lumen, resulted in salt hypersensitivity (50). Similarly, our present work showed that treatment with a low dose (0.3 μg ml⁻¹) of the N-glycosylation inhibitor TM results in severe PCD phenotype in the atbi1-2 mutant as compared with wild-type plants (Fig. 5). Furthermore, under this condition wild-type seedlings can survive, whereas the atbi1-2 mutant cannot (Fig. 6), suggesting that suppression of TM-induced cell death by AtBI1 is required for the survival and/or adaptation under mild ER stress condition.

With respect to the postulated role of the ER in plant PCD, it is notable that Arabidopsis DAD1 (defender against death-1), originally identified in hamster cells as a defender against apoptotic death, shows high homology to OST2, which is one of nine subunits of the yeast OST enzyme complex (51). Interestingly, Arabidopsis seedlings overexpressing AtDAD1 were found to suppress PCD induced by UV-C irradiation (52). These findings suggest that perturbation of ER homeostasis may also mediate PCD induced by other stress signaling pathways in plants such as high salt and UV irradiation. In addition, the importance of proper ER functions for plant defense against microbial pathogens was recently reported (53). An intact and responsive protein secretion pathway with up-regulation of secretion-related genes such as Bip, PDI, CRT, and DADI was shown to be essential for the induction of systemic acquired resistance in Arabidopsis (53). Interestingly, barley BI-1 was recently shown to attenuate the invasion and proliferation of the root endophytic fungus Piriformospora indica, which requires host cell death for successful pathogenicity (54). In this regard, we note that AtBI1 plays a role in the modulation of ER stress-mediated PCD in root cells. Clarifying the regulation process in ER stress-induced cell death and its connection with other biotic and abiotic stress response pathways may thus provide us with new insights into common and distinct mechanisms leading to their tolerance in plants.

Although mammalian and plant BI-1 proteins were reported to localize to the ER membrane, their mode of action is still unclear in any system. It has been proposed that they may form ion-conducting channel either alone or together with other proteins involved in cell death regulation such as BCL-X₁ (5). In plants, BI-1 is induced by a variety of stress stimuli such as pathogen attack, oxidative stress, and heat stress, and its overexpression suppresses cell death activation (6, 7). Potentially, these stresses trigger the accumulation of ROS and increase of cytosolic calcium level in a cell, leading to cell death activation (3, 4). Given its localization in the ER, which is a major intracellular calcium reservoir and an organelle that is sensitive to external and internal stresses, BI-1 may function by regulating cytosolic calcium concentration and/or redox status.

It was shown that AtBI1 overexpression attenuates cell death induced by transgenic expression of BAX or by abiotic stress without affecting production of ROS (10, 55), suggesting that AtBI1 is not responsible for scavenging the oxidant that is a prerequisite for cell death activation under these conditions. We showed here that ROS level in atbi1-2 root cells in response to TM-induced ER stress was significantly higher than that in wild-type root cells (supplemental Fig. S4), indicating that loss of function of AtBI1 results in the enhancement of ER stress-mediated ROS production. Whether AtBI1 functions downstream of ROS production or in parallel pathways affecting execution steps for ER stress-mediated cell death activation remains to be determined. In contrast, it was recently shown that overexpression of mice BI-1 reduces the level of ER stress-associated ROS accumulation in cultured mammalian cells through the modulation of heme oxygenase-1 expression (56). The elevation of heme oxygenase-1 activity following ER stress might be an adaptive response that allows the cells to re-establish ER homeostasis through limiting the oxidative damage that can result in misfolding of proteins in the ER, thereby reducing cell death.
In animal system, accumulating evidence has suggested that both mitochondria-dependent and -independent cell death pathways likely mediate apoptosis in response to ER stress (18). The ER might serve as a site where apoptotic signals are generated through several mechanisms, including BAK/BAX-regulated Ca\(^{2+}\) release from the ER (19). Although BCL-2 family members are thought to function principally at the outer membrane of mitochondria, there is strong evidence that they influence homeostasis and apoptosis signaling from the ER as well (57, 58). Although no obvious BCL-2 family member has been found in plants, plant BAX-like protein, designed as Cdf1, was recently identified using a negative selection approach in yeast cells (59). Cdf1 induces an apoptotic-like cell death of yeast, which is suppressed by co-expression of AtBII1. Interestingly, CDF1 has no sequence homology to any animal proteins, including BAX, but apparently causes a loss of mitochondrial membrane potential in a BAX-like manner, suggesting the existence of cross-talk between the ER- and mitochondria-related cell death pathways in plant cells (59). In addition, it was recently reported that a plant calmodulin (AtCaM7) can interact with AtBII1 in vitro and in vivo and that overexpression of AtBII1 can modulate CPA-induced cell death in tobacco BY-2 cells (60). This suggests the possible connection between AtBII1 and calcium homeostasis. By analogy with animal systems, controlling the calcium content of the ER might be a key process to activate the downstream pathways that promote cell death in plant cells.

In conclusion, our results established the function of AtBII1 as a critical determinant for plant survival under ER stress. Currently, the mechanism by which plant BI-1 suppresses cell death induced by a variety of stress stimuli remains to be clarified. Interestingly, our analysis using the publicly available Arabidopsis transcriptome data base (GENEVESTIGATOR) revealed that expression of AtBII1 as well as UPR genes (AtBip2, AtPDI, and AtCNCX1) is up-regulated under several stress conditions induced by bacterial and fungal pathogens, ozone, norflurazon, or salicylic acid (data not shown). In this regard, it will be interesting to determine whether different unrelated biotic and abiotic stresses trigger similar perturbation of ER homeostasis followed by ER stress response and cell death activation. This proposed unifying theme of stress response and related PCD activation may help explain the observed role of BI-1 under diverse stress conditions (13–16). Future structure/function studies will also provide much needed insight into the mode of action for BI-1 as a survival factor in plants for stress tolerance.

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