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AtBAG7, an *Arabidopsis* Bcl-2–associated athanogene, resides in the endoplasmic reticulum and is involved in the unfolded protein response

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The Bcl-2–associated athanogene (*BAG*) family is an evolutionarily conserved, multifunctional group of cochaperones that perform diverse cellular functions ranging from proliferation to growth arrest and cell death in yeast, in mammals, and, as recently observed, in plants. The *Arabidopsis* genome contains seven homologs of the *BAG* family, including four with domain organization similar to animal BAGs. In the present study we show that an *Arabidopsis BAG*, *AtBAG7*, is a uniquely localized endoplasmic reticulum (ER) *BAG* that is necessary for the proper maintenance of the unfolded protein response (UPR). *AtBAG7* was shown to interact directly in vivo with the molecular chaperone, *AtBiP2*, by bimolecular fluorescence complementation assays, and the interaction was confirmed by yeast two-hybrid assay. Treatment with an inducer of UPR, tunicamycin, resulted in accelerated cell death of *AtBAG7*-null mutants. Furthermore, *AtBAG7* knockouts were sensitive to known ER stress stimuli, heat and cold. In these knockouts heat sensitivity was reverted successfully to the wild-type phenotype with the addition of the chemical chaperone, tauroursodexycholic acid (TUDCA). Real-time PCR of ER stress proteins indicated that the expression of the heat-shock protein, *AtBiP3*, is selectively up-regulated in *AtBAG7*-null mutants upon heat and cold stress. Our results reveal an unexpected diversity of the plant's *BAG* gene family and suggest that *AtBAG7* is an essential component of the UPR during heat and cold tolerance, thus confirming the cytoprotective role of plant *BAGs*.

programmed cell death | chaperones | cytoprotection

As nonmotile organisms devoid of an adaptive immune system, plants must adapt rapidly to changing environmental conditions. To combat environmental constraints, plants have developed various defense strategies (1). At the organism level, abiotic stresses such as extreme temperature can induce both structural (e.g., increased leaf thickness) and developmental (e.g., fruit ripening) changes. Adverse environmental conditions also induce changes at the cellular level (2). Recent research has established that the regulation of cell death pathways is relevant to both abiotic and biotic stress responses in plants (3–5). Although core regulators of programmed cell death are conserved in mammals (e.g., the Bcl-2 family and caspases), they have not been identified in plants. The failure to identify plant homologs of mammalian cell-death regulators results, in part, from a lack of conservation at the primary sequence level. We have used functional genomic approaches in conditional yeast strains and bioinformatics approaches to address this issue and to identify the *Arabidopsis* Bcl-2–associated athanogene (BAG) gene family (5, 6).

The BAG proteins are a broadly conserved gene family with homologs spanning wide evolutionary distances including yeast, animals, and plants. The first identified mammalian BAG gene (*BAG1*) was discovered in a screen of a mouse embryo cDNA library using recombinant human Bcl-2 protein as bait to identify binding partners to Bcl-2 (7). *BAG1* enhanced cell survival synergistically with Bcl-2, suggesting involvement in programmed cell death (PCD) pathways. Subsequent studies uncovered the BAG family and more accurately indicated that BAGs function as molecular cochaperones (8). Members of the BAG family are distinguished by the presence of a conserved C-terminal BAG domain (BD) containing ~110–130 amino acids. The BD is comprised of three α helices of 30–40 amino acids each; the second and third helices mediate direct interaction with the ATPase domain of heat-shock protein 70 (Hsp70)/heat-shock cognate 70 (Hsc70) chaperones (9, 10). Six BAG family members have been identified in humans and were shown to regulate the function of Hsp70/Hsc70 both positively and negatively and to form complexes with a range of transcription factors (11) to modulate numerous physiological processes including apoptosis, tumorigenesis, neuronal differentiation, stress responses, and cell-cycle progression (reviewed in ref. 12).

Far less is known about the function of BAGs in plants. Initial BLAST searches failed to identify BAG homologs in *Arabidopsis* because of low sequence identities. Therefore more sensitive methods were used, including Hidden Markov Model-based approaches and profile–profile algorithms (13, 14). With these approaches, seven BAG homologs were identified in *Arabidopsis*, four of which have domain organization similar to animal members (5). The remaining three members contain a predicted calmodulin-binding motif near the BD, a feature unique to plant Bcl-2–associated athanogene (BAG) proteins (5). Like their mammalian counterparts, the plant BAGs are multifunctional and regulate cytoprotective processes from pathogen attack to abiotic stress and development (5). Our initial studies focused on two structural representative groups of the *Arabidopsis* BAG family, *AtBAG4* (structurally similar to mammalian BAGs) and *AtBAG6* (unique to plants). Transgenic tobacco overexpressing *AtBAG4* provided enhanced tolerance to several abiotic stress stimuli, including cold, salt, and drought (5). Interestingly, *AtBAG6* appears to play a role within basal defense pathways (5). These results suggest that the *Arabidopsis* BAG family may have developed specialized roles for cell regulation in response to various stress stimuli.

Previously, it was shown that mammalian BAGs localize either to the nucleus or to cytoplasm. In plants BAG localization is more diverse (nucleus, vacuole, mitochondria, cytoplasm), and in this study we show that the *Arabidopsis* BAG member *AtBAG7* resides in the endoplasmic reticulum (ER). The ER is essential for the maturation of proteins, performing processes such as correct folding, disulfide bond formation, and protein assembly. The synthesis of steroids, lipids, and cholesterol also are ER-specific processes. Several physiological conditions including nutrient deprivation, alterations in redox balance, and changes in calcium homeostasis can perturb these activities, causing an abundance of unfolded proteins, ER stress, and eventually cell death (15). To alleviate the detrimental effects of an accumulation of misfolded protein, eukaryotes have developed an ER–nucleus signaling pathway termed the "unfolded protein response" (UPR) (15, 16). The

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UPR serves to restore normal function of the cell by halting protein translation and activates signaling pathways that lead to increased production of molecular chaperones involved in protein folding. In contrast to yeast and mammals, the underlying mechanisms of the UPR in plants are not well documented. Recent data, however, suggest that the UPR is important in plants, particularly in response to both abiotic and biotic stimuli where it triggers a variety of processes, including attenuation of protein translation, activation of the ER-associated degradation pathway, or the induction of ER chaperones (17–23).

Here we show that *AtBAG7* localizes to the ER and is involved in the maintenance of the UPR. Consistent with the premise that BAG proteins and ER chaperones are involved in stress adaptation and PCD pathways in plants, *AtBAG7* knockouts were hypersensitive to known ER stress inducers such as heat, cold, and tunicamycin (Tm), a chemical inducer of ER stress. Furthermore, with bimolecular fluorescence complementation (BiFC), *AtBAG7* was shown to interact in vivo with immunoglobulin-binding protein (*BiP*), an Hsp70 paralog and known component of the UPR. Real-time analysis of a range of ER stress proteins demonstrated selective up-regulation of the Hsp *AtBiP3* in *AtBAG7*-null mutants subjected to heat and cold stress. These findings indicate that *AtBAG7* is a functional homolog of the BAG family with a unique ER location and is an important cytoprotective cochaperone for ER stress responses as part of the UPR.

Results

A Member of the Arabidopsis BAG Family Is Localized to the ER. Although there has been extensive research on BAG genes in mammals, far less is known about plant BAG function. In a previous study we used structural criteria rather than primary sequence to search the *Arabidopsis* genome for BAG-like proteins. Seven genes were identified, two of which, *AtBAG4* and *AtBAG6*, have demonstrated involvement in plant PCD regulatory pathways and thus have functional similarity to the mammalian BAG genes (5, 24). In this study we focused on *AtBAG7* (AT5G62390). The coding region of *atbag7* was fused to the C terminus of Enhanced-YFP in the 35S promoter-driven expression cassette, pEarleygate104 (25). Microprojectile bombardment into onion epidermal cells was performed, and YFP-fusion expression was visualized by confocal microscopy. Upon visualization, YFP-*AtBAG7* colocalized with the ER-specific dye, ER-Tracker (Invitrogen), and with *Arabidopsis* wall-associated kinase 2 red fluorescent protein (*AtWAK2*-RFP), a known ER marker (Fig. 1) (26). This localization was confirmed in green tissue using recombinant *Agrobacterium* harboring pBAG704. Three days after infiltration, the leaves were stained with ER-Tracker, and the YFP-*AtBAG7* fusion was visualized by confocal microscopy. Interestingly, the localization of *AtBAG7* to the ER was not predicted by the computer-based localization programs pSORT and signalP, both of which suggested a nuclear localization. However, we cannot exclude the potential translocation of *AtBAG7* to other areas of the cell, including the nucleus, given the appropriate signal.

***AtBAG7* Binds the Hsp70 Paralog, *BiP*.** Distinguishing features of mammalian BAG proteins include the presence of a conserved BD and the ability to bind Hsp70 (7, 8, 10). Previously we showed that a member of the *Arabidopsis* BAG family, *AtBAG4*, is able to bind an *Arabidopsis* Hsp70 (5). Therefore, we considered whether *AtBAG7* also could interact with Hsp70. Because our initial localization studies indicated that *AtBAG7* resides in the ER, we concentrated on the ER-localized Hsp70 paralog, *Arabidopsis* luminal binding protein *BiP2* (AT5G42020). To determine whether *AtBAG7* and *AtBiP2* interact, we performed an in vivo BiFC assay. The coding region of GFP was split (amino acids 1–174 and 175-end) and cloned independently as N-terminal fusions to *AtBiP2* and *AtBAG7* to generate pBIP2080 and pBAG7096, respectively. Once generated, these constructs were cobombarded into onion epidermal cells and analyzed for interaction (GFP fluorescence) by confocal microscopy. As shown in Fig. 2, strong GFP expression was observed in samples comprising pBiP2080

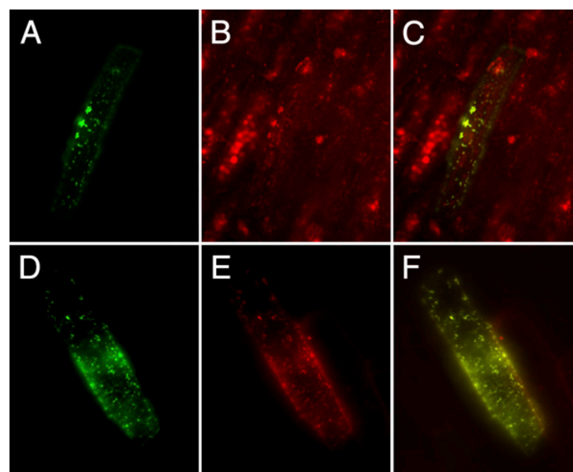

Fig. 1. The N-terminal YFP-*AtBAG7* fusion localizes to the ER. Onion epidermal cells were microprojectile bombarded with YFP-*AtBAG7* (*A–C*) or cobombarded with YFP-*AtBAG7* + *AtWAK2*-RFP (*D–F*). Two days after bombardment, cells were stained with ER Tracker (*A–C*) or visualized directly (*D–F*) by confocal microscopy. (*A*) YFP-*AtBAG7* overlay. (*B*) ER-Tracker overlay. (*C*) YFP/ER-Tracker overlay. (*D*) YFP-*AtBAG7* overlay. (*E*) RFP-*AtWAK2* overlay. (*F*) YFP/RFP overlay.

and pBAG7096. However, cotransformation of pBAG7096 and an empty vector control did not result in a fluorescent signal. These results demonstrate that *AtBAG7* interacts with *AtBiP2* in vivo. This interaction was confirmed further by yeast two-hybrid analysis (Fig. S1).

***AtBAG7* Is Involved in Maintenance of the Unfolded Protein Response.** Recently a role for the ER as a cellular sentinel to detect and alleviate stress as part of plant adaptation pathways has been recognized (27). During ER stress, BiP and other Ca^{2+}-dependent folding proteins, including calreticulin (CRT) and calnexin (CNX), are expressed as part of the UPR to create an optimal environment for proper protein folding (17, 19, 21, 27–31). Because *AtBAG7* interacts with a major mediator of ER stress, *AtBiP*, we considered whether it plays a role within the UPR. We induced ER stress and a UPR in *AtBAG7* knockouts (Salk_065883 and Salk_058247) using Tm, an inhibitor of glycosylation (18, 20). Five-day-old wild-type and *AtBAG7*-knockout seedlings grown on solid Murashige and Skoog (MS) medium were transferred to liquid MS with or without 0.5 μg/mL Tm, were incubated for 6 hours, and were llowed to recover for 5 days in the absence of Tm. In comparison with wild-type plantlets and untreated controls, treated *AtBAG7* knockouts exhibited pronounced growth retardation and chlorosis after 5 days (Fig. 3). These results are consistent with a role for *AtBAG7* within the UPR.

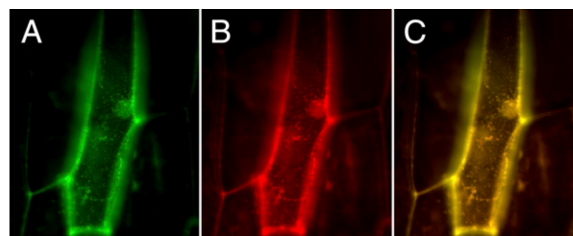

Fig. 2. *AtBAG7* binds to the Hsp70 paralog, *BiP*. Onion epidermal cells were microprojectile bombarded with *AtBAG7*-N(GFP), *AtBIP2*-C(GFP), and RFP-*AtWAK2*. Two days after bombardment, cells were visualized by confocal microscopy. (*A*) *AtBAG7*-N(GFP) + *AtBIP2*-C(GFP) overlay. (*B*) RFP-*AtWAK2* overlay. (*C*) *A* and *B* overlay.

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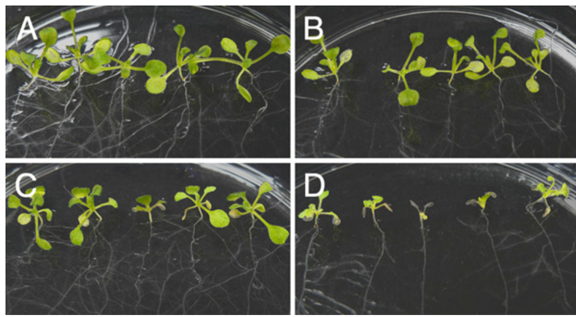

Fig. 3. Tunicamycin accelerates cell death in *AtBAG7* knockouts. Seven-day-old wild-type and *AtBAG7*-mutant plantlets were submerged in 1/2 MS liquid containing, 0.5 μg mL^{-1} Tm for 6 h, washed twice in 1/2 MS containing no antibiotic, and allowed to recover on 1/2 MS agar. (*A*) Wild-type MS-treated plantlets. (*B*) Wild-type Tm-treated plantlets. (*C*) *AtBAG7*Δ MS-treated plantlets. (*D*) *AtBAG7*Δ Tm-treated plantlets. Plants were analyzed for phenotypic differences 5 days posttreatment.

***AtBAG7* Plays an Integral Role in Abiotic Stress Tolerance.** Previously we demonstrated that members of the *Arabidopsis* BAG family play a role in the regulation of PCD pathways induced by abiotic stress, including cold and salt, as well as in pathways induced by biotic challenge (5). Several biotic and abiotic stresses, including excessive heat and cold, are known to induce ER stress (19, 21, 22, 32). Because *AtBAG7* could play a role within ER stress pathways, including the UPR, we investigated whether *AtBAG7* impacts plant tolerance to abiotic stimuli that induce ER stress. Five-day-old *Arabidopsis BAG7*-knockout mutants were subjected to heat or cold stress, and phenotypes were assessed following a recovery period of 4 days. As a positive control for heat sensitivity, *AtBiP2*-knockout plants were analyzed also, because *AtBiP2* is a known component of heat-shock responses (32). As shown in Fig. 4, both *AtBAG7* and *AtBiP2* mutants were more sensitive than wild-type controls to heat treatment(50 °C for 30 min). In addition to heat stress, *AtBAG7* knockouts also were more sensitive than wild-type plants to severe cold stress (−20 °C for 90 min) (Fig. 4). These results correlate with previous reports that *Arabidopsis* BAGs function in cytoprotection. Therefore, *AtBAG7* is localized within the ER and is involved in plant PCD pathways, particularly in response to abiotic stress (5, 33).

Chemical Chaperones Revert the *AtBAG7*-Knockout Heat-Sensitive Phenotype During Heat Stress. During heat stress, unfolded proteins accumulate within the ER, resulting in ER stress and eventual cell death (34). To overcome the abundance of unfolded proteins and to alleviate ER stress, cells increase expression of folding proteins and chaperones including BiP as part of the UPR. Tauroursodexycholic acid (TUDCA), a chemical chaperone that stabilizes protein conformation and promotes proper folding, alleviates ER stress in plants and mammals (22, 35). In light of our BiFC and abiotic stress results indicating a role for *AtBAG7* within the UPR, we attempted to complement the heat-sensitive *AtBAG7* knockouts with the addition of TUDCA. One day before heat treatment, 4-day-old wild-type, *AtBiP2*, and *AtBAG7* plantlets grown on MS medium were transferred to MS medium containing 0.5 mM TUDCA. As shown in Fig. 4 and consistent with previous studies performed on *AtBiP2* mutants, the survival rate of ER-stressed (i.e., heat-stressed) *AtBAG7* knockout plants was increased significantly by pretreatment with TUDCA (Fig. 4). The alleviation of the *AtBAG7* sensitive phenotype by the chemical chaperone TUDCA is consistent with *AtBAG7* chaperone activity during abiotic-induced ER stress.

During Abiotic Stress *BiP3* Transcript Levels Are Increased in the *AtBAG7*-Null Mutant. The previous results demonstrate a chaperoning activity for *AtBAG7* during stress and maintenance of the UPR. Numerous studies have shown increased levels of ER chaperones, including BiP and Ca^{2+}-dependent ER folding proteins, during ER stress and the UPR, (22, 30, 31, 36). Therefore, using real-time PCR, we considered whether *AtBAG7* expression also is induced upon ER stress. In addition to *AtBAG7*, we analyzed the expression of several ER stress markers in the *AtBAG7*-mutant genotype. ER stress was induced in wild-type and *AtBAG7*-knockout mutants by heat treatment (50 °C for 30 min) or cold treatment (−20 °C for 90 min), and the expression patterns of *AtBAG7* and the ER stress markers *BiP1*/*BiP2*, *BiP3*, CNX, CRT, protein disulfide isomerase, and Bax inhibitor (*Bi-1*) were quantified by real-time PCR (Table S1) . Consistent with previous studies, the real-time data demonstrated increases in chaperone expression for both heat and cold treatment in wild-type plants (22, 30, 31, 36). The real-time analysis failed to demonstrate an increase in *AtBAG7* transcript for either heat or cold treatment. This finding suggests that *AtBAG7* activity is regulated posttranscriptionally. Interestingly, expression of *BiP1*/*BiP2*, CRT, protein disulfide isomerase, and *BI-1* was not affected in the *AtBAG7*-null mutants (Fig. S2), *BiP3* expression, however, was induced earlier and to higher levels following both heat and cold stress (Fig. S3). These data provide further evidence of a role for *AtBAG7* within heat and cold stress pathways.

Discussion

The BAG family is a multifunctional group of cytoprotective proteins that are distinguished by a common region, the BD, which mediates interaction with Hsp70 proteins (7, 10–12). BAG genes are broadly conserved, are found in yeasts, plants, and animals, and function as adapter proteins/cochaperones, forming complexes with signaling molecules (e.g., Siah) and molecular chaperones (e.g., Hsc70-interacting protein, Hsp70) (37, 38). The *Arabidopsis* genome contains seven homologs of the BAG family, including four with domain organization similar to animal BAGs (5, 12). Like their mammalian counterparts, plant BAG members are multifunctional and regulate PCD processes, including those induced by pathogen attack, abiotic stress, and development (5). In this study we used a combination of reverse and chemical genetics to demonstrate that *AtBAG7*, an *Arabidopsis* BAG member, is an important component in the mediation of ER–PCD pathways, including the UPR, during abiotic stress adaptation.

The first aim of this study was to localize *AtBAG7* within the cell. Using E-YFP as a marker, an YFP-*AtBAG7* fusion was used to transform transiently onion epidermal cells and *Nicotiana benthamiana* leaf tissue where it colocalized with the ER-specific stain, ER-Tracker, and molecular marker *AtWAK2* (26). This localization was consistent with proteomic studies that suggested an ER localization for the gene product of the At562390 locus which we have identified as *AtBAG7* (39).

The ER has become a major focal point in PCD research in animals and plants, particularly in relation to stress responses through the induction of the UPR (reviewed in ref. 40). A principal component of this research was to determine whether and to what extent *AtBAG7* is involved in ER stress pathways. Using BiFC, we demonstrated direct interaction of *AtBAG7* with the ER-localized Hsp70 paralog, *AtBiP* (32). As a marker of ER stress, *BiP* is well documented as an integral player of the UPR, performing roles including protein translocation, folding, and assembly of ER-modified proteins (32). Although the mechanistic consequences of *AtBAG7*/*BiP* binding are unknown, it is reasonable to hypothesize that this interaction is part of a complex required for proper maintenance of the UPR, especially because *AtBAG7* knockouts are sensitive to ER stress. Alternatively, and consistent with our real-time data, *AtBAG7* may serve a role in the UPR via indirect regulation of *BiP3* expression.

Mammalian BAGs are known cochaperones that are involved in PCD pathways during abiotic and biotic stress responses (41–43). Although the demonstration of *AtBAG7* and *BiP* interaction via BiFC assays suggests a role for *AtBAG7* as a cochaperone for the maintenance of the UPR, further studies were required to confirm this interaction. Tm, a specific inducer of ER stress, has been used to identify

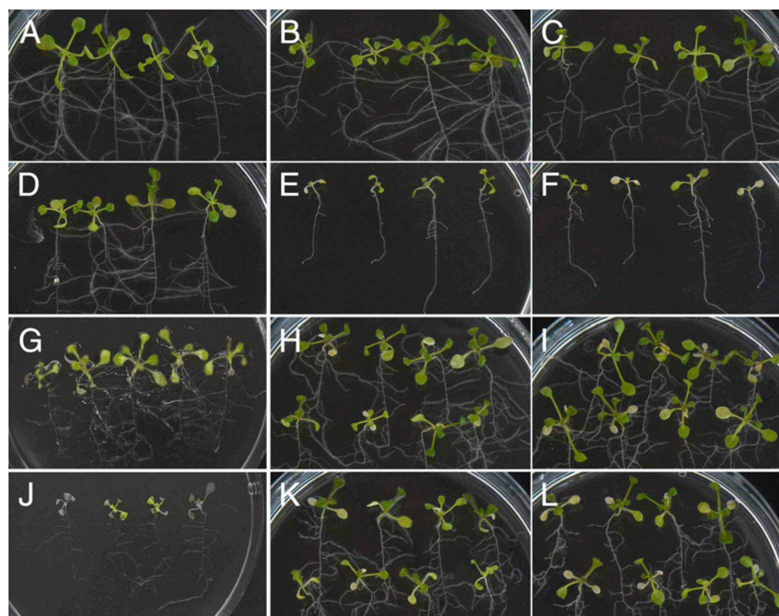

Fig. 4. *AtBAG7* knockouts are susceptible to heat and cold stress. Five-day-old wild-type, *AtBiP2* –mutant (Salk_073202), and *AtBAG7*-mutant (Salk_058247) *Arabidopsis* plants were grown on 1/2 MS, 2% sucrose and subjected to heat treatment at 50 °C for 30 min or cold treatment at –20 °C for 90 min. For complementation of the heat-sensitive phenotype TUDCA was added 1 day before heat treatment, 4-day-old wild-type and *AtBiP2*- and *AtBAG7*-knockout (Salk_073202 and _058247, respectively) plantlets grown on 1/2 MS media were transferred to 1/2 MS media containing 0.5 mM TUDCA. (*A*) Wild-type untreated plantlets. (*B*) *AtBiP2Δ* untreated plantlets. (*C*) *AtBAG7Δ* untreated plantlets. (*D*) Wild-type heat-treated plantlets. (*E*) *AtBiP2Δ* heat-treated plantlets. (*F*) *AtBAG7Δ* heat-treated plantlets. (*G*) Wild-type cold-treated plantlets. (*H*) *AtBiP2Δ* plantlets pretreated with TUDCA, no heat stress. [To serve as a comparative control, wild-type plantlets were used in each of the plates above (*Top Row*).] (*I*) *AtBAG7Δ* plantlets pretreated with TUDCA, no heat stress*. (*J*) *AtBAG7Δ* cold-treated plantlets. (*K*) *AtBiP2Δ* plantlets pretreated with TUDCA, heat stressed*. (*L*) *AtBAG7Δ* plantlets pretreated with TUDCA, heat stressed*. Photographs were taken after a 5-day recovery period.

key regulators of ER stress pathways including *BiP*, *Bi-1*, and the membrane-tethered transcription factors bZIP28 and bZIP60 (22, 28, 30, 31, 36). Consistent with these studies, *AtBAG7* knockouts were significantly more sensitive than wild-type plants to Tm treatment. During Tm treatment unfolded proteins accumulate in the ER; cytoprotective *AtBAG7* may delay ER-induced cell death as part of the UPR.

BiP is a chaperone that directs heat-shock responses (22, 32). To confirm that *AtBAG7* aids *BiP* in the protection of cells during heat-induced ER stress via cochaperone activity, we tested whether pretreatment with the chemical chaperone TUDCA could protect heat-stressed *AtBAG7* mutants. Treatment with TUDCA compensated for the lack of *AtBAG7* and protected *AtBAG7* knockouts undergoing heat stress. Collectively, the Tm and TUDCA results confirm a role for *AtBAG7* as a cochaperone for the coordination of the UPR and delay of ER-induced cell death.

As mentioned, *AtBAG7* also may play a role within the UPR indirectly by regulating *BiP* expression. Our gene-expression analysis using real-time PCR indicates that *BiP3* is significantly induced in the *AtBAG7*-mutant background during heat and cold stress. In the knockouts, the plant may compensate for the lack of *AtBAG7* activity by inducing the expression of another ER chaperone, *BiP3*. Importantly, no other chaperones were specifically induced in the *AtBAG7* knockouts. This finding may indicate that the other chaperones are not involved with *AtBAG7* or that their regulation does not occur at the transcriptional level. *AtBAG7* transcript levels also were not altered upon heat and cold treatment, thus suggesting that *AtBAG7* is regulated at the posttranscriptional level. These observations are in accordance with our bioinformatic studies that failed to identify ER stress elements in the *AtBAG7* promoter. The increased level of *BiP3* in *AtBAG7* knockouts raises the possibility that removal of *AtBAG7* from the ER activates a transcription factor required for *BiP3* expression. This possibility is consistent with studies involving bZIP60, a transcription factor that regulates *BiP3* expression but makes only minor contributions to *BiP1/2* (31). Our results show that *BAG7* may be required for the optimal regulation of *BiP3* under heat and cold stress.

The ER has been associated with biotic responses, and previous studies have shown that *AtBAG6*, another BAG that contains a calmodulin-binding domain, may be involved in basal defense (5). Therefore we assessed whether *AtBAG7* also is involved in biotic stress responses. *AtBAG7* knockouts were challenged with the bacterial pathogen *Pseudomonas syringae* DC3000, and the fungal necrotrophs *Botrytis cinerea*, *Sclerotinia sclerotiorum*, and *Alternaria alternata*. In contrast to *AtBAG6*, no significant differences were observed between the *AtBAG7* knockouts and the wild-type controls. This finding indicates that, unlike *AtBAG6*, *AtBAG7* is involved primarily within abiotic stress pathways.

Plants must adapt continually to the changing environment. An increase in freezing tolerance known as "cold acclimation" typically is observed after plants are exposed to low nonfreezing temperatures. In this study we subjected plants to severe cold without prior acclimation. During freezing temperatures, cell death occurs because of irreversible damage to cellular membranes, particularly during the thawing process. To circumvent this damage, plant membranes, particularly the plasma membrane, undergo structural changes including the accumulation of mono- or diunsaturated species of phosphatidylcholine which increase cryostability (44, 45). Plasma membrane proteins may play an important role during cold acclimation (46–48). A recent study by Kawamura and Uemura (33) identified substantial changes in the protein content for range of plasma membrane proteins during the early stages of cold acclimation. These changes included increased levels of the gene product of locus At5g62390 which we identified as *AtBAG7*. In light of these findings, future studies involving the translocation of *AtBAG7* during cold acclimation are of interest.

In summary, this paper details the investigation of an *Arabidopsis* BAG protein, *AtBAG7*. In contrast to the mammalian BAGs, the *Arabidopsis* BAGs appear to have diverse specificities and are

localized to a variety of subcellular organelles for a range of cellular functions. Here we show that *AtBAG7* is an ER-localized chaperone that is involved in the maintenance of ER stress pathways, a unique role for BAG proteins. Specifically, *AtBAG7* is integral for the survival of heat- and cold-stressed cells by delaying PCD pathways of cells undergoing ER stress. This finding is consistent with our previous data that linked *AtBAG4* with inhibition of PCD, including DNA laddering and TUNEL-positive nuclei, during cold stress. Taken together, these results further demonstrate the importance of the BAG family in plant cell-death pathways and cytoprotection.

Materials and Methods

Materials and Growth Conditions. *Arabidopsis* transfer-DNA insertion mutants of *BAG7* (Salk_065883, 058247) and *BiP* (Salk_073202) in the col-1 background were acquired from the *Arabidopsis* Biological Resource Center (ABRC). Unless stated otherwise, all plants and cells were grown at 22 °C using a 16-h photoperiod. Plasmids comprising RFP localized to the ER (ER-rk CD3-959), the YFP-fusion construct pEarleygate104 (CD3-686), and BiFC constructs pSAT4A-DEST-n (1-174)EYFP-N1 (CD3-1080) and pSAT5A-DEST-c(175-end)EYFP-N1 (CD3-1096) also were acquired from the ABRC.

Microprojectile Bombardment of Onion Cells. Onion inner epidermal peels were plated onto MS medium without vitamins [MS salts, 2% (wt/vol) sucrose, 2% agar] no more than 1 h before bombardment. Gold particles (1 μm) (Biorad) were coated with the respective plasmid DNAs in an equal molar ratio and were introduced transiently into epidermal cells by microprojectile bombardment using a Bio-Rad PDS/1000 helium-driven particle accelerator. Bombardment parameters were essentially as outlined in ref. 49 and included the following: 1,100 psi bombardment pressure, helium vacuum of 27 in. Hg, and a distance of 12 cm from the microcarriers to the sample. All samples were incubated for ∼24–48 h under dark conditions at 22 °C before microscopy analysis.

BiFC Analysis. The *AtBAG7*-containing fragment was subcloned using LR Clonase II (Invitrogen) from pENTRBAG7 into the destination vector pSAT5A-DEST-c(175-end)EYFP-N1 to generate pBAG7097. Simultaneously, the *AtBiP2*-containing fragment was cloned from pENTRBiP2 into pSAT4A-DEST-n(1-174)EYFP-N1 to generate pBiP2089. Once generated, pBAG7096 and pBiP2080 were cobombarded into onion epidermal cells. Three days after bombardment, fluorescent cells were viewed by confocal microscopy as described above using the appropriate GFP filter (excitation 474 nm /emission 510 nm).

Abiotic Stress Assays. Wild-type, *AtBAG7*-knockout (Salk_065883 and 058247), and *AtBiP2*-knockout (Salk_073202) seeds were surface sterilized, vernalized at 4 °C for 48 h, germinated on 1/2 MS containing 2% sucrose, and grown for 5 days. All plantlets were subjected to either heat stress (50 °C for 30 min) or cold stress (−20 °C/90 min) and allowed to recover for 4 days before phenotypic analysis. Each treatment was performed in duplicate, and each experiment was repeated twice.

Chemical Chaperone Protection Assay. Wild-type, *AtBAG*-knockout, and *AtBiP2*-knockout seeds were germinated and grown for 5 days as described above. One day before heat treatment (50 °C for 30 min), five plantlets of each genotype were transferred to fresh MS medium or to MS medium containing 0.5 mM TUDCA. After treatment plants were allowed to recover for 4 days before phenotypic analysis.

Tunicamycin Assays. Wild-type and *Arabidopsis BAG7*-knockout seeds were prepared and grown as described in the abiotic stress assays. One week after germination, five plantlets of each genotype were submerged in 1/2 MS liquid containing, 0.5 μg mL^{-1} Tm for 6 h, washed twice in 1/2 MS containing no antibiotic, and grown for a further 5 days on 1/2 MS agar. Control (MS only) plants and Tm-treated plants were analyzed for phenotypic differences and photographed.

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