Identification and Functional Characterization of the BAG Protein Family in Arabidopsis thaliana

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The genes that control mammalian programmed cell death are conserved across wide evolutionary distances. Although plant cells can undergo apoptosis-like cell death, plant homologs of mammalian regulators of apoptosis have, in general, not been found. This is in part due to the lack of primary sequence conservation between animal and putative plant regulators of apoptosis. Thus, alternative approaches beyond sequence similarities are required to find functional plant homologs of apoptosis regulators. Here, we present the results of using advanced bioinformatic tools to uncover the Arabidopsis family of BAG proteins. The mammalian BAG (Bcl-2-associated athanogene) proteins are a family of chaperone regulators that modulate a number of diverse processes ranging from proliferation to growth arrest and cell death. Such proteins are distinguished by a conserved BAG domain that directly interacts with Hsp70 and Hsc70 proteins to regulate their activity. Our searches of the Arabidopsis thaliana genome sequence revealed seven homologs of the BAG protein family. We further show that plant BAG family members are also multifunctional and remarkably similar to their animal counterparts, as they regulate apoptosis-like processes ranging from pathogen attack to abiotic stress and development.

Programmed cell death (PCD) plays an indispensable role in development and physiology, but it is unclear whether the mechanisms governing PCD in both plants and animals are similar. The genes that control programmed cell death are conserved across wide evolutionary distances from Caenorhabditis elegans to humans (1), although whether such conservation extends to plants is unknown. PCD plays a normal physiological role in many plant processes, and although the biochemical mechanisms responsible for cell suicide in plants are largely unknown, a number of reports suggest similarities to animal PCD (reviewed in Ref. 2). Moreover, ectopic expression of certain animal anti-apoptotic genes in transgenic plants is known to confer protection from pathogen attack and other stresses by death suppression (3–5).

The identification of homologs of animal cell death regulators is of considerable interest. However, to date, few endogenous plant genes that show significant sequence similarity to animal apoptotic genes have been identified. Examination of the completed genome sequence of Arabidopsis thaliana, as well as other partially or nearly complete plant genomes by tools such as BLAST and FASTA, has not revealed any apparent homologs to the core apoptosis regulators. This may be explained by high sequence divergence of functional plant homologs of animal apoptotic proteins. Therefore, one approach to identify candidate PCD modulators in plants is through advanced tools of bioinformatics. We describe the results of such efforts, viz. the identification and characterization of the BAG protein family of Arabidopsis by profile-sequence (Pfam) and profile-profile (FFAS) algorithms.

The BAG proteins are an evolutionarily conserved family of multifunctional proteins that promote cell survival and are distinguished by a conserved BAG domain that directly interacts with Hsp70 and Hsc70 interaction domain (BD). The first BAG gene was discovered in a search for Bcl-2-interacting proteins and was shown to synergistically enhance cell survival with Bcl-2 (6). These proteins share a common Hsp70/Hsc70 interaction domain (BD), but generally differ in the N-terminal region, which imparts specificity to particular proteins and pathways. Two of the six human BAG proteins (BAG1 and BAG6) contain a ubiquitin-like sequence at the N terminus. This domain is probably functionally relevant, as it is conserved in BAG proteins from yeast, plants, and worms. In addition, BAG1 is associated with the proteasome in HeLa cells and thus may serve as a bridge between Hsp70 and the ubiquitin system (7).

Although BAG proteins are being extensively studied in animals, plant homologs have been noted only in comparative sequence studies (10), and nothing is known about the function of BAG-like proteins in plants or to what extent functional similarities exist between the plant and animal families. Our searches of the A. thaliana genome sequence revealed seven homologs of the BAG family, including four with domain organization similar to their animal homologs. Three members contain a calmodulin-binding domain near the BD, a novel feature associated with plant BAG proteins reflecting possible divergent mechanisms involved with plant-specific PCD. Herein, we describe genome organization, molecular phylogeny, and comparative genomics that provide a basis for further functional studies of this multigene family. We further show that plant BAG family members are also multifunctional and regulate processes from pathogen attack to abiotic stress and development.

EXPERIMENTAL PROCEDURES

Computational Methods—Six BD-containing proteins of the A. thaliana genome were found in Pfam and SMART BAG families (accession numbers PF02179 and SM00264, respectively) created via hidden Markov model (HMM)-based data base searches. A. thaliana (At)
BAG7 was identified as a potential match to the BAG protein family. Several criteria such as conservation of the critical amino acids in the BD, similarities in electrostatic potential, and hydrophobic residue distribution on the molecular surface were considered in accepting the putative AtBAG7 member. Representative members of the assembled Arabidopsis BAG family were used to scan expressed sequence tag (EST) data banks of NCBI with the TBLASTN program. The homology models were generated by the SWISS-MODEL web server using the human BAG4 BD solution NMR structure as a template. Each model was generated based on multiple alignments of AtBAG1–7 and human BAG4 BDs. The models were processed by the DeepView/Swiss-PdbViewer program. All BAG proteins were analyzed for the presence of the additional BDs and organellar target sequences using the Pfam and PSORT programs, respectively.

**Plant Assays**—Treated and untreated detached tobacco leaves were chopped with a razor blade into 0.5-cm fragments and stored overnight in ice-cold 70% ethanol, followed by standard paraffin embedding and sectioning procedures. Slides were prepared for TUNEL assays as described (30). Transgenic and wild-type tobacco plants were grown at 25 °C with 16-h light periods in a greenhouse. Knock-out and wild-type tobacco plants were grown at 25 °C with 16-h light periods and 50% humidity. Sur-face-sterilized plants were grown at 23 °C with 16-h light periods and 50% humidity. Surface-sterilized Arabidopsis seeds were plated on one-half Murashige and Skoog medium and grown for 2 weeks at 23 °C with a 16-h light period and 70% humidity. Six-week-old tobacco and 4–5-week-old Arabidopsis plants and detached leaves were used for fungal inoculation. Five-mm-diameter agar plugs containing actively grown hyphal tips from 5-day-old colonies of Botrytis cinerea (ATCC 11542) grown on potato dextrose agar were used for plant inoculation. Inoculated leaves were placed on moistened sterile filter paper in glass Petri dishes and incubated for 2–10 days at 25 °C (tobacco leaves) or 23 °C (Arabidopsis leaves) under high humidity conditions. Inoculated plants were placed in a mist chamber at high humidity at 22–25 °C. To induce cell death by cold treatment, wild-type and low atbag4-expressing tobacco plants were stressed at −20 °C for 10 min. Two hours later, tissue was prepared and evaluated by TUNEL assay as described previously (30).

All experiments were repeated at least three times with a minimum of three independent lines. The following T-DNA insertion lines were used: AtBAG4, SALK_033845, SAIL_418_B09, and SAIL_144_A10; and AtBAG6, SALK_009534 and SALK_058290. T-DNA insertions were confirmed by PCR and sequencing the products.

### RESULTS

**Identification of the Arabidopsis bag Gene Family**—Low sequence identities (13–25%) and similarities (32–46%) between the BDs of Arabidopsis and animal BAG proteins explain the inability of BLAST searches to find animal BAG homologs in the Arabidopsis genome. Therefore, more sensitive methods such as HMM-based protein search tools and profile-profile alignment algorithms were used (11, 12). These methods allow the identification of homologs with large sequence divergence via building probabilistic models of the protein family and comparing the models with available sequences to find new family members. To identify Arabidopsis BD-containing proteins, we used HMM-based protein search tools (Pfam and SMART) and found seven BD-containing proteins in the A. thaliana genome. Sixteen seed alignments of 80% consensus were used by both the Pfam and SMART programs to build an HMM of the BAG protein family. The putative BAG proteins were annotated as “unknown” or “hypothetical” proteins in the data bases. We named this family the bag gene family and adopted the previously reported numbering system for Arabidopsis bag genes (32).

Upon application of this HMM to search the available A. thaliana genome sequence, we identified putative Arabidopsis BAG homologs with high statistical significance (E values are in the interval 2.7e-06 – 8.3e-26) except for AtBAG7 (which was classified as a potential match). Several criteria, including conservation of critical amino acids in the BD, similarities in electrostatic potential, and hydrophobic surface residue distribution, were considered in evaluating AtBAG7 as a member of the BAG protein family. Our additional bioinformatic studies (see below) revealed that this protein is most likely also a member of the Arabidopsis BAG family. The bag gene products were assigned to distinct subcellular locations (Table 1) based on the predictions of the PSORT program (13). The BD amino acid sequences of the putative Arabidopsis BAG proteins share similarities of between 50 and 90%. However, the BDs of several protein pairs within the Arabidopsis BAG family share no easily recognizable sequence similarity, suggesting that at least two subgroups of Arabidopsis BAG proteins emerged from early duplication, followed by divergent or independent evolution.

To examine the relationships of Arabidopsis BAG proteins, a tree was built based on multiple alignment of the full-length BAG protein sequences (Fig. 1A). According to sequence similarity, the Arabidopsis BAG proteins cluster into two groups. Group I is represented by AtBAG1–4, which are predicted to localize in the cytoplasm (except AtBAG2, the predicted localization of which is the microbody); and as
with some mammalian BAG proteins, ubiquitin-like domains are located in the N terminus. Thus, the BAG proteins of Group I are similar to animal counterparts. The Arabidopsis BAG proteins that are predicted to localize in the nucleus (AtBAG6 and AtBAG7) compose Group II. AtBAG5 does not belong to either group.

A phylogenetic tree of the BAG protein family was constructed (Fig. 1B). The first major branch splits the predicted cytosolic Arabidopsis BAG protein family members from the organelar members. Notably, the Arabidopsis cytosolic members compose the plant origin cluster, including putative plant BAG proteins from Oryza sativa and Cicer arietinum. The nuclear members (AtBAG6 and AtBAG7) are in a separate cluster from the putative mitochondrial member AtBAG5. Interestingly, AtBAG6 and AtBAG3 cluster with the BD-containing protein Samui from Bombyx mori, which is induced in response to cold (14).

**Gene and Domain Organization—Arabidopsis BAG genes encoding proteins predicted to localize in the same cellular compartment tend to have similar exon-intron organization (supplemental Fig. S1). The common feature of each Arabidopsis BAG proteins is the presence of the BD in the C terminus (Fig. 1C). As in several mammalian BAG proteins, four of the predicted plant BAG family members possess ubiquitin domains in the N terminus, whereas three plant BAG proteins (AtBAG5–7) contain a calmodulin-binding motif near the BD, and are predicted to localize in the same subcellular compartment.**

*FIGURE 1. A, phylogenetic tree of A. thaliana BAG proteins. The full-length protein sequences were aligned using the ClustalW multiple sequence alignment program (Version 1.83). The tree was built using the PHYLIP package (parsimony method). The lengths of branches are proportional to evolutionary distances between species. B, phylogenetic tree of Arabidopsis BAG family proteins. The tree was built using the PHYLIP package (parsimony method). The Arabidopsis BAG family members are clustered in separate regions of the tree as indicated by different colors. AtBAG5 is not clustered with any other proteins and supposedly is of more ancient origin. The predicted subcellular localization of Arabidopsis BAG proteins is also shown. The GI numbers of the protein sequences analyzed are as follows: A. thaliana proteins AtBAG1 (GI:18423349), AtBAG2 (GI:10176928), AtBAG3 (GI:21537107), AtBAG4 (GI:3068705), AtBAG5 (GI:3157923), AtBAG6 (GI:3702325), and AtBAG7 (GI:15241803); O. sativa (Osat) rice proteins Q9SC76 (GI:6624711), Q9LW5S (GI:6983875), and Q8S234 (GI:3049393); the C. arietinum (Cicer; chickpea) BAG protein (GI:18034497); the Saccharomyces cerevisiae (yeast) Snl1p protein (GI:6322173); Schizosaccharomyces pombe (fission yeast) proteins 1B (GI:7491690) and 1A (GI:19113552); C. elegans (C. elegans) proteins CPH1 (GI:11359735) and UNCC2 (GI:168927); the B. mori protein Samui (GI:13442776); Drosophila melanogaster proteins CG2130-PA (GI:24663861), CG2130-PC (GI:24663866), and CG32130-PC (GI:24663865); Homo sapiens (Hsap) proteins BAG1 (GI:17384436), BAG1L (GI:12831857), Bag2 (GI:12831857), Bag5H (GI:12831857), RIKEN (GI:14318608), and SimtoBAG2 (GI:16740710); and the Rhodospirillum rubrum (R. rubrum) protein (AAC45124; outgroup). C, domain structure of the Arabidopsis BAG proteins. The positions of the BD (red), ubiquitin-like domain (yellow), calmodulin-binding motif (green), and nuclear localization sequences (blue) in Arabidopsis BAG family members are shown. A similar domain organization for proteins that are predicted to localize in the same subcellular compartment was found.
FIGURE 2. A, crystal structure of the human BAG1M-Hsc70 complex (data taken from the Protein Data Bank) (15). The BD is shown in yellow, and the Hsc70 ATPase domain is shown in green. B, a ribbon representation of the AtBAG4 BD homology model and the human BAG4 BD solution NMR structure (Protein Data Bank code 1M7K). The α helices shown as yellow ribbons compose a short antiparallel triple-helix bundle in both human and Arabidopsis BDs. The molecules are oriented so that second and third helices (the Hsc70-binding surface) are facing up front; residues of the human BAG4 BD that interact with the N- and C-terminal lobes and the corresponding residues in the AtBAG4 BD are colored red and blue, respectively. C, secondary structure-based sequence alignment of BDs from AtBAG4 and human BAG4 and BAG1. D, alignment of BD amino acid sequences from seven Arabidopsis BAG Family members.

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Multiple alignment of BD sequences from A. thaliana BAG proteins and human BAG4 and BAG1 revealed that most residues of mammalian BAG proteins that are required for Hsc70 interaction are conserved in Arabidopsis BAG proteins (Fig. 2D). The structure of the human BAG1–Hsc70 complex is shown in Fig. 2A. The crystal structure reveals that Glu211, Asp222, Arg237, and Gln245 in human BAG1 participate in an electrostatic interaction with the ATPase domain of Hsc70 (15). These key interaction residues are conserved and, in rare cases, are conservatively replaced in the BAG proteins of Arabidopsis with the exception that Gln245 is not found in the BDs of AtBAG2, AtBAG5, and AtBAG6 (Fig. 2D). However, based on the overall similarity of the plant BD interaction surfaces to equivalent mammalian BAG protein BD surfaces, it is reasonable to assume that Arabidopsis BAG proteins bind Hsc70 in a manner analogous to mammalian BAG proteins.

The plant BAG/Hsc70 interaction was tested experimentally by pull-down assays using AtBAG4 and Arabidopsis Hsc70. In vitro translated 35S-labeled AtBAG4 was examined for its ability to bind glutathione S-transferase-Hsc70 fusion protein produced in Escherichia coli. AtBAG4 bound to glutathione S-transferase-Hsc70, but not to the glutathione-Sepharose beads (supplemental Fig. S2). Hence, AtBAG4 is a structural and possible functional homolog of the BAG protein family.

Comparison of the BDs of Arabidopsis BAG Proteins and Human BAG4—Similar to mammalian BAG proteins, AtBAG4 binds to Hsc70. Therefore, we compared the surface of the reported BD solution NMR structure of human BAG4 with a three-dimensional model of the BD of AtBAG4. The three-dimensional structure of the human BAG4 BD was chosen as a template for homology modeling because, of all available Protein Data Bank three-dimensional BD structures, the human BAG4 BD shows the highest identity/similarity (21.2%/42.4%) to the AtBAG4 BD. In this way, we found that the Hsc70-binding surfaces (a2 and a3 helices) of the BDs from AtBAG4 and human BAG4 are very similar in charge distribution (Fig. 2E). Similar to the BD of human BAG4, the BD of AtBAG4 (acidic residues Asp175 and Glu179 from the a2 helix; Asp189, Glu192, and Glu194 from the connecting loop; and Glu213 and Asp216 from the a3 helix) contributes to the negative charge of the Hsc70-interacting surface. Basic residues (Lys198, Arg200, Lys202, and Lys209) in the N terminus of the a3 helix of the AtBAG4 BD contribute to the positive charge and are clustered in the upper left corner of the surface. The positions of the residues that are important for Hsc70 interaction in the AtBAG4 BD homology model are also remarkably similar to those in the three-dimensional structure reported for the human BAG4 BD (Fig. 2B). The Hsc70-interacting surface similarity of the AtBAG4 and human BAG4 BDs is consistent with AtBAG4 binding to Hsc70. To confirm binding of AtBAG4 to Hsc70, two site-directed BD mutants (deletion and point mutants) were generated. The results of in vitro pull-down experiments show that binding of AtBAG4 to Hsc70 was abolished in these mutants, indicating that Arabidopsis BAG4 BD is required for interaction with Hsc70, similar to human BAG proteins (supplemental Fig. S2).

The central hydrophobic region composed of a2 helix residues (Leu185, Leu186, and Leu188) and a3 helix residues (Val205 and Ile208) of the BD surface of AtBAG4 is very similar to that of human BAG4 (Fig. 2E). However, the a2 and a3 helix surface of the AtBAG4 BD appears to be more hydrophobic than that of the human BAG4 BD. Thus, despite distant homology between the BDs of AtBAG4 and human BAG4 (21.2% identity), a prediction for AtBAG4/Hsc70 interaction is consistent with the experimental data. This can be explained by the higher degree of BD similarity in the a2 and a3 helices, the analysis of which was used for the AtBAG4/Hsc70 interaction prediction. We therefore generated homology models for all A. thaliana BAG BDs as described above and compared their a2 and a3 helix surface residues with those of the AtBAG4 and human BAG4 BDs (Fig. 3). High similarities with respect to both charge and hydrophobic residue distributions were found between the a2 and a3 helix residues of the AtBAG4 protein and those of the AtBAG1–3, AtBAG6, and AtBAG7 proteins. All these proteins share acidic residues and a basic cluster in the domain as well as hydrophobic residues in the a2 and a3 helices (Fig. 3, first and third rows). On the basis of this analysis, it is likely that AtBAG1–3, AtBAG6, and AtBAG7 bind Hsc70 in a manner similar to AtBAG4. Interestingly, the Hsc70-interacting surface of AtBAG5 was found to be quite different from those of the other BAG family members with respect to charge distribution. The a2 and a3 helix surface of the AtBAG5 BD is more positively charged, with an acidic cluster in the left bottom corner. However, with respect to hydrophobic residue distribution, the Hsc70-interacting surface of the AtBAG5 BD is very similar to that of the human BAG4 BD. Thus, it is difficult to predict AtBAG5 interaction with Hsc70 based on comparative models.

Expression Patterns of atbag4 and atbag6—A representative of each phylogenetic group (atbag4 and atbag6) was chosen to assess expres-
Arabidopsis BAG Family

ission patterns in various tissues during development and under stress conditions. Reverse transcription-PCR indicated that varying levels of atbag4 transcripts were detected in stems, leaves, flowers, and roots, with expression in roots and flowers being higher than in other tissues (supplemental Fig. S3). Similar expression patterns were detected for atbag6 transcripts, except that atbag6 transcript expression was not detectable in leaves. We also analyzed transcript abundance of atbag4 and atbag6 in Arabidopsis leaf tissue prior to and during flowering. Both genes had much higher levels of expression in leaf tissue at younger, actively growing stages of development (supplemental Fig. S3).

The clustering of AtBAG6 with the cold-induced protein Samui from B. mori as revealed by our phylogenetic analysis of BAG proteins suggested a possible role for AtBAG6 in cold responses. Thus, atbag6 induction was analyzed in the leaf tissue of Arabidopsis plants at 0, 20, and 90 min after exposure to −20 °C for 10 min (supplemental Fig. S3). In parallel, we analyzed the induction kinetics of atbag4. atbag4 induction was detected 20 min after cold stress. In contrast, atbag6 transcripts were not observed until 90 min after cold treatment, indicating that atbag3 is a more rapidly induced cold-responsive gene compared with atbag6.

Because BAG proteins interact with Hsp70/Hsc70, bag gene responsiveness to heat shock was of interest. We analyzed induction of the atbag4 and atbag6 genes in Arabidopsis leaf tissue over a time course of 30-min intervals following exposure to 37 °C (supplemental Fig. S3). In these experiments, heat treatment strongly induced expression of atbag6 at all chosen time points, with a peak at 60 min of treatment, whereas no induction was detected for atbag4 under the same conditions. Thus, expression patterns during heat stress are distinct for these two genes.

Phenotypes of the atbag4 and atbag6 Knock-out Lines—Transgenic lines with T-DNA insertions in the exons of atbag4 and atbag6 were obtained from the Arabidopsis Biological Resource Center and the Syngenta Arabidopsis Insertion Library. Knock-out plants were grown under long-day conditions (16 h of light/8 h of dark). All heterozygous knock-out lines exhibited earlier flowering and shorter vegetative and reproductive phases, producing more branched roots and inflorescences compared with wild-type plants (Fig. 4A). These plants senesced earlier than the wild-type plants, indicating that BAG proteins influence plant growth and development.

AtBAG4-overexpressing Transgenic Tobacco Plants Are Stress-tolerant—AtBAG4 cDNA was overexpressed in tobacco plants under the control of the cauliflower mosaic virus 35S promoter. Seventeen independent transgenic plant lines were generated. T2 generations of atbag4 expression were tested by reverse transcription-PCR (data not shown). atbag4 expression was not detected in wild-type tobacco. Thus, tobacco plants either lack atbag4 or have diverged in sequence. We selected representative transgenic lines expressing atbag4 at high, intermediate, or low levels for additional experiments.

UV irradiation induced chlorosis in wild-type leaf discs of control plants 48 h after exposure to UVB light (32 kJ/m²). The leaf discs of the high level atbag4-expressing lines were similar to those of control plants. However, the leaf discs of the low level atbag4-expressing lines remained green during the 1-week time course, whereas those of the intermediate level atbag4-expressing lines exhibited chlorosis 1 week after recovery (supplemental Fig. S4). Therefore, the level of UVB resistance was inversely proportional to the level of atbag4 expression. Because the high level atbag4-expressing plants responded similarly to the wild-type phenotype in this and subsequent experiments, this line was not used further. UV resistance was also detected in intact transgenic plants of the low level atbag4-expressing expression lines. Two days after irradiation (10.7 kJ/m²), severe tissue damage was observed in the wild-type tobacco seedlings, and these seedlings died within 2 weeks. In contrast, low level atbag4-expressing seedlings wilted and were slightly damaged 2 days after irradiation, but completely recovered with new normal growth and leaf development (Fig. 4B).

We then tested the overexpressing tobacco lines for response to the following oxidants: menadione, paraquat, acifluorfen, and H2O2. When detached leaves of 8-week-old wild-type plants were treated with 5 mM menadione, chlorosis was observed 2 weeks after treatment, followed by rapid leaf death. In the intermediate and (more obviously) low level atbag4-expressing lines, inhibition of chlorosis was observed compared with wild-type plants (supplemental Fig. S5). Through the entire experimental time period (>2 weeks), the leaves of the low level atbag4-expressing lines remained turgid and green after menadione treatment. When the leaf discs of the low and intermediate level atbag4-expressing lines were floated on 10 μM paraquat solution for 48 h, the leaf tissue appeared normal and unaffected, whereas the leaf discs of wild-type plants became chlorotic and died (supplemental Fig. S6). Similar results were obtained with H2O2. 25 μM acifluorfen caused lesions on the upper part of leaves after 24 h of treatment, whereas the leaves of the low level atbag4-expressing lines remained intact with no visible damage (Fig. 4C).

Because cold enhances the expression levels of atbag4 and because several bag gene ESTs derived from cold- and drought-treated plants were identified (see below), atbag4-expressing plants were tested for freezing and drought tolerance. Following cold stress (−20 °C for 10 min), patches of dead tissue appeared on wild-type leaves 10 h after treatment. The leaves of the low level atbag4-expressing lines remained intact. The total chlorophyll content of transgenic plant leaves was ~2-fold higher than that of wild-type leaves 24 h after cold stress (sup-
Enhanced Susceptibility to B. cinerea in AtBAG6 Mutants—As described below, we identified three putative plant BD EST’s induced by pathogen stress, suggesting that BAG proteins may function in response to pathogen attack. To determine whether AtBAG4 and AtBAG6 affect plant responses to pathogen challenge, we analyzed searches, we found three additional proteins from Arabidopsis BAG Family

C. arietinum beet ESTs from abiosis-stressed seedling mRNAs, and one grape EST from drought-stressed leaf mRNAs, two sugar beet ESTs from cold-stressed seedling shoot mRNAs, two sorghum ESTs from water-stressed plant mRNAs (supplemental Table S1). Moreover, two barley ESTs are derived from cold-stressed Arabidopsis, 2-week-old wild-type and atbag4 knock-out plants were transferred to Murashige and Skoog agar medium with 100 mM NaCl. atbag4 knock-out plants were severely damaged 3 weeks after transfer and eventually died within 6 weeks (Fig. 5A). In contrast, wild-type plants tolerated the salt stress and grew and developed normally. Thus, these observations are consistent with the data from tobacco AtBAG4-overexpressing mutants and indicate a role for AtBAG4 in resistance/tolerance to salt stress.

Cold Resistance and Apoptosis—To determine whether the cell death observed in tobacco plants shares features associated with mammalian apoptosis and whether AtBAG4 protects tobacco plants by inhibiting a programmed cell death, cold-stressed leaf cells of wild-type and low level atbag4-expressing plants were analyzed for markers of apoptosis. Genomic DNA was isolated from tobacco leaves after cold stress and assayed for the presence of characteristic oligonucleosome-sized fragments by agarose gel electrophoresis and DNA fragmentation using the TUNEL reaction. Chromatin condensation was visualized by propidium iodide staining by fluorescence microscopy.

DNA isolated from wild-type leaves 2 h after cold stress was fragmented and formed characteristic apoptosis-like DNA ladders (supplemental Fig. S8). Similar leaf samples 2 h after cold treatment were fixed and sectioned for the TUNEL reaction. TUNEL-positive nuclei were observed in wild-type leaf cells (Fig. 5C). Additionally, the appearance of apoptosis-like bodies was observed in wild-type cells. Thus, before visible tissue injury occurred, cold stress induced apoptosis-like cell death in wild-type tobacco leaves as evidenced by the presence of fragmented DNA and TUNEL-positive cells. In contrast, DNA isolated from low level atbag4-expressing tobacco leaves did not exhibit any laddering at the same time period or even at later time points (data not shown). TUNEL-positive nuclei and apoptosis-like structures were also absent in cold-resistant low level atbag4-expressing cells (Fig. 5C), and such tissue also remained viable. Therefore, low levels of atbag4 expression confer resistance to apoptosis-like death imposed by cold stress.

BAG-like Genes Are Widely Distributed in Plants—We searched EST data banks for Arabidopsis bag sequences. Eight tissue-specific ESTs from A. thaliana (root, flower, inflorescence) and one water-stressed EST were identified (summarized in Table 2). In the course of the Arabidopsis BAG searches, we found three additional proteins from O. sativa and one protein from C. arietinum assigned to the BAG family by Pfam and SMART tools. In parallel, we found that several ESTs from various plants show 43–79% amino acid homology to the BDs of Arabidopsis BAG proteins (supplemental Table S1). Moreover, two barley ESTs are derived from cold-stressed seedling shoot mRNAs, two sorghum ESTs from water-stressed plant mRNAs, one soybean EST from drought-stressed leaf mRNAs, two sugar beet ESTs from abiosis-stressed seedling mRNAs, and one grape EST from abiosis-stressed berry mRNAs (supplemental Table S1). Therefore, induction by abiotic stresses such as cold, drought, high salt, and oxidative stress appears to be a common feature associated with bag genes. Three ESTs from barley, grape, and legume (supplemental Table S1) expressed in response to biotic stress were also found, suggesting bag gene induction
**Arabidopsis BAG Family**

### Table 2

| Gene and EST accession no. | Library description                          |
|---------------------------|----------------------------------------------|
| atbag1                    |                                                                 |
| A0959956                  | Inflorescence tissue                         |
| AV556585                  | Green siliques                               |
| atbag2                    |                                                                 |
| AU228495                  | Dark-grown plant                             |
| AU237430                  | Dark-grown plant                             |
| AV567680                  | Green siliques                               |
| atbag3                    |                                                                 |
| A094398                   | Whole plant                                  |
| T76428                    | Mixed tissues                                |
| T44437                    | Mixed tissues                                |
| T41725                    | Mixed tissues                                |
| T76731                    | Mixed tissues                                |
| T22997                    | Mixed tissues                                |
| T13965                    | Mixed tissues                                |
| AV784569                  | Dehydration-treated plant                    |
| atbag4                    |                                                                 |
| N65910                    | Mixed tissues                                |
| AV440831                  | Above-ground organ                           |
| AV442804                  | Above-ground organ                           |
| AV518795                  | Above-ground organ                           |
| AV534399                  | Flower buds                                  |
| atbag5                    |                                                                 |
| atbag6                    |                                                                 |
| AV543670                  | Roots                                        |
| AV546796                  | Roots                                        |
| atbag7                    |                                                                 |
| R90357                    | Mixed tissues                                |
| H76671                    | Mixed tissues                                |
| T49897                    | Mixed tissues                                |
| AV538293                  | Roots                                        |
| AV551882                  | Roots                                        |
| AV790264                  | Plants at various developmental stages from   |
|                           | germination to mature seeds                  |

upon pathogen challenge. Supplemental Table S1 also shows a number of plant ESTs associated with development. These findings indicate that BAG proteins are widely distributed through plant genomes and that their expression is likely to be regulated in response to environmental stimuli.

**DISCUSSION**

The mammalian BAG family proteins participate in several cellular processes, including apoptosis, proliferation, differentiation, and stress signaling (e.g. Refs. 16 and 17). Remarkably, the functional versatility of this family appears to be maintained in the model plant *A. thaliana* and quite possibly in other plants as well. Using HHMs and profile-profile alignments, we have identified seven bag genes in *Arabidopsis*. Because animal pro- and anti-apoptotic homologs in plants appear to have highly diverged, general tools such as BLAST may not be particularly useful for identifying functional but distantly related proteins. Applying more sensitive protein search tools such as Pfam, SMART, and FFAS, we were able to identify multiple animal BAG homologs in plants, which have low sequence identities (13–25%) and cannot be reliably predicted by BLAST searches. The identification of cell death modulators with extreme levels of sequence divergence is not unprecedented. Although several human Bcl-2 family proteins such as Bcl-2 and BID are known to share striking three-dimensional structural similarity (18), the primary amino acid sequences of these proteins lack any apparent similarity. Thus, diverse primary amino acid sequences cannot preclude a common function for cell death regulation, perhaps explaining the inability to detect Bcl-2 homologs in plant genomes based on standard sequence comparisons.

Analysis of the anti-apoptotic genes of animal viruses has also demonstrated that cytoprotective proteins can be identified in viral genomes that share very little primary amino acid sequence similarity with their cellular counterparts, but that nevertheless serve the same function. For example, the E1b-19kD protein of adenovirus is a functional analog of the Bcl-2 family of proteins (19). E1b-19kD binds to and neutralizes the activity of pro-apoptotic Bcl-2 family proteins such as Bax and can substitute for Bcl-2 in multiple in vitro models of cell death (20). This protein has <15% amino acid sequence identity to Bcl-2. Whether this is an example of extreme sequence divergence between distant homologs or an example of functional convergence between non-homologous proteins is presently unclear. By analogy, we hypothesize that plant genomes may have functional equivalents of cytoprotective Bcl-2 family proteins that share little or no sequence similarity with animal counterparts. The generation of sensitive protein search tools such as HMM-based Pfam and SMART and profile-profile alignment algorithms applies probabilistic models or profiles of the entire protein family to find sequentially divergent but still homologous proteins (11, 12). In this study, we have demonstrated the utility and power of these search tools for the identification of diverged BAG family members in the *A. thaliana* genome, which is further supported by experimental data.

The identification of plant homologs of animal cell death modulators such as Bax inhibitor-1 (21, 22) indicates a degree of conservation of cell death regulators between plants and animals, although it is important to note that none of the core machinery members (e.g. caspases, Bcl-2 family) have been identified. Other similarities of animal and plant PCD include DNA fragmentation, cell shrinkage, chromatin condensation, cysteine protease activation, reactive oxygen species generation (e.g. Ref. 23), indicating the existence of common elements in both systems. Our identification and characterization of these new homologs of animal cell death modulators represent an important additional example of common players that mediate stress responses.

Of the seven *Arabidopsis* BAG proteins identified, three (AtBAG5–7) have unique features found only in plant BAG proteins. These *Arabidopsis* bag genes differ from animal BAG genes in that a predicted calmodulin-binding motif is present near the BD; and thus, these proteins may be regulated by calmodulin and possibly Ca\(^{2+}\). In support of this, we found that atbag5 and atbag6 (two of the genes encoding proteins containing a calmodulin-binding motif), but not atbag4, are selectively induced by Ca\(^{2+}\), but not other divalent cations (Supplemental Fig. S9C). However, two-hybrid analyses using calmodulin and AtBAG7 were unsuccessful, although it is possible that the necessary protein conformation required for binding did not occur.

The results presented here establish experimentally that AtBAG4 interacts with Hsc70, consistent with the mammalian BAG family. Comprehensive analysis of BDs from *Arabidopsis* BAG family members using homology modeling and charge and hydrophobic residue distributions revealed remarkable structural similarity between the BDs of animal BAG proteins and AtBAG1–4, AtBAG6, and AtBAG7. As the BAG/Hsc70 interaction is a noncovalent ionic interaction, it is plausible that AtBAG1–3, AtBAG6, and AtBAG7 bind Hsc70 in a manner similar to AtBAG4, although this possibility needs to be experimentally addressed before firm conclusions can be drawn. Because the AtBAG5 charge distribution of the Hsc70-interacting surface appears to be slightly different compared with human BAG4 and AtBAG4, it is difficult to make predictions regarding the AtBAG5 interaction with Hsc70 based on these comparisons. Furthermore, based on secondary structure alignment (Fig. 2C), all *Arabidopsis* BAG BDs appear to be the short BD version, found in animals and fungi, and thus presumably more ancient (24). Based on current data, the long BDs are limited to vertebrates and nematodes.

Understanding the function(s) of individual members of the *Arabidopsis* BAG family represents a significant challenge. Multiple...
T-DNA insertion mutants for atBag4 and atBag6 were used in this study and were consistent in their phenotypes, indicating that inactivation of a given bag gene is responsible. The atBag4 and atBag6 knock-out lines exhibited early flowering and a branched inflorescence phenotype, suggesting a role in plant development. When BAG expression was inhibited, senescence was hastened, consistent with the idea that BAG proteins function in cell survival. An additional phenotype associated with these knock-out lines was hypersensitivity to light (Fig. 4A).

The results of the B. cinerea inoculation assays indicate that AtBAG6, but not AtBAG4, has a role in limiting pathogen colonization and spread. In addition, atBag6 transcript levels were elevated after treatment with salicylic acid, supporting involvement in host defense mechanisms (supplemental Fig. S9B). We also observed strong induction of the atBag4 and atBag6 genes, as well as atBag5 and atBag7, in response to abscisic acid treatment (supplemental Fig. S9B), suggesting that abscisic acid may be important for Arabidopsis bag gene regulation. Furthermore, abscisic acid is known as a negative growth regulator controlling adaptive responses to environmental stresses such as drought, cold, and high salinity (25). Thus, Arabidopsis bag genes may coordinate stress-induced hormone signaling pathways.

The BAG protein family has been proposed to recruit molecular chaperones to target proteins and to modulate their functions by altering protein conformation under stress conditions (10). Therefore, BAG proteins may link signaling to molecular chaperones via their BDs and to the proteasome via the N-terminal ubiquitin domain found in several animal and plant BAG proteins. Thus, BAG proteins can link signaling to molecular chaperones as well as to the proteasome. We report here that overexpression of AtBAG4 in tobacco plants confers tolerance to a wide range of biotic stresses such as UV light, cold, oxidants, and salt treatments. These observations parallel animal studies in which overexpression of BAG1 provided resistance against a variety of external stresses, including heat shock, hypoxia, radiation, and chemotoxic drugs (26).

Curiously, only low constitutive levels and, in some cases, intermediate constitutive levels of AtBAG4 expression in tobacco plants correlated with resistance/tolerance to abiotic stresses. It was recently found that low concentrations of human BAG1 activate the refolding activity of Hsp70/Hsc70 under physiological conditions, whereas high concentrations of BAG1M inhibit the refolding activity of the chaperones (27). Thus, the concentration of a given BAG protein in the cell relative to the concentration of Hsp70 may be critical for optimal chaperone activity, reflecting the need for balanced access of various complementary co-chaperones that govern the ATP hydrolysis cycle of Hsp70 and that perform other functions (28). Under stress conditions, the refolding activity of Hsp70/Hsc70 is of particular importance for cell survival. We suggest that low concentrations of AtBAG4, functioning as a co-chaperone, may be necessary for the resistant/tolerant phenotype. Our experimental results are consistent with the proposed function of BAG family members to coordinate signals for cell growth and to induce cell survival pathways and/or to inhibit PCD pathways in response to stress.

To investigate the latter possibility, cold-stressed leaf tissues of wild-type and AtBAG4-overexpressing transgenic plants were examined for the presence/absence of TUNEL-positive nuclei and oligonucleosome DNA fragmentation. Our findings indicate that cold stress induces these apoptosis-like features subsequent to tissue demise. Notably, AtBAG4 overexpression ameliorates cold stress injury and prevents the onset and presence of PCD features. It will be of considerable interest to identify BAG-interacting partners and to characterize the responsible pathways involved in mediating stress tolerance.

The identification of plant genes that functionally resemble mammalian core regulators of cell death regulatory pathways has remained elusive, raising legitimate questions regarding the extent to which conserved cell death mechanisms occur in plants and animals. It is evident that, if such plant genes exist, they will need to be identified by functional screens and/or advanced bioinformatic approaches. We have used both functional screens (29, 30) and advanced bioinformatic tools to identify candidate plant genes that function in modulating plant cell death. The Arabidopsis bag gene family shows remarkable structural and functional similarity to its mammalian counterparts. Through a variety of methods, the plant bag genes clearly function in cell protection under stress and inhibit plant programmed cell death that shares features associated with apoptosis, supporting the idea that at least some mechanisms for programmed cell death regulation are conserved between plants and animals. Elucidating the similarities and identifying the differences of these processes in plants and animals represent a challenge that offers new solutions for therapeutic design against plant and human apoptosis-related diseases and stresses.

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