The BAG2 and BAG6 Genes are Involved in Multiple Abiotic Stress Tolerance in *Arabidopsis Thaliana*

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Abstract

Background: The BAG (Bcl-2 associated athanogene) family is a multi-functional group of proteins that perform functions ranges from apoptosis to environmental stress tolerance. The BAG proteins are found in yeast, plants and animals. In plants, especially in *Arabidopsis thaliana* (*Arabidopsis*), BAG proteins were found to play roles both in abiotic and biotic stresses tolerance including heat, cold, salt, drought, and pathogens. However, the function of *Arabidopsis* BAG2 remains largely unknown. Whereas BAG6 was found to play important roles in plants response to biotic stresses, it remains unknown whether BAG6 is involved in plants tolerance to abiotic stresses.

Results: In this study, we have characterized functions of the *Arabidopsis* BAG2 and BAG6 genes.

Promoter: GUS analysis results show that both BAG2 and BAG6 genes are expressed in various tissues in *Arabidopsis thaliana*. Expression of both BAG2 and BAG6 genes is induced by salt, mannitol, and heat stress treatments and by the stress-related hormones including ABA, ethylene, and SA, while BAG6 expression is additionally induced by JA. Germination of the *bag2* and *bag6* single and *bag2 bag6* double mutant seeds are less sensitive to ABA than that of the wild type (WT). Both *bag2* and *bag6* single mutant and *bag2 bag6* double mutant show more survival rate than WT in drought treatment but display less survival rate on 45 °C heat stress experiment. Consistently, transcription levels of stress-related genes such as *RD29A*, *RD29B* and *NCED3* are higher in the mutant than that in the WT. Furthermore, these mutants exhibit lower content of reactive oxygen species (ROS) after drought and ABA treatment but higher ROS accumulation after heat treatment than the WT.

Conclusion: These results suggest that BAG2 and BAG6 genes are negatively involved in drought stress but play a positive role in heat stress in *Arabidopsis*.

Background

The BAG (Bcl-2 associated athanogene) family is a group of multi-functional chaperone proteins that contain a conserved BAG domain at their C-terminal [1,2,3]. The BAG proteins are evolutionarily conserved with homologs present in yeast, plants and animals. They perform functions ranges from apoptosis to response to biotic and abiotic stresses. Seven BAG genes were identified in the model plant *Arabidopsis thaliana* (*Arabidopsis*) ranges from BAG1 to BAG7 [4,5]. The AtBAG1-4 proteins contain a BAG domain and an Ubiquitin-like domain (UBL), whereas the AtBAG5-7 proteins contain an IQ motif, which preferentially binds to Ca²⁺-free calmodulin (CaM) in addition to a BAG domain [5,6,7]. Phylogenetic analysis by MEGA7 software clearly revealed that the plant BAG family proteins can be divided into two subgroups, Group I including the BAG1-4 proteins, and Group II including the BAG5-7 proteins (Fig. S1). Overall, plant BAG proteins have roles in programmed cell death (PCD), in plants response to biotic and abiotic stresses and in development [1, 3].

AtBAG1 was found to be localized in the cytosol and interact with the chaperone Hsc70-4 to cooperatively mediate proteasomal degradation of misfolded cytosolic proteins and unimported plastid proteins [8].

Physiologically, *AtBAG1* overexpression transgenic seedlings were more sensitive to salt treatment, suggesting that *AtBAG1* might be involved in plants response to abiotic stress [8]. It has been reported that under normal growth conditions the *atbag2* mutant exhibited slightly better growth than the WT, implying that *AtBAG2* might participate in plants PCD or response to environmental stresses [6]. *AtBAG3* expression was upregulated by salt stress and by the stress-related hormones ABA and ACC, but downregulated by cold stress [1]. *AtBAG3* protein is localized to the cytosol [8]. However, so far the physiological function of *AtBAG2* and *AtBAG3* remains unknown.

Among the plant *BAG* genes, *BAG4* is one of the most studied. Overexpression of the *AtBAG4* in tobacco plants confers tolerance to UVB irradiation and cold, oxidant and salt treatments [5]. In contrast, the *atbag4* mutant is more sensitive to salt treatment [2]. In rice, *OsBAG4* functions as a positive regulator of disease resistance, and an E3 ligase EBR1 targets *OsBAG4* for proteasome degradation to balance growth and immune response [9]. Recently, *AtBAG4* was found to interact with the potassium channel protein KAT1 and help its localization to the plasma membrane [10].

Our previous studies show that *AtBAG5* acts as a hub linking calcium signaling and the Hsc70 chaperone to regulate leaf senescence [7,11]. *AtBAG6* expression is induced by the stress-related plant hormone SA and ABA, and by heat stress and *atbag6* mutant is sensitive to pathogen infection and non-acclimated heat stress [5,1,13]. However, another report found that *atbag6* mutant exhibited mild thermotolerance to non-acclimated heat stress compared to the WT plants [12]. Although in acclimated heat stress treatment, *atbag6* mutant showed no different phenotype from those of the WT, it could increase the thermotolerance of the *fes1a* mutant [13]. Overexpression of *AtBAG6* induced cell death [14]. Aspartyl protease-mediated cleavage of *AtBAG6* is necessary for autophagy and fungal resistance in plants [15]. *AtBAG7* localized in ER and is involved in unfolded protein response and heat tolerance [16,17]. It has been reported that salt treatment downregulated expression of *AtBAG6* and *AtBAG7*, but ACC treatment could reverse the effect of salinity [18]. Whereas *BAG6* was found to play significant role in biotic (pathogens) and abiotic stresses (heat), it remains unknown whether *BAG6* is involved in other stressors or not.

Here, we have focused on function of *BAG2* and *BAG6* in *Arabidopsis* to analyze their expression pattern in response to various hormones and stresses and especially abiotic stress treatments such as drought, ABA and heat stress and the genes involved in it. Our results showed that both *BAG2* and *BAG6* genes are expressed in various tissues in *Arabidopsis*. Expression of both *BAG2* and *BAG6* genes is induced by salt, mannitol, and heat treatments and by the stress-related hormones including ABA, ethylene and SA, while *BAG6* is additionally induced by JA. Germination of the *bag2* and *bag6* single and *bag2 bag6* double mutant seeds are less sensitive to ABA than that of the wild type (WT). Both *bag2* and *bag6* single mutant and *bag2 bag6* double mutant showed more survival rate than WT in drought treatment but displayed less survival rate on 45 °C heat stress experiment. Consistently, these mutants displayed higher transcription levels of multiple stress-related genes such as *RD29A*, *RD29B* and ABA biosynthesis genes *NCED3* than that of the WT in response to ABA treatment. Finally, when compared to wild type plants, the

mutants exhibited higher content of reactive oxygen species (ROS) after heat stress and less contents accumulated after drought and ABA treatment.

Results

***BAG2* and *BAG6* expression during Arabidopsis development**

To determine expression patterns of the *BAG2* and *BAG6* genes, we generated Arabidopsis transgenic lines expressing the *GUS* reporter gene under control of the promoter fragments of the *BAG2* and *BAG6* genes, respectively (*ProBAG2:GUS* and *ProBAG6:GUS*) and analyzed the expression of the *GUS* reporter gene in various tissues. Strong *GUS* staining was observed in most tissues throughout the *ProBAG2:GUS* and *ProBAG6:GUS* transgenic plants (Fig. 1 A-T). In 7-day-old seedlings, *ProBAG2:GUS* activity is strongly detected in cotyledons, hypocotyl, and root vascular tissues, with the strongest signal at the lower part of the hypocotyl (Fig. 1A and B), while *ProBAG6:GUS* expression is strongly detected in all of the tissues of the whole seedling (Fig. 1 K and L). In 2-week-old plants and bolting plants, both *ProBAG2:GUS* and *ProBAG6:GUS* are highly expressed in rosette leaves and cauline leaves (Fig. 1 C-E and M-O), with *ProBAG6:GUS* showing additional *GUS* activity in stems (Fig. 1P). In flowers, both *ProBAG2:GUS* and *ProBAG6:GUS* are expressed in sepals, anther filaments, pollen grains, and style (Fig. 1 F-H and Q-R), with *ProBAG6:GUS* showing additional expression in ovules (Fig. 1R). In siliques, both *ProBAG2:GUS* and *ProBAG6:GUS* are expressed in young siliques with *ProBAG6:GUS* showing stronger *GUS* signals (Fig. 1I and S), while in old siliques both genes expression are very weak (Fig. 1J and T).

***BAG2* and *BAG6* expression response to abiotic stress and plant hormones**

We further investigated whether *BAG2* and *BAG6* expression is regulated by abiotic stressors or plant hormones. The results show that *ProBAG2:GUS* expression is strongly increased by mannitol, salt (NaCl), heat and ABA treatments (Fig. 2A-B; Fig. S2 A) and its expression is also slightly increased in response to other stressors and hormones such as PEG, SA and ACC, respectively. Similarly, *ProBAG6:GUS* showed significant enhanced *GUS* staining in response to JA, ABA, mannitol, salt, and heat treatment, while showed slight increase in response to ACC, PEG and SA (Fig. 2C-D; Fig. S2 B-D). These results are similar to a previous report that had analyzed *AtBAG* genes response to abiotic stressors such as cold, heat, mannitol, and salt and hormones such as ABA, ACC, MeJA and SA by reverse transcription quantitative PCR (RT-qPCR) [1]. Together, these results suggest that *AtBAG2* and *AtBAG6* genes are involved in plants response to environmental stress.

Loss-of-function of *BAG2* and *BAG6* in *Arabidopsis* improves tolerance to ABA and drought Stresses

To explore the function of these genes, transfer DNA (T-DNA) insertion mutants were isolated for *BAG2* and *BAG6* within the fourth exon (SALK_030285, *bag2*; Fig. 3A) and within the first exon (SALK_004760, *bag6*; Fig. 3C), respectively. These mutants were crossed to generate the *bag2 bag6* double mutant. The *bag2* and *bag6* mutants had been reported previously [6,12]. RT-PCR analyses revealed that weak *BAG2* transcript was detected in the homozygous *bag2* mutant (Fig. 3B; Fig. S3A-B) and no *BAG6* transcript was detectable in the homozygous *bag6* mutant (Fig. 3D; Fig. S3C-D), indicating that the *bag2* mutant is a knockdown allele and the *bag6* mutant is a null allele. Under normal growth conditions, the *bag2* and *bag6* single and *bag2 bag6* double mutants exhibited slightly larger rosette size than the WT (Fig. S4).

To study the response of *bag2* and *bag6* single and *bag2 bag6* double mutants to the plant stress hormone abscisic acid (ABA), these mutants and wild type (WT) seeds were sown on Murashige and Skoog (MS) medium supplemented with different concentrations of ABA to compare the germination and greening rate between WT and mutants lines. Without ABA, the WT and mutants showed similar germination rate and greening rate, but when ABA was applied, higher germination and greening rate were observed in the *bag2* and *bag6* single and *bag2 bag6* double mutants (Fig. 4). When compared germination and greening rate on 0.75 μ M ABA, we found a significant difference i-e WT germination rate (72%) and greening rate (46%) while *bag2* 75% and 84%, *bag6* 79% and 88% and *bag2 bag6* 81% and 91%, respectively (Fig. 4B-C). Similarly, when compared on 1 μ M ABA, WT exhibited 57% seeds germinated and after 7 days 29% greening rate were found but *bag2* single mutant exhibited 65% germination while 49% greening rate were observed, *bag6* also showed more germination and greening rate than WT (i-e 68% and 55%, respectively) (Fig. 4B-C). Interestingly *bag2 bag6* double mutant germination rate was more than each single mutant and WT which is 75% and 71%, respectively (Fig. 4B-C). Finally, when compared on 2 μ M ABA, WT exhibited 37% germination and 24% greening rate, while *bag2* exhibited 50% and 36%, *bag6* 55% and 36% and *bag2 bag6* 62% and 49%, respectively (Fig. 4B-C). Thus these mutants appeared to enhance the ABA tolerance of *Arabidopsis* during germination and the early vegetative growth period.

To study drought stress tolerance, 21-day-old WT and mutants plants were used, and 9 plants per pot were sown and 12 pots were prepared for each genotype (108 plants in total for each genotype). After withholding water for 14 days, almost all the WT and mutants plants were wilted and near to die (Fig. 5A). Then the plants were re-watered for three days. On third day a significant difference were noticed between WT and mutants plants (Fig. 5A); *bag2* showed 43%, *bag6* showed 44%, *bag2 bag6* showed highest survival rate i-e 73% while WT showed less survival rate i-e 29 % (Fig. 5B). To further verify these results, we performed water loss experiment with different time interval. Consistently, the water loss in mutants plants rosette leaves was slower than that of the WT (Fig. 5C). These results suggest that *BAG2* and *BAG6* play a negative role in *Arabidopsis* response to ABA and drought treatments.

Loss-of-function of *BAG2* and *BAG6* in *Arabidopsis* compromises tolerance to heat stress

Our *ProBAG2:GUS* and *ProBAG6:GUS* expression results and previously reported RT-qPCR results showed that *BAG2* and *BAG6* genes were upregulated during heat stress [1,13]. So here in this study we have studied the effect of heat stress on *bag2* and *bag6* single and *bag2 bag6* double mutants. The results showed that the *bag2* and *bag6* single and *bag2 bag6* double mutants are sensitive to heat stress when compared to WT seedlings (Fig. 6). The survival rates of these mutants were one third less than WT (Figs. 6B). Also we measured chlorophyll content in mutants and WT on control untreated seedlings (22 °C) and 45 °C heat treated seedlings. Significant difference was observed (Figs. 6C). Total chlorophyll levels decreased as consequence of the heat treatment both in WT and mutants, but the mutants retained less chlorophyll contents (Fig. 6C). These results suggest that *BAG2* and *BAG6* play a positive role in *Arabidopsis* response to heat stress.

Stress related genes are upregulated in the *bag2* and *bag6* mutants

To further investigate the role of *BAG2* and *BAG6* in plants response to ABA, heat and drought treatment, we performed RT-qPCR analysis on several stress-related genes such as *RD29A*, *RD29B* (stress related), and *NCED3* (ABA biosynthesis genes). We found that the expression of *RD29A* and *RD29B* genes was upregulated in *bag2* and *bag6* single mutants and *bag2 bag6* double mutant compared to WT when treated with ABA (Fig. 7A-B), while expression of the ABA biosynthesis gene *NCED3* was slightly upregulated in these mutants compared to WT (Fig. 7C). These results further support the heat, ABA and drought phenotypic data.

ROS accumulations in WT and mutant plants

The accumulation of H_2O_2 and superoxide anion ($O_2^{\cdot -}$) was detected using 3,3-diaminobenzidine (DAB) and nitro blue tetrazolium (NBT) staining in seedling shoots and roots. Upon 20 μ M ABA treatment, the cotyledon and root of the mutant plants displayed lighter color than that of the WT, which clearly indicates lower accumulation of $O_2^{\cdot -}$ and H_2O_2 , respectively in the mutant seedlings (Fig. 8A-B), whereas upon heat stress treatment, the mutants exhibited higher accumulation of $O_2^{\cdot -}$ than the WT (Fig. 8C). Finally DAB staining was also applied to drought stressed rosette leaves of WT and mutant plants. We got the same results that leaves of mutant lines accumulated lower H_2O_2 than that of the WT (Fig. S5). Combined these results indicate that mutant lines had a lower accumulation of ROS when treated with ABA and drought while higher accumulation when treated with heat stress. These results corresponded well with the respective drought- and heat-tolerance phenotypes of these mutants.

Discussion

In plants especially in *Arabidopsis* BAG proteins are a topic of interest and they mediate multiple abiotic, biotic and developmental processes. Actually, most research on plant BAGs so far are focused on their functions in abiotic and biotic stress tolerance. The expression patterns of *AtBAG* genes and their roles during plant development remain largely unknown. In this study, we analyzed the expression patterns of *AtBAG2* and *AtBAG6* genes during development by using *ProBAG2:GUS* and *ProBAG6:GUS* transgenic lines. Interestingly, the results show that both *AtBAG2* and *AtBAG6* genes are expressed in various tissues with overlapping or specific expression patterns during the whole developmental process. For example, *ProBAG6:GUS* is expressed in all of the root tissues with stronger expression in columella root cap cells and root vascular tissue, but *ProBAG2:GUS* is only expressed in the root vascular tissue. In shoots, both *ProBAG2:GUS* and *ProBAG6:GUS* are strongly expressed in rosette leaves and cauline leaves, but they again show differences in flower tissues. Mutant analysis showed that *bag2* and *bag6* single mutants and *bag2 bag6* double mutant plants are a bit larger than the WT, and they show no developmental defect. These results suggest that the *AtBAG2* and *AtBAG6* genes and other *AtBAG* genes may have redundant and specific functions during *Arabidopsis* development. Analyzing the other *AtBAG* genes expression patterns and generating multiple *atbag* mutants by crossing or by using CRISPR/Cas9 gene editing methods will provide more information of the role of the *AtBAG* family during *Arabidopsis* development.

Previous RT-qPCR and Northern blot analysis had shown that *AtBAG2* transcript level was significantly upregulated by ABA and salt treatment and *AtBAG6* transcript level was significantly upregulated by ABA, SA, H₂O₂ and heat treatment [1,5,13]. Consistently, our *ProBAG2:GUS* and *ProBAG6:GUS* expression results show that they both are upregulated by heat, salt and osmotic treatment and by stress-related plant hormones including ABA, SA and ACC treatment, and *ProBAG6:GUS* is additionally induced by JA. These expression results suggest that the *AtBAG2* and *AtBAG6* genes may be involved in *Arabidopsis* response to multiple environmental stresses. Indeed our stress and hormone treatments results show that *bag2* and *bag6* single mutants and *bag2 bag6* double mutant seeds germination are less sensitive to ABA treatment and their plants are more tolerant to drought stress than the WT, but they are more sensitive to heat stress. Interestingly, in ABA and drought treatments the *bag2 bag6* double mutant shows more tolerance than either of the single mutant, suggesting an additive effect of these two genes in *Arabidopsis* response to ABA or drought stress treatments.

Conclusion

Results in the present study show that the *AtBAG2* and *AtBAG6* genes are broadly expressed in various tissues during *Arabidopsis* development. Also, *AtBAG2* and *AtBAG6* expression is induced by abiotic stresses such as heat, salt and osmotic and by stress-related plant hormones including ABA, SA, JA and ethylene. Seed germination of *bag2* and *bag6* single and *bag2 bag6* double mutant are more tolerant to ABA treatment and their plants are more tolerant to drought stress but sensitive to heat stress. These

findings suggest that *AtBAG2* and *AtBAG6* play important roles in *Arabidopsis* response to multiple abiotic stresses and hormones.

Methods

Plant Materials and Growth Conditions

The T-DNA insertion mutants *bag2* (SALK_030285) and *bag6* (SALK_004760) are in Columbia-0 (Col-0) background. These mutants were obtained from the Arabidopsis Stock Centre and double mutant is obtained by crossing *bag2* and *bag6*. T-DNA insertion was confirmed by RT-PCR analysis. Primers used in this experiment are listed in Supplemental Table S1. Seeds were surface sterilized in 70% ethanol for 5 min and 1% Clorox bleach for 10 min, stratified for 3 days at 4 °C. The seeds were plated on solidified Murashige and Skoog (MS) medium and vertically grown at 22 °C under 16 h light/8 h dark conditions. For ABA treatments experiment, all the single, double mutant and WT seeds were surface sterilized and after 72 hours stratification, sown on plates supplemented with or without ABA. For drought treatments, the stratified seeds were grown vertically on MS plates for seven days and transferred to soil (75% soil and 25% vermiculite) for more 14 days to grow. Nine plants per pots were grown. During each independent experiment, we grew 108 plants in total for each genotype.

Vector construction and generation of transgenic plants

To create the *ProBAG2:GUS* construct, 1886 bp sequence upstream of the *BAG2* gene start codon were PCR-amplified. The resulting fragment was inserted upstream of the *GUS* reporter gene in the pGreenII0229-GUS vector via *XhoI* and *KpnI* sites [19]. Similarly, *ProBAG6:GUS* was also created for this study, 1884 bp sequence upstream of the *BAG6* gene start codon were PCR-amplified. The resulting fragment was inserted upstream of the *GUS* reporter gene in the pGreenII0229-GUS vector via *XhoI* and *KpnI* sites [19]. Primer sequences used in this experiment are available in Supplementary Table S1. These constructs were transformed into *Agrobacterium tumefaciens* strain C58C1 (pMP90/pJICSa-Rep). The *Arabidopsis* transgenic plants were generated using floral dip method [20].

RT-PCR Analysis

RT-PCR was performed to test the transcription levels of the *BAG2* and *BAG6* gene in mutant or wild-type (WT) plants. Total RNA was extracted from 7-day-old seedlings using Trizol reagent (TransGen Biotech, Beijing, China). The first-strand cDNA was synthesized using Easy Script First-Strand cDNA Synthesis Super Mix (TransGen Biotech, Beijing, China). The *ACTIN2* gene was used as an internal control. The primer sequences used in this experiment are listed in Supplemental Table S1.

GUS Histochemical Staining

For GUS staining, various tissues of *ProBAG2:GUS* and *ProBAG6:GUS* transgenic plants were incubated for 12 h at 37 °C in GUS staining buffer as previously described [21]. After staining, the tissues were

cleared in chloral hydrate: distilled water: glycerol (8:3:1, *w:v:v*). Samples were visualized with microscopy (Olympus, bx63, Tokyo, Japan).

Drought and ABA Treatments

bag2 and *bag6* single and *bag2 bag6* double mutants and WT seeds were harvested at the same time and used for ABA treatments. In brief, surface-sterilized seeds were sown on MS medium plates supplemented without or with different concentrations of ABA (Sigma-Aldrich) as indicated. Plates were stratified at 4 °C in the dark for 72 hours and transferred to a growth incubator for normal conditions (16 h light/8 h dark, 22 °C). For quantification of the percentage of seedlings with green cotyledons, seeds were sown on MS medium supplemented different concentrations of ABA and analyzed on the indicated days after stratification as previously described [22]. Pictures were scanned using HP LaserJet P1108 scanner.

For the drought tolerance test, 3-week-old plants grown in soil with sufficient water were treated with natural drought (water was withheld). After 14 days without watering, the drought-treated plants were re-watered and recovery was checked after 3 days. Drought experiments were repeated three times, and at least 9 plants for each individual genotype were used per experiment.

Heat treatment and measurement of Chlorophyll content

For heat treatment, 5-day-old WT, *bag2*, *bag6* and *bag2 bag6* seedling were exposed to 45 °C for 45 minutes. After three days recovery at 22 °C, seedlings survival rate was counted [1]. For the measurement of chlorophyll content, mesophyll cells were spectrophotometrically measured as previously reported [23].

DAB and NBT Staining

DAB and NBT staining were performed as previously described [24]. The experiment was performed three times and each time 20 seedlings of each genotype were stained. Five-day-old seedlings were used in this experiment.

Hormone Treatment

Hormonal treatment of the *ProBAG2:GUS* and *ProBAG6:GUS* seedling was performed as previously described [1].

RT-qPCR

For RT-qPCR analysis, of different stress related genes, we followed previously described protocol [1].

Abbreviations

ABA: Absciscic acid, ACC: 1-aminocyclopropane-1-carboxylic acid, JA: jasmonate, SA: salicylic acid, WT: wild type, GUS: β -glucuronidase, GA: Gibberellic acid

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that there is no conflict of interest.

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Authors' contributions

SM and YS conceived the project. SM planned and designed the research. MA performed experiments with the help of QL, ZL and LL. MA and SM analysed data and wrote the manuscript with contributions of all the authors. All authors have read and approved the manuscript.

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Figures

Figure 1

Expression patterns of the Arabidopsis BAG2 and BAG6 genes. a-j expression of PROBAG2:GUS in 5-day-old seedlings cotyledons and hypocotyl (a), root tip (b), 2-week-old plant (c), rosette leaf (d), cauline leaf (e), inflorescence (f), flower bud (g), opened flower (h), young siliques (i), and mature siliques (j). k-t Expression of ProBAG6:GUS in cotyledons and hypocotyl (k) and root tip (l) of 5-day-old seedling, in 2-week-old plant (m), rosette leaf (n), cauline leaf (o), stems (p), inflorescence (q), opened flower (r), young siliques (s), and mature siliques (t).

Figure 2

Expression of ProBAG2:GUS and ProBAG6:GUS in response to various abiotic stresses and hormones. Five-day-old ProBAG2:GUS (a and b) and ProBAG6:GUS (c and d) seedlings were transferred to MS media (mock) supplemented with 5% PEG, 300 mM mannitol, 200 mM NaCl, 10 μ M JA, 10 μ M ABA, 5 mM SA and 10 μ M ACC and treated for 24 h, then were collected for GUS staining. Shown are representative

images of shoots (a and c) and root tips (b and d) of three independent experiments. Bars = 400 μ m in (a and c) and 100 μ m in (b and d).

Figure 3

Insertion sites and transcription analysis of the *bag2* and *bag6* mutants. a and c Structures of the AtBAG2 (a) and the AtBAG6 (c) genes with T-DNA insertion sites. White boxes at left and right borders represent UTR regions, black boxes indicate exons, lines between black boxes represent introns, flags indicate the T-DNA insertion sites, and arrows indicate the positions of primers used for PCR verification of the insertions and for RT-PCR. b and d RT-PCR analysis of transcription levels of the AtBAG2 (b) and the AtBAG6 (d) genes in WT and *bag2* or *bag6* mutants. The ACTIN2 gene was used as the internal control. Total RNA was extracted from 7-day-old seedlings. The presented images in (b) and (d) were cropped and the original, full-length gel images were provided in the additional file Fig. S3.

Figure 4

bag2 and *bag6* mutants are less sensitive to ABA than the WT. a-c Seed germination rate and greening rate of WT, *bag2* and *bag6* single, and *bag2 bag6* double mutants. Sterilized seeds were sowed on MS medium supplemented without or with different concentrations of ABA. The germination percentage and the greening rate were scored. All the data represent the means of three independent experiments $\pm$ SD. *, represent the significant differences at $p < 0.05$; ** represent $p < 0.01$; ***, represent $p < 0.001$ (Student's t-test).

Figure 5

bag2 and *bag6* mutants are more tolerant to drought. a Phenotypes of WT, *bag2* and *bag6* single, and *bag2 bag6* double mutant plants treated with drought stress. Three-week-old plants were subjected to drought stress for 14 days and re-watered for 3 days. b The survival rate of WT, *bag2*, *bag6*, and *bag2 bag6* plants after 3 days of re-watering following the 14 days drought treatment. Values are means $\pm$ SD ($n = 5$ independent experiments, 108 plants for each genotype in each independent experiment were used for analysis). c Water loss in WT, *bag2*, *bag6*, and *bag2 bag6* plants. Leaves from 3-week-old plants were collected and subjected for water loss at different time interval. All the data represent the means $\pm$ SD of five independent experiments. *, represent the significant differences at $p < 0.05$; **, represent $p < 0.01$; ***, represent $p < 0.001$ (Student's t-test).

A

Untreated

45°C for 45 min

B **C** **Figure 6**

bag2 and bag6 mutants are sensitive to heat stress. a Phenotype of seedlings treated with heat stress. b Survival rates of WT, bag2, bag6, and bag2 bag6 mutants under heat stress. Five-day-old seedlings were placed at 45 °C for 45 minutes and then resumed growth at 22 °C. c Chlorophyll content measured from seedlings grown on normal growth conditions and after 45 °C heat stress. The data represent the means $\pm$ SD of two independent experiments.

Figure 7

The relative expression of stress-related genes. a *RD29A*. b *RD29B*. c *NCED3* were examined under control and ABA conditions after 12 hours. Values are means $\pm$ SD of three replicates.

Figure 8

BAG2 and *BAG6* mutation affects the ROS levels after ABA and heat treatment in *Arabidopsis* seedlings. Five-day-old seedlings were transferred to 1/2 MS agar plates supplemented with 20 μ M ABA for 6 h or were treated with heat stress (37 $^{\circ}$ C) for 3 h, then were stained with NBT (staining for $O_2^{\cdot -}$) (a and c) or DAB (staining for H_2O_2) (b). At least three independent experiments were performed for each analysis

and 15 seedlings of each genotype were examined. Bars = 400 μm in (a) and in the shoot images in (c) and 100 μm in (b) and in the root images in (c).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [BAG26SupplementaryFig.sformatted.pdf](#)