INTRODUCTION

Abscisic acid (ABA) is a phytohormone that plays critical roles in various biological processes. One of its best-characterized roles is in adaptive responses to abiotic stresses, such as high salt and dehydration stress (Cutler and Krochko, 1999; Zeevaart, 1999; Wilkinson and Davies, 2002; Zhu, 2002). Cellular ABA levels are drastically increased, which in turn activates ABA-mediated signaling and adaptive responses (Shinozaki and Yamaguchi-Shinozaki, 2000; Wilkinson and Davies, 2002; Zhu, 2002; Himmelbach et al., 2003). Under osmotic stress conditions, cellular ABA levels are drastically increased, which in turn activates ABA-mediated signaling and adaptive responses (Shinozaki and Yamaguchi-Shinozaki, 2000; Wilkinson and Davies, 2002; Zhu, 2002; Himmelbach et al., 2003).

Cellular ABA levels are determined by two opposing processes: biosynthetic and catabolic pathways increase and reduce ABA levels, respectively (Cutler and Krochko, 1999; Zeevaart, 1999; Wilkinson and Davies, 2002; Zhu, 2002). Biosynthetic and catabolic pathways increase and reduce ABA levels. For instance, ABA is produced in multiple organelles by organelle-specific β-glucosidases in response to abiotic stresses.

The phytohormone abscisic acid (ABA) plays a critical role in various physiological processes, including adaptation to abiotic stresses. In Arabidopsis thaliana, ABA levels are increased both through de novo biosynthesis and via β-glucosidase homolog1 (BG1)-mediated hydrolysis of Glc-conjugated ABA (ABA-GE). However, it is not known how many different β-glucosidase proteins produce ABA from ABA-GE and how the multiple ABA production pathways are coordinated to increase ABA levels. Here, we report that a previously undiscovered β-glucosidase homolog, BG2, produced ABA by hydrolyzing ABA-GE and plays a role in osmotic stress response. BG2 localized to the vacuole as a high molecular weight complex and accumulated to high levels under dehydration stress. BG2 hydrolyzed ABA-GE to ABA in vitro. In addition, BG2 increased ABA levels in protoplasts upon application of exogenous ABA-GE. Overexpression of BG2 rescued the bg1 mutant phenotype, as observed for the overexpression of NCED3 in bg1 mutants. Multiple Arabidopsis bg2 alleles with a T-DNA insertion in BG2 were more sensitive to dehydration and NaCl stress, whereas BG2 overexpression resulted in enhanced resistance to dehydration and NaCl stress. Based on these observations, we propose that, in addition to the de novo biosynthesis, ABA is produced in multiple organelles by organelle-specific β-glucosidases in response to abiotic stresses.
Multiple ABA receptors that localized to different subcellular locations have been identified (Shen et al., 2006; Ma et al., 2009; Pandey et al., 2009; Park et al., 2009). For example, regulatory components of ABA receptor/pyrabactin resistance (RCAR/PYR), which localizes in the cytosol, the Mg-chelatase H subunit ABAR/CHLH, which localizes to chloroplasts, and two homologous G protein-coupled receptor (GPCR)-type G proteins, GTG1 and GTG2, which localize to the plasma membrane, have been identified as ABA receptors, although CHLH and GPCRs are still controversial and need to be confirmed. Thus, it is possible that ABA-mediated cellular processes are regulated by the local concentration of ABA, which is perceived by specific ABA receptors localized to various subcellular locations in the cell, rather than by the overall level of ABA. However, it remains unknown how the ABA levels are controlled at the cellular level. The two ABA-producing pathways, which occur in different compartments, need to be coordinated to maintain homeostasis of the cellular ABA levels. In addition, the ABA-specific transporters localized to the plasma membrane may also contribute to the homeostasis of the cellular ABA levels in the plant cells (Kang et al., 2010; Kuromori et al., 2010). Furthermore, the catabolic pathways in the cytosol also play a critical role in the regulation of cellular ABA levels. Therefore, a mechanism must exist for coordinating these various cellular processes to achieve the cellular level of ABA that is required for adaptation of the cellular processes to maintain homeostasis, including those that function in biosynthetic and catabolic pathways, as well as in transport between compartments.

Here, we report that an Arabidopsis β-glucosidase homolog, which localized to the vacuole, has the ability to hydrolyze ABA-GE to produce ABA and thereby plays an important role in the osmotic stress response.

## RESULTS

### A Previously Uncharacterized β-Glucosidase Homolog, BG2, Is Involved in the Dehydration and NaCl Stress Responses in Arabidopsis

To identify the genetic locus that controls the osmotic stress response, we mutagenized Arabidopsis plants that express the firefly luciferase reporter gene (LUC) driven by the osmotic stress–responsive RD29A promoter, RD29Ap (Ishitani et al., 1997), using Agrobacterium tumefaciens–mediated T-DNA transformation. T4 plants were used to screen for mutants that exhibited altered expression of LUC in response to high NaCl stress. One mutant, los15, showed decreased LUC expression relative to the wild-type plants at high NaCl concentration (Figure 1A), indicating that los15 had a defect in the response to salt stress. Sequence analysis of the PCR product obtained by thermal asymmetric interlaced–PCR (Li et al., 1995) revealed that the T-DNA was inserted into the promoter region of Arabidopsis locus At2g32860, which encodes a β-glucosidase (Figures 1B and 1D; see Supplemental Figure 1 online). We thus named this gene BG2. Since los15 had the T-DNA inserted into the promoter region of BG2, we obtained another mutant that had a T-DNA insertion in its coding region (Figures 1B and 1D). The original los15 mutant was renamed bg2-1, and the new mutant was named bg2-2. The absence of the BG2 transcript in both bg2 mutant alleles was confirmed by RT-PCR (Figure 1C). Sequence analysis revealed that BG2 showed 38.9% amino acid sequence identity to BG1, a recently identified β-glucosidase that hydrolyzes ABA-GE to produce ABA (see Supplemental Figure 1 online) (Lee et al., 2006). Phylogenetic analysis revealed that the large number of β-glucosidase homologs in Arabidopsis can be grouped into six subfamilies (see Supplemental Figure 2A and Supplemental Data Set 1 online) (Xu et al., 2004) and that BG2 belongs to the same subfamily as BG1 (see Supplemental Figure 2B and Supplemental Data Set 2 online); within this clade, however, BG2 was related most distantly to BG1. Despite the overall sequence similarity, BG2 contains two prominent differences from BG1 in its primary structure: BG2 lacks an ER retention signal at the C terminus and contains N- and C-terminal extensions (see Supplemental Figure 1 online).

To test whether BG2 is involved in osmotic stress responses, as indicated by the defect in induction of the RD29Ap:LUC transgene in bg2-1 mutants by NaCl stress, we examined the expression of three osmotic stress–inducible genes, RD29A, RD29B, and COR47, using quantitative RT-PCR (qRT-PCR). Total RNAs were prepared from wild-type and bg2-1 and bg2-2 mutant seedlings treated with 100 μM ABA or 300 mM NaCl for 3 h. BG2:HA/bg2-2 plants expressing BG2:HA from the BG2 promoter on the background of the bg2-2 mutation (see below) were included in the analysis. qRT-PCR was performed using gene-specific primers. Both mutants showed a defect in NaCl-mediated induction of RD29Ap, RD29B, and COR47 expression in both alleles (Figure 1E; see Supplemental Figure 3 online), supporting the idea that they represent two different alleles of the same gene. However, the mutants showed slight differences in the amount of induction, which is likely due to the difference in ecotype. In addition, this defect in bg2-2 plants was rescued by BG2:HA. By contrast, these mutants did not exhibit any defect in ABA-mediated induction of these genes. In fact, the increase mediated by ABA was slightly higher in plants with the bg2-1 allele than that in the wild-type plants. These results strongly suggest that BG2 is involved in NaCl stress responses.

To gain further insight into the biological role of BG2, we examined the phenotype of both bg2 mutant alleles. They did not display any noticeable phenotype under normal growth conditions. Since the bg2-1 mutant was originally identified as a mutant with a defect in the NaCl stress signaling pathway, we further tested the response to NaCl stress of these bg2 mutants. Plants grown on Murashige and Skoog (MS) plates that had the same root length were transferred to half-strength MS plates supplemented with 125 mM NaCl, and then root growth was examined. Both alleles of bg2 mutants displayed reduced root growth at 125 mM NaCl compared with their respective wild-type plants C24 and Columbia-0 (Col-0) (Figures 2A and 2B), confirming that the phenotype is caused by the mutation in BG2. However, the degree of sensitivity to NaCl stress was slightly different between the two alleles. In addition, the NaCl-mediated root growth defect was rather mild. To exclude the possibilities
that this was caused by two different mutations at other loci and that the mild defect was caused by the mutations, we identified two new alleles, bg2-3 and bg2-4, which had a T-DNA insertion at BG2 (see Supplemental Figure 4A online) and examined their responses to NaCl stress. bg2-3 and bg2-4 showed significant defects in development, including root length, dry weight of the aerial parts, and survival rates under high NaCl stress (Figure 2C; see Supplemental Figures 4B and 4C online), thus supporting the idea that BG2 is involved in osmotic stress responses. To obtain additional support for this, BG2:HA under control of the BG2 promoter was introduced into bg2-2 mutant plants, and we examined whether the transgene could rescue the high degree of sensitivity to NaCl stress of bg2-2 plants. Indeed, the two independent lines of pBG2:BG2:HA/bg2-2 (BG2:HA) that had been treated with or without 100 μM ABA or 300 mM NaCl for 3 h and used for qRT-PCR analysis. The expression level of RD29A was then compared between plants. The expression levels of RD29A in plants treated with NaCl (300 mM) or ABA (100 μM) were presented as relative values to untreated control samples. Error bars indicate SD (n = 3). ACTIN2 was used as internal control for the qRT-PCR.

Figure 1. Screening of the bg2-1 Mutant, Which Displays a Defect in the Induction of RD29Ap:LUC Expression in Response to High NaCl.

(A) Mutant screening. Charge-coupled device (CCD) camera images (right panels) were taken of 10-d-old bg2-1 (T4) and wild-type (C24) seedlings (left panels) that had been exposed to a high concentration of NaCl (300 mM) for 4 h.

(B) Identification of T-DNA insertion sites. The T-DNA insertion sites were determined by PCR analysis using the indicated primers. Top panel, bg2-1; bottom panel, bg2-2. The primer sites are indicated in (D), C24, C24 wild type harboring RD29Ap:LUC; Col-0, wild-type Columbia plants; LP, BG2-LP; RP, BG2-RP; P1, promoter primer 1; P2, promoter primer 2; LB, left border primer.

(C) RT-PCR analysis of transcripts. Total RNA from C24, Col-0, bg2-1, and bg2-2 plants was used for RT-PCR analysis. Primers R1 and R2 were used for bg2-1 plants, and primers BG2-LP and BG2-RP were used for bg2-2 plants. TUBULIN transcripts and 18S rRNA were used as controls for RT-PCR analysis.

(D) Schematic presentation of BG2 and the T-DNA insertion sites. P1, P2, LP, RP, R1, and R2 indicate BG2-specific primer sites. LB and RB indicate the left border and right border of T-DNA. LP, BG2-LP; RP, BG2-RP; P1, promoter primer 1; P2, promoter primer 2; LB, left border; RB, right border; box, exon.

(E) qRT-PCR analysis of the transcript levels of ABA- and NaCl-inducible genes. Total RNA was extracted from the wild type, bg2-1, bg2-2, and pBG2:BG2:HA/bg2-2 (BG2:HA) that had been treated with or without 100 μM ABA or 300 mM NaCl for 3 h and used for qRT-PCR analysis. The expression level of RD29A was then compared between plants. The expression levels of RD29A in plants treated with NaCl (300 mM) or ABA (100 μM) were presented as relative values to untreated control samples. Error bars indicate SD (n = 3). ACTIN2 was used as internal control for the qRT-PCR.
Indeed, these transgenic plants displayed enhanced resistance to 150 mM NaCl compared with wild-type (Col-0) plants (Figure 3A). We examined the effect of BG2 overexpression on dehydration stress by determining the seed output of Col-0, bg2-2, and BG2:HA plants as an indicator of the dehydration stress response of plants grown without water for 2 weeks. Under normal growth conditions, the seed yield of the mutant plants was 90% that of wild-type (Col-0) plants (Figure 3B, No stress). Under dehydration conditions, the seed output of the bg2-2 mutant was 30% that of the wild type (Col-0), indicating that the bg2-2 mutant is more sensitive to dehydration stress than wild-type plants. By contrast, the seed output of BG2:HA plants was increased to 150% that of the wild-type (Col-0) plants under the same dehydration conditions (Figure 3B, Dehydration). Together,
these results strongly suggest that BG2 plays a role in the dehydration stress response. We examined the expression of BG2. Total RNA was isolated from plants treated with 150 mM NaCl and 100 μM exogenous ABA (Piao et al., 1999), and the transcript levels of BG2 were examined by qRT-PCR analysis. BG2 transcript levels increased to a maximum level 30 min after ABA treatment and then declined to a lower level. By contrast, BG2 transcript levels increased continuously to higher levels until 1 h after NaCl treatment (see Supplemental Figure 5A online). The positive control, RD29A, was also induced under these conditions (see Supplemental Figure 5A online). The induction level of BG2 under these conditions was lower than that of RD29A. The spatial and temporal expression patterns were examined using transgenic plants harboring an BG2p::GUS chimeric gene consisting of the promoter region of BG2 (BG2p, 2000 bp) and the GUS coding region. In seeds, the expression of BG2 was confined to the radicle (see Supplemental Figure 5B online, panel a). In seedlings, BG2 was expressed in the veins of cotyledon and leaf tissues, the vascular bundles of the hypocotyl, and primary and secondary root tissues (see Supplemental Figure 5B online, panels b to d). The high levels of BG2 expression in the vasculature of plants were similar to those of BG1 and many genes involved in the production of ABA (Cheng et al., 2002; Koiwai et al., 2004; Lee et al., 2006).

BG2 Hydrolyzes ABA-GE to ABA in Vitro

The bg2 mutant phenotype strongly suggested that BG2 is involved in ABA-mediated processes. We examined whether BG2 possesses the ability to hydrolyze ABA-GE to ABA, as observed for BG1 (Lee et al., 2006). BG2::HA was transiently expressed in protoplasts (Jin et al., 2001), and BG2::HA was immunopurified from protoplast extracts using the anti-HA antibody, as described previously (Lee et al., 2006). As a negative control, protein extracts from protoplasts transformed with an empty expression vector, R6, were included in the immunopurification. Purification of BG2::HA was confirmed by immunoblot analysis using anti-HA antibody (Figure 4A, left panel). In addition, that equal amounts of anti-HA antibody were used for immunopurification experiments was confirmed by the amount of IgG heavy chain detected in the immunoblot by Coomassie blue. Next, BG2::HA was incubated with ABA-GE in vitro, and the reaction products were fractionated by high-pressure liquid chromatography. The amount of ABA released was determined from these fractions by ELISA using an antibody that specifically recognizes ABA, but not ABA-GE (Banowetz et al., 1994; Xu et al., 2002; Lee et al., 2006). BG2::HA produced ABA from ABA-GE with a specific activity of 150 pmol/mg·min (Figure 4A, right panel). By contrast, the control did not produce a detectable level of ABA. To obtain supporting evidence for this, a BG2 mutant protein, BG2[Δ303], which had a deletion at the C-terminal region, was expressed and tested for the hydrolysis of ABA-GE in vitro. BG2[Δ303] produced almost undetectable levels of ABA, confirming that BG2 is involved in ABA-GE hydrolysis (Figure 4B). Next, we compared the activity of BG2 with that of BG1 in the production of ABA from ABA-GE. The specific activity of BG2 was slightly lower than that of BG1 (Figure 4C). These results demonstrate that, similar to BG1, BG2 hydrolyzes ABA-GE.
to produce ABA in vitro. In addition, these results are consistent with those showing that knockout and overexpression of BG2 gave rise to the increased sensitivity and enhanced resistance to dehydration and high NaCl stresses, respectively. The expression data we obtained in this study were also consistent with data obtained from a public database (http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi).

**Figure 4.** BG2 Hydrolyzes ABA-GE in Vitro.

(A) Hydrolysis of ABA-GE by BG2:HA. Protoplasts were transformed with BG2:HA or empty expression vector (Control), and protein extracts from transformed protoplasts were used to immunopurify BG2:HA using anti-HA antibody as described in Methods. Immunopurified proteins were analyzed by immunoblotting using anti-HA antibody (top left panel). To confirm whether equal amounts of anti-HA antibody were used for immunopurification experiments of each sample, the amount of IgG heavy chain (IgG-H) was detected in the immunoblot by Coomassie blue staining (bottom left panel). Immunopurified proteins were incubated with ABA-GE (1 nmol). Released ABA was fractionated by HPLC and analyzed by ELISA using anti-ABA antibody. The specific activity was determined based on the total protein used for immunopurification of BG2:HA. Error bars indicate SD (n = 3). Control, empty expression vector. IP, immunopurification of BG2:HA.

(B) Lack of ABA-GE hydrolyzing activity in the BG2[Δ303] mutant. Protoplasts were transformed with BG2[Δ303], BG2, or R6 (Control), and protein extracts were analyzed by immunoblotting using an anti-HA antibody (left panel). BG2 proteins were purified using the anti-HA antibody and incubated with ABA-GE as described in (A). ABA levels were measured as described in (A) (right panel).

(C) Comparison of ABA-GE hydrolyzing activities between BG1 and BG2. Protoplasts were transformed with BG1:HA or BG2:HA. BG1:HA and BG2:HA were immunopurified from protein extracts of transformed protoplasts and used for the ABA-GE hydrolyzing assay. Immunopurified proteins were analyzed by immunoblotting using anti-HA antibody (left panel). The activity was measured as described in (B) and normalized based on the protein intensity of the immunoblot. The specific activity was determined based on the total proteins used for immunopurification of BG1:HA and BG2:HA (right panel). IgG-H, IgG heavy chain stained with Coomassie blue. Error bars indicate SD (n = 3).

**Figure 5.** Transiently Expressed BG1 and BG2 in Protoplasts Hydrolyze Exogenous ABA-GE.

(A) ABA-GE hydrolyzing activity of BG1:HA and BG2:HA in protoplasts. Protoplasts from wild-type plants were transformed with the indicated constructs or empty vector (R6) and incubated for 24 h followed by an additional 4 h incubation in the presence of exogenous ABA-GE (10 μM). ABA was extracted from protoplasts using 40% methanol, and ABA levels were determined by ELISA using an anti-ABA antibody. As a control, ABA levels were determined in protoplasts that had been treated the same way but without ABA-GE. ABA levels were measured and expressed as described in (A). Control protoplasts were not treated with ABA-GE. Error bars represent the SD (n = 3).

(B) Effect of mutations in BG1 and/or BG2 on ABA-GE hydrolyzing activity. Protoplasts were prepared from the indicated plants and incubated with 10 μM ABA-GE for 4 h at room temperature. ABA levels in the protoplasts were measured and expressed as described in (A). Control protoplasts were not treated with ABA-GE. Error bars represent the SD (n = 3).
BG2 Hydrolyzes ABA-GE to ABA in Vivo

The in vitro activity of BG2 and the phenotype of the bg2 mutants raised the possibility that BG2 hydrolyzes ABA-GE in vivo. To test this, we examined whether transiently expressed BG2 was able to hydrolyze ABA-GE in protoplasts when ABA-GE was supplied exogenously. Protoplasts transformed with BG2, BG2[E426/Q]:HA, BG1:HA, or empty vector (R6) were incubated for 24 h to allow the expression of these proteins followed by an additional 4 h incubation with 10 μM of exogenous ABA-GE. Subsequently, ABA was extracted from the transformed protoplasts and the ABA levels were determined by ELISA using an ABA-specific antibody (Lee et al., 2006). BG2[E426/Q]:HA contained a mutation at Glu-426, a residue thought to be critical for catalytic activity (Lee et al., 2006). BG1:HA was included as a positive control. In protoplasts transformed with the empty vector (R6), ABA levels increased twofold compared with those in the control without ABA-GE treatment (Figure 5A), indicating that exogenously supplied ABA-GE was hydrolyzed by endogenous ABA-GE hydrolyzing activity in the protoplasts. This is consistent with previous results showing that Arabidopsis contains ABA-GE-hydrolyzing activity mediated by BG1 (Lee et al., 2006). In protoplasts transformed with BG2:HA, ABA levels increased markedly by ninefold (Figure 5A). A similar result was obtained with BG1:HA, indicating that the overexpression of BG1:HA and BG2:HA in protoplasts mediated the hydrolysis of exogenously supplied ABA-GE to ABA. By contrast, BG2[E426/Q]:HA did not increase ABA levels over those in the control R6-transformed protoplasts, indicating that the ninefold increase in ABA levels resulted from BG1:HA or BG2:HA. To support these findings further, we performed similar experiments using protoplasts from wild-type, bg2-2, bg1, or bg1 bg2-2 double mutant plants. Compared with wild-type plants, ABA levels were reduced slightly in bg2-2 plants but were reduced much more significantly in bg1 plants (Figure 5B). Furthermore, bg1 bg2-2 double knockout mutants did not show any noticeable ABA-GE hydrolyzing activity, indicating that BG1 and BG2 mediated the intracellular ABA-GE hydrolyzing activity. These results provide additional evidence that BG2 has ABA-hydrolyzing activity in vivo.

BG2 Localizes to the Central Vacuole

The fact that BG2 hydrolyzes ABA-GE to ABA, as observed for BG1, prompted us to determine the localization of BG2 and thereby establish the site of ABA production by BG2 in plant cells. In plant cells, ABA is known to be produced in two different subcellular locations, the cytoplasm and ER, through the de novo biosynthetic pathway and hydrolysis of ABA-GE by BG1, respectively (Nambara and Marion-Poll, 2005; Lee et al., 2006). To determine the subcellular localization of BG2, we next generated a green fluorescent protein (GFP) reporter construct, BG2:GFP, by inserting GFP downstream of the hydrophobic leader sequence at the N terminus of BG2. The BG2:GFP construct was placed under the control of the CsVMV promoter in a binary vector. Subsequently, transgenic plants expressing BG2:GFP were generated, and the GFP signals were examined. Most of the GFP signal was detected in the central vacuole (Figure 6A), indicating that BG2 localizes primarily to the central vacuole. The expression of BG2:GFP was examined by immunoblot analysis using anti-GFP antibody. A protein band was detected at the 98 kD position, which is the expected position of the BG2:GFP fusion protein (Figure 6A).

To confirm further the targeting of BG2 to the vacuole, we examined the amino acid sequence of the N-terminal region of BG2 and found a sequence motif (NADARP) that is similar to the vacuolar sorting motif (NPIR) found in the N-terminal region of sporamin (Figure 6B) (Matsuoka and Nakamura, 1991; Hwang, 2006). To test whether this motif plays a role in vacuolar targeting, we generated a chimeric GFP construct, whose N-terminal segment contained the first 40 amino acid residues of BG2 (AtBG2N40:GFP), and a mutant construct (BG2N40[4A]:GFP) with Ala substitutions in the sequence motif (NADARP) (Figure 6B). Protoplasts were transformed with BG2N40:GFP or BG2N40[4A]:GFP, and their localization was examined under a fluorescence microscope. BG2N40:GFP was targeted to the vacuole, but BG2N40[4A]:GFP produced a network pattern (Figure 6C), indicating the ER localization. It has been proposed that sorting of vacuolar proteins is initiated at the ER (Niemes et al., 2010). These results strongly suggest that the sequence motif NADARP located at the N-terminal region of BG2 is responsible for the vacuolar targeting of BG2.

BG2 Overexpression Results in Complementation of the Loss of BG1 in Vivo

To obtain independent evidence for its localization, we purified the vacuoles from transgenic plants expressing BG2:HA by ultracentrifugation using a Picoll gradient (Kim et al., 2005) and analyzedvacuolar proteins together with total protein extracts of protoplasts by immunoblotting using anti-HA antibody. Consistent with the in vivo localization using BG2:GFP, ~80% of total BG2:HA was detected in the vacuolar fraction (Figure 6D). These results confirm that the majority of BG2 localizes to the vacuole. The localization of BG2 differs from that of BG1, which localizes to the ER (Lee et al., 2006). In addition, these results suggest that ABA may be produced in the vacuole.
novo biosynthetic pathway, results in high levels of ABA in plants (Thompson et al., 2000). The leaf yellowing phenotype, one of the most visible phenotypes of \textit{bg1} plants, was no longer observed in either transgenic plant (Figures 7B and 7C), indicating that overexpression of \textit{BG2}:HA or \textit{NCED3}:HA complemented the leaf yellowing phenotype of \textit{bg1} plants. The degree of complementation was quantified by measuring the chlorophyll contents, which showed that overexpression of \textit{BG2}:HA increased chlorophyll contents from 60 to 80% that of wild-type (Col-0) plants (Figure 7D), indicating that overexpression of \textit{BG2}:HA partially complemented the leaf yellowing phenotype of \textit{bg1}. By contrast, \textit{NCED3}:HA overexpression resulted in complete complementation of the leaf yellowing phenotype (Figures 7B and 7D), consistent with previous results that showed that NCED greatly enhances the production of ABA (Thompson et al., 2000). The expression of \textit{BG2}:HA and \textit{NCED3}:HA was determined by immunoblot analysis using anti-HA antibody (Figure 7E). The difference in the degree of complementation of the \textit{bg1} phenotype between \textit{BG2}:HA and \textit{NCED3}:HA may reflect the amount of ABA produced by these proteins. Another possibility is that the ABA produced in the vacuole by \textit{BG2} may contribute indirectly to the active pool of ABA involved in ABA-mediated signaling; ABA must be transported from the vacuole to the cytosol to induce the signaling. These results suggest that higher activity in one pathway of ABA production can complement the loss of ABA production in other pathways.

To gain further insight into the relationship between \textit{BG1} and \textit{BG2}, we generated a \textit{bg1 bg2} double mutant (Figure 8A) and examined the phenotype in terms of water relations. The aerial parts of wild-type Col-0, \textit{bg1}, \textit{bg2}, and \textit{bg1 bg2} plants were excised and their water loss was examined under dehydration conditions. As observed previously (Lee et al., 2006), \textit{bg1} plants displayed an increased water loss compared with the wild-type plants (Figures 8B and 8C). The \textit{bg2} mutant also showed a slight

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Figure 6. \textit{BG2} Localizes to the Vacuole.

(A) Localization of \textit{BG2}:GFP. Protoplasts were prepared from transgenic plants harboring \textit{BG2}:GFP or \textit{GFP} alone, and localization of \textit{BG2}:GFP and \textit{GFP} alone was examined. Total proteins from these transgenic plants together with wild-type plants were also analyzed by immunoblot analysis using anti-GFP antibody. Asterisk indicates a nonspecific band detected by anti-GFP antibody. CH, red autofluorescence of chlorophyll; WT, nontransformed plants. Bars = 20 μm.

(B) Schematic presentation of N-terminal fusion constructs. The N-terminal segment containing the first 40 amino acid residues was fused to GFP to give \textit{BG2N40}:GFP. The sequence motif NADARP was substituted with Ala residues to give \textit{BG2N40}[4A]:GFP.

(C) Localization of \textit{BG2N40}:GFP and \textit{BG2N40}[4A]:GFP. Protoplasts from wild-type plants were transformed with \textit{BG2N40}:GFP or \textit{BG2N40}[4A]:GFP, and the localization of these proteins was examined. Bars = 20 μm.

(D) Localization of \textit{BG2}:HA in the vacuole. Protoplasts were prepared from transgenic plants expressing \textit{BG2}:HA and subjected to ultracentrifugation on a Picoll step gradient. Proteins were prepared from the top fraction that contained vacuoles and analyzed by immunoblotting using anti-HA antibody. Total protein extracts from protoplasts were included. As controls for the fractionation, BiP and AALP were detected with anti-BiP and anti-AALP antibodies, respectively.
increase in water loss compared with the wild-type (Col-0) plants. Moreover, water loss was additive in the bg1 bg2 double mutant, indicating that the two β-glucosidases contribute independently to the dehydration stress response. One possible explanation for the higher water loss of bg2-2, bg1, and bg1 bg2-2 double knockout plants could be the impairment of dehydration stress–induced ABA production, which leads to defective stomata closure in response to drought stress conditions. To confirm this finding, Col-0, bg1, bg2, and bg1 bg2 plants grown for 3 weeks in the soil under normal growth conditions were kept in a greenhouse without watering for 10 d. Two days after rewatering, the survival rate was determined (Figures 8D and 8E). Among these plants, the bg1 bg2 double mutant plants were most sensitive to dehydration stress followed by bg1 plants, consistent with the water loss results in these plants.

BG2 Exists as a High Molecular Weight Form under Normal Growth Conditions, and Its Levels Increase upon Dehydration Stress

Next, we investigated the biochemical properties of BG2. In a previous study, it was shown that BG1 undergoes polymerization into high molecular mass complexes under dehydration stress (Lee et al., 2006). The protein extracts of transgenic plants expressing BG2:HA were separated by gel filtration, and these fractions were analyzed by immunoblotting using anti-HA antibody. The large subunit of the ribulose-1,5-bis-phosphate carboxylase/oxygenase complex (RbcL) stained with Coomassie blue was used as a loading control. The large size of the RbcL marker confirmed that the gel filtration was successful. The protein extracts of transgenic plants expressing BG2:HA under control of the CsVMV promoter were analyzed by immunoblotting using anti-HA antibody. The amount of BG2:HA increased fivefold upon dehydration stress (Figure 9B) compared with that in normal growth conditions. As controls, endogenous binding protein (BiP) and aleurain-like protein (AALP), which localize to the ER and vacuole, respectively, were detected using anti-BiP and anti-AALP antibodies, respectively (Sohn et al., 2003). These proteins were present at similar levels in both growth conditions, indicating that a dehydration stress–induced increase in protein level was specific to BG2:HA. Since BG2:HA in the transgenic plants was under the control of the CsVMV promoter, which is not affected by dehydration stress, the increase in BG2:

Figure 7. Overexpression of BG2 Partially Complements the Mutant Phenotype of bg1 Plants.

(A) ABA levels in bg2-2 and wild-type plants. Plants grown on MS plates for 14 d were used to measure the total ABA level. Endogenous ABA levels of whole plants were determined as described in Methods. Error bars indicate sd (n = 3); FW, fresh weight of leaf tissues. (B) to (D) Complementation of the leaf yellowing phenotype by BG2 overexpression. BG2:HA under control of the CsVMV promoter was introduced into the bg1 mutant. As a control, NCED3:HA was introduced into the bg1 mutant. The two independent lines of BG2:HA/bg1 and NCED3:HA/bg1 transgenic plants were grown on MS plates. Images of single plants (B) or whole plates (C) were taken 10 d after planting. To quantify the leaf yellowing phenotype, chlorophyll a/b contents were measured (D). Error bars indicate sd (n = 10). Col-0, wild-type plants; P1 < 0.01 and P2 < 0.05 (P1 and P2 by Student’s t test). (E) Expression of BG2:HA and NCED3:HA. Protein extracts from transgenic plants were used for immunoblot analysis using the anti-HA antibody. The large subunit of the ribulose-1,5-bis-phosphate carboxylase/oxygenase complex (RbcL) stained with Coomassie blue was used as a loading control.
HA was likely caused by a posttranslational mechanism. One possibility is that BG2:HA in the vacuole is subjected to continuous degradation under normal growth conditions but is protected from degradation under dehydration stress.

**DISCUSSION**

Among the large number of β-glucosidases found in *Arabidopsis*, BG2 belongs to the same subfamily as BG1 that consists of 16 members (Xu et al., 2004; Lee et al., 2006). Similar to BG1, BG2 hydrolyzes ABA-GE to produce ABA. However, it has not been tested whether or not other β-glucosidase homologs in this BG1 clade have ABA-GE hydrolyzing activity. These β-glucosidase homologs can be divided into two groups: Seven members with an ER retrieval signal constitute one group, to which BG1 belongs, and the other nine members without the ER retrieval signal constitute another group, to which BG2 belongs. Among these proteins, At1g66270 (PSR3.2) (Malboobi and Lefebvre, 1997), At3g09260 (PYK10) (Matsushima et al., 2003), and At3g60140 (DIN2) (Fujiki et al., 2001) are thought to be involved in phosphate starvation, defense response, and senescence, respectively. Furthermore, At1g75940 (ATA27) is an anther-specific gene (Rubinelli et al., 1998). These results imply that these β-glucosidase homologs are involved in diverse cellular responses. Their exact physiological role may

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**Figure 8. bg1 bg2 Double Mutants Show Higher Sensitivity to Dehydration Stress.**

(A) Genotyping of the bg1 bg2 double mutant. Total genomic DNA from the indicated plants was used for PCR-based genotyping using gene-specific primers.

(B) and (C) Comparison of water loss among various types of plants. The aerial part of Col-0 (the wild type), bg1, bg2, and bg1 bg2 double mutants grown on MS plates for 2 weeks were excised and dehydrated for 20 min after excision.

(B) Images of the indicated plants exposed to dehydration stress for 20 min after excision.

(C) To quantify water loss, the weight of the plant tissues was measured at the indicated time points. Error bars indicate SD (n = 10).

(D) and (E) Comparison of dehydration stress sensitivity among various types of plants.

(D) Col-0, bg1, bg2, and bg1 bg2 plants grown for 3 weeks in the soil under normal growth conditions (watered) were kept in a greenhouse without watering for 10 d (drought) and then grown for 2 d after rewatering (Re-watered).

(E) Survival rate was calculated from the results of five independent experiments. Error bars indicate SD (n = 5).
depend on the biochemical reactions performed by these individual proteins. Sequence similarity may not provide any clue for their biochemical reactions and substrates of these proteins. In fact, BG2 that is most distantly related to BG1 in this clade hydrolyzes ABA-GE, the conjugated inactive form of ABA that is stored in the vacuole (Cutler and Krochko, 1999; Zeevaart, 1999; Seo and Koshiba, 2002). Interestingly, they localize to two different compartments: the ER and vacuole. One possible explanation is that the amino acid sequence difference between BG1 and BG2 may reflect the difference in their subcellular localization. For example, a protein localized to the vacuole contains a vacuolar targeting signal, whereas an ER-localized protein contains an ER retrieval motif, which may result in divergence in amino acid sequence. Indeed, BG2 contains a sequence motif at the N-terminal region for the vacuolar targeting. This conclusion is based on the results of in vivo localization of BG1, which localizes to the ER, BG2 localizes to the vacuole. In addition, these organelles may provide different conditions for biochemical reactions. In fact, the vacuole and the ER have acidic and neutral pH, respectively. These differences may also cause sequence divergence.

The ABA-hydrolyzing activity of BG2 was confirmed using two different approaches. The first showed that immunopurified BG2:HA was able to hydrolyze ABA-GE to ABA in vitro, and the second showed that overexpression of BG2:HA, as well as BG1:HA, in protoplasts resulted in increased ABA levels via the hydrolysis of exogenously supplied ABA-GE. Furthermore, protoplasts from bg2, bg1, or bg1 bg2 mutant plants showed low levels of ABA-GE hydrolyzing activity. The significance of the ABA-GE hydrolyzing activity of BG2 in plants was demonstrated using bg2 mutants. Four different alleles of bg2 displayed increased sensitivity to NaCl and dehydration stresses. By contrast, overexpression of BG2:HA under the strong constitutive promoter CsVMV gave rise to enhanced resistance to NaCl and dehydration stresses. However, the cellular levels of ABA in bg2-2 plants under normal growth conditions did not differ significantly from those of wild-type plants. One possible explanation for this is that the ABA produced by BG2 may not contribute significantly to the total cellular ABA pools. Consistent with this notion, bg2 mutants displayed a much milder phenotype than did bg1 mutants. A second possible explanation is that the cellular ABA levels in bg2-2 are maintained at wild-type levels by adjusting other biosynthetic pathways to compensate for the loss of BG2. This possibility was supported by the fact that overexpression of BG2:HA complements the bg1 mutant phenotype, as observed for the complementation of the bg1 mutant phenotype by overexpression of NCED3:HA.

The identification of a second β-glucosidase homolog that hydrolyzes ABA-GE to produce ABA in cells raises many interesting questions, such as why plants have multiple β-glucosidase homologs for the hydrolysis of ABA-GE. In contrast with BG1, which localizes to the ER, BG2 localizes to the vacuole. This conclusion is based on the results of in vivo localization of BG2:GFP and subcellular fractionation of BG2:HA. Furthermore, the fact that the N-terminal region of BG2 contains a sequence motif NADARP for vacuolar targeting supports this conclusion. Of course, we cannot completely exclude the possibility that overexpression of BG2:HA or BG2:GFP from the strong CsVMV promoter may result in their vacuolar localization in transgenic plants. Thus, the different subcellular locations of BG1 and BG2, to the ER and central vacuole, respectively, may provide a clue to answer the question of why plant cells contain multiple ABA-GE hydrolyzing β-glucosidases (Lee et al., 2006). Perhaps the local concentration of ABA, rather than the overall cellular ABA level, is critical for initiating ABA-mediated signaling in particular processes. In this hypothesis, organelle-specific BG isoforms may be involved in the rapid fine-tuning of the local concentrations to meet the cellular demand under constantly changing environmental and developmental conditions (Lee et al., 2006). For example, BG1 and BG2 may be responsible for increasing
ABA levels in the ER and vacuole, respectively. Extracellular ABA-GE hydrolyzing activity, the details of which remain unknown, may be responsible for increasing the ABA level in the apoplast (Dietz et al., 2000; Lee et al., 2006). Clearly, the one-step hydrolysis of ABA-GE to ABA is a fast process compared with the lengthy de novo biosynthetic pathway (Cutler and Krochko, 1999; Zeevaart, 1999; Seo and Koshiaba, 2002; Nambara and Marion-Poll, 2005); thus, this pathway is better suited to meet the rapid demands for ABA at the local level. The local ABA concentration may be perceived by ABA receptors localized to particular subcellular compartments. In fact, plant cells are thought to contain multiple types of ABA receptors in multiple subcellular compartments (Anderson et al., 1994; Allan et al., 1994; Levchenko et al., 2005; Razem et al., 2006; Shen et al., 2006; Ma et al., 2009; Pandey et al., 2009; Park et al., 2009). Among these, RCAR/PYR and their homologs are localized to the cytosol, and the GPCR-type G proteins GTG1 and GTG2 are localized to the plasma membrane. In addition, the Mg-chelatase H subunit, which binds to ABA and functions in ABA-mediated signaling, localizes to the chloroplasts (Shen et al., 2006; Wu et al., 2009). Interestingly, the effect of mutations in BG1 and BG2 appears to be additive in responses to dehydration stress. However, how ABA produced in the ER or vacuole activates the ABA-mediated signaling in plant cells is currently not known.

How are the biosynthetic pathways of ABA coordinated to maintain the homeostasis of cellular ABA levels? ABA is produced by two mechanisms: the de novo biosynthetic pathway and the hydrolysis of ABA-GE to ABA by β-glucosidases (Cutler and Krochko, 1999; Zeevaart, 1999; Seo and Koshiaba, 2002; Lee et al., 2006). All steps in the de novo ABA biosynthetic pathway occur in plastids, with the exception of the last two steps, which occur in the cytosol (Cutler and Krochko, 1999; Zeevaart, 1999; Seo and Koshiaba, 2002). By contrast, the hydrolysis of ABA-GE to ABA by β-glucosidases takes place in two endomembrane compartments: the ER and the vacuole (Lee et al., 2006; this study). Interestingly, a loss of one ABA production pathway can be compensated for by the overexpression of genes in other ABA production pathways, as demonstrated by the fact that BG2 or NCED3 overexpression can complement the bg1 mutant phenotype. Thus, these results raise an intriguing possibility that multiple ABA production pathways need to be coordinated to achieve certain cellular ABA levels. An interesting feature of BG1 is its dehydration stress–induced activation, which is mediated by its assembly into high molecular weight forms (Lee et al., 2006). In contrast with BG1, BG2 exists as a high molecular weight form under normal growth conditions, which implies that it employs a different mechanism to increase ABA levels in the vacuole in response to dehydration or high NaCl stresses. BG2 appears to be degraded continuously in the vacuole and thus is maintained at a low level under normal growth conditions. This is not surprising, since many proteases are active in the vacuole (Rojo et al., 2003). However, the levels of BG2-HA were greatly increased in response to dehydration stress through an unknown mechanism. High levels of BG2 can increase cellular ABA levels through hydrolysis of stored ABA-GE. Currently, it is not fully understood how the degradation of BG2 in the vacuole is inhibited under dehydration stress conditions.

In conclusion, we provide evidence that BG2 localizes to the vacuole and assists in the production of ABA from ABA-GE under dehydration stress conditions. Based on these results and previous results relating to BG1, we propose that multiple isoforms of β-glucosidases that are localized to the ER, the central vacuole, or possibly the apoplast increase local ABA concentrations through the hydrolysis of ABA-GE in these compartments, which in turn results in a response to specific developmental and environmental signals.

METHODS

Plant Growth Conditions

Arabidopsis thaliana plants (ecotype Col) were grown either on MS plates at 20°C in a culture room or in a greenhouse with 70% relative humidity and a 16-h/8-h light/dark cycle. Various parts of the plants were harvested and frozen immediately in liquid nitrogen. For NaCl or ABA treatment, plants grown in MS liquid medium for 1 week were treated with 150 mM NaCl or 100 μM ABA, respectively, for the indicated periods of time, as described previously (Piao et al., 1999).

For growth measurements, 5-d-old seedlings grown on MS plates containing 3% Suc and 0.8% agar, pH 5.7, were transferred to half-strength MS plates supplemented with 125 mM NaCl. Root growth was measured 9 d after the plants were transplanted. For the dehydration stress experiment, 3-week-old plants grown in soil under normal watering conditions were kept in a greenhouse without watering for 10 d. Plants were then allowed to recover for 2 d after rewatering. Isolation of bg1 mutant plants was described previously (Lee et al., 2006).

Construction of Plasmids

The BG2 cDNA was isolated from a cDNA library by PCR using the gene-specific primers BG2-5 and BG2-3 (see Supplemental Table 1 online). The HA epitope was added to the C terminus of BG2 by PCR using the primers BG2-HA-5 and BG2-HA-3. To generate the BG2-HA-5 and BG2-HA-3 constructs, the BG2 cDNA was subcloned into 326-GFP (Jin et al., 2001) vector using the BamHI site. The BG2-[303] mutant construct was produced by PCR using the primers BG2-3/5 and BG2-3/3, and the point mutant BG2-[E426/Q] was produced by PCR using the primers BG2-E/Q5-5 and BG2-E/Q5-3, and BG2-E/Q3-5 and BG2-E/Q3-3. Both of the PCR fragments were inserted into pBl21 using XbaI and BamHI sites. To generate BG2N40[4A]:GFP, the N-terminal segment containing the first 40 amino acid residues was obtained by PCR using primers BG2-N40-5 and BG2-N40-3. To construct BG2N40[4A]:GFP, Ala substitutions were introduced into BG2N40 by PCR using primers BG2N40-4/4A-5 and BG2N40-4/4A-3. The resulting PCR fragments were fused to the N terminus of GFP by inserting the PCR fragments using XbaI and BamHI sites of 326-GFP (Jin et al., 2001). In addition, NCED3-HA was generated by PCR using primer NCED3-HA-5 and NCED3-HA-3. These constructs were transferred to a pCsV1300 (Invitrogen) binary vector containing the CsMV promoter using XbaI and BamHI restriction enzyme sites. The nucleotide sequences of all of the PCR products were confirmed.

Screening of Arabidopsis Mutants and RT-PCR Analysis of Transcripts

A T-DNA insertion population of Arabidopsis plants (ecotype C24) expressing the transgene RD29Ap:LUC (Ishitani et al., 1997; referred to as the C24 wild type) was generated using Agrobacterium tumefaciens–mediated transformation. The T4 seeds of transgenic plants were used to screen for mutants that exhibit altered expression of RD29Ap:LUC in
response to NaCl stress, using a luminescence imaging system, as described (Ishitani et al., 1997).

Genomic DNA was prepared from los15, a putative mutant and used to isolate a flanking region to the T-DNA insertion site by the thermal asymmetric interlaced-PCR approach using the primer LB3 and gene-specific primers BG1p (forward primer) and BGP2 (reverse primer). The nucleotide sequence of the PCR product was then determined. In the case of bg2-2, left border primer and gene-specific primers (BG2-RP2 and BG2-LP2 for BG2) were used to confirm the insertion of T-DNA (Liu et al., 1995). bg2-2 mutants were crossed to wild-type plants (Col-0) to clean up the background. Additional bg2 mutant alleles were screened by PCR using the primers BG2-LP3 and BG2-RP3 for bg2-3 and BG2-LP4 and BG2-RP4 for bg2-4.

Total RNA was isolated from leaf tissues, and BG2 transcript levels were determined by RT-PCR using BG2-specific primers BG2-15 and BG2-13. As controls, tubulin and 18S rRNA levels were determined using the primers Tub-5 and Tub-3 for tubulin and 18S-5 and 18S-3 for 18S rRNA. For RT-PCR analysis, a reverse transcription reaction was performed with 1 µg of total RNA using Superscript RT (Gibco BRL) and 100 ng of a random primer in a 20-µL reaction volume. PCR was performed in a 50-µL reaction volume using 2 µL of the RT reaction with conditions of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s for a total of 15 to 35 cycles, depending on the expression levels of the genes.

To generate a bg1 bg2 double mutant, bg1 was crossed to bg2 and double mutants were screened by PCR genotyping using the gene-specific primers BG1-5 and BG1-3 for BG1 and BG2-t25 and BG2-t23 for BG2.

Generation of Transgenic Plants

To generate BG2::GFP transgenic plants, the BG2::GFP fragment was amplified by PCR using primers BG2-GFP-5′ and BG2-GFP-3′, and the product was inserted into a binary vector pCvs1300 (Invitrogen) using the BglII site. To generate BG2::HA transgenic plants, the BG2 cDNA was amplified by PCR using primers BG2-HA-5′ and BG2-HA-3′, and the product was inserted into pCvs1300 using the BamHI site. To generate the BG2::GUS construct, the 2.0-kb promoter region of BG2 was amplified by PCR using the gene-specific primers BG-p5 and BG-p3, and the BG2 promoter was inserted into pCAMBIA3301 (Invitrogen) using BamHI and NcoI sites. To generate bg1 plants that harbor NCED3::HA, NCED3::HA was generated by PCR using primers NCED3-HA-5′ and NCED3-HA-3′, and the product was inserted into a binary vector pCvs1300 (Invitrogen) using XbaI and BamHI restriction enzyme sites. These binary vectors were used to transform wild-type or bg1 plants, according to a published protocol (Clough and Bent, 1998). Transgenic plants were screened on MS plates supplemented with hygromycin (25 mg/L) or phosphinothricin (25 mg/L).

In Vivo Localization of BG2 in Protoplasts

To investigate the subcellular localization of BG2::GFP, protoplasts were prepared from transgenic plants expressing BG2::GFP, and the localization of BG2::GFP was examined using fluorescence microscopy (Jin et al., 2001; Bae et al., 2008). Images were processed using Adobe Photoshop.

Preparation of Protein Extracts and Immunoblot Analysis

Total protein extracts were prepared from transformed protoplasts or protoplasts isolated from transgenic plants as described previously (Jin et al., 2001; Hyunjong et al., 2006). To purify vacuoles, protoplasts from transgenic plants expressing BG2::HA were subjected to ultracentrifugation on a step Picoll gradient as described previously (Kim et al., 2005). The top fraction, which contained the central vacuole, was collected, separated from the gradient, and used to prepare vacuolar proteins.

Immunoblot analysis was performed using anti-GFP (Bio-Application), anti-HA (Roche), anti-BiP (Park et al., 1997), and anti-AALP (Sohn et al., 2003) antibodies as described previously (Jin et al., 2001). The protein blots were developed with an ECL Detection Kit (Amersham Pharmacia Biotech), and images were obtained using an LAS3000 image capture system (FUJIFILM).

Fractionation of BG2::HA Using Gel Filtration Column Chromatography

To fractionate BG2::HA from BG2::HA plants, leaf tissue was homogenized in homogenization buffer (50 mM sodium phosphate, pH 7.0, 0.15 M NaCl, 0.02% NaN3, 1.0 mM DTT, and 0.1% Triton X-100). Homogenates were centrifuged at 14,000g for 5 min to remove debris, and the supernatant was applied to a Sephacyr S-300 high-resolution column (Park et al., 1997; Kim et al., 2001). Proteins were eluted using the homogenization buffer without Triton X-100 at a flow rate of 0.5 mL/min in a fraction volume of 3.0 mL. Fractions were analyzed by immunoblotting using an anti-HA antibody. BSA and endogenous RbcL (the large subunit of the ribulose-1,5-bis-phosphate carboxylase/oxygenase complex) were used as markers for the position of eluted proteins in the gel and visualized by Coomassie Brilliant Blue staining.

Hydrolyzing Activity Assay of BG2 on ABA-GE in Vitro and in Protoplasts

Immunopurification was performed as described previously (Cutler, 2004; Park et al., 2005). Briefly, protein extracts (100 µg of total protein or 30 µg of vacuolar proteins) in immunoprecipitation buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.0 mM EDTA, 0.2 mM PMSF, and 1% [v/v] Triton X-100) supplemented with an EDTA-free protease inhibitor cocktail were first incubated with Protein A-Sepharose (CL-4B, Amersham) for 30 min and then centrifuged at 10,000g for 5 min at 4°C for preclearing. Subsequently, 4 µg of anti-HA antibody (12CA5; Roche Diagnostics) was added to the supernatant and incubated for 3 h at 4°C. To precipitate the immunocomplexes, protein A-agarose was added to the protein/antibody mixture and further incubated for 1 h at 4°C. The pellet was then washed three times with immunoprecipitation buffer and suspended in homogenization buffer. The precipitates were used to determine ABA-GE hydrolyzing activity and for immunoblot analyses.

The ABA-GE hydrolyzing activity of BG2 in vitro was determined as described previously (Lee et al., 2006). The immunopurified proteins were incubated with ABA-GE (1 nmol) (Apex Organics) in 1.0 mL of 100 mM citrate buffer, pH 5.5, for 1.5 h at 37°C, and reaction mixtures were subsequently extracted with chloroform/methanol and fractionation by HPLC using a prepacked RT 250-4 column (Merck) containing 40% methanol, 0.1 M acetic acid, and 10 mM butylated hydroxytoluene as the solvent. The fractions were analyzed by ELISA using anti-ABA antibody.

To examine the hydrolyzing activity of ABA-GE by BG2 in vivo, protoplasts (1 × 104) from various types of plant were transformed with BG1 (Lee et al., 2006), BG2, or empty expression vector R6 (Kim et al., 2005). The transformed protoplasts were incubated for 24 h to allow expression of the proteins and then further incubated in the presence of exogenous ABA-GE (10 µM) for an additional 4 h at room temperature. ABA was extracted using 80% methanol, and ABA levels were determined by ELISA using an anti-ABA antibody (Lee et al., 2006).

Real-Time qRT-PCR

Plants grown in a liquid culture were subjected to 300 mM NaCl stress and 100 µM ABA treatment as described previously (Jang et al., 1998). Total RNA was extracted from 2-week-old whole plant using a Qiagen RNeasy Plant Mini Kit and then digested with TURBO DNase (Ambion). Extracted RNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time RT-PCR was performed using a SYBR Green Kit (Applied Biosystems) to
detect BG2 levels. ACT2 was used as an internal control. Primer sequences were as follows: BG-135 and BG-133 for BG2, ACT2-5 and ACT2-3 for ACT2 (At3g18780), RD29A-5 and RD29A-3 for RD29A, RD29B-5 and RD29B-3 for RD29B, and COR47-5 and COR47-3 for COR47.

Measurement of Endogenous ABA Levels

Approximately 100 mg of leaf tissue was used for the ABA measurements. Leaf samples were lyophilized before extraction. Samples were homogenized with 1 mL of 80% acetone:20% water (v/v) containing 1% (v/v) acetic acid in a 2-mL round-bottom tube using 3-mm ceramic beads and a vortex. After d6-ABA (Isotec) was added as an internal standard, the homogenate was incubated for 12 h at 4°C in darkness and then centrifuged at 14,000 g for 10 min at 4°C. The precipitate was then re-extracted for 1 h, and the combined supernatant was dried under vacuum. Following resuspension in 1 mL 99% isopropanol:1% acetic acid (v/v) by vortexing and sonication, samples were centrifuged (14,000 g for 5 min, 4°C), and the supernatant was transferred to a fresh tube and then dried again. Samples were dissolved in 50 μL of methanol and a further 450 μL of 1% acetic acid solution (v/v) was added. Oils in the samples were again removed to a fresh tube and dried by centrifugation under vacuum. Following resuspension in 1 mL 99% isopropanol:1% acetic acid (v/v) by vortexing and sonication, samples were centrifuged (14,000 g for 5 min, 4°C), and the supernatant was transferred to a fresh tube and then dried again. Samples were dissolved in 50 μL of methanol and a further 450 μL of 1% acetic acid solution (v/v) was added. Oils in the samples were again removed to a fresh tube and dried by centrifugation under vacuum. Extracts were dissolved in 100 μL of methanol, and 900 μL of 1% acetic acid solution (v/v) was added. Oasis HLB 1-mL solid-phase extraction cartridges (Waters) were conditioned with 1 mL of acetonitrile followed by 1 mL of methanol and equilibrated with 1 mL of 1% acetic acid solution (v/v). Samples were loaded onto the cartridges and then washed with 1 mL of 1% acetic acid solution (v/v). ABA was eluted with 1 mL 50% acetonitrile, 49% water:1% acetic acid (v/v) before samples were dried by centrifugation under vacuum. Oasis cartridges (Waters) were conditioned with 1 mL of acetonitrile followed by 1 mL of 1% acetic acid solution (v/v) was added. Oasis HLB 1-mL solid-phase extraction cartridges (Waters) were conditioned with 1 mL of acetonitrile followed by 1 mL of 1% acetic acid solution (v/v). ABA was eluted with 1 mL 50% acetonitrile, 49% water:1% acetic acid (v/v) before samples were dried by centrifugation under vacuum. The resulting sample mixture was dissolved in a final volume of 20 μL of water, and 10 μL was injected into the cartridge. ABA was quantified using a liquid chromatography–tandem mass spectrometry system (Agilent 1200 series, Agilent 6410). The chromatograph, which was equipped with a 2.1 × 50-mm, 1.8-μm ZORBAX XDB C18 column (Agilent), was used with a binary solvent system comprising water containing 0.01% acetic acid (A) and acetonitrile containing 0.05% acetic acid (B). Separations were performed using a gradient of increasing solvent B content with a flow rate of 0.2 mL min⁻¹. The gradient was increased linearly from 3% B to 50% B over a 15-min period and then to 98% B at 16 min. After 5 min at 98% B, the initial condition was restored and allowed to equilibrate for 5 min. The retention time of ABA was 10.8 min. tandem mass spectrometry transition conditions were as follows: gas temperature (°C) = 300, gas flow (L/min) = 9, nebulizer (p.s.i.) = 30, capillary (V) = 4000, fragmentor (V) = 140, collision energy (V) = 8.0, and tandem mass spectrometry transition (mass-to-charge ratio): 263/153 (ABA), 269/159 (D6-ABA). The amount of each compound was calculated using the spectrometer software (MassHunter Qualitative Analysis; Agilent).

Measurement of Chlorophyll a/b Content

To extract chlorophyll from leaf tissue, leaf tissue from 2-week-old plants was treated with 50 volumes of 95% ethanol for 20 min at 80°C. The amount of chlorophyll was calculated as described by Vernon (1960).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: BG2, At2g32860; BG1, At1g52400; NCED3, At1g14440; RD29A, At1g52310; RD29B, At1g52300; COR47, At1g20440; ACTIN2, At1g18780; TUBULIN, At1g04820; 18s rRNA, At2g01010; BiP, At1g9080; and AALP, At5g60360.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Sequence Alignment of At-BG2 with At-BG1.

Supplemental Figure 2. Phylogenetic Tree of β-Glucosidase Homologs in Arabidopsis.

Supplemental Figure 3. Expression Levels of Osmotic Stress-Inducible Genes in Two Different Alleles of the bg2 Mutants.

Supplemental Figure 4. Screening of New bg2 Mutant Alleles and Their Phenotype under High NaCl Stress.

Supplemental Figure 5. BG2 Is Induced by Exogenously Applied ABA and NaCl Stress and Shows a Tissue-Specific Expression Pattern.

Supplemental Table 1. Sequences of Primers Used in This Study.

Supplemental Data Set 1. Text File of the Alignment Used for the Phylogenetic Analysis in Supplemental Figure 2A.

Supplemental Data Set 2. Text File of the Alignment Used for the Phylogenetic Analysis in Supplemental Figure 2B.

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AUTHOR CONTRIBUTIONS

Z.-Y.X., K.H.L., and I.H. conceived the project and designed the research strategies. Z.-Y.X. conducted the majority of experiments. K.H.L. isolated bg2-2 and performed experiments for ABA-GE hydrolizing activity of At-BG2. T.D., S.Y.K., and S.Y.K. carried out the real-time qRT-PCR and complementation of bg7 with NCED3 and BG2. R.A.B. and D.-J.Y designed osmotic stress-defective mutant screening strategies. J.C.J. and J.B.J. isolated the original mutant los15 and characterized the phenotype of los15. Y.K. and M.S. performed the measurement of endogenous ABA levels. I.H. and Z.-Y.X. wrote the article. All authors contributed to writing the article.

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