Mutations in the Hyperosmotic Stress-Responsive Mitochondrial *BASIC AMINO ACID CARRIER2* Enhance Proline Accumulation in Arabidopsis[1][C][W]

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Mitochondrial carrier family proteins are diverse in their substrate specificity, organellar location, and gene expression. In Arabidopsis (*Arabidopsis thaliana*), 58 genes encode these six-transmembrane-domain proteins. We investigated the biological role of the basic amino acid carrier Basic Amino Acid Carrier2 (BAC2) from Arabidopsis that is structurally and functionally similar to ARG11, a yeast ornithine and arginine carrier, and to Arabidopsis BAC1. By studying the expression of *BAC2* and the consequences of its mutation in Arabidopsis, we showed that BAC2 is a genuine mitochondrial protein and that Arabidopsis requires expression of the *BAC2* gene in order to use arginine. The *BAC2* gene is induced by hyperosmotic stress (with either 0.2 M NaCl or 0.4 M mannitol) and dark-induced senescence. The *BAC2* promoter contains numerous stress-related cis-regulatory elements, and the transcriptional activity of *BAC2:β-glucuronidase* is up-regulated by stress and senescence. Under hyperosmotic stress, *bac2* mutants express the *P5CS1* proline biosynthetic gene more strongly than the wild type, and this correlates with a greater accumulation of Pro. Our data suggest that BAC2 is a hyperosmotic stress-inducible transporter of basic amino acids that contributes to proline accumulation in response to hyperosmotic stress in Arabidopsis.

In plants, mitochondria are essential, as they provide energy through respiration and supply metabolites and precursors for sugar, organic acid, amino acid, and vitamin metabolism (Lunn, 2007; Smith et al., 2007). Through the photorespiratory pathway, mitochondria also link the carbon and nitrogen metabolism of the plant cell (Douce et al., 2001). Plant responses to stress and adaptation also involve mitochondria (Matos et al., 2007; Atkin and Macherel, 2009; Skirycz et al., 2009). These functions rely on the efficient transport of molecules between the cytosol and the mitochondrial matrix by protein transporters. The six-transmembrane-domain (6TM) mitochondrial carrier family (MCF) is a remarkably diverse group of transporter proteins. In Arabidopsis (*Arabidopsis thaliana*), there are over 58 genes encoding putative MCF members (Picault et al., 2004; Haferkamp, 2007).

MCF proteins are found in all eukaryotic cells. The primary structure of an MCF protein is of three imperfect tandem repeats each of about 100 amino acids, which form a secondary structure of two transmembrane helices separated by loops. Each odd-numbered helix harbors the signature motif PX[DE]XX[RK]. A structural model based on the ADP/ATP transporter from bovine heart mitochondria reveals that the MCF proteins are monomer transporters with selective binding sites at conserved positions on the even-numbered helices (Kunji and Robinson, 2006).

In vitro reconstitution of transport function has been invaluable in demonstrating biochemical functions of putative transporter molecules. For example, the adenylate translocator, dicarboxylic acid and tricarboxylic acid transporters, and ion carriers have been characterized this way (Palmieri et al., 2006a). Mitochondrial transporters in plants have been assessed by their similarities with known transporters from animal and yeast and by complementation of mutant cells: yeast or mammalian cells deficient in a known transporter function. However, complementation of nonplant mutants with plant cDNAs does not necessarily reflect the genuine cellular location of plant transporters. The yeast *Δmir1 Δpic2* mitochondrial phosphate transport mutant was complemented by two Arabidopsis phosphate carriers, mPiC1 and mPiC2 (Hamel et al., 2004), but in proteomic analysis, mPiC1 (At5g14040) was found in the chloroplast (Kleffmann et al., 2004). Mammalian folate-deficient mutant cells can also be complemented with the chloroplast-located folate transporter from Arabidopsis (Bedhomme et al., 2005).

Identification of the *Saccharomyces cerevisiae arg11* mutant, defective in the mitochondrial transport of Arg and Orn, has led to the discovery of basic amino

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acid transporters in fungi, mammals, and plants, even though the biology and physiology of basic amino acids differ in these organisms. The ARG11 protein has been described as an Orn, Arg, and Lys antiport protein (Palmieri et al., 1997), important for Orn export from the mitochondria and Arg synthesis in the cytosol (Crabeel et al., 1996). The Arabidopsis *BASIC AMINO ACID CARRIER1* (*BAC1*) and *BAC2* genes were identified by complementation of the yeast *arg11* mutation (Catoni et al., 2003; Hoyos et al., 2003). Both proteins have been confirmed to be basic amino acid carriers in vitro (Hoyos et al., 2003; Palmieri et al., 2006b). An interesting difference between BAC1 and BAC2 is in their substrate specificity, BAC2 being less specific for L-amino acids. Both genes are expressed at a low level during germination and seedling establishment (Catoni et al., 2003).

Major differences in basic amino acid metabolism in yeast, animals, and plants make it unlikely that carriers of basic amino acids play identical physiological roles in the mitochondria of different organisms. While in yeast ARG11 is involved in Arg synthesis, which occurs in the cytosol from Orn synthesized in the mitochondria, in plants Orn and Arg are synthesized from Glu. Most of the biosynthetic pathway in plants is plastidial. Orn can also be formed in mitochondria from the degradation of Arg by arginase (for review, see Slocum, 2005).

To investigate the compartmentalized nature of basic amino acid metabolism in plants, we investigated the importance of the Arabidopsis *BAC2* gene in basic amino acid transport in mitochondria. First, we found that *BAC2* gene expression is restricted to aerial parts of plants and is modulated by hyperosmotic stress. We showed that BAC2 is located in mitochondria and that overexpression of *BAC2* in transgenic plants allows plants to use Arg as a source of nitrogen. The involvement of *BAC2* in one aspect of the stress response was revealed by the altered amino acid homeostasis and Pro accumulation in *bac2* mutants. More Pro accumulates in *bac2*, the result of higher Pro biosynthesis gene expression. Taken together, our results indicate a unique role for basic amino acid mitochondrial transport in the hyperosmotic stress response.

RESULTS

BAC2 Is a Distinct Member of the MCF

The *BAC2* basic amino acid carrier gene (At1g79900; Catoni et al., 2003; Hoyos et al., 2003; Palmieri et al., 2006b) encodes a 1,455-bp transcript with a 296-codon open reading frame, the amino acid sequence being similar to 6TM MCF members. There is a single 185-bp intron in the *BAC2* gene at positions 575 to 759, 237 bp downstream of the ATG (Fig. 1A). BAC2, BOU (Lawand et al., 2002), and BAC1 (Hoyos et al., 2003) form a group of sequences distinct from other Arabidopsis mitochondrial carriers (Catoni et al., 2003; Hoyos et al., 2003; Picault et al., 2004). We compared BAC2 with related MCF proteins, some of known function, from Arabidopsis and yeast. The BAC2 amino acid sequence is no more closely related to the yeast basic amino acid carrier ARG11 than to the yeast carnitine carrier CRC1 or to YMC1 and YMC2, whose functions are unknown (Fig. 1B). Therefore, this makes it difficult to deduce BAC2 function based on sequence-based analysis alone but suggests that the function of BAC2 (and BAC1 and BOU) is distinct from the functions of other MCF members.

BAC2 Is a Mitochondrial Protein

It is important to verify the organellar localization of predicted mitochondrial proteins like BAC2, as MCF members may be mitochondrial, plastidial, or peroxisomal (Ferro et al., 2003). We transformed wild-type Arabidopsis plants with a fusion construct of *BAC2* and GFP coding sequences under the control of the 35S cauliflower mosaic virus promoter. Confocal microscopy of anther epidermal cells from transgenic plants showed GFP fluorescence in organelles of similar size and shape to mitochondria, clearly distinct from autofluorescing chloroplasts (Fig. 2, A–D). The GFP fluorescence was confirmed as being in mitochondria, as in leaf mesophyll cell protoplasts it colocalized with the mitochondria-specific fluorescent label MitoTracker (Fig. 2, E–H). This demonstrates that BAC2 is a mitochondrial protein.

BAC2 Transcription Is Up-Regulated by Stress

To assess how BAC2 might function in the whole plant, *BAC2* expression in wild-type Arabidopsis was analyzed in different physiological conditions. *BAC2* mRNA levels are low in all vegetative tissues, with higher expression in flowers and siliques (Supplemental Fig. S1). During germination and seedling establishment at the cotyledon stage, *BAC2* mRNA is barely detectable (Supplemental Fig. S2) but is more readily detected in dark-grown than in light-grown seedlings (Fig. 3A). Digital northern analysis of microarray hybridization data processed by the Genevestigator database (Zimmermann et al., 2004) revealed strong expression of *BAC2* in response to biotic and abiotic stresses, in response to abscisic acid, and during senescence (Supplemental Fig. S3). To verify the microarray data, we subjected 12-d-old wild-type seedlings to hyperosmotic (0.4 M mannitol) or saline (0.2 M NaCl) stress. *BAC2* mRNA accumulates in aerial parts of hyperosmotically stressed plants (Fig. 3B). Both treatments caused a sharp increase in *BAC2* mRNA levels after 3 h, with the increase being greater in response to hyperosmotic stress than to saline stress (Fig. 3B).

To investigate how *BAC2* transcription may be regulated, its promoter sequence was analyzed using PLACE (http://www.dna.affrc.go.jp/PLACE/; Higo et al., 1999) and putative cis-regulatory elements were drawn on the sequence map. A combination of cis-

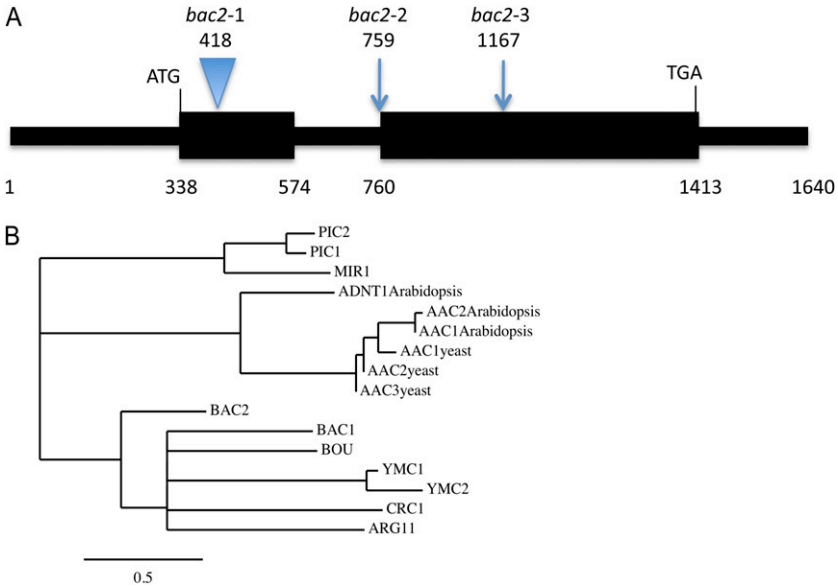

Figure 1. *BAC2* sequence analysis. A, Schematic representation of *BAC2*, including the position of the coding sequence (solid boxes). Numbers indicate the nucleotide positions from the transcription start. The positions of mutations are shown. The *bac2-1* T-DNA insertion is indicated by the triangle. Arrows indicate positions of the *bac2-2* and *bac2-3* point mutations. B, Maximum likelihood tree of selected mitochondrial carriers from yeast and Arabidopsis. The phylogenetic reconstruction was performed using the PhyML algorithm (Guindon and Gascuel, 2003) online (http://www.phylogeny.fr/version2_cgi/phylogeny.cgi; Dereeper et al., 2008) based on a multiple sequence alignment generated with MUSCLE (Edgar, 2004). All the branches are supported by bootstrap values higher than 50%. The tree was mid point rooted. The bar corresponds to 0.5 substitutions per site. Sequence references are as follows. Phosphate carriers: PiC2 (At3g48850), NP190454.1 (Arabidopsis); PiC1 (At5g14040), NP196908.1 (Arabidopsis); MIR1, NP012611.1 (*Saccharomyces cerevisiae*). ATP carriers: Aac1, NP013772.1 (*S. cerevisiae*); Aac2, NP009523.1 (*S. cerevisiae*); Aac3, NP009642.1 (*S. cerevisiae*); AAC1, NP850541.1 (Arabidopsis); AAC2, NP196853.1 (Arabidopsis). Basic amino acid carriers: BAC2 (At1g79900), NP178108.1 (Arabidopsis); BAC1 (At2g33820), NP180938.2 (Arabidopsis); ARG11, NP014773.1 (*S. cerevisiae*). Carnitine carrier-like: Crc1, NP014743.1 (*S. cerevisiae*); BOU (At5g46800), NP568670.1 (Arabidopsis). Others: YMC1, NP015383.1 (*S. cerevisiae*); YMC2, NP009662.1 (*S. cerevisiae*). [See online article for color version of this figure.]

regulatory elements was found in the *BAC2* promoters MycAtErd1, ABRELAtErd1, and ACGTErd1. These correspond to cis-acting elements found in the *ERD1* promoter that are known to be dehydration stress- and dark-induced senescence-responsive elements (Simpson et al., 2003; for review, see Yamaguchi-Shinozaki and Shinozaki, 2005). Three AS-1 motifs (Redman et al., 2002) were also found in the *BAC2* promoter, which could indicate that *BAC2* transcription is regulated by abiotic stresses. In the *BAC2* promoter sequence, there are also several pollen-specific cis-regulatory elements (Bate and Twell, 1998) and I-box cis-elements (GATAA; Terzaghi and Cashmore, 1995) that are involved in the response to light (Fig. 4A). This map of the *BAC2* promoter, therefore, suggests that the gene is likely to be regulated by biotic and abiotic stimuli, possibly those related to stress.

To study the promoter activity in vivo, wild-type Arabidopsis was transformed with a construct with the *BAC2* upstream sequence fused to the GUS gene. In *BAC2*:*GUS* transgenic plants grown in normal conditions, no staining was detected in vegetative tissues. However, GUS staining was observed in aerial parts of 12-d-old seedlings after 24 h of hyperosmotic stress (0.4 M mannitol; Fig. 4B); the cotyledons were GUS stained, but the immature first pair of leaves showed no GUS activity. Leaves detached from 4-week-old plants showed GUS staining when subjected to hyperosmotic (0.4 M mannitol) or saline (0.2 M NaCl) stress or dark-induced senescence (senescence induced by wrapping leaves in aluminum foil for 4 d), unlike the control (Fig. 4C). In flowers from soil-grown transgenic plants, anthers and pollen grains at stages 10 to 12 of flower development (Smyth et al., 1990) were GUS stained (Fig. 4D; Catoni et al., 2003). Later, when stamens dehisced, which correlates with natural senescence of the filament at stage 16 of flower development, the stamen filament showed strong GUS staining (Fig. 4D).

Three Mutant Lines Have a Defective *BAC2* Gene

To learn more about the in vivo function of BAC2, we looked for Arabidopsis lines in which the gene is mutated. We screened the T-DNA Express Web site (http://signal.salk.edu/cgi-bin/tdnaexpress) and found one line that has an insertion in the *BAC2* gene. This mutant allele was named *bac2-1*. We confirmed the

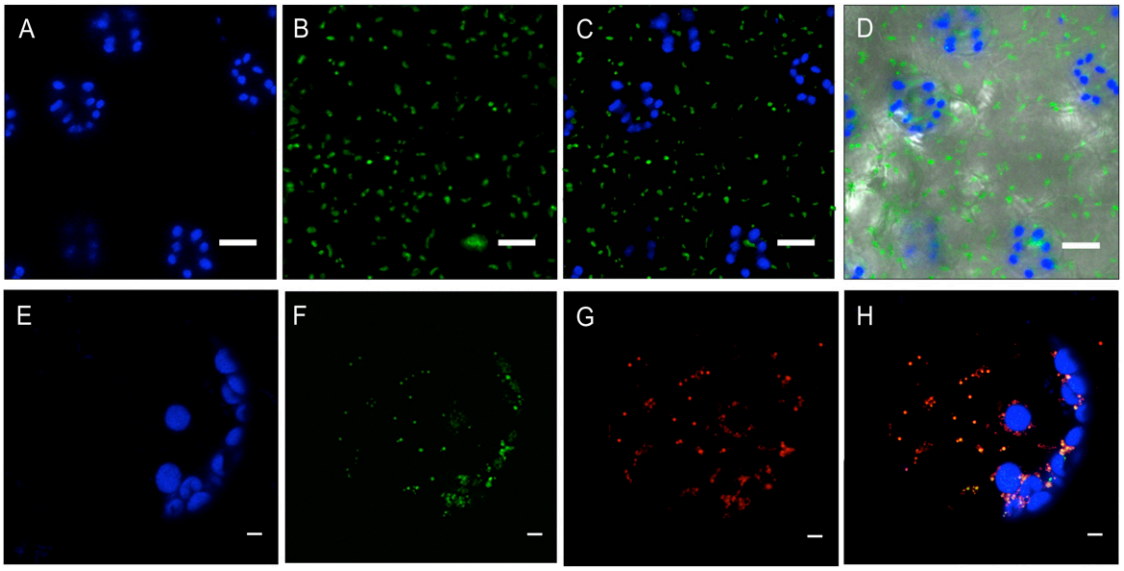

Figure 2. Localization of BAC2 in mitochondria. Transgenic BAC2:GFP-expressing plants display GFP fluorescence in mitochondria. The top row shows stamen epidermis of transgenic Arabidopsis. Bars = 50 μm. The bottom row shows mesophyll protoplast from stably transformed Arabidopsis. Bars = 5 μm. A and E, Chlorophyll autofluorescence in chloroplasts. B and F, GFP fluorescence of the BAC2:GFP fusion protein. C, Merged images of stamen epidermis fluorescence. D, Fluorescence images merged with bright-field image. G, Mesophyll cell mitochondria labeled with MitoTracker Red fluorescence. H, Mesophyll cell merged image showing orange (red plus green)-labeled mitochondria.

presence of a T-DNA insertion in the first exon of the gene (Fig. 1A) located 418 bp and 80 bp from the transcription and translation start sites, respectively. We also found two independent point mutations in *BAC2* induced by ethyl methanesulfonate mutagenesis using the TILLING method (McCallum et al., 2000a, 2000b; Henikoff et al., 2004). The *bac2-2* allele has a G-to-A transition at position 759, changing the intron 3′ splice donor site from the AG consensus sequence to AA (Fig. 1; Supplemental Fig. S4). We sequenced the *bac2-2* cDNA, as it is commonly observed that mutations modifying the AG consensus sequence result in unspliced or misspliced mRNA (Brown, 1996). The *bac2-2* cDNA sequence has 18 bp deleted relative to the wild-type cDNA. A cryptic GA junction site located 18 bp downstream from the site of the mutation must have been used by the splicing machinery as an alternative splicing site. This leads to the deletion of six codons (codons 80–85) from within transmembrane domain 2 of the BAC2 protein (Fig. 5A). The sequence FQNAMV, deleted in this allele, is at the predicted substrate-binding site I in helix H2 found in the ADP/ATP translocator and is conserved in MCF proteins (Kunji and Robinson, 2006). The deletion is also close to a conserved motif, FXXY, that appears in other MCF proteins. The mutation in *bac2-2*, therefore, deletes a substrate-binding site and disorganizes helix II, altering the relative position of other conserved sequences that might result in a nonfunctional protein. The *bac2-3* allele is a G-to-A transition at position 1,167, located within the coding sequence at 645 bp from the ATG, that converts codon 215, TGG (Trp), into a TGA (stop) codon (Fig. 5A; Supplemental Fig. S4).

The effect of mutations on *BAC2* gene expression was assessed by reverse transcription (RT)-PCR on RNA extracted from seedlings under conditions that trigger *BAC2* gene expression in the wild type. *BAC2* is not expressed in the T-DNA insertion allele *bac2-1* (Fig. 5A). In *bac2-2* and *bac2-3* seedlings under hyperosmotic stress (0.4 M mannitol), *BAC2* mRNA is detectable (Fig. 5B). Overall, this shows that all three mutations are defective in *BAC2* because of either the loss of inducible transcription or the disruption or truncation of the proteins encoded.

BAC2 Expression Is Positively Correlated with the Ability to Use Arg

bac2 mutants do not have a distinguishable phenotype in standard growth conditions either in vitro or in soil. Plant stature and the flowering time were similar to the wild type (data not shown). BAC2 has been shown in vitro to transport Arg, citrulline (Cit), and Orn into proteoliposomes (Palmieri et al., 2006b). Wild-type and *bac2* mutant plants were grown on medium with Arg as the sole source of nitrogen (Arg medium). Under these conditions, wild-type seedlings grew very slowly, producing just one pair of leaves over a period of 2 weeks. *bac2-1* seedlings grew even more slowly, failing to fully develop the first pair of leaves after 2 weeks. To test whether *BAC2* is a limiting factor in Arg utilization in vivo (Fig. 6; Supplemental Fig. S5), we used transgenic wild-type plants expressing *BAC2*

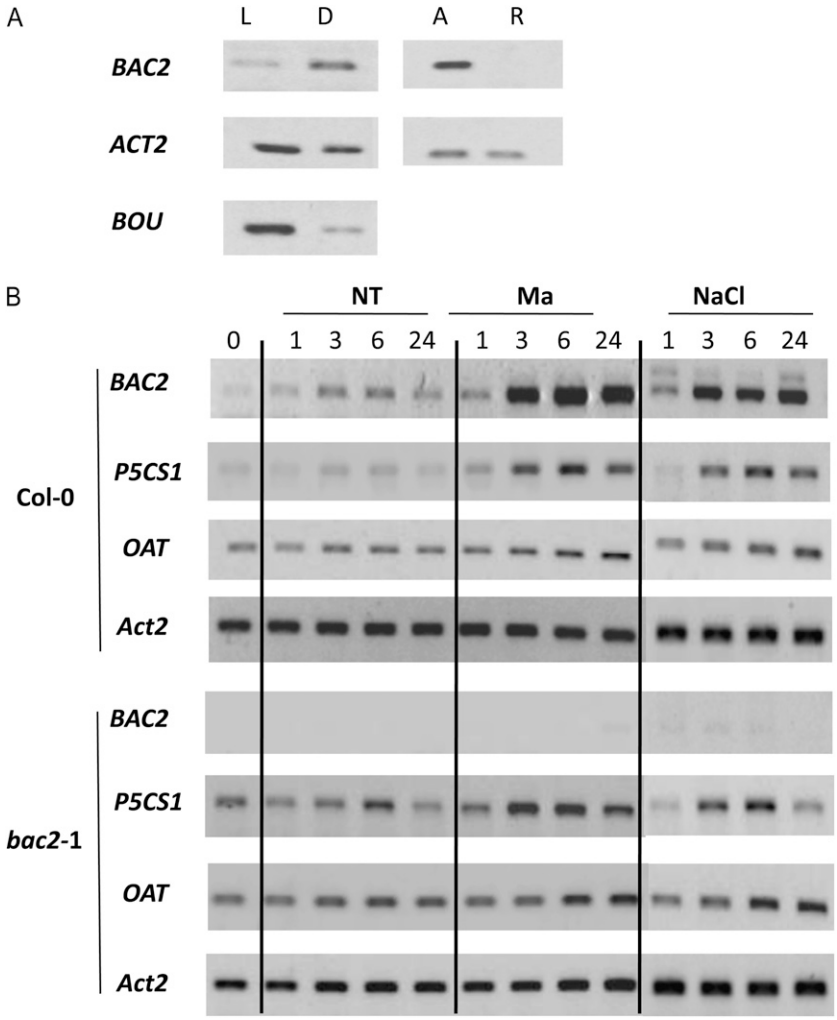

Figure 3. RT-PCR analysis of *BAC2* and Pro biosynthetic gene expression in Col-0 and *bac2-1*. A, *BAC2* gene expression in light-grown (L) or dark-grown (D) 5-d-old seedlings and in aerial parts (A) and roots (R) of Arabidopsis seedlings treated with 0.4 M mannitol for 24 h. *ACT2*, Actin control gene, *BOU*, At5g46800 gene used as a light-induced control gene. B, Time course of gene expression in Arabidopsis seedlings after water stress. NT, Nontreated seedlings; Ma, seedlings treated with 0.4 M mannitol; NaCl, seedlings treated with 0.2 M NaCl. Amplification of *BAC2* (30 cycles) and Pro biosynthetic genes *P5CS1* and *OAT* (26 cycles) was measured. Amplification (26 cycles) of *ACT2* was used as a control.

under the control of the 35S cauliflower mosaic virus promoter. Seedlings overexpressing *BAC2* or the *BAC2*:*GFP* translational fusion grew faster than the wild type on Arg medium. The rosettes were larger and three extra leaves were formed after 2 weeks (Fig. 6; Supplemental Fig. S5). In the same conditions, neither the wild type nor overexpressors nor mutants could grow with Cit or Orn as the sole source of nitrogen. Therefore, we can conclude that *BAC2* expression is a limiting factor for mitochondrial Arg transport in vivo.

bac2 Mutants Accumulate Excess Pro When Stressed

Free amino acid metabolism varies in response to hyperosmotic stress (Less and Galili, 2008). *BAC2* is a hyperosmotic stress-induced amino acid carrier gene, so we compared the free amino acid content of wild-type and *bac2-1* seedlings in response to such stress (Fig. 7). In wild-type plants, mainly Gln and Pro accumulate in response to stress. The most abundant free amino acid, Gln (21 μmol g^{-1} fresh weight), accumulated 2.3-fold after treatment with 0.4 M mannitol. However, the concentration of Pro increased relatively more, from trace amounts in normal conditions to 3 μmol g^{-1} fresh weight after stress. Other amino acids accumulated to low levels; Gly, Ala, Ser, and Asn concentrations in wild-type seedlings increased 3-fold upon treatment. Stress-induced changes in amino acid content after treatment were not the same in the *bac2-1* mutant as in the wild type. Less Gln accumulated, but the Pro concentration reached up to 5.5 μmol g^{-1} fresh weight in *bac2-1*, 180% of the concentration observed in the wild type (Fig. 7A). Concentrations of basic amino acids, Orn and Cit, did not differ significantly between the mutant and the wild type whatever the conditions (Supplemental Fig. S6).

We analyzed in more detail the variation in free Pro content after hyperosmotic stress in all three *bac2* alleles. After 24 h of treatment with 0.4 M mannitol, *bac2* plants all accumulated higher amounts of Pro, up to 160% of the wild-type amount (Fig. 7B). When stress was relieved by returning plants to control conditions without mannitol, Pro levels decreased both in wild-type and mutant plants (data not shown). In order to

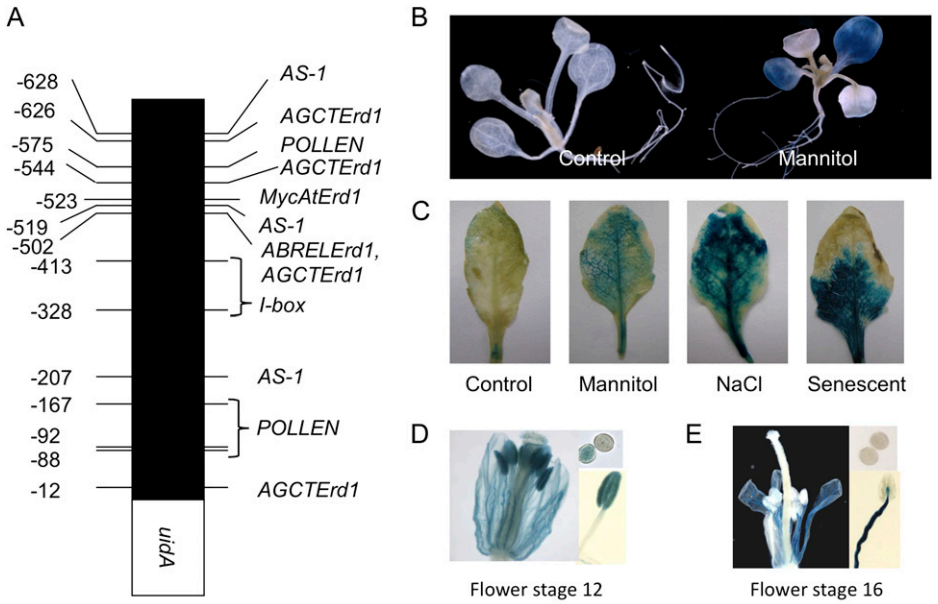

Figure 4. GUS staining of Arabidopsis plants transformed with the *BAC2* promoter-GUS fusion. A, Schematic representation of positions of cis-elements found in the *BAC2* promoter sequence (black box) fused to the *uidA* gene (white box). The numbers on the left indicate the positions of cis-elements relative to the ATG. The cis-elements identified are as follows: AS-1, response to salicylic acid, TGACG; MycAtErd1, dehydration stress and dark-induced senescence, CATGTG; ABRELAtErd1, dehydration stress and dark-induced senescence, ACGTG; ACGTErd1, etiolation, ACGT; I-box, light regulated, GATAA; POLLEN, pollen-specific activating element from tomato *lat52* gene, AGAAA. B to E, Histochemical staining of transgenic *BAC2* promoter-GUS fusion plants. B, Nontreated control and mannitol-treated 12-d-old seedlings. C, Detached leaves from 4-week-old plants. From left to right: nontreated, treated with 0.4 M mannitol, treated with 0.2 M NaCl, and dark-induced senescent. D, Flower stage 12, with stamen and isolated pollen grains. E, Dehiscent flower at stage 16 with stamen and individual pollen grains. [See online article for color version of this figure.]

verify that Pro transport and degradation were not affected in the mutant, mitochondria isolated from 12-d-old wild-type and *bac2-1* seedlings were tested for their ability to oxidize Pro in stress-relieved conditions after 24 h of stress. The Pro oxidation rate is similar in the wild type and the *bac2-1* mutant in these conditions (Supplemental Fig. S7). To summarize, these results show that BAC2 has an exaggerated stress response in terms of Pro accumulation.

Expression of a Pro Biosynthesis Gene Is Up-Regulated in *bac2*

Pro biosynthesis in response to water stress is dependent on the transcriptional activation of the *P5CS1* gene, encoding pyrroline-5-carboxylate (P5C) synthetase, the enzyme that catalyzes the reduction of Glu to Δ1-pyrroline-5-carboxylate (Savouré et al., 1995; Yoshiba et al., 1995; Székely et al., 2008). Although *P5CS1* mRNA was detectable in the absence of stress, more accumulated after 3 h of hyperosmotic (0.4 M mannitol) or saline (0.2 M NaCl) stress, reaching a maximum after 6 h and then decreasing after 24 h (Fig. 3B). *P5CS1* induction was estimated by quantitative RT-PCR to increase 100-fold in response to hyperosmotic stress in both ecotype Columbia (Col-0) and the *bac2-1* mutant. However, there was always twice as much *P5CS1* mRNA in *bac2-1* mutant seedlings than in the wild type (also measured by quantitative RT-PCR; Supplemental Fig. S8). We then focused on Orn-δ-aminotransferase (OAT), which catalyzes the conversion of Orn to P5C (Roosens et al., 1998; Funck et al., 2008). Although up to three times more *OAT* mRNA accumulates during hyperosmotic or saline stress (treatment with 0.4 M mannitol or 0.2 M NaCl, respectively), we found no difference in mRNA levels between the wild type and *bac2* (Fig. 3B; Supplemental Fig. S8). For comparison, we found no induction of the *P5CS2* gene, which encodes a P5C synthetase and does not respond to hyperosmotic stress (Székely et al., 2008) in either the wild type or *bac2-1* (data not shown). This shows that *P5CS1* mRNA levels are up-regulated in the *bac2-1* mutant in both hyperosmotic stress and control conditions.

DISCUSSION

Distinct Biological Function of BAC2

Among the 58 Arabidopsis genes that encode 6TM MCF proteins, the two basic amino acid carriers BAC1

A

MDFWPEFMATSWGREFVAGGFGGVAGIISGYPLDTLRIRQ
QQSSKSGSAFSILRRMLAIEGPSSLYRGMAAPLASVT**FQN**
AMVFQIYAIFSRSFDSSVPLVEPPSYRGVALGGVATGAVQ
SLLLTPVELIKIRLQLQQTKSGPITLAKSILRRQGLQGLY
RGLTITVLRDAPAHGLYFWTYEYVRERLHPGCRKTGQENL
RTMLVAGGLAGVAS**W**VACYPLDVVKTRLQQGHGAYEGIAD
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RCLFNQSPSPDIVTGD

B

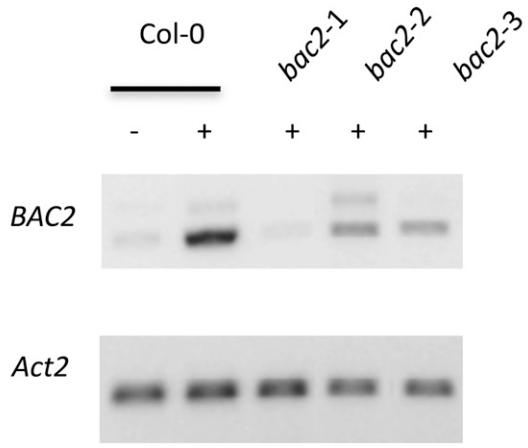

Figure 5. Characterization of *bac2* mutant lines. A, BAC2 amino acid sequence with the positions of transmembrane domains underlined. The alterations to the coding sequence in mutant alleles are shown in larger font; FQNAMV are deleted in *bac2-2* and a stop codon replaces the Trp in *bac2-3*. B, RT-PCR analysis of *BAC2* gene expression in wild-type and mutant alleles. −, Nontreated seedlings; +, seedlings treated for 24 h with 0.4 M mannitol to trigger *BAC2* gene expression. *BAC2*, *BAC2* cDNA; *Act2*, actin 2 control gene.

and BAC2 are able to complement the yeast *arg11* mutant, which is defective in the transport of Orn and Arg across the mitochondrial envelope (Catoni et al., 2003; Hoyos et al., 2003). Both BAC proteins have been shown to function in vitro as Arg and Orn carriers (Hoyos et al., 2003; Palmieri et al., 2006b). The in vitro substrate and pH specificities of BAC2 are different from those of the yeast Arg/Orn carrier ARG11 and Arabidopsis BAC1, BAC2 being less specific toward L-amino acids and more active at a higher pH in vitro. In addition, BAC2 is able to transport Cit (Palmieri et al., 2006b). Interestingly, BAC2 transport characteristics are quite similar to those of the human isoform of the Orn carrier ORC2 (Fiermonte et al., 2003; Palmieri et al., 2006b).

To assign a biological role to BAC2 in the plant cell, it is important to know its cellular localization, particularly as it has been shown that some chloroplast MCF proteins can complement yeast or mammalian mutant cells that have no chloroplasts (Hamel et al., 2004; Kleffmann et al., 2004; Bedhomme et al., 2005). We show here that BAC2 is genuinely located in Arabidopsis mitochondria. This and its gene expression pattern indicate that BAC2 is involved in basic amino acid transport to mitochondria during the Arabidopsis stress response. In plants, the biosynthesis of Arg is plastidial (Slocum, 2005), making it unlikely that BAC2 is an Orn/Arg carrier involved in Arg synthesis like ARG11 in yeast.

In order to clarify the biological role of BAC2, we tested its transport function in vivo by providing Arg as the sole source of nitrogen to the wild type, the *bac2-1* mutant, and a *BAC2* overexpressor. In our experimental conditions, *BAC2* expression correlated with the plant's ability to use Arg as a nitrogen source. Mitochondria contain arginase, an enzyme that can hydrolyze Arg to give urea, which, in turn, is a source of ammonia through the action of cytoplasmic urease (Polacco and Holland, 1993). It has been shown that urease activity is a route of nitrogen supply in Arabi-

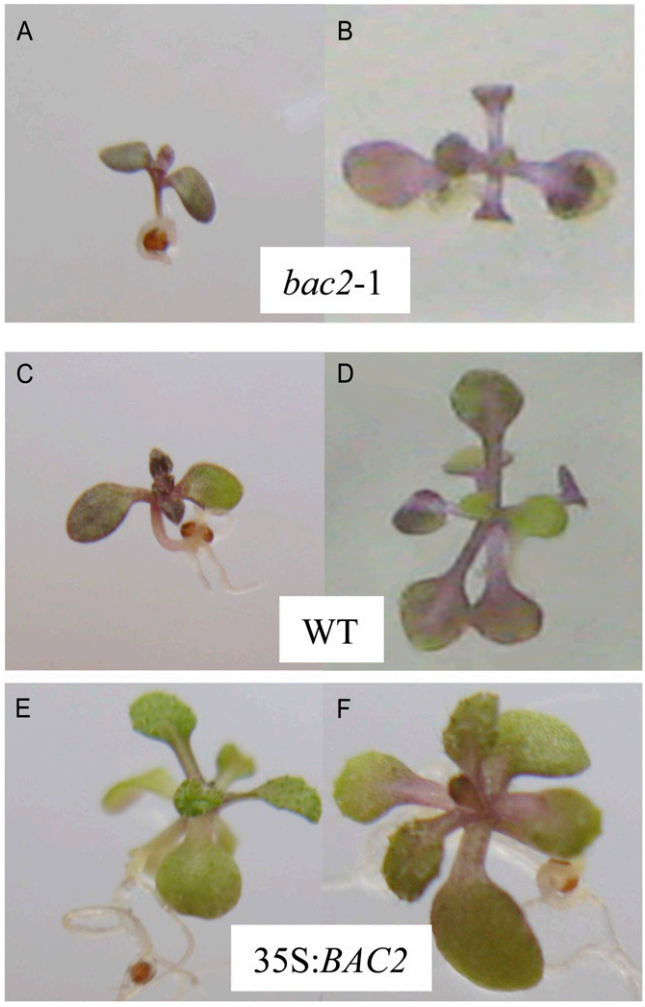

Figure 6. Growth of Arabidopsis with Arg as sole source of nitrogen. *bac2-1* mutant seedlings can grow on Arg medium (A and B), although it develops more slowly than the wild type (WT; C and D), and *BAC2* overexpressor (E and F) develops faster than the wild type. A, C, and E, Two-week-old seedlings. B, D, and F, Three-week-old seedlings. [See online article for color version of this figure.]

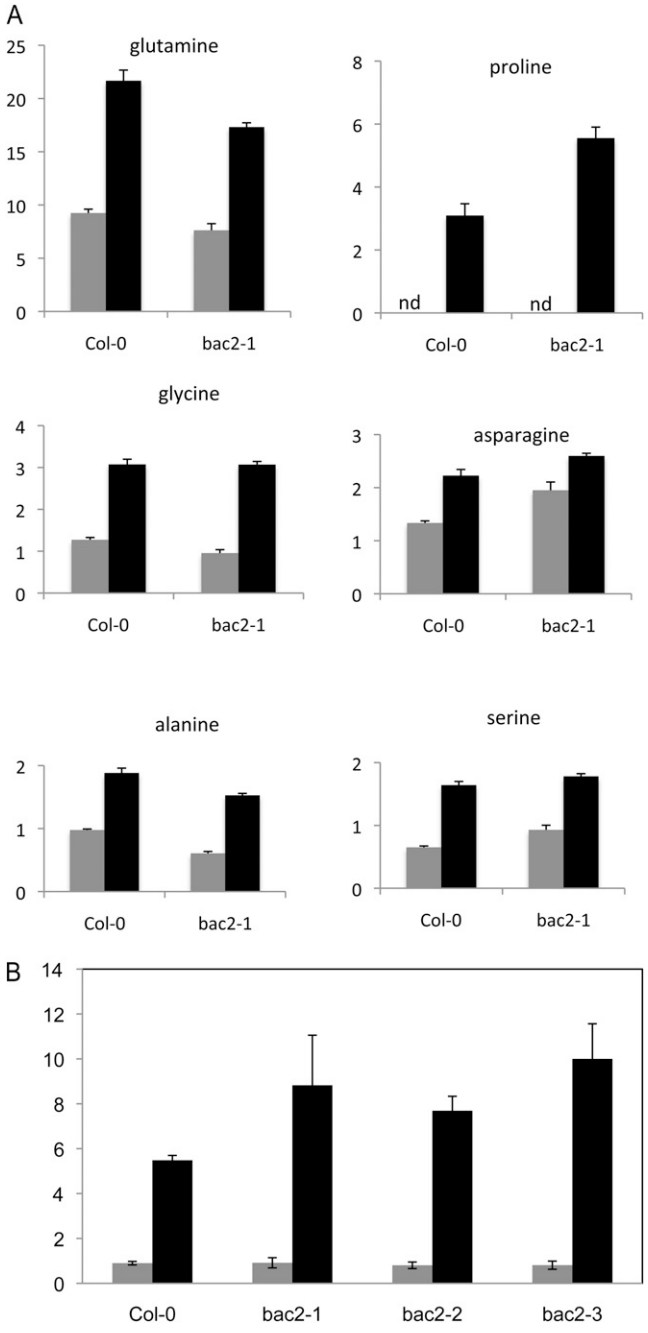

Figure 7. Differential amino acid accumulation caused by water stress. Twelve-day-old seedlings were treated for 24 h with 0.4 M mannitol to cause hyperosmotic stress. Free amino acids were quantified in micromoles per gram fresh weight (*y* axis). A, Changes in free amino acid contents during water stress. B, Stress-induced Pro accumulation in *bac2* mutant alleles compared with wild-type seedlings. Gray bars represent nontreated seedlings, and black bars represent water-stressed seedlings.

dopsis seedlings (Zonia et al., 1995) and that exogenous urea triggers gene responses allowing plants to adapt to urea as a sole nitrogen source (Mérigout et al., 2008). We hypothesize that once transported to mitochondria, Arg is dissimilated into urea, a source of ammonia for amino acid synthesis. Although Arg is not an efficient source of nitrogen for general plant growth, Arg import to mitochondria should be vital for mitochondrial translation. *bac2* mutants do not have a phenotype when grown in soil or in vitro with ammonia and nitrate as sources of nitrogen, so we deduce that BAC2 is not the main supplier of Arg to the mitochondria during vegetative growth. Genetic redundancy between *BAC2*, *BAC1*, or another transporter might account for postgerminative Arg supply to mitochondria and could explain the residual growth of the *bac2* mutant on Arg. Chloroplasts have been previously identified as the location of Arg and Cit dissimilation in Arabidopsis based on L-Arg iminohydrolase enzymatic activities and L-[guanido-^{14}C]Arg uptake by intact chloroplasts (Ludwig, 1993); this metabolic capacity might also be responsible for the growth of the *bac2* mutant on Arg medium.

BAC2 and Pro Response to Stress

The hyperosmotic stress response in Arabidopsis involves cross talk between several pathways such as abscisic acid, phospholipase, or calcium-dependent signaling. Pro accumulation via biosynthetic gene activation and catabolism repression is regulated via lipid and calcium signaling (for review, see Verbruggen and Hermans, 2008; Szabados and Savouré, 2010). *BAC2* transcriptional regulation may depend on the same signaling pathways. Our data suggest that hyperosmotic stress is a major inducer of *BAC2* transcriptional activation in leaves only. The promoter of *BAC2* harbors motifs recognized by stress-related transcription factors also found in the promoter of the stress/senescence-regulated gene *ERD1* (MycAtErd1, ABRELAtErd1, and ACGTErd1; Yamaguchi-Shinozaki and Shinozaki, 2005) and AS-1 motifs that modulate the response to stress (Redman et al., 2002).

There are vast changes in the Arabidopsis transcriptome in response to stress, and amino acid metabolism genes respond acutely to abiotic stress (Less and Galili, 2008). We observed a striking overaccumulation of Pro in *bac2* mutants, 1.6 times that of the wild type Col-0, in response to hyperosmotic stress. In plants, Pro biosynthesis in response to hyperosmotic stress has been speculated to take place mainly in the chloroplast (Szabados and Savouré, 2010). In Arabidopsis, it occurs mainly through the activation of *P5CS1* transcription (Savouré et al., 1995; Yoshiba et al., 1995). A P5CS1-GFP fusion is located in chloroplasts of Arabidopsis leaf mesophyll cells subjected to salt or osmotic stress (Székely et al., 2008). The last step of Pro biosynthesis in plants, which is performed by P5CR, may also take place in chloroplasts. P5CR activity has been associated with chloroplasts in pea (*Pisum sativum*), soybean (*Glycine max*), and spinach (*Spinacia oleracea*; Rayapati et al., 1989; Szoke et al., 1992; Murahama et al., 2001). In Arabidopsis, there is another P5C synthetase-encoding gene, *P5CS2*, that is

vital for embryogenesis and whose expression is stimulated in response to biotic stress. *P5CS2* does not play a major role in hyperosmotic stress Pro synthesis, and the gene product is cytosolic (Székely et al., 2008). We found that twice as much *P5CS1* mRNA accumulates in the *bac2* mutant than in the wild type. In *bac2*, the *P5CS1* gene is induced in a similar way, starting from a basal level of expression in the absence of hyperosmotic stress that is twice as high as in the wild type. A higher rate of transcription or greater stability of *P5CS1* mRNA might explain the overaccumulation of Pro in *bac2*.

There is a speculative link between Pro synthesis and basic amino acid transport into the mitochondria. Arg transported into the mitochondria can produce Orn and urea via the action of arginase. Orn can also be transported to the mitochondria and transaminated by OAT, leading to the formation of P5C, the substrate for Pro synthesis. In young (12-d-old) Arabidopsis seedlings, OAT mRNA accumulation and OAT activity correlate with high Pro accumulation after NaCl stress. Conversely, older plantlets (4 weeks old) accumulate less OAT mRNA, have lower OAT activity, and accumulate less Pro after NaCl stress (Roosens et al., 1998). However, OAT mutants grown on increasing concentrations of NaCl over a few weeks do not produce less Pro than the wild type (Funck et al., 2008). It is speculated, therefore, that the OAT pathway is not a route for Pro biosynthesis but rather for Glu synthesis from Arg (Funck et al., 2008; Szabados and Savouré, 2010). In the *bac2* mutant background, there was little variation in OAT mRNA levels. There was little difference in Arg, Orn, or Cit accumulation in *bac2* compared with Col-0, either before or after hyperosmotic stress induced by mannitol. In plants, Gln is the main nitrogen source for the production of amino acids, including Glu, the substrate for Pro biosynthesis, so we speculate that the Glu biosynthetic pathway of Pro is overstimulated in the mutant because of overexpression of the *P5CS1* gene. Therefore, Pro overaccumulates in *bac2* at the expense of Gln.

CONCLUSION

We have found that *BAC2* expression is modulated by hyperosmotic and saline stresses and dark-induced senescence. An overstimulation of the Pro biosynthetic pathway observed in *bac2* mutants in response to hyperosmotic stress results in the accumulation of more Pro. In Arabidopsis, basic amino acid metabolism, especially Arg metabolism, has been linked to stress adaptation and stress metabolism (Watson et al., 1998; Kasinathan and Wingler, 2004; Tun et al., 2006; Flores et al., 2008). As we showed that BAC2 is a basic amino acid carrier located in mitochondria, we speculate that BAC2 may have a role as a carrier of precursors of metabolic or signaling components of the hyperosmotic stress response in Arabidopsis.

MATERIALS AND METHODS

Characterization of T-DNA Insertion in Arabidopsis Lines

Arabidopsis (*Arabidopsis thaliana* Col-0) line WiscDsLox498_08F (*bac2-1*) harbors a T-DNA insertion of the pDs-Lox construct from the University of Wisconsin (Woody et al., 2007). The position of the insertion was deduced from the sequence of insertion-flanking genomic DNA, which is located within the first exon. Twelve seedlings from the T-DNA insertion line were tested for the presence of the insertion. DNA was extracted by grinding a single leaf in extraction buffer (0.2 M Tris-HCl, pH 7.5, 0.25 M NaCl, 0.025 M EDTA, and 0.5% SDS). After a 1-min centrifugation (10,000*g*), the supernatant was precipitated with 1 volume of isopropanol and pelleted for 1 min at 10,000*g*. The pellet was dissolved in 100 μL of deionized water. PCR was performed on 2 μL of the genomic DNA solution. Primers hybridizing at each end of the coding sequence were used to amplify the whole of At1g79900. T-DNA-specific primers were used to amplify border sequences including the P745 primer specific for the left border of the pDs-Lox T-DNA construct (Woody et al., 2007). Plants showing the insertion within the gene and for which no intact copy of the gene could be amplified were selfed, and their progeny were tested for homozygosity of the insertion allele.

Identification of Point Mutations in *BAC2*

Two point mutations in *BAC2* were identified using the TILLING method (McCallum et al., 2000a, 2000b; Henikoff et al., 2004) by the TILLING laboratory at the Department of Botany of the University of Washington in Seattle. Primers were determined using Primer3 software (Rozen and Skaletsky, 2000). Primers that discriminate between mutant and wild-type sequences were used in order to identify homozygous mutant plants. All selected homozygous mutant lines were further confirmed by DNA sequencing after PCR amplification of the *BAC2* gene with primers (BOULA and BOULATG, SPLICEref and SPLICEalt, W215*ref and W215*alt). Primers BLSS and BLWS were used for DNA sequencing of the mutated region. For details of primers, see Supplemental Table S1.

RT-PCR Analysis

Seedlings (up to 100 mg) were ground in extraction buffer (Tris-HCl, pH 7.5, 0.25 M NaCl, 25 mM EDTA, and 0.5% SDS) with an equal volume of citrate-buffered (pH 4) phenol:chloroform (1:1, v/v). After 1 min of homogenization and 5 min of centrifugation at 10,000*g*, the upper aqueous phase was extracted twice with phenol:chloroform. RNA was selectively precipitated in 2 M LiCl overnight at 4°C, pelleted for 5 min at 10,000*g*, dissolved in 300 μL of water, and precipitated in 2 M LiCl for 6 h at 0°C. After centrifugation at 10,000*g* for 10 min, the RNA pellet was rinsed with 70% ethanol, dried, and dissolved in water.

cDNAs were synthesized from 1 μg of RNA using the Omniscript reverse transcriptase mix (Qiagen) according to the manufacturer's instructions. PCR was carried out on 2 μL of the cDNA mixture in the Programmable Thermal Controller 100 (MJ Research) in a final volume of 25 μL, containing 200 μM of each deoxynucleoside triphosphate, 1 μM of each primer, and 2.5 units of Hot Start Taq polymerase with the manufacturer's buffer (Qiagen). The number of cycles used depended on the expression level of each gene, 30 cycles for *BAC2* and 26 cycles for all the other genes.

Quantitative RT-PCR

RNA was extracted with the RNeasy Plant Mini kit (Qiagen) from 100 mg of 12-d-old seedlings with a DNase treatment during purification. cDNAs were generated as described above. The RT-PCR was performed using the iCycler automated PCR machine from Bio-Rad. PCR products were detected using the Power SYBR Green PCR Master Mix according to the manufacturer's instructions (Applied Biosystems). The following PCR program was used: 13 min, 30 s at 95°C and 40 cycles of 15 s at 95°C, 30 s at 57°C, and 30 s at 72°C. The purity of PCR products was checked by the fusion curves between 55°C and 95°C that showed a 0.5°C increase every 10 s. The relative quantification was calculated according to Pfaffl (2001). The *APT1* gene was used as a control (Gutierrez et al., 2009).

Cloning of 35S:BAC2:GFP

BAC2 cDNA was amplified by PCR from Arabidopsis cDNA using BOULATG2 and BOULSTOP primers, which contain *Xba*I and *Sal*I restriction

sites, respectively, cloned into the pGEM T-Easy vector, and sequenced. The *Xba*I/*Sal*I fragment was cloned downstream of the 35S promoter and upstream of the *GFP* gene in a pCP60 binary vector (pCP60 is derived from pBIN19 and contains the 35S promoter of the *Cauliflower mosaic virus*); this construct was named pBG5. We confirmed by sequencing that BAC2 cDNA is in frame with *GFP*.

Cloning of *BAC2* for Overexpression in Planta

The pBG5 vector was digested with *Sal*I, and a stop codon was introduced at the end of *BAC2* cDNA by ligating the digested vector to annealed oligonucleotides BOUL-STOP-XBA-R and BOUL-STOP-XBA-F (for primer sequences, see Supplemental Table S1). The construct named pB5 was checked by sequencing.

Cloning of the *BAC2* Promoter Sequence

The full genomic sequence of *BAC2* was amplified by PCR from plant genomic DNA (Col-0), cloned into a pGEM T-Easy vector (Promega), and sequenced. This was used as a template to amplify the *BAC2* promoter using pBOUL619 and pBOULATG2 primers, introducing *Hin*dIII and *Sal*I restriction sites, respectively. This sequence was cloned into pGEM T-Easy and sequenced. The released *Hin*dIII/*Sal*I fragment was cloned upstream of the *uidA* reporter gene in the *Hin*dIII/*Sal*I-digested binary vector pPR97 (Szabados et al., 1995).

Histochemical Staining

Plantlets were incubated in a substrate buffer containing 0.5 mg mL^{-1} X-glucuronide (Euromedex), 100 mM sodium phosphate buffer, pH 7, 10 mM EDTA, pH 8, 0.1% Triton X-100, 1 mM potassium ferricyanide, and 1 mM potassium ferrocyanide. The substrate buffer was infiltrated into the samples by applying a vacuum for 5 min. Samples were then incubated at 37°C for 16 h. Plantlets were fixed for 30 min in 1.5% formaldehyde, 4% acetic acid, and 28.5% ethanol followed by three washes with absolute ethanol to remove chlorophyll.

Plant Transformation

Binary vectors were used to transform *Agrobacterium tumefaciens* LBA4404. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). T3 homozygous plants were selected by kanamycin resistance and analyzed.

Physiological Stress Conditions

Arabidopsis (Col-0) seeds were surface sterilized (20% sodium hypochloride, 80% ethanol) for 10 min, rinsed once in ethanol, and dried. Seeds were grown on 0.5× Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) with 1% saccharose and 0.8% agar on metal filters (0.8 mm mesh). After overnight imbibition at 4°C to synchronize germination, seedlings were grown at 22°C under continuous light (60 μmol photons m^{-2} s^{-1}) for 12 d. The meshes were transferred into 0.5× liquid MS medium for 1 h and then to 0.5× liquid MS medium supplemented with either 0.2 M NaCl or 0.4 M mannitol for the duration of the stress (1, 3, 6, or 24 h). Aerial parts of seedlings were collected, frozen immediately in liquid nitrogen, and stored at –80°C for further analysis. Whole leaves were detached and treated for 24 h in control MS medium or MS medium supplemented with 0.2 M NaCl or 0.4 M mannitol. Dark-induced senescence was triggered by wrapping a single leaf in aluminum foil for 4 d.

Amino Acid Analysis

Frozen plant tissues were ground in liquid nitrogen. Total free amino acids were extracted in a solution of 2% 5-sulfosalicylic acid according to Ferrario-Méry et al. (1997). Amino acids were analyzed by ninhydrin labeling (Rosen, 1957). Total amino acid content was assayed using Gln as a control. Individual amino acids were identified and quantified by ion-exchange chromatography using the AminoTac JLC-500/V amino acid analyzer according to the manufacturer's instructions (JEOL-Europe). Pro was quantified separately after ninhydrin labeling according to Bates et al. (1973). For each amino acid, the mean value of three 100-mg samples from three independent experiments was calculated.

Sample Preparation and Confocal Microscopy

Arabidopsis mesophyll protoplasts were prepared from 3-week-old 35S:*BAC2*:*GFP* transgenic plants following the protocol described by Yoo et al. (2007). Protoplasts in 4 mM MES (pH 5.7), 0.4 M mannitol, and 15 mM $MgCl_2$ (MMG) buffer were stained with 400 nM MitoTracker Red (Molecular Probes) for 30 min at room temperature in the dark. Stained protoplasts were washed twice with MMG buffer and then examined. Anthers from 6-week-old 35S:*BAC2*:*GFP* plants were mounted in 1 M sodium phosphate buffer, pH 7, and examined. GFP, MitoTracker Red, and chlorophyll fluorescence in the above samples were visualized by confocal laser microscopy using a Leica SP5 microscope and a Plan-APO CS 63.0 × 1.40 oil objective. Protoplast samples were excited at 488 and 561 nm. Fluorescence emission of protoplasts was acquired sequentially using PMT1 500 to 546 nm for GFP, PMT2 590 to 657 nm for MitoTracker Red, and PMT3 687 to 751 nm for chlorophyll. For the anther epidermis, excitation was at 488 nm and PMT values were PMT1 493 to 589 nm for GFP and PMT2 671 to 735 nm for chlorophyll. Image analysis and overlaying were done using Image J (National Institutes of Health) and Metamorph.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GU967412 and GU967413.

Supplemental Data

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

Supplemental Figure S1. *BAC2* expression in Arabidopsis organs.

Supplemental Figure S2. *BAC2* expression during Arabidopsis germination.

Supplemental Figure S3. *BAC2* expression in response to stresses taken from the Genevestigator database.

Supplemental Figure S4. Point mutations in *bac2-2* and *bac2-3* alleles.

Supplemental Figure S5. Mutant and overexpressor *BAC2* seedlings grown on Arg.

Supplemental Figure S6. Amino acid accumulation data.

Supplemental Figure S7. Pro respiration during hyperosmotic stress.

Supplemental Figure S8. Quantitative RT-PCR in response to hyperosmotic stress.

Supplemental Table S1. Oligonucleotide sequences.

Supplemental Data S1. Gene and protein sequences described in Figure 1.

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