BT2, a BTB Protein, Mediates Multiple Responses to Nutrients, Stresses, and Hormones in Arabidopsis[1][C][W][OA]

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The Arabidopsis (*Arabidopsis thaliana*) gene *BT2* encodes a 41-kD protein that possesses an amino-terminal BTB domain, a central TAZ domain, and a carboxyl-terminal calmodulin-binding domain. We previously demonstrated that *BT2* could activate telomerase expression in mature Arabidopsis leaves. Here, we report its distinct role in mediating diverse hormone, stress, and metabolic responses. We serendipitously discovered that steady-state expression of *BT2* mRNA was regulated diurnally and controlled by the circadian clock, with maximum expression in the dark. This pattern of expression suggested that *BT2* mRNA could be linked to the availability of photosynthate in the plant. Exogenous sugars decreased *BT2* expression, whereas exogenous nitrogen increased expression. *bt2* loss-of-function mutants displayed a hypersensitive response to both sugar-mediated inhibition of germination and abscisic acid (ABA)-mediated inhibition of germination, thus supporting a role of ABA in sugar signaling in germination and development. Moreover, constitutive expression of *BT2* imparted resistance to both sugars and ABA at germination, suggesting that *BT2* suppresses sugar and ABA responses. In support of the previously described antagonistic relationship between ABA and auxin, we found that *BT2* positively regulated certain auxin responses in plants, as revealed by knocking down *BT2* expression in the high-auxin mutant *yucca*. Accumulation of *BT2* mRNA was affected by a variety of hormones, nutrients, and stresses, and *BT2* was required for responses to many of these same factors. Together, these results suggest that BT2 is a central component of an interconnected signaling network that detects and responds to multiple inputs.

Plants have evolved sophisticated mechanisms to perceive and transduce diverse environmental signals. Changes in light, the circadian clock, and nutrient status serve as major inputs to modulate the diurnal expression of networks of genes that regulate growth and development (Blasing et al., 2005; Gutierrez et al., 2008; Usadel et al., 2008). The circadian clock further serves as an input to regulate or "gate" the expression of multiple genes involved in metabolism, growth, and development, thereby rendering a physiological advantage for plant growth and survival (for review, see McClung, 2006). In addition to the intrinsic clock function, diurnal changes in nutrient status modulate the expression of several genes (Blasing et al., 2005). The availability of sugars activates "feast" genes involved in growth and biosynthesis, while low sugar concentrations activate "famine" genes that mobilize carbon from primary reserves or other cellular components (Koch, 1996; Yu, 1999). Similarly, changes in nitrogen status modulate the expression of numerous transcription factors, protein kinases/phosphatases, and enzymes involved in nitrate reduction and assimilation, amino acid biosynthesis, glycolysis, and iron and sulfate metabolism (Scheible et al., 2004; Wang et al., 2004).

Sugars and nitrates primarily affect plant growth by serving as building blocks for anabolic metabolism. They also function as signaling molecules that interact with light, hormones including abscisic acid (ABA) and ethylene, and stress signals to control vital processes of growth and development (Zhou et al., 1998; Stitt, 1999; Stitt and Krapp, 1999; León and Sheen, 2003; Wang et al., 2004). Sugars normally promote growth; however, high sugar concentrations suppress germination and postgermination development. Interestingly, these inhibitory effects are antagonized by nitrates, suggesting interplay between carbon and nitrogen status in the control of germination (Moore et al., 2003; Bi et al., 2005). Using screens for either resistance or sensitivity of germination to high sugar concentrations, sugar-insensitive or -hypersensitive mutants have been identified (Zhou et al., 1998; Laby

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et al., 2000; Pego et al., 2000; Rolland et al., 2002). Surprisingly, many sugar-insensitive mutants, such as *sugar-insensitive4/glucose-insensitive1* (*sis4/gin1*) and *sis5/gin6*, are allelic to ABA synthesis (*aba2*) and ABA-insensitive (*abi4*) mutants, respectively (Arenas-Huertero et al., 2000; Cheng et al., 2002). Moreover, exogenous Glc specifically increases the expression of ABA synthesis genes and affects endogenous ABA concentrations, revealing an intimate connection between ABA and sugar signaling (Cheng et al., 2002; Price et al., 2003). ABA itself mediates seed dormancy, leaf senescence, stomatal closure, and several other plant stress responses (Fedoroff, 2002; Gubler et al., 2005). ABA signaling also has antagonistic interconnections with other hormones, including auxin and ethylene. The ABA-hypersensitive mutant *hyponastic leaves1* (*hyl1*) is simultaneously resistant to auxin and cytokinin (Lu and Fedoroff, 2000). During lateral root initiation, auxin promotes initiation by down-regulating cell cycle inhibitors such as Kip-related proteins (Richard et al., 2001; Himanen et al., 2002). In contrast, ABA inhibits lateral root initiation by activating Kip-related proteins (Verkest et al., 2005). Also, several genes involved in promoting lateral root initiation, including *AUXIN INDUCED IN ROOT CULTURES12* and *INDOLE-3-ACETIC ACID19*, are repressed by ABA (Hoth et al., 2002).

Although there has been significant progress in understanding how plants perceive light, nutrient, hormone, and stress signals, major questions persist regarding how plants simultaneously integrate and transduce these different signals. Global gene expression studies in Arabidopsis (*Arabidopsis thaliana*) have revealed that specific signals modulate extensive networks of genes. These networks typically include genes encoding putative transcription factors and protein kinases, along with genes involved in protein synthesis and ubiquitin-mediated protein degradation (Wang et al., 2004; Blasing et al., 2005; Gutierrez et al., 2008; Usadel et al., 2008). Members of the bZIP family of transcription factors characterized as G box (CACGTG)-binding factors (GBFs), such as bZIP2/GBF5 and bZIP11/ATB1, together with Snf1-related kinases (SnRK), KIN10/11, orchestrate synergistic transcriptional networks in response to sugar, energy deprivation, and diverse stresses (Baena-Gonzalez et al., 2007; Hanson et al., 2008). However, the molecular mechanisms of the components downstream of bZIP/SnRK that affect the adaptive responses remain elusive.

We previously identified BT2 (At3g48360) as an activator of telomerase in mature leaves of Arabidopsis (Ren et al., 2007). BT2 is an approximately 41-kD protein with an N-terminal BTB/POZ (for Broad-Complex, Tramtrack, and Bric-a-Brac/Poxvirus and Zinc finger) domain, a central TAZ (for Transcriptional Adaptor Zinc finger) domain, and a C-terminal calmodulin-binding domain. The Arabidopsis genome encodes four additional proteins with a similar domain structure (Du and Poovaiah, 2004). Recently, members of this BT family, including BT2, were demonstrated to play crucial roles in gametophyte development in Arabidopsis and were further shown to compensate for the loss of one another by reciprocal transcriptional regulation (Robert et al., 2009). Here, we report distinct and broader functions of BT2 in responding to changes in light signals, nutrient status, hormones, and certain stresses. During the diurnal cycle, *BT2* expression peaked in the dark, and its expression was regulated by the circadian clock. Nutrient status also modulated *BT2* expression: sugars repressed *BT2* expression, while nitrates increased *BT2* expression. Using *bt2*-null mutants and constitutively expressing *BT2* lines, we demonstrated that *BT2* modulated hormone responses. *BT2* negatively regulates ABA- and sugar-mediated inhibition of germination. Loss of *BT2* in the auxin-accumulating mutant *yucca* suppresses many of the phenotypes associated with high auxin concentrations. This result confirms our previous conclusion from *BT2*-overexpressing lines that *BT2* potentiates auxin responses in postgermination and vegetative development (Ren et al., 2007). Furthermore, *BT2* expression was modulated by multiple abiotic and biotic stresses, including ABA, cold, methyl jasmonate, and hydrogen peroxide (H_2O_2). Loss of BT2 function resulted in sensitivity to H_2O_2. Because *BT2* expression is affected by multiple physiological and environmental conditions, and because it is also required for responses to many of these same conditions, the BT2 protein appears to be a key element in an interconnected network that detects and integrates responses to diverse signals.

RESULTS

Circadian and Light Regulation of *BT2*

In our previous characterization of BT2's role in activating telomerase in mature leaves (Ren et al., 2007), we noticed that the level of *BT2* message fluctuated among RNA samples harvested at different times. To uncover the cause of this fluctuation, we analyzed *BT2* mRNA levels throughout the diurnal cycle. *BT2* was highly expressed in the dark phase (19, 24, 43, and 48 h) and was almost undetectable in the light phase (7 and 31 h; Fig. 1A; Supplemental Fig. S1). However, its abundance increased slightly toward the end of the light phase (14 and 38 h), in apparent anticipation of the dark phase. Rhythmic expression of *BT2* suggested that it may be under the control of a circadian clock. To test this hypothesis, 3-week-old light/dark-entrained plants were either transferred to continuous light or kept in a normal diurnal cycle, and RNA samples were subjected to quantitative real-time (qRT)-PCR analysis. The rhythmic pattern of *BT2* expression seen in control plants under a normal diurnal cycle was maintained in plants transferred to continuous light (Fig. 1B).

We then performed two experiments to determine whether light alone could modulate *BT2* expression.

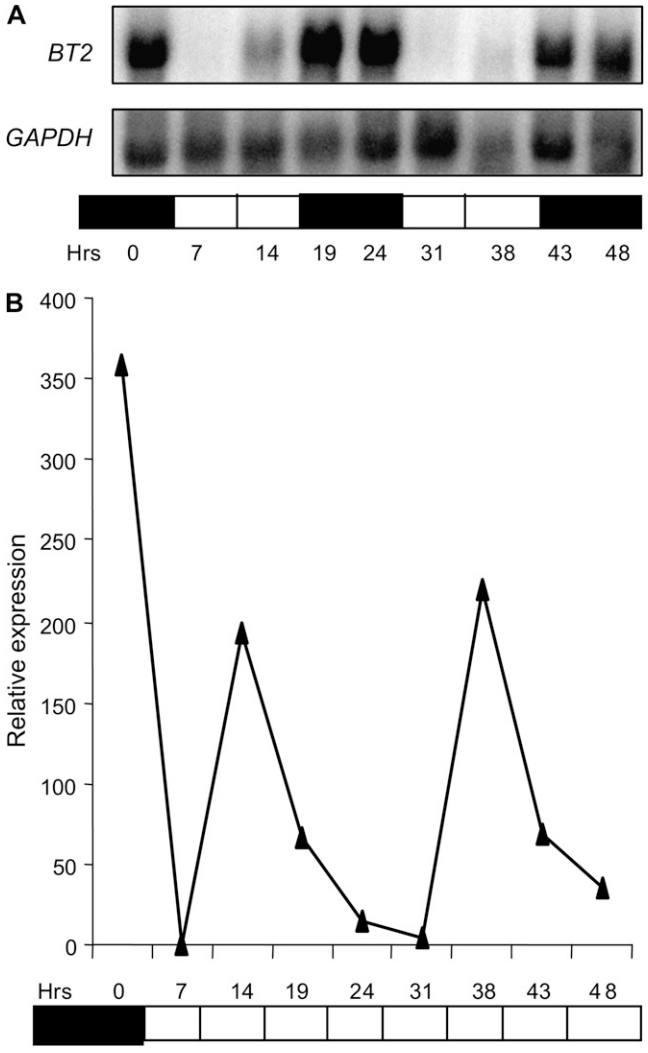

Figure 1. *BT2* expression is diurnally regulated and controlled by a circadian clock. After entrainment of wild-type plants to 14-h/10-h light/dark cycles for 3 weeks, plants were either held in light/dark or transferred to continuous light. Total RNA was extracted from rosette leaves harvested at the indicated times and was subjected to RNA gel blot analysis (A) or qRT-PCR (B). mRNA of *GAPDH* was used as a loading control for the RNA gel blot. *EIF-4A2* was used to normalize the qRT-PCR data. Expression values plotted for *BT2* in B are averages of two biological replicates and are relative to the minimum value, which occurred at the 7-h time point. White and black bars at the bottom of each panel represent light and dark conditions, respectively.

First, we subjected 3-week-old light/dark-entrained plants to an extended dark treatment. RNA samples were harvested at −1, 0, +1, and +2 h into the extended dark phase, along with control samples that were harvested from plants in a normal light/dark cycle, and subjected to qRT-PCR analysis. *BT2* expression was at its highest when the plants were in the dark at −1 and 0 h. As expected, *BT2* expression was down-regulated at +1 and +2 h in control samples that were transferred to light. However, in plants that stayed in extended darkness, *BT2* expression remained higher (Fig. 2A).

In the second experiment, 3-week-old light/dark-entrained plants were exposed to either 1 h of dark during the light phase or 1 h of light during the dark phase and *BT2* expression was analyzed by RNA gel blots. *BT2* mRNA was increased by the brief exposure to dark during the light phase but decreased by the brief exposure to light during the dark phase (Fig. 2, B and C). Together, these results indicate that expression of *BT2* is modulated by light and also is under the control of a circadian clock.

BT2 Responds to Changes in Nutrient Status of the Plant

To understand the functional significance of the diurnal regulation of *BT2*, we investigated whether its expression was correlated with metabolic changes. One of the major metabolic changes associated with a diurnal cycle is the concentration of sugars, which peak during the light and diminish in the dark. To test whether *BT2* expression responded to sugars, 3-week-old light/dark-entrained plants that were in the middle of a dark phase were treated with either Suc or mannitol (as an osmotic control) for 3 h in the dark. All samples were then subjected to RNA gel blot analysis. *BT2* transcript was repressed by Suc even in the absence of the light (Fig. 3A), suggesting that the low-sugar status of plants is a strong signal for *BT2* induction. Glc also repressed *BT2* expression (Fig. 3B). Mannitol had no effect on *BT2* mRNA concentrations (Fig. 3D).

Similar to sugars, changes in nitrogen status can affect resource allocation, growth, and development in plants. Nitrogen status also modulates the circadian clock by serving as an input (Scheible et al., 2004; Gutierrez et al., 2008). Moreover, the carbon-nitrogen ratio in plants is tightly regulated, with interconnected sensing and signaling mechanisms (Coruzzi and Zhou, 2001). For example, addition of nitrates reverses sugar-mediated repression of gene expression (Moore et al., 2003). Because *BT2* expression was modulated by the circadian clock and responded to carbon signals, we wanted to determine whether nitrogen also modulated *BT2* expression. Three-week-old light/dark-entrained plants were treated with either KNO_3 or KCl for 3 h in the middle of a light phase. *BT2* repression during the light phase was reversed by the addition of nitrates (Fig. 3C). Interestingly, nitrate induction of *BT2* was also observed when plants were treated during their dark phase (Fig. 3C). KCl had no effect on *BT2* mRNA concentrations (Fig. 3D).

BT2 Suppresses Sugar Signaling

Sugars, in addition to their metabolic roles, act as signaling molecules and control key aspects of plant growth and development. High sugar levels early in plant development can inhibit germination and cotyledon emergence (Smeekens, 2000; Gazzarrini and McCourt, 2001; Moore et al., 2003). Because *BT2* expression was modulated diurnally and by the sugar

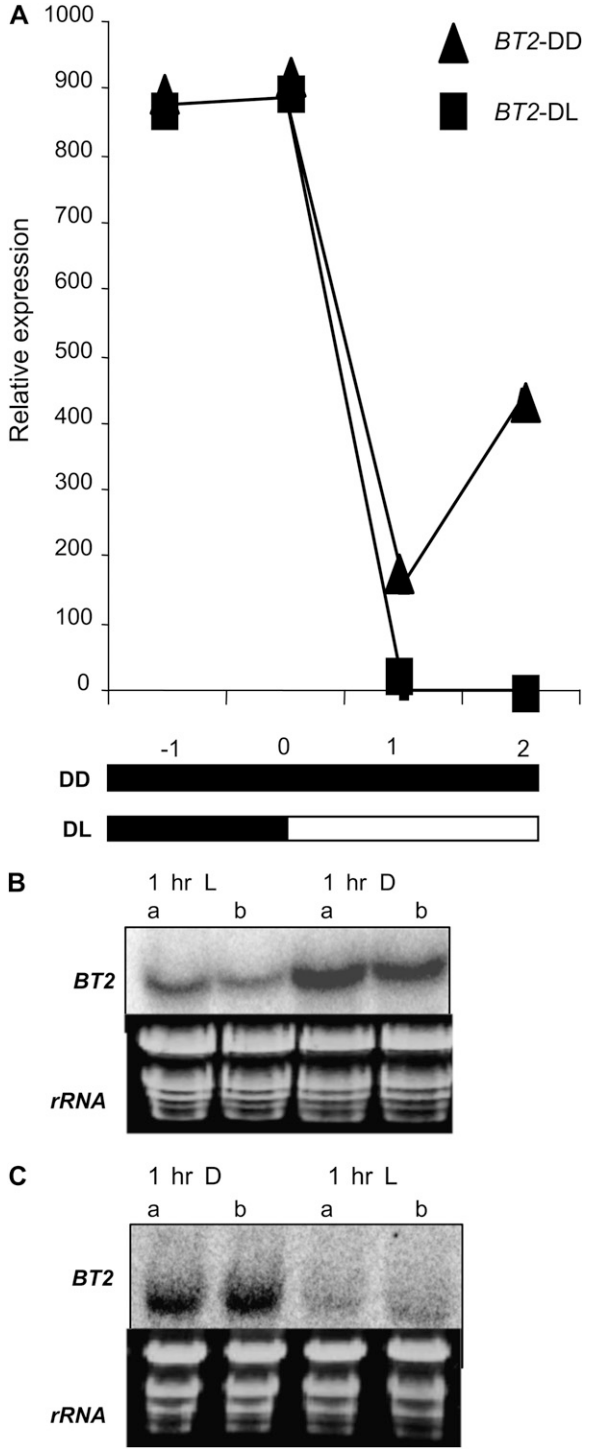

Figure 2. Light modulates *BT2* expression, independent of circadian regulation. A, After entrainment of wild-type plants to 14-h/10-h light/dark cycles for 3 weeks, plants were either held in extended darkness (DD) or transferred to light (DL). Total RNA was extracted from rosette leaves harvested at the indicated times and subjected to qRT-PCR analysis. *18S rRNA* was used to normalize the qRT-PCR data, and the expression values plotted for *BT2* are averages of two biological replicates. Values are relative to the minimum value, which occurred at the +1-h time point. White and black bars at the bottom represent light and dark conditions, respectively. B and C, Three-week-old wild-type plants were either exposed to 1 h of dark (D) during the middle of light phase (B) or 1 h of light (L) during the middle of dark phase (C). Rosette leaves were harvested and analyzed by RNA gel blots. The first two lanes in both blots represent controls that remained in their respective light and dark conditions. Replicate samples are indicated by a and b. Ethidium bromide-stained rRNA was used as a loading control.

status of the plant, we predicted a role for *BT2* in some aspects of sugar signaling. To test this hypothesis, seeds from *bt2*-null (*bt2-1*), constitutively expressing *BT2* (*BT2OE*), and wild-type lines were germinated on various concentrations of Glc (4%, 5%, and 6%) or mannitol (5%) and the percentage of seedlings with normal cotyledon emergence was determined. All lines had nearly 100% germination on 5% mannitol; however, concentrations of 4% and 5% Glc were sufficient to inhibit wild-type germination. At similar concentrations, *bt2-1* seeds were hypersensitive and *BT2OE* seeds were resistant to Glc inhibition of germination (Fig. 4). Higher concentrations of Glc (6%) inhibited germination of all lines equally. These results suggest that *BT2* suppresses sugar signaling during germination and early vegetative development.

BT2 Modulates Hormone Responses in Plants by Suppressing ABA Signaling while Enhancing Auxin Signaling

Because ABA inhibits germination in a manner similar to sugars (Arenas-Huertero et al., 2000; León and Sheen, 2003), we performed two experiments to determine whether BT2 also affected ABA signaling and responses at germination. First, we germinated seeds from *bt2-1*, *BT2OE*, and wild-type lines on various concentrations of ABA (0, 1, 2.5, and 5 μM) and determined the percentage of seedlings with normal cotyledon emergence. In parallel to the results obtained for sugars, ABA concentrations as low as 2.5 μM reduced germination of the wild type. However, at similar concentrations, the *bt2-1* line was sensitive while the *BT2OE* line was resistant to ABA inhibition (Fig. 5A), leading us to conclude that BT2 suppresses certain ABA signals or responses at germination. Next, we asked whether selected ABA signaling genes were differentially expressed in *bt2-1*, *BT2OE*, and wild-type lines when grown in the presence of high sugars (5% Glc). The results from RT-PCR experiments performed on *ABA-insensitive3* (*ABI3*), *ABI4*, and *ABI5* indicate that the relative abundance of the respective transcripts in the different lines remained unaffected (Fig. 5B).

We previously reported that *BT2* potentiates some responses to auxin. *bt2-1* seedlings are resistant to exogenous auxin, while constitutive expression of *BT2* in the high-auxin mutant *yucca* exacerbates its phenotype (Ren et al., 2007). Here, we found that loss of BT2 in *yucca* specifically suppressed its characteristic high-

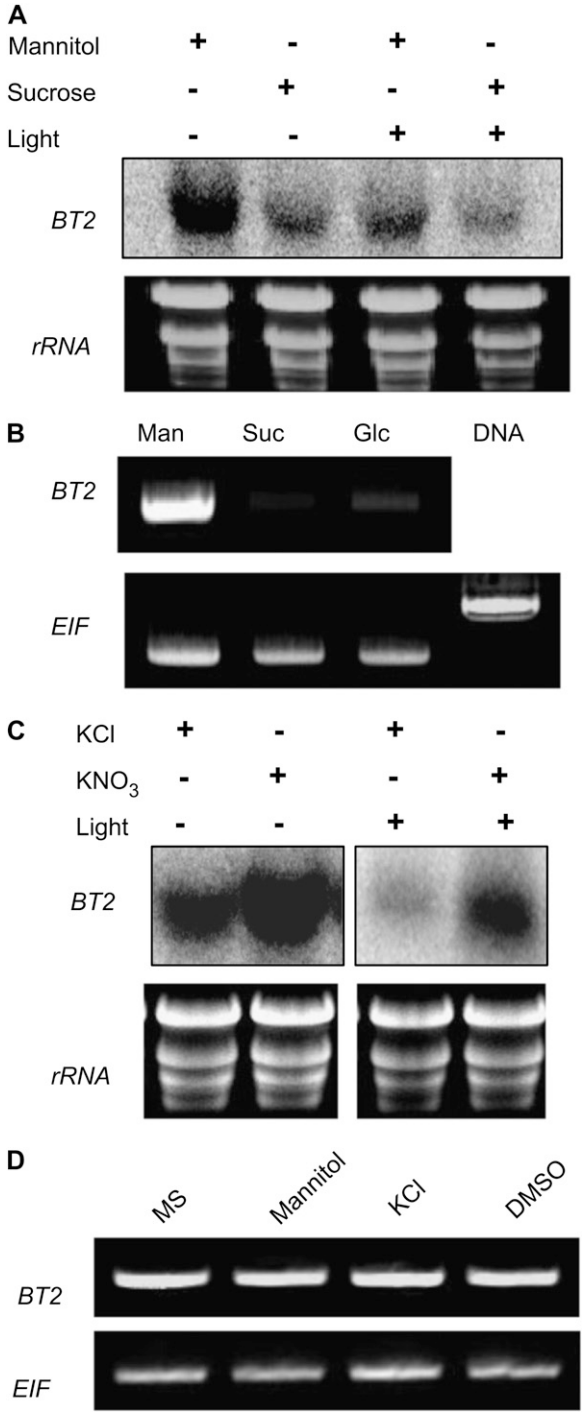

Figure 3. *BT2* is repressed by sugars and induced by nitrates. A, Three-week-old wild-type plants were treated with mannitol or Suc in the dark or the light for 3 h. B, One-week-old seedlings, grown in continuous light, were treated with mannitol, Suc, or Glc for 3 h. Total RNA was isolated and subjected to RT-PCR analysis. *EIF4-A2* expression was used as a loading control. C, Three-week-old wild-type plants were treated with KCl (control) or KNO_3 in the dark (lanes 1 and 2) or the light (lanes 3 and 4) for 3 h. D, Conditions used as controls for this figure and for Figure 7A had no effect on *BT2* mRNA concentrations. DMSO, Dimethyl sulfoxide.

auxin phenotype of epinastic cotyledons, epinastic leaves, shorter primary roots, excess root hair, and delayed development (Fig. 6). However, the elongated hypocotyl and petioles were less affected. Taken together, our findings here further strengthen the role of BT2 in enhancing certain auxin responses while suppressing ABA and sugar responses in plants.

BT2 Appears to Integrate Multiple Stress Signals

Because sugar and hormone signaling affect several responses to abiotic and biotic factors, and because BT2 has a role in both hormone and sugar signaling, we asked whether it also was required for stress responses. We started by analyzing changes in *BT2* expression in response to different stress signals by treating 3-week-old plants with ABA, methyl jasmonate (Me-JA), cold, wounding, and H_2O_2. Treatment with ABA and cold lowered *BT2* mRNA levels (Fig. 7A). Me-JA, which is antagonistic to ABA and mediates plant-pathogen defense signaling (Anderson et al., 2004), induced *BT2* expression. Wounding did not affect the expression of *BT2* (Fig. 7A). *BT2* expression was induced by H_2O_2, a major reactive oxygen species (ROS) in plants, but not by treatment with methyl viologen, an electron transport inhibitor that also leads to oxidative stress (Fig. 7B). To ask whether loss of BT2 had any effect on plants challenged with free radical stress generated by H_2O_2, we grew *bt2-1*, *BT2OE*, and wild-type plants on media with and without 2 mM H_2O_2. Initially, H_2O_2 suppressed the growth of all lines equally (i.e. smaller leaves, shorter petioles, and an overall inhibition of vegetative growth). However, after a period of approximately 3 weeks in the presence of H_2O_2, *bt2-1* plants were significantly smaller, whereas *BT2OE* plants were indistinguishable from wild-type plants (Fig. 7C).

DISCUSSION

We previously identified BT2 as an activator of telomerase activity in mature Arabidopsis leaves (Ren et al., 2007). Here, we report its roles in sugar signaling, its connections to hormone signaling, and its apparent function in integrating diverse biotic and abiotic stress signals. We showed that *BT2* expression was diurnally regulated. However, this is not surprising, since 30% to 50% of Arabidopsis genes are diurnally regulated and the circadian clock and sugar status are the major inputs driving the diurnal regulation (Blasing et al., 2005). *BT2* mRNA was more abundant (more than 100-fold) in the dark relative to the light (Fig. 1). *BT2* expression was controlled by the circadian clock, because the diurnal anticipation of *BT2* expression at 14 and 38 h (Fig. 1) in entrained plants persisted even in a continuous light cycle. In light/dark conditions, *BT2* mRNA begins to accumulate at 14 and 38 h, in a possible anticipation of the dark phase (Fig. 1A). An initially puzzling feature of *BT2*

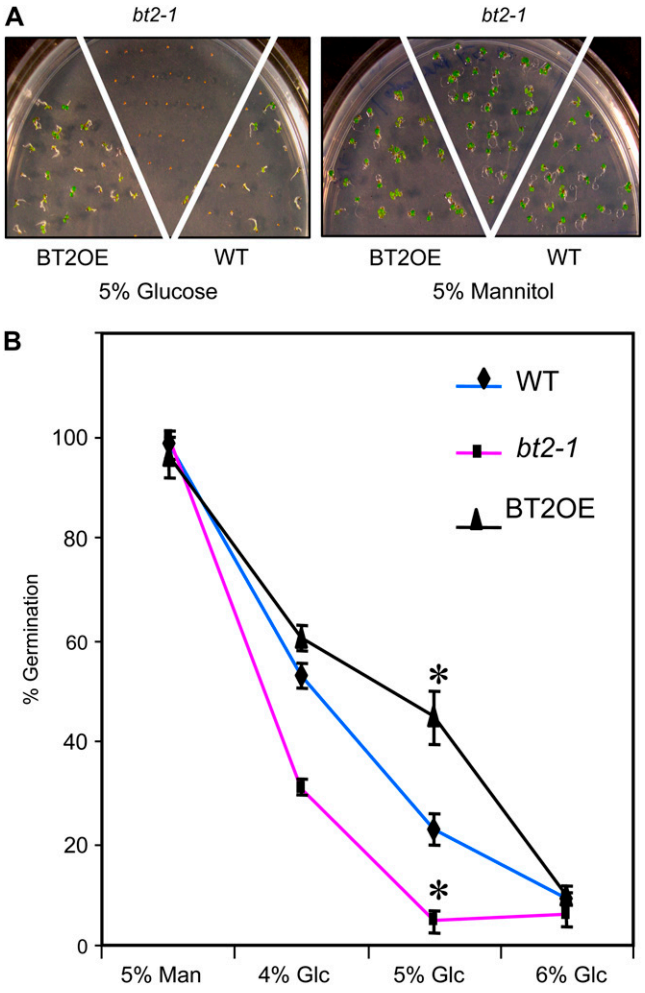

Figure 4. BT2 suppresses sugar-mediated inhibition of germination. A, Visible phenotypes of 6-d-old wild-type (WT), *bt2-1*, and *BT2OE* lines grown on either 5% Glc or 5% mannitol. B, Quantification of germinated seedlings with normal cotyledons of wild-type, *bt2-1*, and *BT2OE* lines grown on various concentrations of Glc (4%, 5%, and 6%) or mannitol (5%). Approximately 30 seedlings per line per plate were assayed, and three plates per treatment were used. Error bars indicate SD. Asterisks indicate significant differences compared with the wild type ($P < 0.05$), as calculated by Student's *t* test.

expression in continuous light was that, instead of peaking at the end of the dark period (24 and 48 h), the peaks of *BT2* mRNA occurred at the end of the light period at 14 and 38 h (Fig. 1B). *BT2* transcript failed to accumulate after the anticipatory period in continuous light, possibly because of the continuous presence of the abnormal light signal or another metabolic signal. We also found that *BT2* was repressed and induced by brief exposure to light and dark alone, respectively (Fig. 2). These results suggest that light can also modulate *BT2* expression independent of the circadian control. However, under prolonged exposure to an inappropriate signal, as in our continuous light experiment, the circadian regulation can override the abnormal signal, in an apparent attempt to restore an appropriate level of *BT2* transcript.

Although the circadian clock drives the diurnal regulation of many genes, diurnal changes in sugar concentration also play a major role (Blasing et al., 2005) and *BT2* expression was repressed by sugars (Fig. 3A). This result was also consistent with our finding that relative expression of *BT2* was lowest during the light phase, possibly because of repression caused by higher levels of sugars produced by photosynthesis. In the dark, however, expression of *BT2* was induced, possibly in response to sugar depletion. We also found that *BT2* was induced by nitrates during

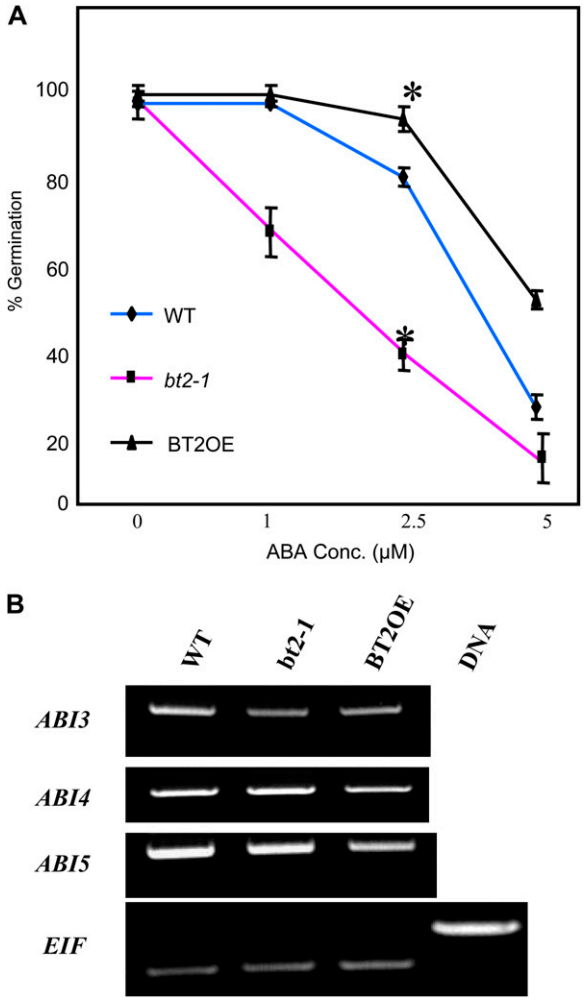

Figure 5. BT2 suppresses ABA-mediated inhibition of germination. A, Quantification of the percentage of germinated seedlings with normal cotyledons of wild-type (WT), *bt2-1*, and *BT2OE* lines grown on various concentrations (Conc.) of ABA (0, 1, 2.5, and 5 μM). Approximately 30 seedlings per line per plate were assayed, and three plates per treatment were used. Error bars indicate SD. Asterisks indicate significant differences compared with the wild type ($P < 0.05$), as calculated by Student's *t* test. B, Ten-day-old wild-type, *bt2-1*, and *BT2OE* lines were grown on 5% Glc. Total RNA was isolated and subjected to RT-PCR analysis (25 cycles) to determine expression of *ABI3*, *ABI4*, and *ABI5*. *EIF4-A2* expression was used as a loading control. [See online article for color version of this figure.]

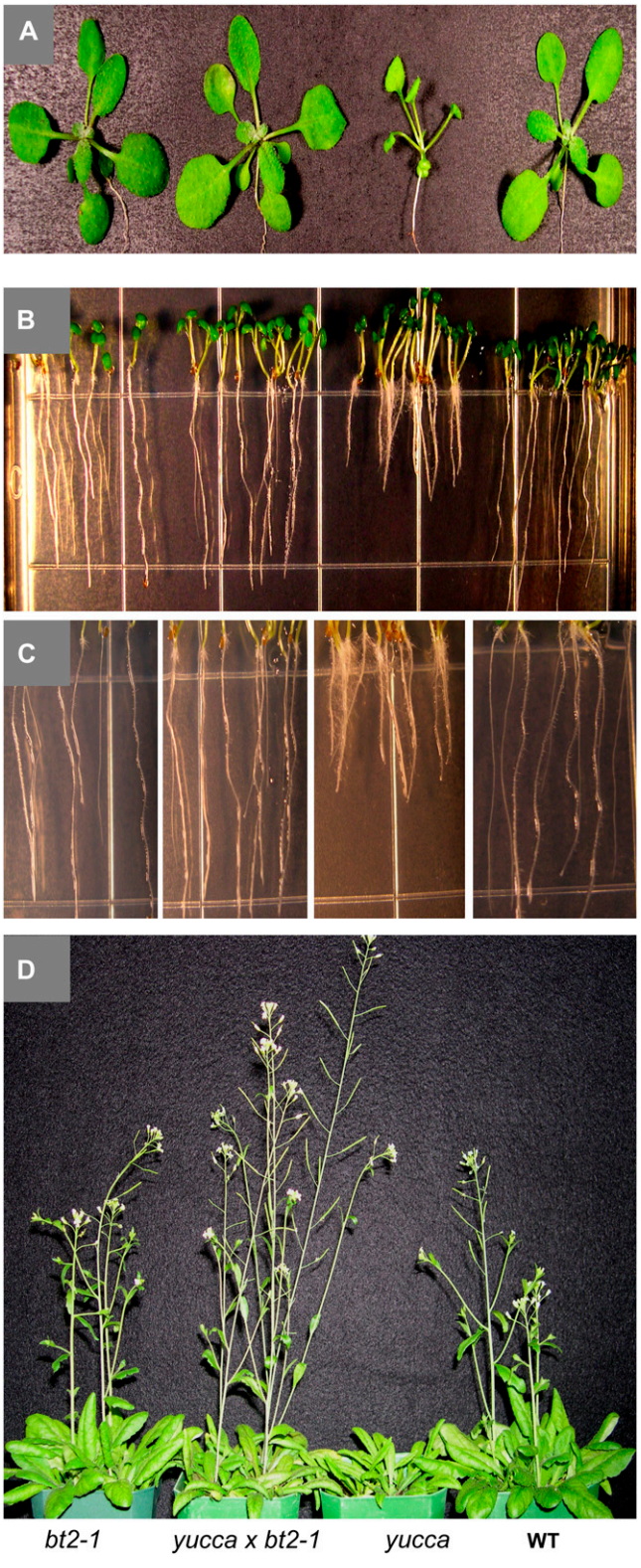

Figure 6. Loss of BT2 suppresses the high-auxin phenotypes in *yucca*. Suppression of the epinastic cotyledon and leaf morphology (A), shorter primary root (B), excess root hairs (C), and delayed flowering (D) of *yucca* in the double mutant, *yucca* × *bt2-1*. WT, Wild type.

both the light and dark phases (Fig. 3C). Addition of nitrates antagonizes sugar repression of gene expression (Moore et al., 2003). This antagonism could be due, in part, to competition for a limited amount of carbon in the cell that can be diverted either to produce organic acids and amino acids by nitrogen metabolism or to produce sugars and starches by carbon metabolism (Stitt and Krapp, 1999). Our results are consistent with gene expression databases, which indicate that *BT2* is repressed by sugars and induced by nitrates (Scheible et al., 2004; Wang et al., 2004; Blasing et al., 2005; Usadel et al., 2008).

To determine whether BT2 was involved in responses to sugar signaling, we analyzed germination in the presence of inhibitory Glc concentrations and found that constitutive overexpression of BT2 imparted resistance to inhibition of germination and early vegetative development by Glc. In contrast, loss of BT2 resulted in significantly increased sensitivity to inhibitory Glc levels (Fig. 4).

High concentrations of sugars impart their inhibitory effect on germination by modulating ABA signaling (León and Sheen, 2003). Also, several mutations identified as Glc-insensitive or sugar-insensitive, such as *gin1*, *gin5*, *sis4*, *sis7*, and *sis10*, are allelic to ABA-insensitive/deficient mutations (Arenas-Huertero et al., 2000; Cheng et al., 2002). After identifying a role for BT2 in modulating sugar signaling/responses at germination, we wanted to determine whether ABA responses also were modulated by BT2. We found that, parallel to the sugar responses, *bt2-1* was sensitive to ABA inhibition of germination, while *BT2OE* lines were resistant (Fig. 5A). Again, similar to sugars, ABA repressed the expression of *BT2* (Fig. 7A). This pattern of reciprocal negative feedback (where BT2 suppresses ABA signaling and ABA suppresses *BT2* expression) suggests that BT2 may normally function to prevent inappropriate signaling at low concentrations of ABA, but this function can be abrogated at higher concentrations of ABA.

BT2 does not appear to affect expression at the mRNA level for ABA signaling genes. This conclusion is supported by our previous microarray studies performed on the *tac1-1d* mutant line, which has increased *BT2* expression (Ren et al., 2007). When compared with the wild type, *tac1-1d* lines did not display any significant changes in transcript levels for genes in ABA signaling pathways. This lack of influence on ABA signaling genes could be due to either insufficient expression of BT2 at the protein level in *35S::BT2* and *tac1-1d* lines or the well-documented redundancy among BT family members (Robert et al., 2009) in the *bt2-1* null mutant. Alternatively, BT2 itself may be a downstream target of the ABA signaling genes. Regardless of the mechanism, our results here strongly support a role for BT2 in modulating sugar and ABA responses at germination.

Cold signaling is intricately associated with ABA, and cold stress and treatment with ABA repressed *BT2* expression (Fig. 7A). In fact, treatment with cold leads

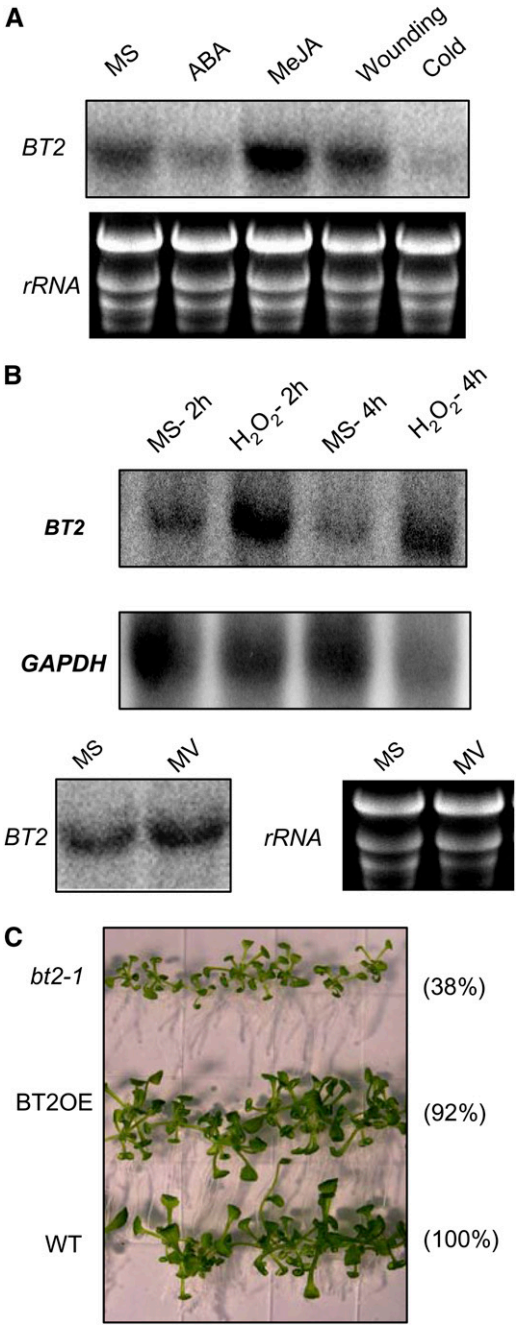

Figure 7. *BT2* expression is modulated by multiple stress signals. A, Three-week-old wild-type plants were grown on MS or treated with either ABA (lane 2) or Me-JA (lane 3) for 3 h and subjected to either wounding stress (lane 4) or cold stress (lane 5) for 3 h. (B) The plants from A were then challenged with oxidative stress caused by either H_2O_2 or methyl viologen (MV) for the indicated times. Total RNA was extracted from rosette leaves and subjected to RNA-blot analysis. Ethidium bromide-stained *rRNA* and *GAPDH* were used as loading controls. C, Visible phenotypes of 3-week-old wild-type, *bt2-1*, and *BT2OE* lines grown on MS medium containing H_2O_2 (2 mM). Total fresh weight (mg) of 30 seedlings from two different plates and ratio of weights relative to the wild type (WT; in parentheses) are shown on the right.

to an increase in the levels of ABA (Lee et al., 2001). Hence, it is possible that the cold repression of *BT2* was an indirect effect of increased ABA levels and/or signaling. Jasmonate is antagonistic to ABA in modulating defense gene expression (Anderson et al., 2004) and salt stress-inducible gene expression in rice (*Oryza sativa*; Moons et al., 1997). In contrast to ABA and cold, Me-JA induced *BT2* expression, thus suggesting a possible role of BT2 in jasmonate signaling/pathogen defense. However, further experiments are necessary to directly implicate BT2 in defense signaling.

ABA, often dubbed "the universal stress hormone," is associated with the response to ROS and cross talks with multiple hormones and biotic and abiotic signals (Roitsch, 1999; Fedoroff, 2002; Couee et al., 2006). *BT2* is induced by H_2O_2, a major ROS in plants, and loss of BT2 renders the plants sensitive to external H_2O_2 (Fig. 7). This sensitivity, however, was not observed when the *bt2-1* lines were subjected to a different ROS stress, superoxide anion generated by methyl viologen (data not shown). Moreover, *BT2* expression was not induced by ROS stress caused by methyl viologen (Fig. 7B), suggesting that the response of *BT2* to H_2O_2 is specific and not due to general ROS-related stress.

We previously reported that BT2 enhances certain auxin responses (Ren et al., 2007). Here, we present additional evidence for its role in potentiating auxin responses in plants. Loss of BT2 in the high-auxin mutant *yucca* reversed several of its high-auxin phenotypes, including its characteristic epinastic cotyledons, epinastic leaves, shorter primary root, excess root hair, and delayed flowering (Fig. 6). Auxin is often antagonistic to ABA, perhaps because of its opposite effects on cytosolic pH and regulation of Ca^{2+} concentrations (Gehring et al., 1990). Mutations in genes such as *HYPONASTIC LEAVES1* result in hypersensitivity to ABA while simultaneously rendering resistance to auxin (Lu and Fedoroff, 2000). From our results here and previous studies (Ren et al., 2007), BT2 seems to potentiate auxin responses and suppress ABA responses, consistent with antagonism between auxin and ABA.

Although BT2's initially described function was in regulating telomerase activity in mature leaves (Ren et al., 2007) and it was recently shown to function in gametophyte development along with other BT family genes (Robert et al., 2009), it now appears to play a much broader role. The gene itself responds to multiple biotic and abiotic signals, including light, circadian clock, phytohormones, and nutrients, and BT2 is required for the appropriate response to many of these same signals. We propose that BT2 occupies an integral position in a complex signaling network that perceives, integrates, and responds to multiple, and sometimes competing, signals. Preliminary results from our laboratory indicate that, similar to *BT2* responses, expression of *BT1* and *BT5* is also responsive to sugars and nitrates (K.K. Mandadi, unpublished data), consistent with previous reports of functional redundancy in the BT gene family (Robert et al., 2009).

It is not yet clear how BT2 affects multiple signaling pathways. Earlier studies from other laboratories, using recombinant proteins, in vitro pull-down assays, or yeast two-hybrid screens, identified BT2, along with other BT family members, as interacting with either CULLIN3 (Figueroa et al., 2005) or with the BET9 and BET10 bromodomain proteins (Du and Poovaiah, 2004). Although BT2's in vivo partners are yet to be identified, we hypothesize that it assembles in multiprotein complexes. If the complex requires CULLIN3 or a similar protein, it may function as a ubiquitin ligase and target specific proteins for degradation. Alternatively, if the BT2 complex requires the BET9 or BET10 bromodomain proteins, the complex may work by recognizing the chromatin state of target gene promoters. Identification of proteins that interact with BT2 in vivo will be required to resolve the possible modes of action.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Wild-type Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) or mutant plants were grown in soil in 14-h-light/10-h-dark cycles at 21°C and a light intensity of approximately 120 to 130 μmol m^{-2} s^{-1} with 70% relative humidity. For germination assays, seeds were surface sterilized with 50% (v/v) bleach and 0.1% (v/v) Triton X-100 for 7 min, cold treated at 4°C for 3 to 4 d, and then grown on Murashige and Skoog (MS) medium (Sigma) with 0.8% (w/v) phytagar under continuous low light (approximately 30 μmol m^{-2} s^{-1}). All media contained 1% Suc, unless stated otherwise. *BT2* overexpression lines and the *bt2-1* null line were described previously (Ren et al., 2007). To examine the effect of loss of BT2 on the high-auxin phenotype of *yucca* (Zhao et al., 2001), we generated and examined the progeny of a *bt2-1* × *yucca* cross.

Treatments and Expression Analysis

For circadian experiments and light/dark treatments, whole rosettes of 3-week-old wild-type plants (prior to flowering) were harvested at the indicated times of the diurnal cycle. Two biological replicates each containing two rosettes were harvested and subjected to RNA gel blot and qRT-PCR analysis. Total RNA was isolated using TRI reagent following the manufacturer's protocol (Ambion). For RNA gel blots, 20 μg of total RNA from each sample was separated on a 1.2% formaldehyde denaturing gel and transferred to a Hybond N^+ membrane (Amersham). Blots were then probed with ^{32}P-labeled PCR products obtained from amplification of *BT2* cDNA using the primers listed (Supplemental Table S1). Subsequently, the blot was stripped and reprobed for *GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE* (*GAPDH*) mRNA as a loading control. For qRT-PCR, 1 μg of RNA was used to make cDNA using the SuperScript first-strand cDNA synthesis kit (Invitrogen). Amplification by PCR was performed as described previously (Guo et al., 2008) using Power SYBR Green Master Mix (Applied Biosystems) and the ABI Prism 7500 sequence detection system (Applied Biosystems). The primers used for qRT-PCR are listed (Supplemental Table S1). *EIF-4A2* (At1g54270) and *18S rRNA* (At2g01010) were used to normalize the expression, and fold changes of *BT2* expression were calculated following the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

To determine the effects of sugars and nitrates, leaves from 3-week-old wild-type plants were excised at the indicated times. To maintain transpiration flow, petioles were immediately recut in liquid medium supplemented with 100 mM Suc or mannitol and 50 mM KNO_3 or KCl (Chiou and Bush, 1998). Treatments were conducted for 3 h in the appropriate light conditions, and the samples were subsequently subjected to RNA gel-blot analysis as described above to detect *BT2* expression. Ethidium bromide-stained rRNA was used as a loading control. For Glc treatments, seedlings were grown in MS liquid medium for 5 d in continuous light and later transferred into medium without any sugars for 2 d. After day 7, the medium was supplemented with 50 mM Glc, 50 mM Suc, or 50 mM mannitol and the seedlings were treated for 3 h (Scheible et al., 2004; Blasing et al., 2005). Subsequent analysis of *BT2* expression was performed by RT-PCR using 5 μg of total RNA to prepare cDNA. To determine the expression of *ABI3*, *ABI4*, and *ABI5*, 10-d-old wild-type, *bt2-1*, and *BT2OE* lines were grown on 5% Glc and analyzed by RT-PCR (25 cycles). The primers used for RT-PCR are listed (Supplemental Table S1).

To determine the effects of various stresses on *BT2* expression, 3-week-old wild-type plants were subjected to various stress stimuli during the light phase. Cold treatment was performed by floating leaves in MS liquid medium on ice for 3 h; for wounding, leaves were punctured with forceps at several places and transferred to MS liquid medium for 3 h; for stress hormones, leaves were treated for 3 h in MS liquid media consisting of ABA (100 μM, mixed isomers) and Me-JA (100 μM) or dimethyl sulfoxide (0.1%); for oxidative stress, leaves were treated for the indicated times in MS liquid media consisting of H_2O_2 (10 mM) and methyl viologen (100 μM). *BT2* expression was analyzed by RNA gel-blot analysis using 20 μg of total RNA as described above. Ethidium bromide-stained rRNA was used as a loading control. All treatments were repeated at least twice at different periods, and the results described are representative of consistent data obtained in replicate experiments. Dimethyl sulfoxide did not affect *BT2* expression.

Glc, ABA, and H_2O_2 Sensitivity Assays

For Glc inhibition assays, wild-type, *bt2-1*, and *BT2OE* lines were germinated on solid MS media with various concentrations of Glc (4%, 5%, and 6%, w/v) or mannitol (5%, w/v), as described previously (Bi et al., 2005). After 5 or 6 d, seedlings with normal cotyledons were counted. For ABA inhibition assay, wild-type, *bt2-1*, and *BT2OE* lines were germinated on MS solid media with various concentrations of ABA (0, 1, 2.5, and 5 μM), as described previously (Xiong et al., 2002). After 5 or 6 d, seedlings with normal cotyledons were counted. Three replicate plates for each treatment were used to calculate the percentage germination rates, and significant differences were determined by Student's *t* test. For the H_2O_2 sensitivity assay, wild-type, *bt2-1*, and *BT2OE* lines were germinated on MS solid medium with or without 2 mM H_2O_2 (Miao et al., 2006) and were kept vertically in continuous low light (approximately 30 μmol m^{-2} s^{-1}) for 3 weeks. Mean fresh weight of the seedlings was determined from averages of two replicate plates.

Arabidopsis Genome Initiative locus numbers for the genes used in this article are as follows: *BT1*, At5g63160; *BT2*, At3g48360; *BT3*, At1g05690; *BT4*, At5g67480; *BT5*, At4g37610; *ABI3*, At3g24650; *ABI4*, At2g40220; *ABI5*, At2g36270; *EIF-4A2*, At1g54270; *18S* rRNA, At2g01010; *GAPDH*, At3g04120.

Supplemental Data

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

Supplemental Figure S1. Quantification of diurnal fluctuation of *BT2* mRNA.

Supplemental Table S1. Primers used.

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