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Roles of pepper bZIP transcription factor CaATBZ1 and its interacting partner RING-type E3 ligase CaASRF1 in modulation of ABA signalling and drought tolerance

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SUMMARY

Ubiquitination is a eukaryotic protein modulation system for identifying and affecting proteins that are no longer needed in the cell. In a previous study, we elucidated the biological function of CaASRF1, which contains a RING finger domain and functions as an E3 ligase. We showed that CaASRF1 positively modulates abscisic acid (ABA) signalling and drought stress tolerance by modulating the stability of subgroup D bZIP transcription factor CaAIBZ1. We performed yeast two-hybrid (Y2H) screening to identify an additional target protein of CaASRF1. In this study, we identified pepper CaATBZ1 (*Capsicum annuum* ASRF1 target bZIP transcription factor 1), which belongs to the subgroup A bZIP transcription factors. We investigated the biological function of this protein using virus-induced gene silencing (VIGS) in pepper plants and by generating overexpressing transgenic Arabidopsis plants. Our loss-of-function and gain-of-function studies revealed that CaATBZ1 negatively modulates ABA signalling and drought stress response. Consistent with *CaATBZ1*-silenced pepper plants, *CaASRF1*/*CaATBZ1*-silenced pepper plants displayed drought-tolerant phenotypes via ABA-mediated signalling. Our results demonstrated that CaASRF1-mediated ubiquitination plays a crucial role in regulating the stability of CaATBZ1. These findings provide valuable insight into the post-translational regulation of transcriptional factors.

Keywords: ABA, bZIP transcription factor, degradation, drought, E3 ligase, ubiquitination.

INTRODUCTION

Water deficit is an important environmental stress caused by drought, high salinity, and cold, and it results in significant losses of crop yield. To adapt to water-deficit conditions, plants have evolved various defence mechanisms to minimize transpirational water loss from stomata and maximize water uptake from root tips (Apse and Blumwald, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). Physiological and molecular mechanisms functioning under water-deficit conditions have been extensively investigated (Zhu, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). The plant defence response to water-deficit conditions is a complex phenomenon, and the precise adaptive processes induced by water deficit remain unclear. Nevertheless, the phytohormone abscisic acid (ABA) is known to be involved in the drought stress response. ABA is a key plant hormone integrating growth and abiotic stress responses. Under water-deficit conditions, ABA facilitates adaptation via several strategies, including promotion of stomatal closure, induction of defence-related genes, and accumulation of various protective metabolites (Lee and Luan, 2012). Simultaneously, ABA biosynthesis and ABA biosynthesis-related genes are upregulated (Tan *et al.*, 2003). Several 9-*cis*-epoxycarotenoid dioxygenases (*NCED*s) genes are associated with ABA biosynthesis (Nambara and Marion-Poll, 2005). *NCED* gene expression is induced by osmotic stresses, including drought, high salinity, and low temperature (Iuchi *et al.*, 2000; Yang and Guo, 2007). Genetic evidence has revealed that *NCED* genes are positive regulators of ABA biosynthesis and abiotic stress tolerance. *NCED*-overexpressing mutants of several higher plants showed increased ABA contents and enhanced tolerance to drought and high salinity (Iuchi *et al.*, 2001; Qin and Zeevaart, 2002; Zhang *et al.*, 2009). In contrast, *NCED3* null mutants exhibited drought-sensitive phenotypes characterized by reduced ABA levels (Iuchi *et al.*, 2001). However, the precise molecular events occurring upstream of ABA biosynthesis remain unclear.

In signal transduction pathways, specific genes are regulated by the interaction between *cis*-acting elements in the promoter region and transcription factors (Thatcher *et al.*, 2012; Llorca *et al.*, 2014). Transcription factors have several characteristics, including nuclear localization, transcriptional activation or repression, and DNA binding to promoter regions of the target gene, which is essential for inducing target gene expression (Eulgem *et al.*, 2000; Jakoby *et al.*, 2002; Llorca *et al.*, 2014). Many transcription factors are involved in adaptation to stress conditions, including drought, high salinity, and temperature stresses (Lee *et al.*, 2006; Froidure *et al.*, 2010; Fu and Dong, 2013). Knockout or overexpressing mutants exhibited tolerant or sensitive phenotypes to different stresses. Hence, several transcription factor families – each containing a distinct type of *cis*-acting element (including ERF, MYB, RAV, and bZIP) – have been implicated in plant adaptive responses (Lee *et al.*, 2006; Sohn *et al.*, 2006; Kesarwani *et al.*, 2007; Li *et al.*, 2014; Zhu *et al.*, 2014). Plant bZIP proteins were clustered into 10 groups (subgroup A–I and S) according to sequence similarities of basic region and additional conserved motifs (Jakoby *et al.*, 2002). Several studies have demonstrated that the functions of bZIP factors are diverse biological processes, including seed maturation, senescence, and adaptive biotic and abiotic stress responses (Jakoby *et al.*, 2002; Zhang *et al.*, 2003; Alves *et al.*, 2013). Involvement of bZIP factors in plant adaptive abiotic stress responses is well established (Blanco and Judelson, 2005; Lee *et al.*, 2006; Pitzschke *et al.*, 2009). In particular, a few bZIP transcription factors from subgroup A – such as ABI5 and AREBs – have been intensively characterized for their roles in regulating drought tolerance or ABA signalling (Finkelstein and Lynch, 2000; Uno *et al.*, 2000; Jakoby *et al.*, 2002). Moreover, subgroup D proteins participate in the defence response to pathogen infection (Jakoby *et al.*, 2002).

A rapid and effective alternative mechanism for plant adaptation to abiotic stress is post-translational modification. Ubiquitination – a type of post-translational modification – is involved in many plant cellular developmental and stress response processes (Ding *et al.*, 2015a). Upon attachment of several ubiquitins, substrate proteins undergo proteolysis by the 26S proteasome; this process is mediated by three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) (Ciechanover and Schwartz, 1998). E3 ligase determines specificity and recruits target proteins. Genome sequencing has identified more than 1400 E3 ligases in Arabidopsis (Vierstra, 2009).

In a previous study, we isolated and characterized RING-type E3 ligase CaASRF1, which functions as a positive modulator of ABA signalling and the drought stress response via modulation of subgroup D bZIP transcription factor CaAIBZ1 (Joo *et al.*, 2019). Here, we identified CaASRF1 interacting subgroup A bZIP transcription factor CaATBZ1, which is a substrate of CaASRF1. CaATBZ1 is associated with drought sensitivity via regulation of the stomatal aperture. Our findings indicated that CaASRF1 positively modulates ABA signalling and drought response via its E3 ligase activity by influencing CaATBZ1 stability.

RESULTS

Identification of CaATBZ1 as a target protein of CaASRF1

To identify an additional target protein of CaASRF1, we performed yeast two-hybrid (Y2H) screening and identified CaATBZ1 (*Capsicum annuum* ASRF1 target bZIP transcription factor 1). We confirmed the direct interaction of CaATBZ1 with CaASRF1 using Y2H, *in vitro* pull-down, and bimolecular fluorescence complementation (BiFC) assays (Figure 1). The growth of yeast with CaATBZ1 and CaASRF1 was detected on the selective medium, indicating that CaATBZ1 interacts with CaASRF1 (Figure 1a). Bacterial expressed MBP–CaASRF1 and GST–CaATBZ1 recombinant proteins were co-incubated with a glutathione sepharose. MBP–CaASRF1 was pulled down from the glutathione sepharose resin by GST–CaATBZ1, indicating the physical interaction of CaASRF1 with CaATBZ1 (Figure 1b). Co-expression of *CaATBZ1*-CYCE with *CaASRF1*-VYNE resulted in yellow fluorescence predominantly in the nuclei (Figure 1c). Moreover, the CaASRF1 RING domain is important

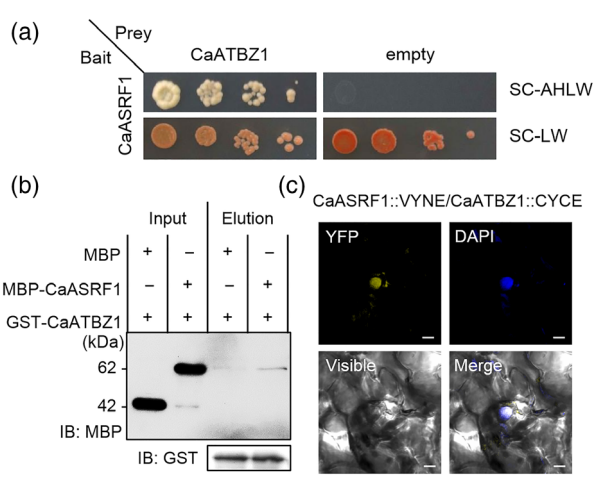

Figure 1. CaASRF1 interacts with CaATBZ1. (a) Yeast two-hybrid assay of interaction between CaASRF1 and CaATBZ1. Growth on the selection medium (SC–adenine–histidine–leucine–tryptophan (AHLW)) was used as an indication of interaction (upper row). Growth on SC–leucine–tryptophan (LW) medium was used as a control (lower row). (b) Pull-down assays show the direct interaction between MBP-CaASRF1 and GST-CaATBZ1 fusion proteins *in vitro*. MBP-CaASRF1 and MBP proteins were incubated with glutathione *S*-transferase (GST)-CaATBZ1 proteins, and immunoprecipitated fractions were detected using MBP and GST antibodies. (c) Bimolecular fluorescence complementation (BiFC) assay of interactions between CaASRF1 and CaATBZ1. *CaASRF1-VYNE* was co-expressed with *CaATBZ1-CYCE* in the leaves of *Nicotiana benthamiana*. White bars, 10 μm.

for interaction with CaATBZ1 (Supporting Information Figure S1).

To further investigate whether CaATBZ1 is a target of CaASRF1, we performed *in vivo* ubiquitination and cell-free degradation assays (Figure 2). To examine CaASRF1-mediated polyubiquitination of CaATBZ1 in the plant cell, *35S:HA-CaATBZ1* was co-expressed with *35S:GFP* or *35S:CaASRF1-GFP* in tobacco leaves. As shown in Figure 2(a), CaATBZ1 was polyubiquitinated by CaASRF1; therefore, we postulated that the turnover of CaATBZ1 is modulated by CaASRF1 in a 26S proteasome-dependent manner. To confirm this possibility, we performed a cell-free degradation assay. When GST–CaATBZ1 was co-incubated with *CaASRF1*-silenced pepper plant extracts (TRV:*CaASRF1*) or control pepper plant extracts (TRV:00), the level of GST–CaATBZ1 protein was less markedly decreased in the *CaASRF1*-silenced pepper plant extracts than in the control pepper plant extracts (Figure 2b). Moreover, supplementation with MG132 inhibited the degradation of CaATBZ1 in TRV:*CaASRF1* pepper plant extracts and TRV:00 pepper plant extracts. In contrast, when GST–CaATBZ1 was co-incubated with *CaASRF1*-overexpressing (OX) Arabidopsis plant extracts or wild-type plant extracts, the level of GST–CaATBZ1 protein was much more decreased in the *CaASRF1*-OX plant extracts than in the wild-type plant extracts (Figure 2c). These results indicated that CaASRF1 is associated with CaATBZ1 degradation through interaction and ubiquitination of CaATBZ1.

Expression of *CaATBZ1* in pepper leaves and subcellular localization of CaATBZ1

CaATBZ1 is composed of 1056 bp nucleotides, and the deduced amino acid sequence encodes a protein of 351 amino acids (Figure S2a). CaATBZ1 consists of a basic leucine zipper (BLZ) domain at the C-terminal region and has high similarity with the amino acid sequence of other species (Figure S2a,b). The BLZ domain, which is composed of 63 amino acids and is predicted to specifically bind to promoter regions and activate target genes, has been characterized in various plant proteins, functioning as signalling transcription factors (Finkelstein and Lynch, 2000; Uno *et al.*, 2000; Jakoby *et al.*, 2002).

Previous studies have showed that group A bZIP transcription factors play a role to form homodimers (Deppmann *et al.*, 2006; Lim *et al.*, 2015). The CaATBZ1 belongs to A group bZIPs (Figure S3); therefore, we verify its homodimerization using the BiFC assay (Figure S2c). Co-expression of *CaATBZ1*-CYCE with *CaATBZ1*-VYNE resulted in yellow fluorescence predominantly in the nuclei, indicating that CaATBZ1 forms a homodimer. Moreover, CaATBZ1 also interacts with CaAIBZ1 (Figure S4), which suggests that CaATBZ1 and CaZIBZ1 probably function together under stress conditions.

CaASRF1 expression was induced by ABA, drought, and high salinity (Joo *et al.*, 2019); therefore, we performed quantitative RT-PCR (qRT-PCR) analysis of *CaATBZ1* using leaves harvested from six-leaf stage pepper plants that had been treated with ABA, drought, or NaCl (Figure 3). After ABA treatment, *CaATBZ1* transcripts were downregulated at 2 h and continued to be suppressed after 24 h (Figure 3a). After drought or NaCl treatment, the *CaATBZ1* transcripts were weakly induced at 2 h, reached peak levels after 6 h, and then gradually decreased to the basal level within 24 h. CaATBZ1 is a bZIP transcription factor, therefore we predicted that CaATBZ1 localizes and functions in the nucleus. To confirm subcellular localization of CaATBZ1, we fused the full-length coding region of *CaATBZ1* cDNA with *GFP* under the control of the 35S promoter (Figure 3b). The 35S:*CaATBZ1-GFP* fusion protein generated GFP signals in the nucleus of *N. benthamiana* epidermal cells, indicating that CaATBZ1 functions in the nucleus.

Enhanced drought tolerance of *CaATBZ1*-silenced pepper plants

To investigate the biological function of CaATBZ1, we performed loss-of-function analysis using virus-induced gene silencing (VIGS) (Figure 4). To confirm the efficiency of VIGS, we monitored the expression levels of *CaATBZ1* using quantitative RT-PCR. The levels of *CaATBZ1* transcripts were lower in *CaATBZ1*-silenced pepper plants (TRV:*CaATBZ1*) than in control pepper plants (TRV:00) (Figure 4a). We monitored the transpirational water loss by measuring the fresh weight loss of detached rosette leaves. At 10 h after detachment, the transpirational water loss was significantly lower in *CaATBZ1*-silenced pepper plants (16.3%) than in control pepper plants (23.2%; Figure 4b). We subjected pepper plants to drought stress by withholding watering for 17 days and then re-watering for 3 days. In comparison with control pepper plants, *CaATBZ1*-silenced pepper plants displayed a drought-tolerant phenotype (Figure 4c, middle and right panels). At 3 days after re-watering, the survival rate of *CaATBZ1*-silenced pepper was 86.67%, whereas only 25.00% of control pepper plants survived (Figure 4c). To examine whether the enhanced drought tolerance of *CaATBZ1*-silenced pepper plants is associated with ABA response, we monitored leaf temperatures and stomatal apertures (Figure 4d,e). After 5 h of ABA treatment, the leaf temperatures of *CaATBZ1*-silenced pepper plants were significantly higher than those of control pepper plants (Figure 4d). To monitor ABA-mediated stomatal closure, we measured the stomatal apertures. Before ABA treatment, we observed no significant differences in stomatal pore size between control and *CaATBZ1*-silenced pepper plants. In the presence of ABA, the stomatal apertures of *CaATBZ1*-silenced pepper plants were significantly

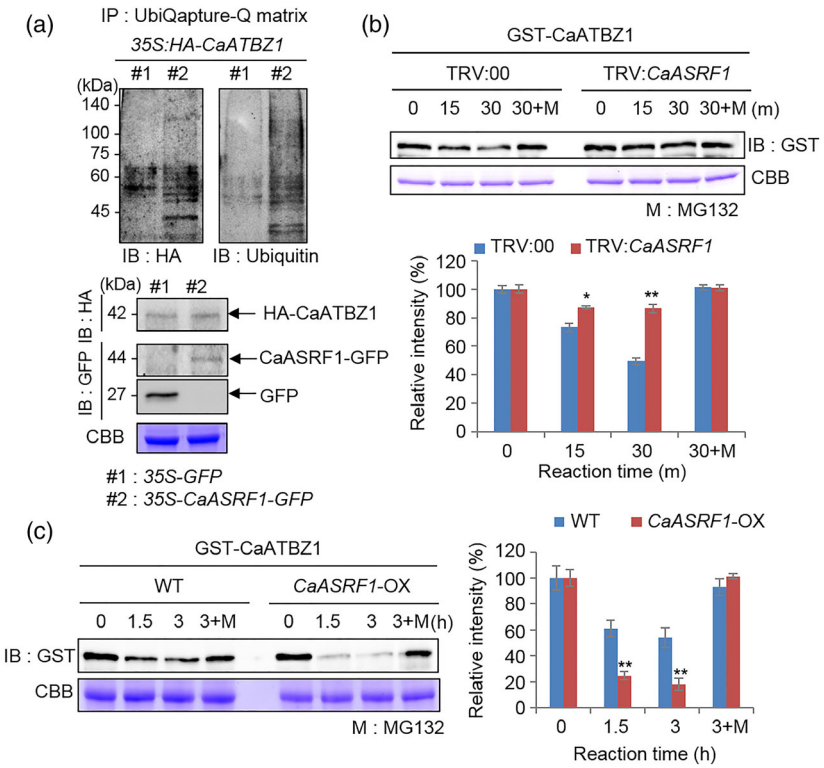

Figure 2. CaASRF1 is associated with CaATBZ1 degradation. (a) CaASRF1-mediated ubiquitination of CaATBZ1 *in vivo*. In the tobacco leaves, *35S:HA-CaATBZ1* was co-expressed with *35S:GFP* or *35S:CaASRF1-GFP*. Ubiquitination of HA-CaATBZ1 was detected using anti-HA or anti-ubiquitin antibodies; shifted bands indicated the attachment of ubiquitin molecules. Coomassie blue staining (CBB) indicated equal loading of protein extract. (b) Cell-free degradation assay for CaATBZ1. The GST–CaATBZ1 protein was incubated for 15 and 30 min with crude extracts prepared from the leaves of empty vector control pepper plants (TRV:00) and *CaASRF1*-silenced pepper plants (TRV:*CaASRF1*). Asterisks indicate significant difference compared with control pepper plants (Student's *t*-test; $*P < 0.05$; $**P < 0.01$). (c) Cell-free degradation assay using wild-type and *CaASRF1*-OX Arabidopsis plants. The GST–CaATBZ1 protein was incubated for 1.5 h or 3 h with crude extracts prepared form the leaves of wild-type and *CaASRF1*-OX Arabidopsis plants. Asterisks indicate significant difference compared with wild-type plants (Student's *t*-test; $**P < 0.01$).

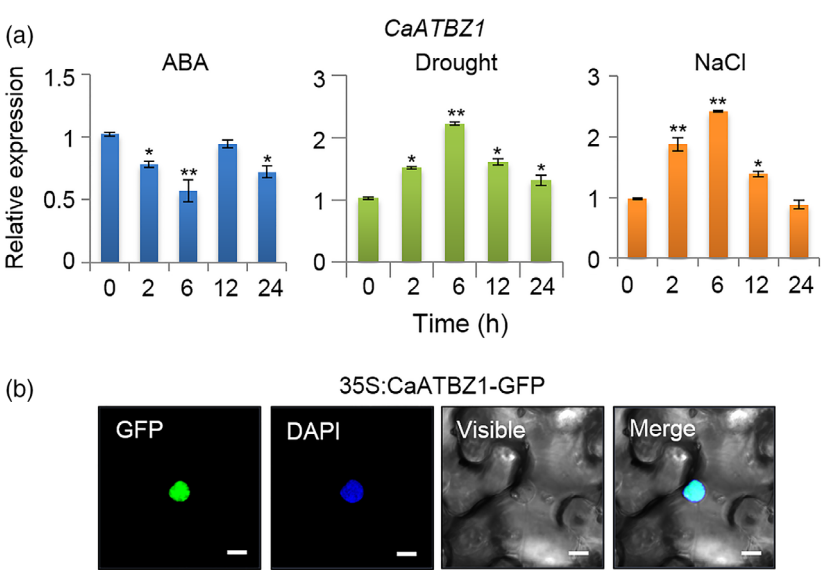

Figure 3. (a) The expression pattern of the *CaATBZ1* gene was analyzed in the leaves of pepper plants after abscisic acid (ABA) (100 μM), drought stress, or NaCl (200 mM) treatments. The *CaACT1* gene was used as an internal control. ($*P < 0.05$; $**P < 0.01$; Student's *t*-test). (b) Subcellular localization of CaATBZ1 based on transient expression of the GFP fusion protein in *Nicotiana benthamiana*. White bars, 10 μm.

smaller than those of control pepper plants, indicating that ABA-induced stomatal closure was enhanced in the *CaATBZ1*-silenced pepper plants. To investigate whether the drought-tolerant phenotype was affected by altered expression of stress-related genes, we conducted qRT-PCR analysis of stress-related genes, including *CaOSM1*, *CaOSR1*, and *CaNCED3* (Figure 4f). The expression levels of stress-related genes were significantly higher in *CaATBZ1*-silenced pepper plants than in control pepper plants. CaATBZ1 interacts with CaAIBZ1 (Figure S4). Therefore, we investigated drought phenotypes of *CaATBZ1*/*CaAIBZ1*-silenced pepper (TRV:*CaATBZ1*/TRV:*CaAIBZ1*) plants (Figure S5). *CaATBZ1*/*CaAIBZ1*-silenced pepper plants showed tolerant phenotypes to drought stress compared with control, *CaATBZ1*-silenced pepper and *CaAIBZ1*-silenced pepper plants.

Reduced ABA sensitivity of *CaATBZ1*-OX plants

We generated *CaATBZ1*-OX Arabidopsis transgenic plants and used these in our subsequent genetic analysis. First, we monitored *CaATBZ1* overexpression using a semiquantitative RT-PCR assay. Expression of *CaATBZ1* transcripts was detected in two independent T_3 homozygous transgenic lines, but not in wild-type plants (Figure S6). We compared the phenotypes of wild-type and transgenic plants in response to ABA. In the absence of ABA, we observed no significant phenotypic differences between wild-type and transgenic plants. However, in the presence of ABA, the germination rate of *CaATBZ1*-OX seeds was significantly higher than that of wild-type seeds (Figure 5a). Next, we compared seedling establishment and root growth of wild-type and *CaATBZ1*-OX plants. Consistent with the germination rate, the numbers of green cotyledons and root growth were significantly higher and longer, respectively, in *CaATBZ1*-OX plants than in wild-type plants (Figure 5b–e). To investigate whether the ABA insensitivity of seedling growth is caused by different response at germination stage, we conducted a postgermination assay. As shown in Figure S7, *CaATBZ1*-OX plants also showed ABA-insensitive phenotypes compared with wild-type plants. To investigate whether the ABA-insensitive phenotype was affected by altered expression of stress-related genes, we conducted qRT-PCR analysis of stress-related genes, including *DREB2A*, *RD29A*, *RD20* and *RD22* using ABA-treated Arabidopsis plants (Figure 5f). The expression levels of stress-related genes were significantly lower in *CaATBZ1*-OX plants than in wild-type plants. These results indicated that enhanced expression of *CaATBZ1* led to reduced ABA response at the germination and seedling stages.

Reduced drought tolerance of *CaATBZ1*-OX plants

To examine the influence of *CaATBZ1* overexpression on drought response, we subjected 4-week-old wild-type and *CaATBZ1*-OX plants to drought stress by withholding watering for 11 days and then re-watering for 1 day (Figure 6a). In comparison with wild-type plants, *CaATBZ1*-OX plants displayed a drought-sensitive phenotype (Figure 6a, middle and right panel). Moreover, the survival rate of *CaATBZ1*-OX plants was significantly lower than that of wild-type plants. Next, we monitored the transpirational water loss by measuring the fresh weight loss of rosette leaves 10 h after detachment (Figure 6b). The transpirational water losses in *CaATBZ1*-OX and wild-type plants were 53.5–56.0% and 49.4%, respectively. Finally, we measured leaf temperatures and stomatal apertures of *CaATBZ1*-OX and wild-type plants (Figure 6c,d). In the presence of ABA, the leaf temperatures of *CaATBZ1*-OX plants were significantly lower (Figure 6c) and the stomatal apertures were significantly larger than those of wild-type plants (Figure 6d). These results indicate that the lower water retention displayed by *CaATBZ1*-OX plants results from reduced ABA sensitivity. To investigate whether the drought-sensitive phenotype was affected by altered expression of stress-related genes, we conducted qRT-PCR analysis of stress-related genes, including *RD29B*, *RD26*, and *RAB18* (Figure 6e). The expression levels of stress-related genes were significantly lower in *CaATBZ1*-OX plants than in wild-type plants. However, the transcript level of *KIN2* did not differ significantly between wild-type and *CaATBZ1*-OX plants. These results suggested that CaATBZ1 functions as a negative modulator of the ABA-mediated drought response.

Enhanced drought tolerance of *CaASRF1*- and *CaATBZ1*-double silenced pepper plants

To investigate whether CaASRF1 functions in the same pathway as CaATBZ1 during the drought response, we performed a double VIGS assay (Figure 7). The transcript levels of *CaASRF1* and *CaATBZ1* were downregulated in *CaASRF1*/*CaATBZ1*-silenced pepper (TRV:*CaASRF1*/TRV:*CaATBZ1*) than in control pepper plants (TRV:00) (Figure 7a). Under well watered conditions, we observed no phenotypic differences between control and *CaASRF1*/*CaATBZ1*-silenced pepper plants (Figure 7b, upper panel). However, after drought stress, *CaASRF1*/*CaATBZ1*-silenced pepper plants displayed drought-tolerant phenotypes (Figure 7b, middle and lower panels). In addition, the survival rate of *CaASRF1*/*CaATBZ1*-silenced pepper plants (79.2%) was significantly higher than that of control pepper plants (37.5%). To verify whether the drought-tolerant phenotypes are derived from different capacities for water retention, we monitored the transpirational water loss by measuring the fresh weight loss of detached leaves (Figure 7c). After 10 h, the transpirational water losses were significantly lower in *CaASRF1*/*CaATBZ1*-silenced pepper plants than in control pepper plants. To investigate whether the enhanced water retention of *CaASRF1*/*CaATBZ1*-silenced pepper plants is associated with ABA response, we monitored the leaf temperatures and stomatal apertures (Figure 7d,e). The leaf temperatures of *CaASRF1*/*CaATBZ1*-silenced pepper plants were significantly higher than those of control pepper plants (Figure 7d). Moreover, the stomatal apertures of *CaASRF1*/*CaATBZ1*-silenced pepper plants were significantly smaller than those of control pepper plants, indicating that ABA-induced stomatal closure was enhanced in the *CaASRF1*/*CaATBZ1*-silenced pepper plants (Figure 7e). These results suggested that CaASRF1 functions upstream of CaATBZ1 in the ABA-signalling-mediated drought response.

DISCUSSION

ABA plays a key role in the plant adaptive abiotic stress response. Many regulatory genes have been identified as

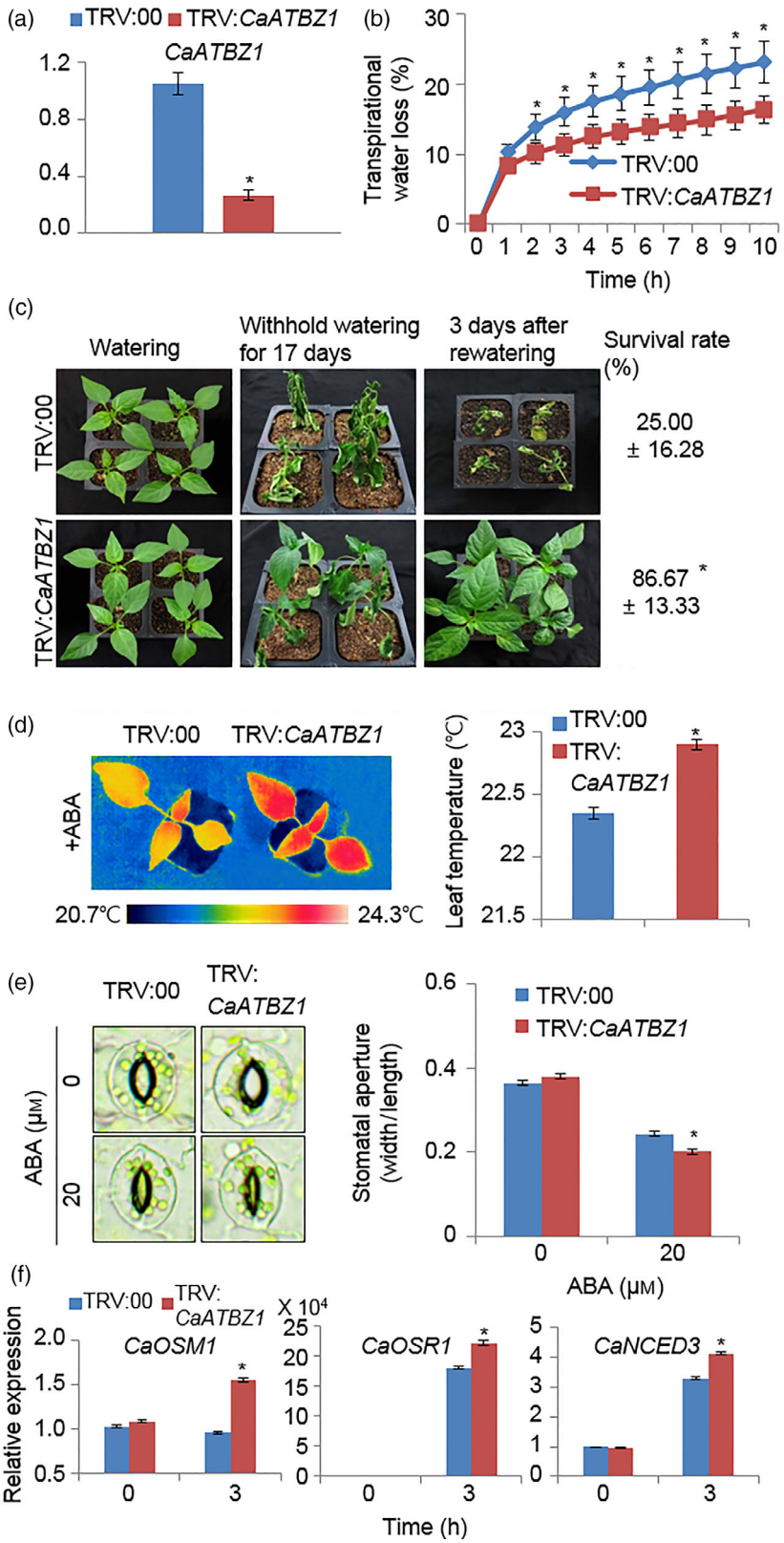

Figure 4. Enhanced drought tolerance of *CaATBZ1*-silenced pepper plants. (a) qRT-PCR analysis of *CaATBZ1* gene expression in the leaves of TRV:00 and TRV:*CaATBZ1* plants. The relative expression (ΔΔCT) of each gene was normalized to that of *CaACT1*, used as an internal control gene. (b) Transpirational water loss from the leaves of empty vector control and *CaATBZ1*-silenced pepper plants at various times after detachment of leaves. (c) The drought-tolerant phenotype of *CaATBZ1*-silenced pepper plants. Empty vector control and *CaATBZ1*-silenced pepper plants were grown in pots for 5 weeks under well watered conditions. Thereafter, watering was withheld for 17 days, followed by re-watering for 3 days. (d) Representative thermographic images of *CaATBZ1*-silenced pepper plants 5 h after treatment with 50 μM ABA. The mean leaf temperature was measured using 10 plants of each line. (e) Stomatal apertures in control and *CaATBZ1*-silenced pepper plants treated with ABA. Leaf peels were harvested from 3-week-old plants of each line and incubated in stomatal opening solution (SOS) buffer containing 0 and 20 μM ABA. Representative photographs were taken under a microscope and the stomatal apertures were measured. (f) qRT-PCR analysis of stress-related genes in the *CaATBZ1*-silenced pepper plants in response to drought stress at 0 and 3 h after detachment. The relative expression (ΔΔCT) of each gene was normalized to that of *CaACT1*, used as an internal control gene. Data represent the mean ± standard error of three independent experiments. Asterisks indicated significant differences between three independent experiments (Student's *t*-test; $*P < 0.05$).

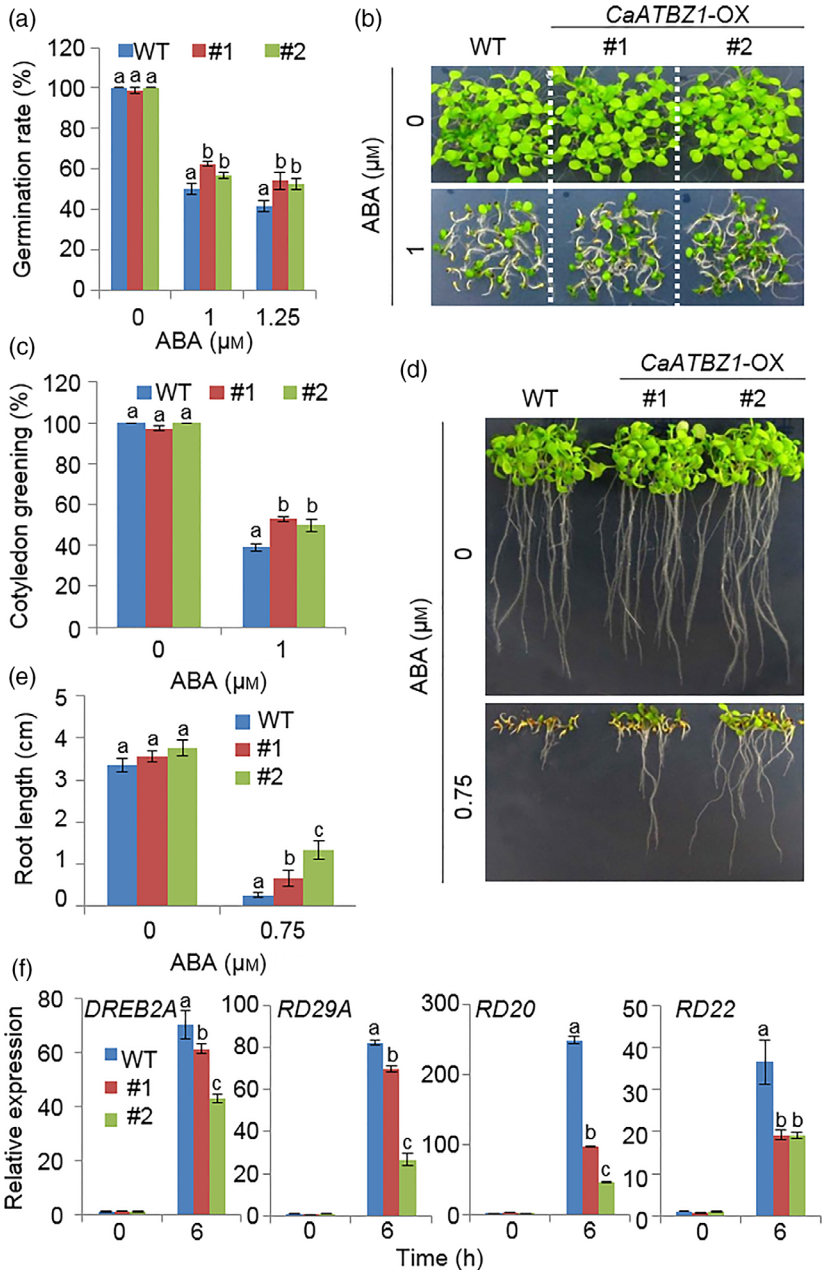

Figure 5. Decreased sensitivity of *CaATBZ1*-overexpressing (OX) transgenic Arabidopsis plants to ABA during germination and seedling growth. (a) Seed germination of wild-type (WT) and transgenic plants in response to ABA. Seeds were germinated on 0.5× MS agar plates containing various concentrations of ABA. (b, c) Growth of WT and transgenic seedlings on 0.5× MS agar plates containing 0 or 1 μM ABA. Representative photographs were taken 15 days after plating. Data represent the mean ± standard error of three independent experiments. (d, e) Root elongation of WT and transgenic plants in response to ABA. The root length of each plant was measured 13 days after plating. (f) qRT-PCR analysis of stress-related genes in the *CaATBZ1*-OX mutant in response to ABA treatment. The relative expression (ΔΔCT) of each gene was normalized to that of *Actin8*, used as an internal control gene. Data represent the mean ± standard error of three independent experiments. Different letters indicate significant differences in three independent experiments (ANOVA; $P < 0.05$).

crucial components of the molecular network functioning in ABA biosynthesis and signal transduction (Seo and Koshiba, 2002; Cutler *et al.*, 2010; Wind *et al.*, 2013; Li *et al.*, 2016), and the involvement of transcription factors in the abiotic stress response is well established. Moreover, the processes of post-translational modification – including acetylation, phosphorylation, and ubiquitination – are known to be directly and indirectly involved in the plant response to abiotic stress (Ding *et al.*, 2015a; Tian *et al.*, 2015; Chen *et al.*, 2018). Several previous studies have demonstrated that the target proteins of ubiquitination are transcriptional activators or repressors, and that their existence or degradation is the cue for regulation of the stress response (Stone and Callis, 2007; Cheng *et al.*, 2012; Ding *et al.*, 2015b). For example, the transcription factor ABI3 – which is upstream of ABI5 in the ABA response pathway and functions as a repressor of seed germination – has been shown to be a potential target for ubiquitination by AIP2 E3 ligase (Lopez-Molina *et al.*, 2001, 2002; Zhang *et al.*, 2005). In addition, the E3 ligase KEG3 is able to ubiquitinate bZIP transcription factor ABI5, which is an efficient control point in ABA signal transduction (Stone *et al.*, 2006; Liu and Stone, 2010). In a previous study, we clearly demonstrated that a pepper C3H2C3 type RING E3 ligase, CaASRF1, and a subgroup D bZIP transcription factor, CaAIBZ1, function as positive and negative modulators,

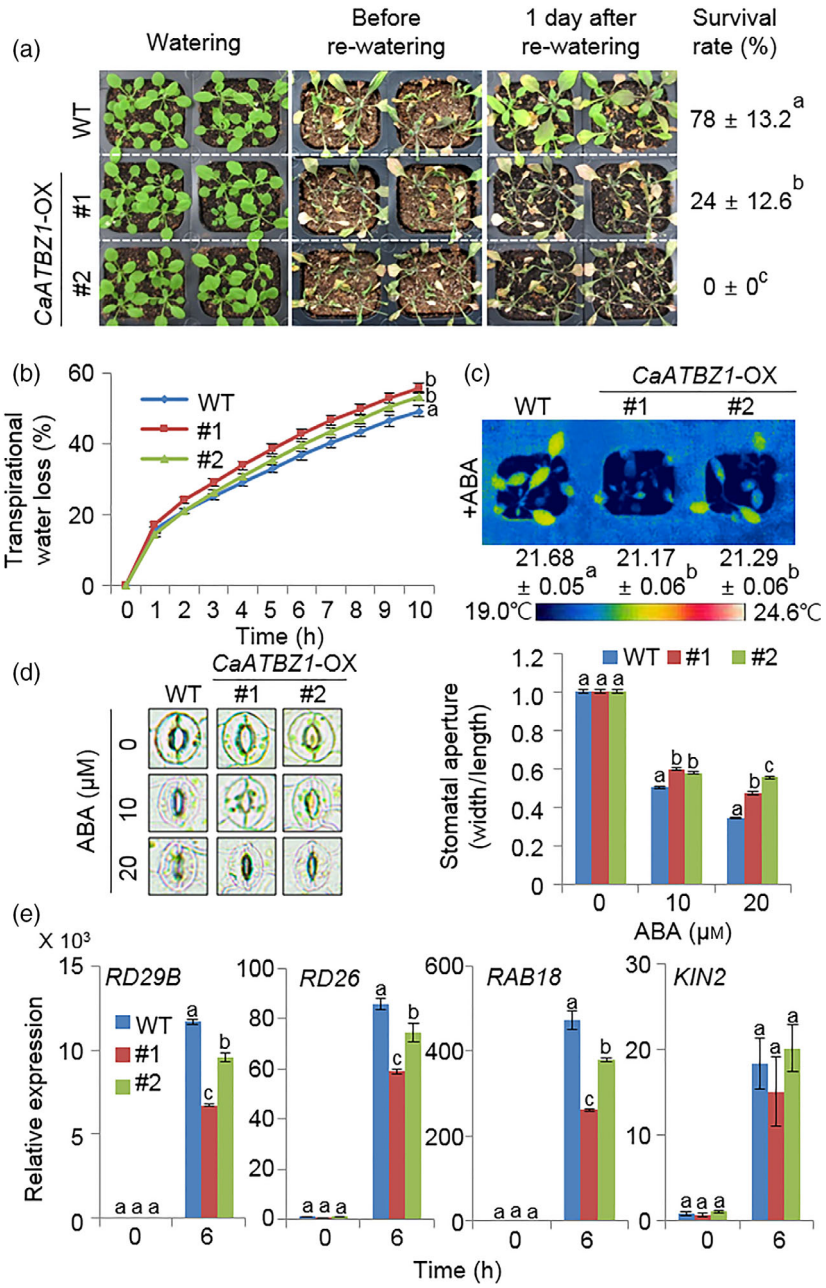

Figure 6. Reduced tolerance of *CaATBZ1*-OX transgenic Arabidopsis plants to drought stress. (a) Increased drought sensitivity of *CaATBZ1*-OX transgenic plants. Four-week-old wild-type and transgenic plants were subjected to drought stress by withholding water for 11 days and then re-watering for 1 day. Survival rates of plants were measured after re-watering. (b) Transpirational water loss from the leaves of wild-type and transgenic plants at various times after detachment of leaves. (c) Decreased leaf temperatures of *CaATBZ1*-OX plants in response to ABA treatment. Leaf temperatures of plants treated with 50 μM ABA were measured using thermal imaging and representative images were taken. (d) Stomatal apertures in wild-type and *CaATBZ1*-OX plants treated with ABA. Leaf peels were harvested from the 3-week-old plants of each line and incubated in SOS buffer containing 0, 10, or 20 μM ABA. Representative photographs were taken under a microscope and the stomatal apertures were measured. Data represent the mean ± standard error of three independent experiments. (e) qRT-PCR analysis of drought-inducible or ABA-responsive genes in the *CaATBZ1*-OX mutant in response to drought stress at 6 h after detachment. The relative expression (ΔΔCT) of each gene was normalized to that of *Actin8*, used as an internal control gene. Data represent the mean ± standard error of three independent experiments. Different letters indicate significant differences in three independent experiments (ANOVA; $P < 0.05$).

respectively, in ABA signalling and the drought stress response (Joo *et al.*, 2019). In the present study, we provide evidence that CaASRF1 ubiquitinates subgroup A bZIP transcription factor CaATBZ1, which is degraded via the 26S proteasome pathway. Moreover, CaATBZ1 negatively modulated the drought stress response via ABA-mediated signalling.

Several previous studies have suggested that a single E3 ligase modifies several substrates and that more than one E3 ligase modulates the stability of a single target protein depending on the physiological context (Kong *et al.*, 2015; Wu *et al.*, 2016; Baek *et al.*, 2017; Lim *et al.*, 2017; Kelley, 2018). Therefore, we cannot exclude the possibility that an additional E3 ligase regulating CaAIBZ1 and CaATBZ1 stability exists in different tissues. Subcellular localization analysis showed that CaASRF1 is localized in the nucleus and cytosol, whereas CaAIBZ1 and CaATBZ1 are localized in the nucleus. Moreover, interaction between these proteins occurs predominantly in the nucleus (Figure 1c). These data suggest that: (i) CaASRF1 is generated in the cytosol and moves to the nucleus for its function and (ii) CaASRF1 modulates protein stability in the nucleus and cytosol. Therefore, CaASRF1 probably modulates other cytosolic target proteins. Moreover, the expression patterns of CaATBZ1 and CaAIBZ1 are similar in response to drought and high salinity, which suggested that the

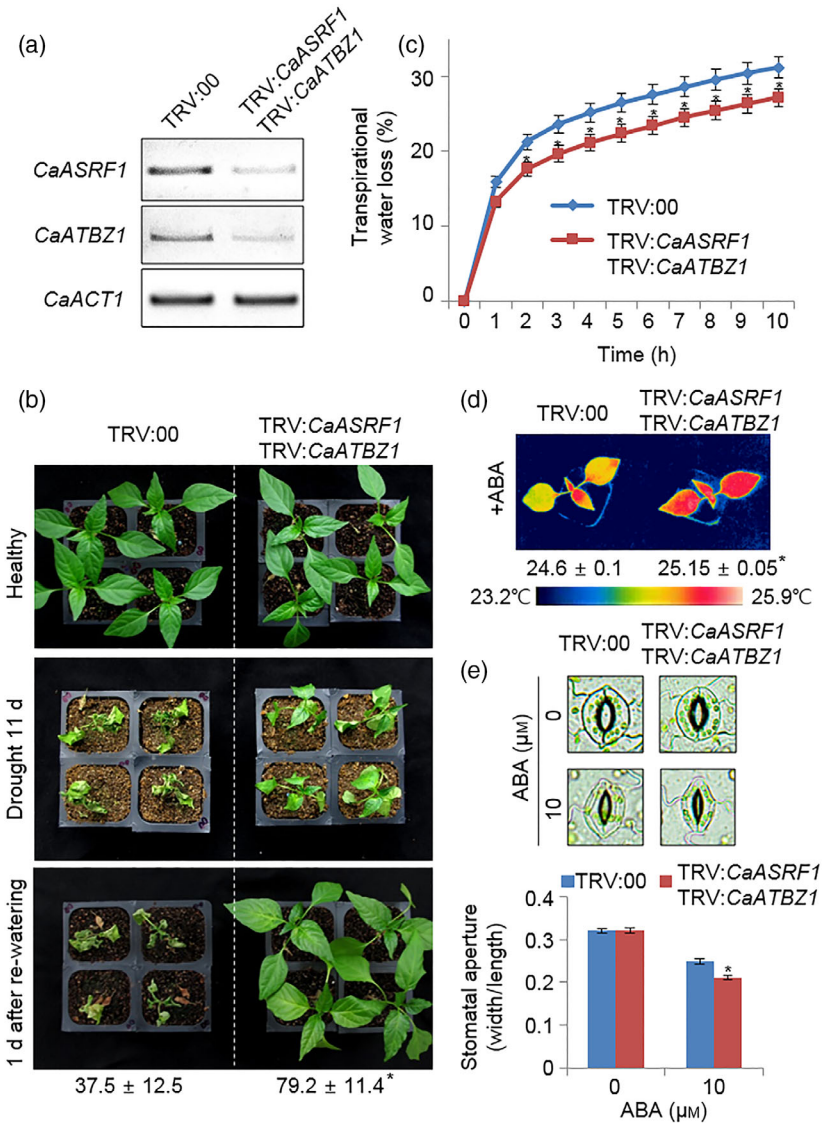

Figure 7. Enhanced drought tolerance of *CaASRF1*- and *ATBZ1*-double silenced pepper plants. (a) RT-PCR analysis of *CaASRF1* and *CaATBZ1* gene expression in the leaves of TRV:00 and *CaASRF1*- and *CaATBZ1*-double silenced pepper (TRV:*CaASRF1*/TRV:*CaATBZ1*) plants. *CaACT1* was used as an internal control gene. (b) The drought-tolerant phenotype of TRV:*CaASRF1*/TRV:*CaATBZ1* plants. Plants were grown in pots for 5 weeks under well watered conditions. Thereafter, watering was withheld for 11 days, followed by re-watering for 1 day. (c) Transpirational water loss from the leaves of TRV:00 and TRV:*CaASRF1*/TRV:*CaATBZ1* plants at various times after detachment of leaves. (d) Representative thermographic images of TRV:00 and TRV:*CaASRF1*/TRV:*CaATBZ1* plants at 5 h after treatment with 50 μM ABA. The mean leaf temperature was measured using 10 plants of each line. (e) Stomatal apertures in control and *CaASRF1*- and *ATBZ1*-double silenced pepper plants treated with ABA. Leaf peels were harvested from 3-week-old plants of each line and incubated in SOS buffer containing 0 and 10 μM ABA. Representative photographs were taken under a microscope and the stomatal apertures were measured. Data represent the mean ± standard error of three independent experiments. Asterisks indicate significant differences between three independent experiments (Student's *t*-test; $*P < 0.05$).

functions of CaATBZ1 and CaAIBZ1 are similar in stress conditions.

In the drought stress response, altered expression levels of *CaASRF1* and *CaAIBZ1* affect ABA-induced stomatal closure (Joo *et al.*, 2019), and this phenomenon also occurred in *CaATBZ1*. In comparison with control pepper plants, *CaATBZ1*-silenced pepper plants showed smaller stomatal apertures after treatment with ABA. Overexpression of *CaATBZ1* led to contrasting phenotypes with those of *CaASRF1*-OX plants. Our data revealed that CaATBZ1 and CaASRF1 contributed negatively and positively, respectively, to drought tolerance, via ABA-induced stomatal closure. However, it remains unclear whether *CaATBZ1* is directly involved in stomatal regulation. When plants encounter drought stress, endogenous ABA is rapidly synthesized and subsequently triggers stomatal closure in guard cells as the early event for preventing transpirational water loss (Schroeder *et al.*, 2001). Several studies have shown that enhanced stomatal closure is associated with increased drought tolerance (Saez *et al.*, 2006; Aubert *et al.*, 2010; Lim *et al.*, 2017). Therefore, we cannot rule out the possibility that drought tolerance in *CaATBZ1* mutants is mainly influenced by stress-responsive gene expression.

RING-type E3 ligases are conserved throughout eukaryotic organisms, however the functions and underlying mechanisms of these enzymes in abiotic stress responses via regulation of target proteins are not completely understood. Our studies revealed that CaASRF1 plays a crucial role in the drought stress response by modulating the stability of bZIP transcription factors. In summary, we propose a potential route for fine-tune regulation of the ABA-signalling pathway and drought stress response in pepper plants via CaASRF1 and CaATBZ1. Our findings demonstrated that the RING-type E3 ligase CaASRF1 interacts

with and ubiquitinates bZIP transcription factors, which function as negative modulators of drought stress and ABA signalling. It is not clear from the present study whether other target proteins exist. However, the upstream partner CaASRF1 modulates CaAIBZ1 and CaATBZ1 at post-translational levels by promoting degradation of these proteins via the ubiquitination-26S proteasome system, leading to enhanced ABA sensitivity and increased drought tolerance. Identification of additional E3 ligases and target proteins of CaASRF1 will help to clarify the route for fine-tune regulation of the drought stress response via the ABA-signalling pathway.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Pepper (*Capsicum annuum* L., 'Nockwang') and tobacco (*Nicotiana benthamiana*) seeds were sown in a compost soil mix (peat moss, perlite, and vermiculite, 5:3:2, by volume), sand, and loam soil (1:1:1, by volume), which was sterilized by steam. The pepper plants were grown in a growth room at 27 ± 1°C under white fluorescent light (80 μmol photons m^{-2} sec^{-1}) with a 16 h light/8 h dark cycle. The tobacco plants were raised in a growth chamber at 25 ± 1°C under a 16 h light/8 h dark cycle. *Arabidopsis thaliana* (ecotype Col-0) seeds were germinated on MS medium supplemented with 1% sucrose and Microagar (Duchefa Biochemie). All seeds were vernalized at 4°C for 2 days before they were placed in the growth chamber. The plates were incubated in a growth chamber at 24°C and under a 16 h light/8 h dark cycle. The Arabidopsis seedlings were grown in a steam-sterilized compost soil mix (peat moss, perlite, and vermiculite, 9:1:1, by vol.) under controlled environmental conditions as follows: 24°C and 60% relative humidity under fluorescent light (130 μmol photons m^{-2} sec^{-1}) with a 16 h light/8 h dark cycle.

Virus-induced gene silencing

To generate *CaASRF1* and *CaATBZ1* knockdown pepper plants, the VIGS system with the tobacco rattle virus was used. A 165–368 bp fragment of the *CaASRF1* and a 2–300 bp fragment of the *CaATBZ1* cDNAs were inserted into the pTRV2 vector. *Agrobacterium tumefaciens* strain GV3101 containing pTRV1, pTRV2:00, pTRV:*CaASRF1*, and pTRV:*CaATBZ1* was co-infiltrated into the cotyledons of pepper plants (OD_{600} = 0.2 for each construct). The plants were maintained at 25°C in a growth room for spread of the virus.

Generation of *CaASRF1*-OX and *CaATBZ1*-OX transgenic Arabidopsis plants

Full-length *CaASRF1* and *CaATBZ1* cDNAs were combined into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA). They were then inserted into the pK2GW7 binary vector using the LR reaction for constitutive expression of the *CaASRF1* and *CaATBZ1* genes under the control of the cauliflower mosaic virus (CaMV) 35S promoter in Arabidopsis. The 35S:*CaASRF1* and 35S:*CaATBZ1* constructs were transformed into *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium*-mediated transformation was conducted using the floral dip method (Clough and Bent, 1998). Seeds harvested from the putative transformed plants were sown on MS agar plates containing 50 μg mL^{-1} kanamycin for selection of transgenic lines.

Yeast two-hybrid assay

The full-length cDNAs of *CaASRF1* or *CaATBZ1* were subcloned into pGBKT7 or pGADT7 vectors, respectively. The obtained constructs were transformed into yeast strain AH109, using the lithium acetate-mediated transformation method (Ito *et al.*, 1983). After selection on synthetic complete (SC)−leucine−tryptophan medium, transformant candidates were transferred to SC−adenine−histidine−leucine−tryptophan medium to evaluate their growth; this growth evaluation provided an indication of protein−protein interactions. Next, 10-fold serial dilutions were prepared from each yeast cell culture (OD_{600} = 0.5) and 5 μl of each sample was spotted onto SC−leucine−tryptophan medium or SC−adenine−histidine−leucine−tryptophan medium.

Subcellular localization analysis

The coding regions of *CaASRF1* or *CaATBZ1* without stop codon were inserted into the GFP-fused binary vector p326GFP. Each construct was introduced into *A. tumefaciens* strain GV3101 and combined with strain p19 (1:1 ratio; OD_{600} = 0.5). Then it was co-infiltrated into fully expanded leaves of 5-week-old tobacco plants. At 2 days after infiltration, microscopic analysis was performed as described previously.

In vivo ubiquitination assay

For the *in vivo* ubiquitination assay, *35S:HA-CaATBZ1* and *35S:CaASRF1-GFP* or *35S:GFP* as an empty vector control were co-expressed in the leaves of tobacco plants. To prevent protein degradation, 50 μM MG132 was infiltrated 12 h before sampling. Each leaf sample was extracted with native extraction buffer (50 mM Tris-MES (pH 8.0), 0.5 M sucrose, 1 mM $MgCl_2$, 10 mM EDTA, 5 mM dithiothreitol (DTT), and complete protease inhibitor (Roche, Basal, Switzerland)) (Liu and Stone, 2010). Following immunoprecipitation with UbiQapture-Q matrix (Enzo Life Sciences, Farmingdale, NY, USA), purified proteins were subjected to SDS-PAGE and immunoblot analysis with anti-HA (Santa Cruz Biotechnology, CA, USA) and anti-UBQ11 (Agrisera, Vännäs, Sweden) antibodies.

Pull-down assay

For the pull-down assay, 5 μg of GST–CaATBZ1 and 5 μg of either MBP or MBP–CaASRF1 were incubated for 1 h at 4°C with constant rotating in glutathione *S*-transferase (GST) resin and 0.5 mL of binding buffer (1 mM EDTA, 200 mM NaCl, 20 mM Tris−HCl (pH 7.5), 0.5% Tween-20). Mixed proteins were boiled at 97°C for 5 min, eluted, and analyzed using SDS-PAGE, followed by western blotting and immunodetection with anti-MBP and anti-GST antibodies.

Cell-free degradation assay

Crude proteins were extracted from the leaves of 4-week-old pepper plants that had been dehydrated for 2 h using extraction buffer (10 mM ATP, 5 mM DTT, 10 mM $MgCl_2$, 10 mM NaCl, 25 mM Tris−HCl (pH 7.5), and 0.1% Triton X-100). The GST−CaATBZ1 fusion proteins (500 ng) were incubated with crude protein extracts (50 μg of total protein) for 0.5 h and additionally for 0.5 h with 50 μM MG132. Then immunoblotting was performed using anti-GST. All assays were independently repeated three times with two replicates per trial.

ABA, drought and NaCl treatments

To examine the expression pattern of the *CaATBZ1* gene in response to abiotic stress, ABA, NaCl, and drought were treated

on six-leaf stage pepper plants. The plants were sprayed with 100 μM ABA or control solution. For NaCl treatment, pepper plants were irrigated with 200 mM NaCl solution. The pepper plants were carefully removed from the soil to avoid injury and then placed on 3 mm paper (Whatman) for drought treatment. Leaves were harvested at the indicated time points and RNA was extracted from the leaves and RT-PCR analysis was performed.

Phenotypic analyses

For the seedling growth test, 36 seeds per genotype were sown on plates containing MS agar medium supplemented with various concentrations of ABA. One-week-old seedlings from the wild-type and *CaASRF1*-overexpressing (OX) or *CaATBZ1*-OX transgenic Arabidopsis lines were randomly planted in a pot containing soil mixture and were grown under normal watering conditions for 2 weeks. To impose drought stress, watering was withheld for 10–11 days and the survival rates of plants with rehydrated leaves were calculated after 1–2 days of re-watering. For pepper, drought stress was imposed on four-leaf stage plants by withholding watering for 11–17 days and the survival rates of plants with rehydrated leaves were calculated after 2–3 days of re-watering. The drought tolerance was determined quantitatively by measuring transpirational water loss. Leaves were detached from four-leaf stage pepper and 3-week-old Arabidopsis plants and placed in Petri dishes. The dishes were kept at 40% relative humidity in a growth chamber, and the loss of fresh weight was determined at the indicated time points. All the experiments were repeated at least three times.

For thermal imaging analysis, 4-week-old pepper plants having fully expanded first and second leaves and 3-week-old or 4-week-old Arabidopsis plants were treated with 50 μM ABA. Thermal images were obtained using an infrared camera (FLIR systems; T420) and leaf temperature was measured with FLIR Tools + v.5.2 software.

The stomatal aperture bioassay was performed as described previously (Lim and Lee, 2016). Briefly, leaf peels were collected from the rosette leaves of 3-week-old plants and were floated in a stomatal opening solution (SOS; 50 mM KCl, 10 mM MES-KOH, 10 μM $CaCl_2$, pH 6.15). The peels were incubated for 3 h to obtain >80% stomatal opening in Arabidopsis and pepper plants. The buffer was replaced with fresh SOS containing various concentrations of ABA. Leaf peels were then incubated for a further 2 h. In each sample, 100 stomata were randomly observed under a Nikon Eclipse 80i microscope and the width and length of each stomata were measured. Each experiment was conducted in triplicate.

DATA STATEMENT

All data referred to are included in the manuscript or supplementary materials

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

HJ and SCL conceptualized and designed the study. HJ and CWL performed all the experiments. SCL supervised the project. HJ, CWL and SCL wrote the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Yeast two-hybrid assay between CaATBZ1 and truncated form of CaASRF1.

Figure S2. Amino acid sequence analysis of pepper CaATBZ1.

Figure S3. Phylogenetic tree analysis of CaATBZ1 protein.

Figure S4. Yeast two-hybrid assay between CaATBZ1 and CaAIBZ1.

Figure S5. Enhanced drought tolerance of *CaATBZ1*/*CaAIBZ1*-silenced pepper plants.

Figure S6. RT-PCR analysis of *CaATBZ1* expression in wild-type plants and *CaATBZ1*-OX transgenic lines.

Figure S7. Reduced ABA sensitivity of *CaATBZ1*-overexpressing (OX) transgenic Arabidopsis plants during post-germinative growth.

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