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The Pepper RING-Type E3 Ligase, CaAIP1, Functions as a Positive Regulator of Drought and High Salinity Stress Responses

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Plant adaptive responses to osmotic stress are co-ordinated by restriction of growth and developmental processes and by molecular and physiological activities. The phytohormone ABA is the primary regulator that induces and responds to osmotic stress, and its sensitivity markedly influences osmotic stress tolerance levels. Several E3 ubiquitin ligases act as positive or negative regulators of ABA, thereby mediating sensitivity to osmotic stress in higher plants. Here, we report that the C3H2C3-type RING finger E3 ligase, CaAIP1, regulates osmotic stress responses via ABA-mediated signaling. *CaAIP1* contains a RING finger motif, which functions during attachment of ubiquitins to the target proteins. Expression of *CaAIP1* was induced by ABA, drought and NaCl treatments, suggesting its role in the osmotic stress response. *CaAIP1*-silenced pepper plants displayed a drought-sensitive phenotype characterized by a high level of transpirational water loss in the drought-treated leaves. *CaAIP1*-overexpressing (OX) plants exhibited increased sensitivity to ABA, but an NaCl- and mannitol-tolerant phenotype during seed germination and seedling growth. *CaAIP1*-OX plants further displayed enhanced tolerance to drought stress, characterized by low levels of transpirational water loss via increased stomatal closure and leaf temperature. Our data indicate that *CaAIP1* is a positive regulator of the osmotic stress tolerance mechanism.

Keywords: Abscisic acid • • CaAIP1 • • Osmotic stress • • RING-type E3 ligase • • Ubiquitination.edited-statecorrected-proof.

Abbreviations: CaMV, *Cauliflower mosaic virus*; GFP, green fluorescent protein; MBP, maltose-binding protein; MS, Murashige and Skoog; OX, overexpressing; qRT–PCR, quantitative reverse transcription–PCR; RT–PCR, reverse transcription–PCR; SOS, stomatal opening solution; TRV, *Tobacco rattle virus*; UB, ubiquitin; VIGS, virus-induced gene silencing.

Introduction

Plants are sessile organisms, and they are constantly challenged by environmental stresses such as various pathogens, high salinity and drought. Consequently, they have evolved extraordinary defense mechanisms that enable them to adapt to various environmental stress conditions. Osmotic stress is a common result of water deficit arising from exposure to drought and/or high salinity. Plants reduce the effects of osmotic stress by minimizing transpirational water loss from the leaves and maximizing water uptake from the roots (Apse and Blumwald 2002, Yamaguchi-Shinozaki and Shinozaki 2006). The molecular and physiological mechanisms underlying osmotic stress have been extensively investigated (Zhu 2002, Yamaguchi-Shinozaki and Shinozaki 2006) However, the plant defense response to osmotic stress is a complex phenomenon, and the precise functional modifications induced by osmotic stress remain unclear. To adapt to osmotic stress conditions, plants have evolved various defense mechanisms, including accumulation of the phytohormone ABA (Lee and Luan 2012, Golldack et al. 2014, C.W. Lim et al. 2014). ABA plays a critical role in adaptation to osmotic stress (Osakabe et al. 2014a, Osakabe et al. 2014b). Under stress conditions, ABA induces stomatal closure by regulating turgor pressure via the ABA signal transduction pathway, from the ABA receptor to ion channels in the guard cells (Schroeder et al. 2001, Vahisalu et al. 2008, Lee et al. 2009, Lee et al. 2013). Moreover, a number of stress-related genes involved in plant defense responses are regulated by ABA (Kilian et al. 2007).

Ubiquitination via the 26S proteasome pathway is the dominant selective post-translational modification pathway in eukaryotes (Stone 2014). In plant cells, ubiquitination is involved in various signaling pathways, including growth, development and adaptation to a constantly changing environment (Vierstra 2009, Lee et al. 2011, Sadanandom et al. 2012, Kim and Kim 2013). In Arabidopsis, a large number of genes (comprising almost 6% of the genome) are involved in this mechanism (Hua and Vierstra 2011). The covalent attachment of ubiquitin (Ub) molecules to the target protein is a multistep process that operates via an enzymatic cascade involving three enzymes—E1 (Ub-activating enzyme), E2 (Ub-conjugating enzyme) and E3 (Ub ligase). The process begins with the activation of Ub by ATP and E1, and this is followed by the transfer of Ub to E2; next, target-recruiting E3 interacts with E2, thereby enabling attachment of Ub to the target protein (Ciechanover and Schwartz 1998, Stone 2014). The most important genes in this pathway

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belong to the E3 Ub ligase family, which play a key role in determining the specificity of the target proteins (Vierstra 2009, Sadanandom et al. 2012). Recently, E3 Ub ligases have emerged as important regulators of the plant defense response to various osmotic stresses, including drought, cold and high salinity (Ding et al. 2015, Tian et al. 2015, Zhang et al. 2015). The Arabidopsis genome is known to contain >1,400 E3 Ub ligases (Vierstra 2009), including >470 genes encoding RING (Really Interesting New Gene) domain-containing E3 Ub ligases (Stone et al. 2005, Vierstra 2009); however, the precise role of RING-type E3 ligases has not yet been elucidated. Recent reports indicate that RING-type E3 ligases play an important role in the response to osmotic stress and ABA signaling (Li et al. 2011, Chen et al. 2013). For example, *RHA2a*/*RHA2b* and *SDIR1* function as positive regulators of the drought and salt stress responses, respectively, via ABA signaling (Bu et al. 2009, Li et al. 2011, Zhang et al. 2015). In contrast, *RGLG2* and *HOS1* function as negative regulators of the drought and cold stress responses, respectively, via degradation of the ABA-positive regulators (Dong et al. 2006, Cheng et al. 2012, Ding et al. 2015).

In the present study, we isolated the E3 Ub ligase RING finger protein gene, *CaAIP1* (*Capsicum annuum* ABA-Induced RING finger Protein 1). We found that expression of *CaAIP1* in pepper leaves was induced by ABA, drought and high salinity treatments. The CaAIP1 protein displayed in vitro Ub ligase activity and localized to the nucleus and cytoplasm. Based on the expression patterns of *CaAIP1*, we used silencing and overexpression of *CaAIP1* in pepper and Arabidopsis, respectively, to elucidate the functions of CaAIP1 in the plant defense response to osmotic stress. We found that *CaAIP1*-silenced pepper plants exhibited a drought-sensitive phenotype, and this was characterized by high levels of transpirational water loss, low leaf temperatures and large-sized stomatal pores. However, overexpression of *CaAIP1* resulted in an ABA-hypersensitive phenotype, which displayed enhanced tolerance to drought and high salinity. Our data indicate that CaAIP1 functions as a positive regulator of the ABA, drought and high salinity tolerance mechanisms.

Results

Isolation and sequence analysis of the *CaAIP1* gene

We isolated *CaAIP1* (accession No. KT361852) cDNA from a cDNA library constructed from pepper leaves treated with ABA, according to the differential hybridization method (S. Lim et al. 2014). We found that *CaAIP1* was up-regulated in ABA-treated leaves. The *CaAIP1* cDNA sequence contains a 702 bp open reading frame, and it encodes 233 amino acid residues with a calculated molecular mass of 26.3 kDa and an isoelectric point (pI) of 4.74 (**Supplementary Fig. S1**). The PROSITE (http://expasy.ch/prosite) and SMART (htttp://smart.embl-heidelberg.de) programs showed two predicted coiled-coil domains in the N-terminal (residues 23–51) and C-terminal (residues 202–229) regions, and a C3H2C3-type RING domain in the C-terminal region (residues 153–194); these domains are essential for E3 ligase activity in the Ub–26S proteasome system. In phylogenetic tree analysis, CaAIP1 protein is clustered with putative E3 Ub-protein ligases containing a RING domain from the *Solanaceae* family into the same clade (**Fig. 1A**). The results of multiple sequence alignment analysis revealed that CaAIP1 has relatively high amino acid sequence identity with other RING finger proteins of *Solanum tuberosum* (accession No. XP_006361527.1, 66.6%), *Solanum lycopersicum* (accession No. XP_004245207.1, 66.3%), *Nicotiana sylvestris* (accession No. XP_009760745.1, 64.5%), *Glycine max* (accession No. XP_003544578.1, 51.8%), *Arabidopsis thaliana* (accession No. CAB75509.1, 48.7%), *Oryza sativa* (accession No. XP_015612683.1, 43.7%) and *Triticum aestivum* (accession No. ACN21975.1, 43.3%) (**Supplementary Fig. S1**). The RING domain of CaAIP1 shares 88–93% identity with other plant RING finger proteins and consists of eight conserved cysteine and histidine residues, which are essential for E3 Ub ligase activity (**Fig. 1B**).

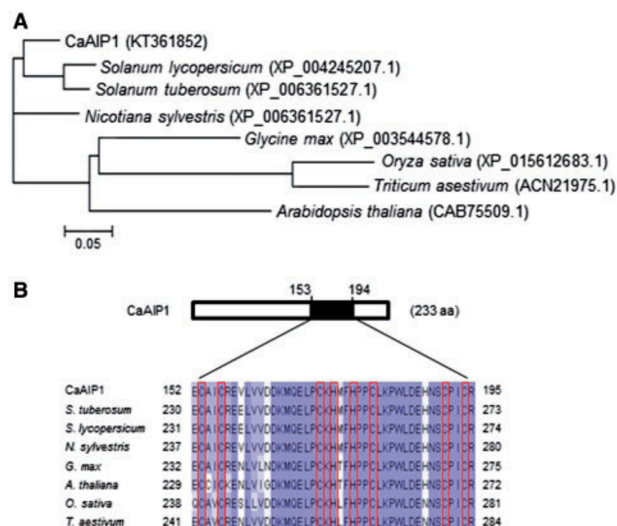

Fig. 1 Amino acid sequence analysis of pepper CaAIP1 (*Capsicum annuum* ABA-Insensitive RING Protein 1). (A) Phylogenetic tree analysis of CaAIP1. Multiple alignment of amino acids in CaAIP1 and its homologous proteins was performed using ClustalW2, and the phylogenetic tree was drawn with MEGA software (version 5.2). (B) Alignment of the RING zinc finger C3H2C3-type domain. Conserved cysteine (C) and histidine (H) residues are indicated using red boxes.

Localization of CaAIP1 protein and expression of the *CaAIP1* gene

To analyze the subcellular localization of the CaAIP1 protein in plant cells, we fused the green fluorescent protein (GFP) gene to the C-terminal region of the *CaAIP1* coding region under the control of the 35S promoter, to produce the 35S:*CaAIP1-GFP* translational fusion. We detected fluorescent signals from CaAIP1–GFP in the nucleus and cytoplasm of *Nicotiana benthamiana* epidermal cells (**Fig. 2A**), suggesting that the CaAIP1 protein functions in the nucleus and cytoplasm.

We examined whether the expression of *CaAIP1* is induced by ABA, drought and high salinity (**Fig. 2B–D**). First, we

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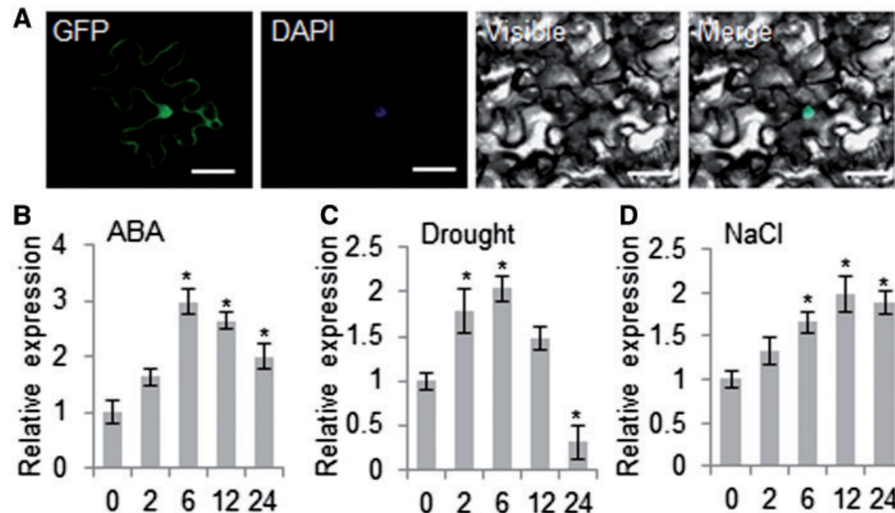

Fig. 2 Localization of CaAIP1 protein and expression of the *CaAIP1* gene. (A) Subcellular localization of CaAIP1 based on transient expression of the GFP fusion protein in *N. benthamiana* epidermal cells. The 35S:*CaAIP1-GFP* construct was expressed using agroinfiltration of *N. benthamiana* leaves and was observed under a confocal laser-scanning microscope. 4′,6-Diamidino-2-phenylindole (DAPI) staining was used as a marker for the nucleus. White bar = 20 μm. The expression pattern of the *CaAIP1* gene was analyzed in the leaves of pepper plants after treatment with 100 μM ABA (B), drought (C) and 200 mM NaCl (D). The relative expression level (ΔΔCT) of each gene was normalized to that of *CaACT1* as an internal control gene. Data represent the mean ± SD of three independent experiments. Asterisks indicate significant differences (Student's *t*-test; $P < 0.05$).

investigated the expression level of *CaAIP1* transcripts in pepper leaves after ABA treatment (Fig. 2B). We found that *CaAIP1* transcripts were weakly detected in the leaf tissues of non-treated pepper plants, but were more strongly induced after 6 h of ABA treatment. We also investigated the induction of *CaAIP1* transcripts in pepper leaves after drought and NaCl treatments. We found that the steady-state levels of *CaAIP1* transcripts were slightly up-regulated by drought and NaCl (Fig. 2C, D). Our findings suggest that abiotic stress signals are involved in the expression of *CaAIP1*.

The CaAIP1 protein functions as an E3 ligase for ubiquitination

Previously, several proteins containing the RING motif were shown to act as E3 ligases in vitro (Ryu et al. 2010, Lee et al. 2011, Zhang et al. 2015). The CaAIP1 protein has a C3H2C3-type RING finger motif. To determine whether this RING finger protein has E3 ligase activity, we performed an in vitro self-ubiquitination assay (Fig. 3). We expressed the CaAIP1 protein in *Escherichia coli* as a fusion protein with maltose-binding protein (MBP), and subsequently purified CaAIP1—MBP. The obtained fusion protein was incubated with Ub, human E1 and Arabidopsis E2 for 3 h, and the ubiquitinated protein was detected using immunoblot analysis with anti-MBP (Fig. 3A) and anti-Ub (Fig. 3B) antibodies. We found that in the presence of Ub, E1 and E2, the CaAIP1 protein exhibited marked Ub ligase activity (Fig. 3).

Increased susceptibility of *CaAIP1*-silenced pepper plants to drought stress

To assess the biological function of *CaAIP1*, we performed virus-induced gene silencing (VIGS)-based gene function analysis, using the *Tobacco rattle virus* (TRV) vector for the loss of function of *CaAIP1* (Lim and Lee 2014). We found that the expression level of *CaAIP1* was lower in *CaAIP1*-silenced pepper plants (TRV:*CaAIP1*) than in empty vector control plants (TRV:00) (Fig. 4A). We investigated the in vivo function of *CaAIP1* in response to drought stress. Under well-watered conditions, we observed no phenotypic differences between wild-type and *CaAIP1*-silenced pepper plants (Fig. 4B, upper panel). However, when control plants and *CaAIP1*-silenced pepper plants were subjected to drought stress induced by withholding water for 10 d, the *CaAIP1*-silenced pepper plants displayed a drought-sensitive phenotype (Fig. 4B, middle panel). Moreover, after re-watering, most of the control plants resumed growth more quickly than the *CaAIP1*-silenced pepper plants (Fig. 4B, lower panel). We monitored the survival rate of each plant after 2 d of re-watering (Fig. 4C). Under these conditions, we found that only 50% of *CaAIP1*-silenced pepper plants resumed growth, whereas 83% of the control plants resumed growth. Our results indicate that suppression of *CaAIP1* expression increases susceptibility to drought stress. To determine whether the drought-sensitive phenotype exhibited by the *CaAIP1*-silenced pepper plants was affected by water loss, we measured the fresh weight of detached pepper leaves and determined the transpiration rate (Fig. 4D). We found that the fresh weight loss of leaf tissues was significantly higher in *CaAIP1*-silenced pepper plants than in control plants, indicating that the increased drought susceptibility of *CaAIP1*-silenced pepper plants was derived from an increased transpiration rate. The expression level of *CaAIP1* was up-regulated by ABA treatment. Moreover, several RING-type E3 ligases are known to function as key regulators of the ABA signal transduction pathway (Bu et al. 2009, Kim and Kim 2013, Bueso et al. 2014). Hence, we measured the leaf temperature and stomatal

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aperture to confirm the alteration of ABA sensitivity with the *CaAIP1*-silenced pepper plants and empty vector control plants (**Fig. 4E, F**). First, we measured the leaf temperatures, which decrease when the stomata open, because of evaporative cooling. We found that after ABA treatment, the leaf temperatures of *CaAIP1*-silenced pepper plants were lower than those of control plants (**Fig. 4E**), implying that CaAIP1 plays a key role in ABA-mediated stomatal closure. Moreover, in the absence of ABA, we determined no significant differences in stomatal pore size between *CaAIP1*-silenced pepper plants and control plants; however, after treatment with 20 μM ABA, the stomatal apertures of *CaAIP1*-silenced pepper plants were larger than those of control plants (**Fig. 4F**).

To investigate the mechanism by which increased ABA sensitivity in *CaAIP1*-silenced pepper plants affects the expression of ABA-responsive genes, we performed quantitative reverse transcription–PCR (qRT–PCR) analysis (**Fig. 4G**). Previously, we showed that the *AtRD29B* homolog *CaOSR1*, *CaAMP1* and *CaOSM1* were significantly induced by ABA and drought stress (Hong et al. 2004, Lee and Hwang 2009, Lim et al. 2015). Consistent with the ABA response, the expression levels of these genes were lower in *CaAIP1*-silenced pepper plants than in control plants.

Enhanced tolerance of *CaAIP1*-OX plants to osmotic stress

Our identification of CaAIP1 as an E3 Ub ligase prompted us to ascertain the function of *CaAIP1* in abiotic stress signaling. To provide detailed evidence for the in vivo function of *CaAIP1*, we created Arabidopsis transgenic plants that overexpressed *CaAIP1* under the control of the strong constitutive *Cauliflower mosaic virus* (CaMV) 35S promoter. We detected no transcripts in wild-type plants. However, we obtained two independent T_3 homozygous lines (*CaAIP1*-OX) that showed high expression of the *CaAIP1* gene (**Fig. 5A**), and we used these lines in our phenotypic analyses. Under favorable conditions, we observed no phenotypic differences between wild-type and *CaAIP1*-OX plants (**Figs. 5–8**). To clarify the possible role of CaAIP1 in the response to abiotic stress, we compared the seed germination and seedling growth of *CaAIP1*-OX plants and wild-type plants subjected to osmotic stress induced by mannitol treatment (**Fig. 5**). In germination assay, *CaAIP1*-OX plants showed a higher tolerance to mannitol than did wild-type plants. In the presence of mannitol, *CaAIP1*-OX seeds germinated more rapidly and showed a much higher germination rate than did wild-type seeds (**Fig. 5B**). Moreover, high levels of mannitol had a suppressive effect on shoot and root growth. To examine the role of CaAIP1 in mannitol-mediated inhibition of root growth, we grew seeds for 8 d in medium containing mannitol and measured the root length. In the absence of mannitol, the root length did not differ significantly between wild-type and *CaAIP1*-OX plants; in contrast, in the presence of mannitol, the roots of *CaAIP1*-OX plants were longer than those of wild-type plants (**Fig. 5C, D**). In addition, cotyledon greening of the wild-type plants was severely impaired by mannitol treatment. We conducted a dose–response

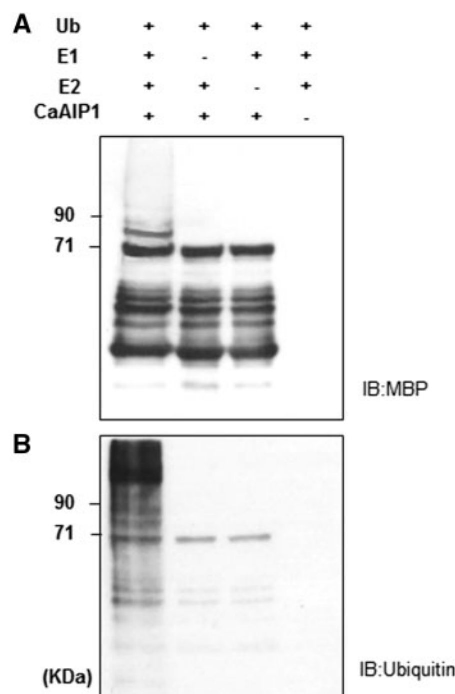

Fig. 3 In vitro self-ubiquitination of CaAIP1. (A) In the presence of ubiquitin (Ub), E1 and E2 (UBC10), maltose-binding protein (MBP)–CaAIP1 fusion proteins displayed E3 Ub ligase activity. Detection of MBP–CaAIP1 auto-ubiquitination. MBP–CaAIP1 fusion proteins were detected using an MBP antibody, and shifted bands indicated the attachment of Ub molecules. (B) Protein bands with attached Ub were detected using anti-Ub immunoblot analysis.

assay by growing seeds for 5 d on medium containing different concentrations of mannitol. In the absence of mannitol, the rate of cotyledon greening did not differ between wild-type and transgenic plants. However, in the presence of mannitol, the rate of cotyledon greening was higher in *CaAIP1*-OX plants than in wild-type plants (**Fig. 5E, F**). Our data indicate that *CaAIP1* functions as a positive regulator of osmotic stress signaling during the seed germination and seedling growth stages.

Enhanced tolerance of *CaAIP1*-OX plants to high salinity

To investigate the function of CaAIP1 in tolerance to high salinity, we compared the seed germination and seedling growth of *CaAIP1*-OX and wild-type plants in the absence or presence of NaCl (**Fig. 6**). We found that at 7 d after plating on media containing NaCl, *CaAIP1*-OX seeds showed germination rates of almost 100%, whereas wild-type seeds showed rates of 69% and 22% in 100 and 150 mM NaCl, respectively (**Fig. 6A**). Moreover, *CaAIP1*-OX plants exhibited enhanced tolerance to NaCl at the seedling stage (**Fig. 6B–E**). We analyzed the root lengths of wild-type and *CaAIP1*-OX plants 8 d after exposure to various concentrations of NaCl. We found that treatment with 100 and 150 mM NaCl resulted in a decrease in root length; however, the roots of *CaAIP1*-OX plants were longer than those of wild-type plants (**Fig. 6B, C**). Moreover, after treatment with 100 and 150 mM NaCl, the greening cotyledon rate was higher in

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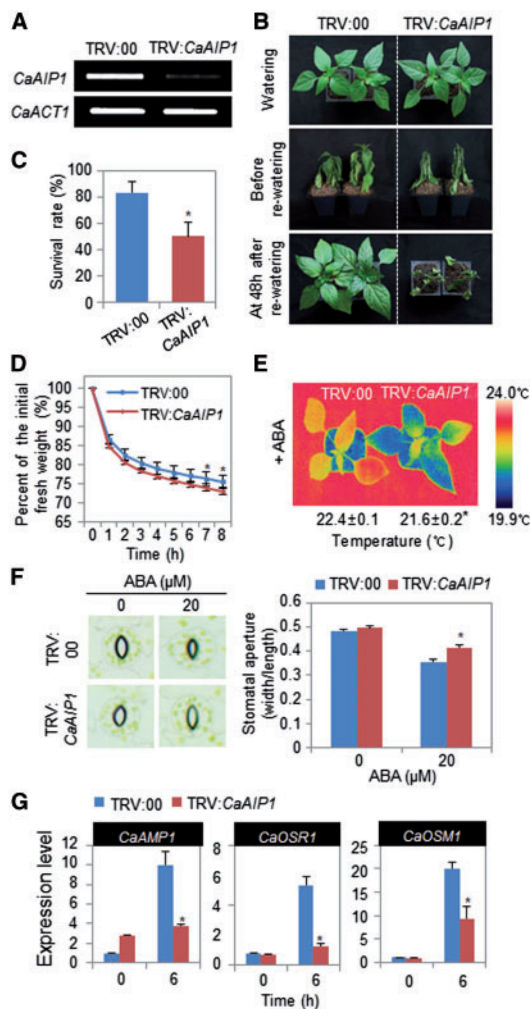

Fig. 4 Decreased tolerance of *CaAIP1*-silenced pepper plants to drought stress. (A) RT–PCR analysis of expression in leaves of pepper plants transfected with the empty vector control (TRV:00) or *CaAIP1*-silenced constructs (TRV:*CaAIP1*). *CaACT1* was used as an internal control gene. (B) Empty vector control and *CaAIP1*-silenced pepper were grown in pots for 5 weeks under normal growth conditions. Thereafter, water was withheld for 10 d, followed by re-watering for 2 d. (C) Percentage of surviving plants after 2 d of re-watering. (D) Transpirational water loss from the leaves of empty vector control and *CaAIP1*-silenced pepper plants at various times after detachment of leaves. (E) Decreased leaf temperatures of *CaAIP1*-silenced pepper plants in response to ABA treatment. (F) Stomatal apertures in control and *CaAIP1*-silenced pepper plants treated with ABA. Leaf peels were harvested from the 3-week-old plants of each line and incubated in stomatal opening solution (SOS) buffer containing 0, 10 or 20 μM ABA. Representative images were taken under a microscope and the stomatal apertures were measured. Data represent the mean ± SE of three independent experiments. (G) qRT–PCR analysis of ABA-responsive gene expression in the leaves of control and *CaAIP1*-silenced pepper plants. Four-week-old plants of each line were treated with 20 μM ABA; after 6 h, leaves of each line were harvested. The relative expression level (ΔΔCT) of each gene was normalized to that of *CaACT1* as an internal control gene. Data represent the mean ± SD of three independent experiments. Asterisks indicate significant differences between the control and the *CaAIP1*-silenced pepper plants (Student's *t*-test; $P < 0.05$).

CaAIP1-OX plants (100% and 83%, respectively) than in wild-type plants (63% and 19%, respectively) (**Fig. 6D, E**). Our findings suggest that *CaAIP1* regulates plant defense responses to high salinity during the germination and seedling growth stages.

Function of the *CaAIP1* gene in response to ABA

To explore the biological function of CaAIP1 in response to ABA, we performed phenotype analysis with *CaAIP1*-OX plants treated with ABA at germinative (**Supplementary Fig. S2**) and post-germinative (**Supplementary Fig. S3**) stages. The germination rates, root growth and cotyledon greening of *CaAIP1*-OX plants were higher than those of wild-type seeds in the presence of ABA (**Supplementary Fig. S2**). Next, to determine whether reduced ABA sensitivity in root growth of *CaAIP1*-OX plants is retained at the post-germinative stage, 5-day-old seedlings of both wild-type and *CaAIP1*-OX seeds germinated on medium without ABA were transferred to medium with 10 and 20 μM ABA and further grown for 6 d. In contrast to the germination stage, root growth was not different between wild-type and *CaAIP1*-OX plants (**Supplementary Fig. S3**). During gaseous exchange via the stomata, stomatal opening/closure is regulated by ABA accumulation (Fujii et al. 2007). To examine the role of CaAIP1 in stomatal opening/closure, we monitored the ABA-mediated stomatal movements and leaf temperatures (**Fig. 7**). In the absence of ABA, we determined no significant differences in stomatal apertures between wild-type and *CaAIP1*-OX plants. In contrast, in the presence of ABA, the stomatal apertures of *CaAIP1*-OX plants were smaller than those of wild-type plants (**Fig. 7A**). After treatment with 20 μM ABA, the stomatal apertures of wild-type and *CaAIP1*-OX plants were reduced by 8.7% and 16.4–20.0%, respectively. Moreover, the leaf temperatures of *CaAIP1*-OX plants were markedly higher than those of wild-type plants (**Fig. 7B**). Our results suggest that CaAIP1 plays a critical role in ABA-mediated regulation of stomatal opening/closure.

To investigate whether this increased ABA sensitivity of *CaAIP1*-OX plants was accompanied by up-regulation of ABA-responsive marker genes, we performed qRT–PCR analysis with *DREB2A*, *RAB18*, *KIN2* and *RD20* (**Fig. 7C**). The expression levels of *RAB18*, *RD29B*, *RD29A* and *DREB2A* in ABA-treated leaves of *CaAIP1*-OX plants plants were higher than in those of wild-type plants.

Function of the *CaAIP1* gene in response to drought stress

The *CaAIP1*-OX lines displayed enhanced sensitivity to ABA (**Fig. 7**) and *CaAIP1*-silenced pepper plants exhibited a drought-sensitive phenotype (**Fig. 4**). Hence, we speculated that *CaAIP1*-OX alters the drought stress response (**Fig. 8**). To examine the drought tolerance of *CaAIP1*-OX plants, we subjected wild-type and *CaAIP1*-OX plants to dehydration treatment by detaching the rosette leaves. In comparison with wild-type plants, *CaAIP1*-OX plants exhibited enhanced dehydration tolerance (**Fig. 8A**). Next, we measured the fresh weight of detached rosette leaves to determine the transpiration rate and thus evaluate

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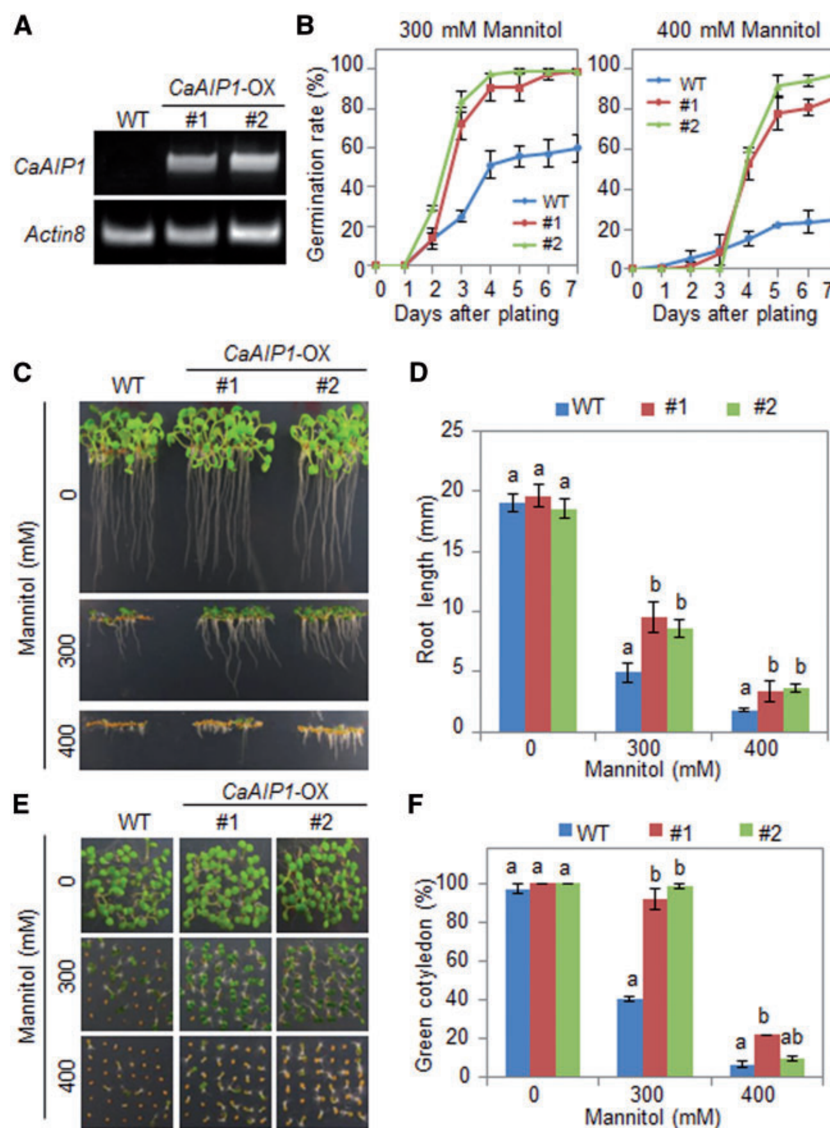

Fig. 5 Enhanced tolerance of *CaAIP1*-overexpressing (OX) transgenic Arabidopsis lines to osmotic stress during germination and seedling growth. (A) RT—PCR analysis of *CaAIP1* expression in wild-type and *CaAIP1*-OX transgenic lines. *Actin8* was used as an internal control gene. (B) Seed germination of wild-type and transgenic lines in response to mannitol after 0—7 d of treatment. Seeds were germinated on 0.5× MS agar plates containing 300 mM or 400 mM mannitol. (C) Root elongation of wild-type and transgenic lines in response to mannitol. (D) The root length of each plant was measured 8 d after plating. Data represent the mean ± SE of three independent experiments. (E) Growth of wild-type and *CaAIP1*-OX seedlings on 0.5× MS agar plates containing 0, 300 or 400 mM mannitol. Representative photographs were taken 5 d after plating. (F) Quantification of green cotyledons in the wild-type and each mutant line was performed at 5 d after plating on 0.5× MS agar plates containing 0, 300 or 400 mM mannitol. Data represent the mean ± SE values obtained after evaluating 72 seeds from three independent experiments. Different letters indicate significant differences between wild-type and transgenic lines ($P < 0.05$; ANOVA followed by Fisher's LSD test).

whether the dehydration-tolerant phenotype exhibited by *CaAIP1*-OX plants was derived from a lower rate of water loss (Fig. 8B). We showed that the fresh weight loss in the leaf tissues of the transgenic lines was significantly lower than that in the wild-type leaves, suggesting that the enhanced tolerance to dehydration stress was attributable to altered leaf transpiration rates. Next, we analyzed the drought phenotype of *CaAIP1*-OX plants. Under well-watered conditions, we observed no phenotypic differences between *CaAIP1*-OX and wild-type plants (Fig. 8C, left panel). In contrast, after drought stress treatment induced by withholding water for 10 d, wild-type and *CaAIP1*-OX plants showed a wilted phenotype, but this phenotype was more pronounced in wild-type plants than in *CaAIP1*-OX plants (Fig. 8C, middle panel). Moreover, after 2 d of re-watering, wild-type plants continued to exhibit a more wilted phenotype than transgenic plants (Fig. 8C, right panel). We determined markedly higher survival rates for *CaAIP1*-OX plants (62.5—65.0%) than for wild-type plants (27.5%) (Fig. 8D). Our findings indicate that overexpression of the *CaAIP1* gene confers enhanced drought tolerance.

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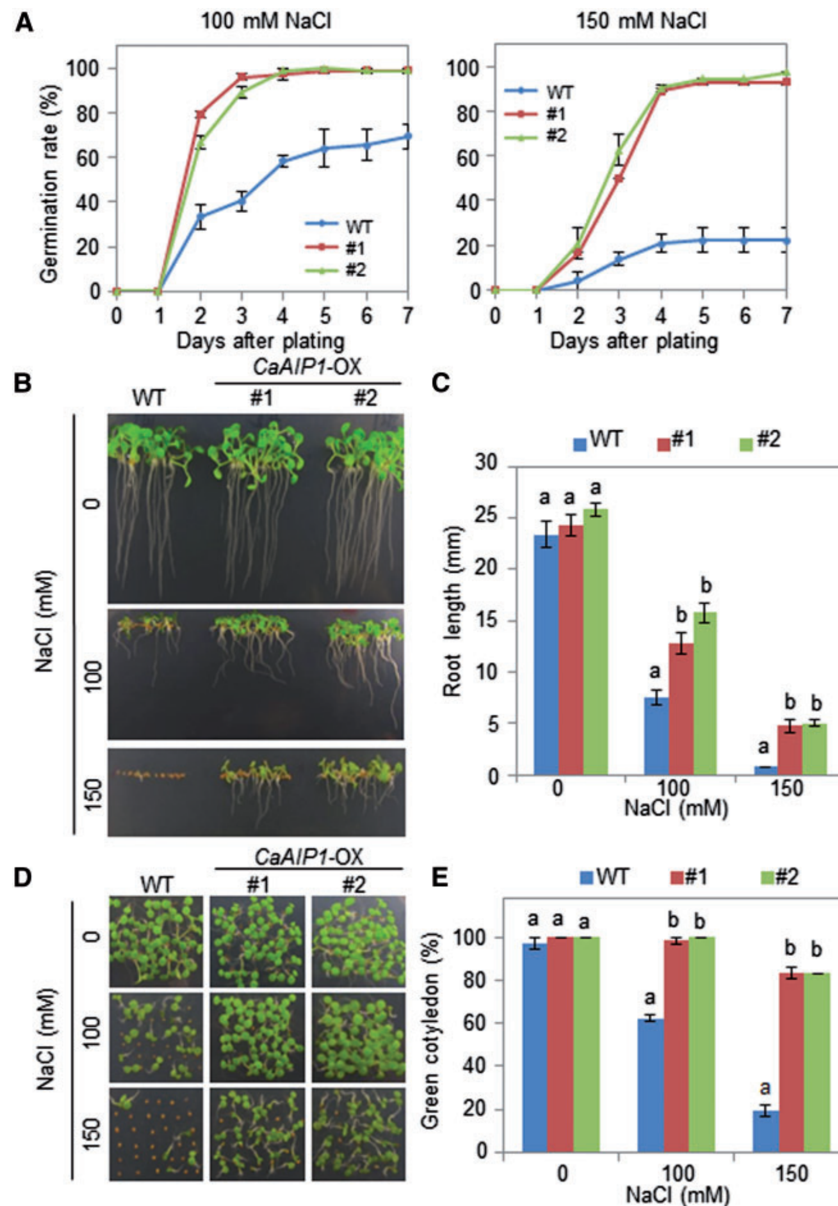

Fig. 6 Enhanced tolerance of *CaAIP1*-OX transgenic Arabidopsis lines to high salinity during germination and seedling growth. (A) Seed germination of wild type and transgenic lines in response to mannitol after 0–7 d of treatment. Seeds were germinated on 0.5× MS agar plates containing 100 or 150 mM NaCl. (B) Root elongation of wild-type and transgenic lines in response to NaCl. (C) The root length of each plant was measured 8 d after plating. Data represent the mean ± SE of three independent experiments. (D) Growth of wild-type and *CaAIP1*-OX seedlings on 0.5× MS agar plates containing 0, 100 or 150 mM NaCl. Representative photographs were taken 5 d after plating. (E) Quantification of green cotyledons in the wild type and each mutant line was performed at 5 d after plating on 0.5× MS agar plates containing 0, 100 or 150 mM NaCl. Data represent the mean ± SE values obtained after evaluating 72 seeds from three independent experiments. Different letters indicate significant differences between wild-type and transgenic lines ($P < 0.05$; ANOVA followed by Fisher's LSD test).

Discussion

In plants, the constitutive defense response to environmental stress is commonly repressed to consume energy for growth and development under favorable conditions. Therefore, the induced defense mechanism is important for energy conservation and protection against various environmental stress conditions. In the present study, we isolated and characterized the RING E3 Ub ligase, CaAIP1, which contains a C-terminal RING-zinc finger domain. The presence of the RING-zinc finger domain in CaAIP1 implies that this protein possesses E3 Ub ligase activity. The CaAIP1 protein examined in the present study showed self-ubiquitination activity, and therefore it may be able to attach ubiquitin to target proteins, which act as positive regulators of plant defense responses. Ubiquitination via the 26S proteasome system is the most widely used mechanism in plant physiological responses to biotic and abiotic stresses (Lee et al. 2011, Guo et al. 2013).

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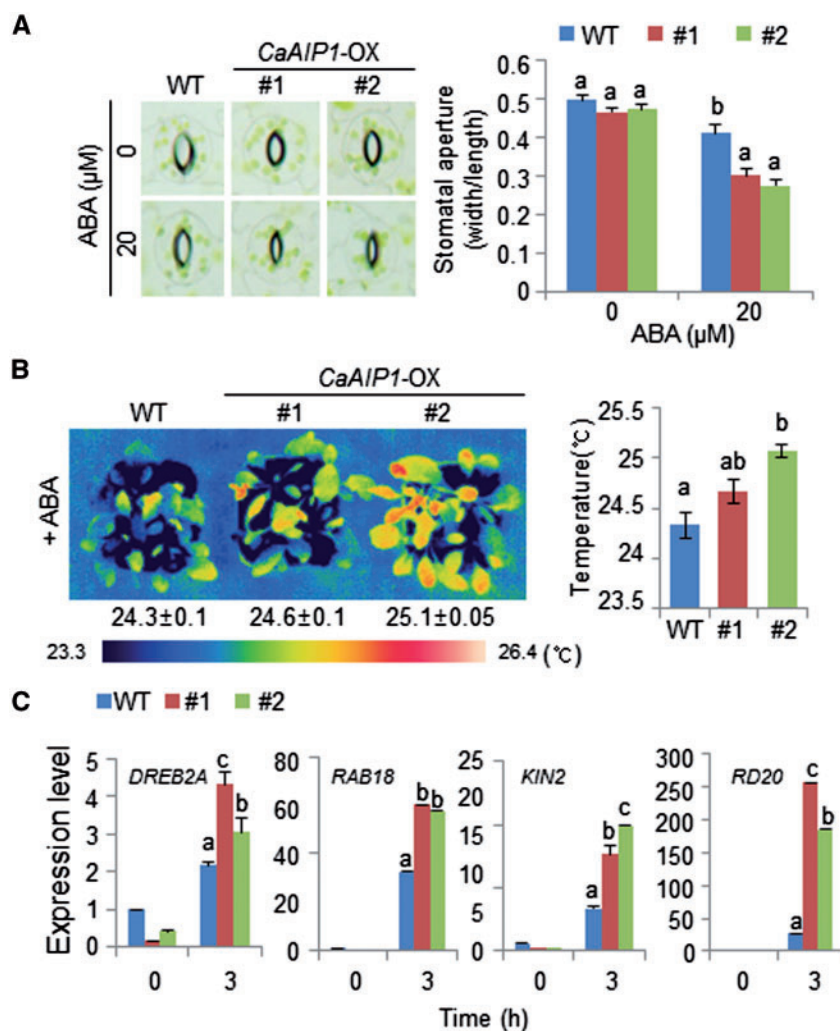

Fig. 7 Enhanced sensitivity of *CaAIP1*-OX transgenic Arabidopsis lines to ABA. (A) Stomatal apertures in wild-type and transgenic lines after treatment with 0 and 20 μM ABA. Leaf peels were harvested from 3-week-old plants and the stomatal apertures were measured under the microscope. Data represent the mean ± SE of three independent experiments, each evaluating 10 plants. (B) Increased leaf temperatures of *CaAIP1*-OX plants in response to ABA treatment. Data represent the mean ± SE of three independent experiments, each evaluating 20 plants. (C) qRT—PCR analysis of ABA signaling genes in the *CaAIP1*-OX mutant after ABA treatment. The relative expression (ΔΔCT) of each gene was normalized to the geometric mean of *Actin8* as the internal control gene. Data represent the mean ± SD from three independent experiments. Different letters indicate significant differences between wild-type and transgenic lines ($P < 0.05$; ANOVA followed by Fisher's LSD test).

Several studies have suggested that RING E3 ligases are associated with cellular responses to ABA and osmotic stresses (Lyzenga et al. 2012, Kim and Kim 2013, Tian et al. 2015). These enzymes function as positive or negative regulators in the response to osmotic stress, via ABA-dependent or ABA-independent pathways (Cheng et al. 2012, Park et al. 2015, Tian et al. 2015, Zhang et al. 2015).

To elucidate the in vivo function of CaAIP1, we examined loss of function via VIGS-based genetic analysis and gain of function by using *CaAIP1*-OX plants.We used these techniques because the transformation efficiency in pepper plants was very low. Our phenotypic analyses revealed that *CaAIP1*-silenced pepper plants exhibited a pronounced drought-sensitive phenotype, and this was characterized by high levels of transpirational water loss via increased stomatal pore size. In contrast, *CaAIP1*-OX plants displayed enhanced tolerance to drought and high salt stresses, indicating that the expression level of *CaAIP1* is positively correlated with osmotic stress tolerance. The observed phenotypic concordance between *CaAIP1*-silenced pepper plants and *CaAIP1*-OX Arabidopsis plants verifies that *CaAIP1* plays an important role in the defense mechanism that enables plants to adapt to osmotic stress. The results of RT—PCR analysis revealed that ABA strongly induces accumulation of *CaAIP1* transcripts in pepper leaves (Fig. 2B), indicating a role for this protein in the response to osmotic stress via the ABA-mediated pathway. ABA constitutes an integral part of adaptive responses to osmotic stresses; moreover, when plants encounter osmotic stress conditions, ABA levels increase in plant tissues, particularly the leaves (Cutler et al. 2010, Hubbard et al. 2010, Zhu 2002). ABA decreases the turgor pressure in guard cells, thereby leading to stomatal closure; this reduces transpirational water loss and

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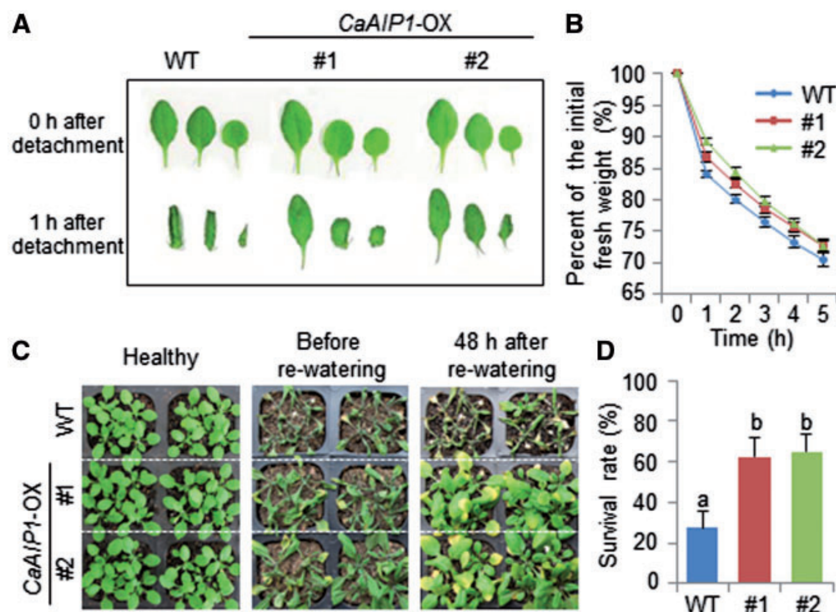

Fig. 8 Enhanced tolerance of *CaAIP1*-OX transgenic Arabidopsis lines to dehydration and drought stresses. (A) Dehydration tolerance of *CaAIP1*-OX transgenic plants. Three-week-old wild-type (WT) and transgenic plants were subjected to dehydration stress by detaching the rosette leaves. (B) Transpirational water loss from the leaves of wild-type and transgenic plants at various times after detachment of leaves. (C) Drought tolerance of *CaAIP1*-OX transgenic plants. Three-week-old WT and transgenic plants were subjected to drought stress by withholding water for 10 d and then re-watering for 2 d. Representative images were taken before (left) and after (middle) drought and after 2 d of re-watering (right). (D) Survival rates of plants after re-watering. Data represent the mean ± SE of three independent experiments, each evaluating 40 plants. Different letters indicate significant differences between wild-type and transgenic lines ($P < 0.05$; ANOVA followed by Fisher's LSD test).

enhances tolerance to osmotic stress (Robertson and Chandler 1994, Murata et al. 2015, Park et al. 2015). In our present study, we showed that *CaAIP1*-OX lines exhibited an ABA-sensitive phenotype (**Fig. 8**). The ABA-hypersensitive phenotype displayed by *CaAIP1*-OX plants is derived from increased stomatal closure and higher leaf temperatures, and these characteristics contribute to a drought-tolerant phenotype. Additionally, *CaAIP1*-OX plants exhibited a salt-tolerant phenotype during the germination and seedling growth stages (**Fig. 7**), but not at maturity. The observed phenotypic discrepancy between the germination and seedling growth stages and the mature stage in *CaAIP1*-OX plants is presumably derived from the different activities of CaAIP1 at different growth stages.

In summary, in the present study, we have identified and characterized the stress-responsive RING-type E3 Ub ligase, CaAIP1. The CaAIP1 protein functions as a positive regulator of osmotic stresses. Moreover, loss-of-function *CaAIP1*-silenced pepper plants and gain-of-function *CaAIP1*-OX plants displayed altered phenotypes to drought stress via changes in the stomatal aperture and leaf temperature. We have demonstrated the functional involvement of CaAIP1 in plant defense responses to osmotic stresses via ABA-mediated signaling; however, the precise mechanism whereby CaAIP1 functions as a positive regulator of osmotic stress responses remains unclear. Further studies to identify the target proteins whose levels are regulated by the CaAIP1–26S proteasome system are required. Nevertheless, by providing a valuable insight into the defense mechanism that operates during osmotic stress signaling, the results of our present study may facilitate the adaptation of plants to various environmental stresses.

Materials and Methods

Plant material and growth conditions

Seeds of pepper (*Capsicum annuum* L., cv. Nockwang) and tobacco (*Nicotiana benthamiana*) were sown in a steam-sterilized compost soil mix (peat moss, perlite and vermiculite, 5 : 3 : 2, by vol.), sand and loam soil (1 : 1 : 1, by vol.). The pepper plants were raised in a growth room at 27 ± 1 °C under white fluorescent light (80 μmol photons m^{-2} s^{-1}; 16 h d^{-1}) as described previously (Lee et al. 2008). The tobacco plants were maintained 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 Murashige and Skoog (MS) salt supplemented with 1% sucrose and Microagar (Duchefa Biochemie); the seeded plates were incubated in a growth chamber at 24 °C and under a 16 h light/8 h dark cycle. The Arabidopsis seedlings were maintained 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} s^{-1}) with a 16 h light/8 h dark cycle. All seeds were vernalized at 4 °C for 2 d before being placed in the growth chamber.

Virus-induced gene silencing

The TRV-based VIGS system was used to generate the *CaAIP1* gene knockdown in pepper plants (Lim and Lee 2014). A 180–390 bp fragment of the *CaAIP1* cDNA was inserted into the pTRV2 vector to generate pTRV:*CaAIP1*. *Agrobacterium tumefaciens* strain GV3101 containing pTRV1, pTRV2:00 and pTRV:*CaAIP1* was co-infiltrated into the fully expanded cotyledons of pepper plants (OD_{600} = 0.2 for each construct). The plants were placed in a growth room and maintained at 26 °C under a 16 h light/8 h dark cycle for growth and spread of the virus.

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ABA, drought and NaCl treatments

To examine the expression pattern of the *CaAIP1* gene in pepper plants after ABA treatment, six-leaf stage pepper plants were sprayed with 100 μM ABA or control solution. For the NaCl and drought treatments, pepper plants were irrigated with 200 mM NaCl solution and were then carefully removed from the soil to avoid injury. The plants were placed onto 3 mm filter paper. Leaves were harvested at 0, 2, 6, 12 and 24 h after each treatment and were subjected to RNA isolation and RT–PCR analysis.

Three-week-old seedlings from wild-type and *CaAIP1*-OX transgenic Arabidopsis lines were randomly planted and were then subjected to drought stress treatment by withholding water for 10 d and then re-watering for 2 d. For pepper plants, drought stress was imposed on four-leaf stage plants by withholding water for 10 d. Plants were re-watered for 2 d to allow recovery, and the survival rate of the plants was then calculated. The drought resistance was determined in a quantitative manner by measuring the transpirational water loss. Leaves were detached from four-leaf stage pepper plants and 3-week-old Arabidopsis plants, and were placed in Petri dishes. The dishes were maintained in a growth chamber at 40% relative humidity, and the loss of fresh weight was determined at the indicated time points. All the experiments were performed at least in triplicate.

Stomatal aperture bioassay

The stomatal aperture bioassay was performed as described previously (Lim and Lee 2014). Briefly, epidermal peels were stripped from the rosette leaves of 3-week-old plants and floated in a stomatal opening solution (SOS: 50 mM KCl and 10 mM MES-KOH, pH 6.15, 10 μM $CaCl_2$) in the light. After incubation for 3 h, the buffer was replaced with fresh SOS containing 20 μM ABA. After an additional 2.5 h of incubation, the stomatal apertures in each individual sample were measured. Each experiment was performed in triplicate.

RNA isolation and semi-quantitative RT–PCR

Total RNA was isolated from leaves of pepper and the Arabidopsis plants, which were treated with various stresses. To remove genomic DNA, all RNA samples were digested with RNA-free DNase. After quantification using a spectrophotometer, 1 μg of total RNA was used to synthesize cDNA using a Transcript First Strand cDNA Synthesis kit according to the manufacturer's instructions. Concomitantly, cDNAs were synthesized without reverse transcriptase and were subjected to semi-quantitative RT–PCR to eliminate the possibility of contamination by genomic DNA in the cDNA samples. Semi-quantitative RT–PCR analysis was carried out using Ex-taq DNA polymerase and specific primers (**Supplementary Table S1**). Each reaction was performed in triplicate. The PCR was programmed as follows: 95 °C for 5 min; 45 cycles at 95 °C for 20 s; 60 °C for 20 s; and 72 °C for 20 s.

Expression of the MBP–CaAIP1 recombinant protein in bacterial cells and in vitro ubiquitination

For expression of the MBP–CaAIP1 recombinant protein, the pMAL-c2X vector (New England Biolabs) harboring the full-length *CaAIP1* cDNA sequence was introduced into the *E. coli* strain c+ cell. The MBP–CaAIP1 fusion protein was induced and purified according to the manufacturer's instruction (New England Biolabs).

For the in vitro ubiquitination assay, the purified MBP–CaAIP1 (500 ng) was mixed with ubiquitination reaction buffer [50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.05 mM $ZnCl_2$, 1 mM Mg-ATP, 0.2 mM dithiothreitol (DTT), 10 mM phosphocreatine and 0.1 U of creatine kinase (Sigma-Aldrich)] containing 250 ng of recombinant human UBE1 (Boston Biochemicals), 250 ng of recombinant his-tagging Arabidopsis E2s and 10 μg of bovine Ub (Sigma-Aldrich). After incubation at 30 °C for 3 h, the reacted proteins were separated using SDS–PAGE and analyzed using immunoblotting with anti-Ub antibody and anti-MBP antibody.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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