Pepper SUMO protease CaDeSI2 positively modulates the drought responses via deSUMOylation of clade A PP2C CaAITP1

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Received: *12 February 2024*
Accepted: *3 June 2024*

New Phytologist (2024)
doi: 10.1111/nph.19920

Key words: ABA, deSUMOylation, drought, protein stability, SUMOylation.

Summary

- Posttranslational modification of multiple ABA signaling components is an essential process for the adaptation and survival of plants under stress conditions. In our previous study, we established that the pepper group A PP2C protein CaAITP1, one of the core components of ABA signaling, undergoes ubiquitination mediated by the RING-type E3 ligase CaAIRE1.
- In this study, we discovered an additional form of regulation mediated via the SUMOylation of CaAITP1. Pepper plants subjected to drought stress were characterized by reductions in both the stability and SUMOylation of CaAITP1 protein.
- Moreover, we identified a SUMO protease, *Capsicum annuum* DeSUMOylating Isopeptidase 2 (CaDeSI2), as a new interacting partner of CaAITP1. *In vitro* and *in vivo* analyses revealed that CaAITP1 is deSUMOylated by CaDeSI2. Silencing of *CaDeSI2* in pepper plants led to drought-hypersensitive and ABA-hyposensitive phenotypes, whereas overexpression of *CaDeSI2* in transgenic *Arabidopsis* plants resulted in the opposite phenotypes. Importantly, we found that the CaAITP1 protein was stabilized in response to the silencing of *CaDeSI2*, and *CaDeSI2* and *CaAITP1* co-silenced pepper plants were characterized by drought-tolerant phenotypes similar to those observed in *CaAITP1*-silenced pepper.
- Collectively, our findings indicate that CaDeSI2 reduces the stability of CaAITP1 via deSUMOylation, thereby positively regulating drought tolerance.

Introduction

Depending upon their distribution, habitats, and climatic conditions, plants are variously exposed to a range of abiotic stresses, among which, drought stress is a particularly important factor restricting plant growth and survival. In response to drought stress, plants activate several defense responses, such as stomatal closure, to minimize water loss. The mechanisms associated with reductions in water loss are primarily mediated via abscisic acid (ABA) signaling, a pathway comprising ABA receptors, group A protein phosphatase 2Cs (PP2Cs), and SNF1-related protein kinase 2s (SnRK2s) (Cutler *et al.*, 2010; Lee & Luan, 2012). In the absence of ABA, ABA co-receptor PP2Cs interact with and dephosphorylate SnRK2s, thereby maintaining the latter in an inactive form (Ma *et al.*, 2009). However, in the presence of ABA, this hormone binds to the ABA receptor PYR/PYL/RCAR (pyrabactin resistance/PYR1-Like/Regulatory Component of ABA Receptor) (Fujii *et al.*, 2009), which thereby alters the conformation of the PYR/PYL/RCAR-PP2C complex, leading the inactivation and subsequent release of phosphatase from the SnRK2 kinase active site (Fujii *et al.*, 2009; Lee & Luan, 2012). By phosphorylating downstream transcription factors and ion channels, the released SnRK2s amplify and deliver the ABA signal (Lee *et al.*, 2009; Vlad *et al.*, 2009).

Among the different types of posttranslational modification, small ubiquitin-like modification (SUMO) conjugation (SUMOylation) has been established to play roles in regulating the stability, localization, and activity of multiple intracellular effectors in plants and other eukaryotic organisms (Hay, 2005, 2013; Vierstra, 2012; Hendriks & Vertegaal, 2016). Moreover, SUMOylation is proving to be an important mechanism associated with the rapid redirection of molecular pathways during stress signaling in plants (Conti *et al.*, 2008; Orosa-Puente *et al.*, 2018). The SUMOylation process is mediated via a series of sequential enzymatic reactions involving activation, conjugation, and ligation catalyzed by SUMO-activating enzyme (E1), SUMO-conjugating enzyme (E2), and SUMO ligase (E3), respectively (Novatchkova *et al.*, 2004). Among these, E3 SUMO ligases catalyze the covalent bond formation between the lysine residue of the target protein and the C-terminal glycine residue of SUMO (Augustine & Vierstra, 2018). The SUMOylation process can also be reversed by the action of SUMO proteases, which have been established to be considerably more numerous than E3 ligases (Yates *et al.*, 2016), and it has accordingly been suggested that these SUMO proteases may contribute to determining the specificity of the SUMO system (Colby *et al.*, 2006; Benlloch & Lois, 2018).

In *Arabidopsis*, the currently identified SUMO proteases are classified as members of the ubiquitin-like protease (ULP) and deSUMOylating isopeptidase (DeSI) superfamilies. SUMO

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proteases, which contain a nucleophilic cysteine residue at the center of the catalytic site triad/dyad, play roles in cleaving the thioester bond between SUMO and target proteins (Morrell & Sadanandom, 2019), and the findings of recent studies have revealed that several SUMO proteases belonging to the ULP superfamily play roles in plant hormone signaling pathways. For example, *Arabidopsis* SUMO protease 1 (ASP1) has been demonstrated to deSUMOylate ABI5 and MYB30, two proteins that function in ABA signaling during early seedling development, thereby enhancing their stability (Q. Wang *et al.*, 2018). A further SUMO protease, EARLY IN SHORT DAYS 4 (ESD4), interacts with NUCLEAR PORE ANCHOR (NUA), and thereby contributes to the negative regulation of ABA signaling (Cui *et al.*, 2022). Similarly, in rice (*Oryza sativa*), the SUMO protease OVERLY TOLERANT TO SALT1 (OsOTS1) has been established to negatively modulate ABA signal transduction (Srivastava *et al.*, 2017). With respect to DeSI proteases, eight putative DeSIs have to date been identified in *Arabidopsis* based on similarity to the sequence of human DeSI1/2, among which, Desi3a has been functionally characterized to be involved in plant innate immunity (Orosa *et al.*, 2018).

In previous studies, we have elucidated the ubiquitination-associated regulation of pepper mediated via group A PP2Cs. Specifically, the pepper RING-type ubiquitin E3 ligase CaAIRF1 was found to interact with and ubiquitinate the pepper group A PP2Cs, CaADIP1 and CaAIPP1, whereas CaAIRE1 similarly interacts with and ubiquitinates CaAITP1 (Baek *et al.*, 2017, 2021; Lim *et al.*, 2017). However, the mechanisms underlying the SUMOylation-associated regulation of PP2Cs have yet to be determined. In this study, we identified a novel pepper SUMO protease, *Capsicum annuum* DeSUMOylating Isopeptidase 2 (CaDeSI2), as an interacting partner of CaAITP1. Based on these findings, we investigated the SUMOylation and deSUMOylation of CaAITP1 and elucidated the role of CaDeSI2 in ABA signaling and drought responses. Our findings provide convincing evidence to indicate that CaDeSI2 influences the drought responses of pepper by deSUMOylating CaAITP1.

Materials and Methods

Plant materials

The pepper (*C. annuum* L. cv Nockwang) were grown in a growth chamber at 27 ± 1°C under a 16 h : 8 h, light : dark cycle, whereas the *Nicotiana benthamiana* were grown in a growth chamber at 25 ± 1°C under the same light conditions. For Arabidopsis (*Arabidopsis thaliana* L., ecotype Col-0), sterilized seeds were placed on Murashige & Skoog (MS) medium with 1% sucrose. To promote germination, all *Arabidopsis* seeds were initially stratified at 4°C for 2 d and transferred to a growth chamber at 24 ± 1°C under a 16 h : 8 h, light : dark cycle.

Yeast two-hybrid assay

As bait for yeast two-hybrid assays, we used the full-length sequence of CaDeSI2 inserted into a pGBKT7 vector, whereas the full-length sequence of CaAITP1 cloned in a pGADT7 vector was used as the prey. Both vector types were used to co-transform cells of the yeast strain Y2HGold using the lithium acetate method. The yeast was grown and selected in synthetic dextrose minimal media.

Co-immunoprecipitation assay

To induce the transient expression of *CaAITP1* and *CaDeSI2*, *Agrobacterium tumefaciens* strain GV3101 cells containing the *35S:HA-CaAITP1* and *35S:CaDeSI2-GFP* constructs were infiltrated to *N. benthamiana* leaves. Leaves of the transformed tobacco were subsequently homogenized, and total protein was extracted using a buffer comprising 10 mM DTT, 1 mM EDTA, 10% glycerol, 2% PVPP, 150 mM NaCl, T150 mM Tris–HCl pH 7.5, 0.2% Triton X-100, and a 1× complete protease inhibitor cocktail. The protein extracts thus obtained were incubated with the GFP-Trap system (Chromotek, Planegg, Germany) at 4°C for 3 h. The precipitated proteins were washed three times, eluted with 4× SDS-PAGE loading buffer, and subjected to immunoblot analysis using anti-HA (1 : 10 000 dilution; Agrisera, Vännäs, Sweden) and anti-GFP (1 : 1000 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies.

Split-luciferase complementation imaging assay

The full-length coding sequences of CaDeSI2 and CaAITP were introduced into the pDEST-NLUC^{GW} and pDEST-CLUC^{GW} vectors, kindly provided by Dr Jörg Kudla of the University of Münster, to generate nLUC-CaDeSI2 and cLUC-CaAITP1 constructs, respectively. *Agrobacterium tumefaciens* GV3101 cells harboring the respective constructs were mixed with strain p19 cells (1 : 1 : 1 ratio; OD_{600} = 0.5) and co-infiltrated into *N. benthamiana* leaves. Three days after infiltration, luciferin solution (0.5% v/v DMSO, 1 mM luciferin 10 mM MES/KOH (pH 5.6), and 10 mM $MgCl_2$) was infiltrated to facilitate the detection of luminescence signals. Before imaging, the plants were maintained in the dark for 30 min to minimize background fluorescence, and luminescence was visualized using a NightSHADE LB 985 plant imaging system, Berthold, Bad Wildbad, Germany, with IndiGO™ software being used for data analysis.

In vivo and *in vitro* SUMO conjugation analyses

For *in vivo* SUMOylation assays, *A. tumefaciens* strain GV3101 cells containing *35S:CaAITP1-GFP* and *35S:HA-CaSUMO1* constructs were infiltrated into 4-wk-old *N. benthamiana* leaves. To determine whether CaDeSI2 mediates the deSUMOylation of CaAITP1, *35S:HA-CaDeSI2 or 35S:HA-CaDeSI2*^{C68S} were also similarly analyzed. Leaves of the transformed tobacco were subsequently homogenized, and total protein was extracted using a buffer containing 1× complete protease inhibitor cocktail, 10 mM DTT, 1 mM EDTA, 10% glycerol, 150 mM NaCl, 150 mM Tris–HCl pH 7.5, 0.2% Triton X-100, and 2% PVPP. The protein extracts thus obtained were incubated using Anti-FLAG® M2 Magnetic Beads (Sigma-Aldrich) at 4°C for 3 h.

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The precipitated proteins were washed at least three times, eluted using 4× SDS-PAGE loading buffer, and subjected to immunoblotting using anti-DDDDK (FLAG) (1 : 10 000 dilution; Abcam, Cambridge, UK), anti-HA (1 : 10 000 dilution; Agrisera), and anti-SUMO1 (1 : 10 000 dilution; Agrisera) antibodies.

For *in vitro* SUMOylation assays, the recombinant proteins MBP-CaAITP1 and GST-CaDeSI2 were purified from *Escherichia coli*. Subsequently, 50 ng purified E1 His-CaSAE1/2, 50 ng purified E2 His-CaSCE1, 1 μg purified His-CaSUMO1, 0.5 μg MBP-CaAITP1, and GST-CaDeSI2 or GST-CaDeSI2^{C68S} were co-incubated in 30 μl of reaction buffer (2 mM ATP, 50 mM Tris–HCl pH 7.5, and 5 mM $MgCl_2$) at 30°C for 3 h. The proteins were separated using SDS-PAGE, followed by immunoblot analysis. The SUMO-conjugated MBP-CaAITP1 was detected using an Anti-SUMO1 antibody, and MBP-CaAITP1 and GST-CaDeSI2 were detected using anti-MBP and anti-GST antibodies, respectively.

Cell-free degradation assay

Cell-free degradation assays were performed as previously described (Baek *et al*., 2017; Lim *et al*., 2017). Briefly, crude protein extracts from pepper leaves were obtained using an extraction buffer comprising 25 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 0.1% Triton X-100, 10 mM ATP, and 10 mM NaCl. Aliquots of these extracts (50 μg total protein) were incubated with MBP-CaAITP1 protein (500 ng) in the presence or absence of 50 μM MG132 (Merck & Millipore, Darmstadt, Germany). Samples were harvested at the indicated time points and subjected to SDS-PAGE, with subsequent immunoblot analysis using an anti-MBP antibody (1 : 10 000 dilution; New England Biolabs, Ipswich, MA, USA) to determine the rate of target protein degradation. The experiment was performed independently three times.

Results

Reduced protein stability and SUMOylation of CaAITP1 under drought stress

In our previous study, we established that CaAIRE1 ubiquitinates the CaAITP1 protein in pepper, thereby resulting in its destabilization and thus contributing to a modulation of the drought responses of these plants (Baek *et al*., 2021). In the current study, we sought to gain an understanding of the mechanisms whereby drought stress affects the stability of the CaAITP1 protein. To this end, we initially performed a cell-free degradation assay using crude extracts obtained from the leaves of healthy and dehydrated pepper plants and accordingly detected a greater degradation of CaAITP1 protein in dehydrated peppers than in healthy peppers following a 2-h reaction (Fig. 1a).

In addition to its ubiquitination function, SUMOylation also serves as an important posttranslational modification process that contributes to modulating protein stability (Geiss-Friedlander & Melchior, 2007) and has been established to play a role in drought stress responses (Benlloch & Lois, 2018). Based on these functional properties, we speculated that SUMOylation may be involved in the regulation of CaAITP1 protein stability. To investigate the SUMO conjugation of CaAITP1, we performed *in vivo* SUMOylation assays using *N. benthamiana* leaves co-expressing the *35S:FLAG-CaAITP1* with *35S:HA-CaSUMO1* or *35S:HA-CaSUMO1*$^{\Delta GG}$ (nonconjugatable SUMO protein) constructs (Fig. 1b). Following immunoprecipitation of CaAITP1 using an anti-FLAG antibody, immunoblot analysis revealed a smear of high molecular weight bands in the presence of CaSUMO1, and notably the intensity of these bands increased as CaSUMO1 expression levels increased. However, this distinctive pattern was not absent in the presence of CaSUMO1$^{\Delta GG}$, thereby indicating that CaAITP1 undergoes SUMOylation. The SUMO conjugation appeared to enhance the stability of CaAITP1 proteins. The C-terminal of CaAITP1 was fused with SUMO1 to mimic its constitutively SUMOylated state, and the fusion proteins were used in a cell-free degradation assay. As shown in Fig. 1(c), CaAITP1-CaSUMO1 fusion protein exhibited less degradation, compared to single CaAITP1 proteins.

Having established this effect, we further investigated how drought stress might influence the SUMOylation of CaAITP1. To this end, we subjected *N. benthamiana* plants co-expressing *35S:FLAG-CaAITP1* and *35S:HA-CaSUMO1* to drought stress and used dehydrated leaves for an *in vivo* SUMOylation assay (Fig. 1d). In the absence of CaSUMO1 expression, drought stress did not affect the SUMOylation of CaAITP1 protein. However, in the presence of CaSUMO1 expression, immunoblot analysis showed SUMO-conjugated CaAITP1 proteins, and notably this SUMOylation was significantly reduced in drought-stressed plants. These findings thus provided evidence suggesting that drought stress not only contributes to reducing the stability of the CaAITP1 protein but also has a negative regulatory effect on the SUMOylation of this protein.

Interaction between CaAITP1 and CaDeSI2

Having verified the SUMOylation of CaAITP1, we subsequently sought to identify an interacting partner responsible for the SUMOylation of this protein. Based on yeast two-hybrid screening, we accordingly identified CaDeSI2 as a candidate CaAITP1-interacting protein, characterized by a putative PPPDE (Permuted Papain fold Peptidases of DsRNA viruses and Eukaryotes) peptidase domain, which is highly conserved in the DeSI proteins identified in other plant species (Supporting Information Fig. S1). To validate the interaction between two proteins, we performed a one-by-one yeast two-hybrid assay, which indeed revealed a strong interaction between CaDeSI2 and CaAITP1 in yeast cells, as shown in Fig. 2. Furthermore, the findings of co-immunoprecipitation and split-luciferase complementation imaging assays tended to confirm the *in vivo* interaction between CaDeSI2 with CaAITP1 (Fig. 2b,c). To gain further evidence in support of this *in vivo* interaction, we also conducted bimolecular fluorescence complementation analysis, which, consistently, indicated that CaDeSI2 interacts with CaAITP1 within the cell nucleus (Fig. 2d). Collectively, these data would thus tend to indicate that CaDeSI2 is a bona fide interacting partner of CaAITP1.

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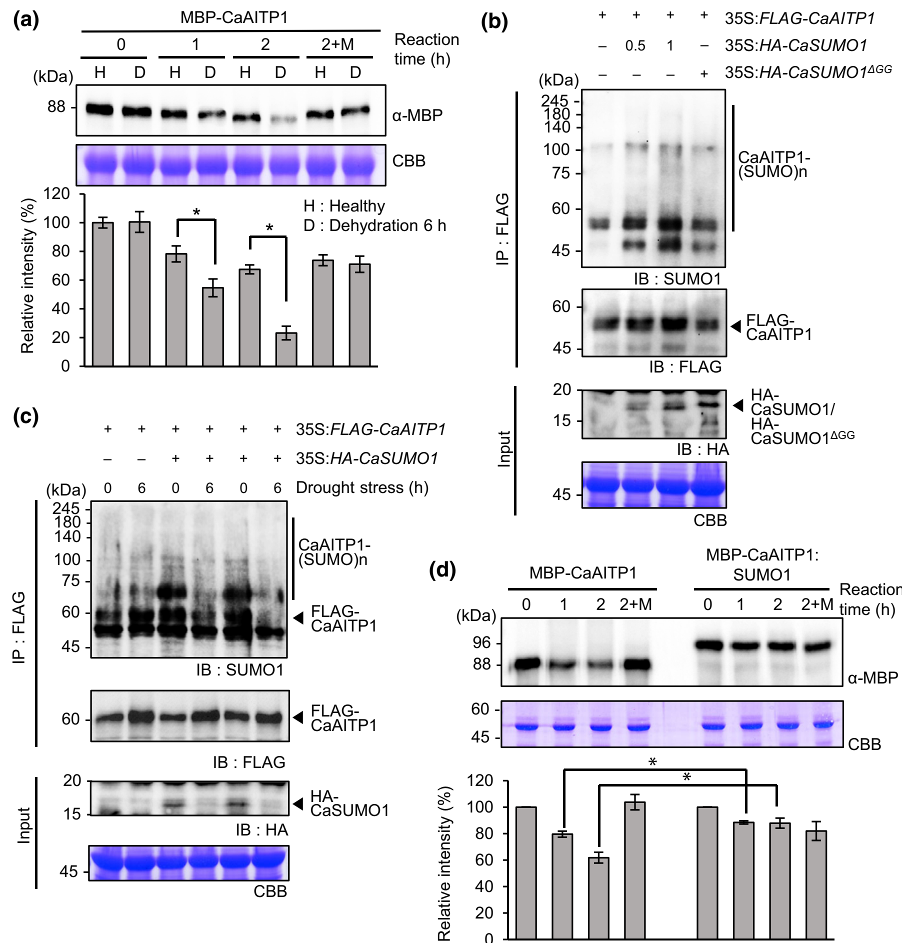

Fig. 1 Drought-induced reduction in the stability and SUMOylation of the CaAITP1 protein. (a) Cell-free degradation assay of CaAITP1 using dehydrated pepper. MBP-CaAITP1 recombinant protein was incubated for 1 and 2 h with crude extracts obtained from the healthy or dehydrated leaves of pepper plants. Immunoblot analysis was performed using an anti-MBP antibody (upper panel). Coomassie Brilliant Blue (CBB) staining indicates equivalent loading of the crude extracts (lower panel). The relative intensity of the MBP-CaAITP1 proteins was measured using ImageJ 1.46r software (http://imagej.nih.gov/ij). Data are presented as the means ± SE of three independent experiments. Asterisks indicate significant differences (Student's *t*-test; $P < 0.05$). M, MG132. (b) SUMOylation of CaAITP1 *in vivo*. Proteins were extracted from the leaves of *Nicotiana benthamiana* plants co-expressing *35S:FLAG-CaAITP1* and *35S:HA-CaSUMO1* or *35S:HA-CaSUMO1*$^{\Delta GG}$ (negative control) and immunoprecipitated with Anti-FLAG Beads. *Agrobacterium* cells harboring *35S:HA-CaSUMO1* were infiltrated into *N. benthamiana* leaves at increasing concentrations OD_{600} = 0.5, and 1.0. Immunoblot analysis was performed using anti-SUMO1, anti-FLAG, and anti-HA antibodies. (c) SUMOylation of CaATIP1 in response to drought stress. Proteins were extracted from the dehydrated leaves of *N. benthamiana* plants co-expressing *35S:FLAG-CaAITP1* and *35S:HA-CaSUMO1* and immunoprecipitated with Anti-FLAG Beads. (d) Cell-free degradation assay of CaSUMO1-tagged CaAITP1. MBP-CaAITP1:CaSUMO1 recombinant protein was incubated for 1 and 2 h with crude extracts obtained from the healthy pepper leaves. As described in (a), immunoblot analysis was performed and the relative intensity of the MBP-CaAITP1:CaSUMO1 proteins was measured. Data are presented as the means ± SE of three independent experiments. Asterisks indicate significant differences (Student's *t*-test; $P < 0.05$). M, MG132.

Molecular characterization of CaDeSI2

Given our assumption that CaDeSI2 interacts with CaAITP1, one of the core components of ABA signaling, we postulated that CaDeSI2 is similarly associated with ABA signaling, based on which, we examined the transcript level of *CaDeSI2* in pepper leaves subjected to ABA treatment or different stress conditions (Fig. 3a). In response to treatment with ABA, we detected a gradual initial increase in *CaDeSI2* expression for up to 12 h, followed by a subsequent reduction when measured at 24 h. Contrastingly, in plants subjected to dehydration, there was an initial reduction in *CaDeSI2* expression until 6 h, followed by a subsequent increase. Moreover, a similar pattern of expression was observed in plants treated with PEG to induce osmotic stress. Furthermore, transcriptional alterations in *CaDeSI2* were evident in plants subjected to other stress conditions, such as those exposed to heat stress, NaCl, and H_2O_2. However, whereas we detected a significant reduction in *CaDeSI2* expression in response to NaCl treatment, increases were observed in plants exposed to heat and H_2O_2.

Given that the subcellular localization of proteins is intricately linked with their functional roles, we examined the subcellular

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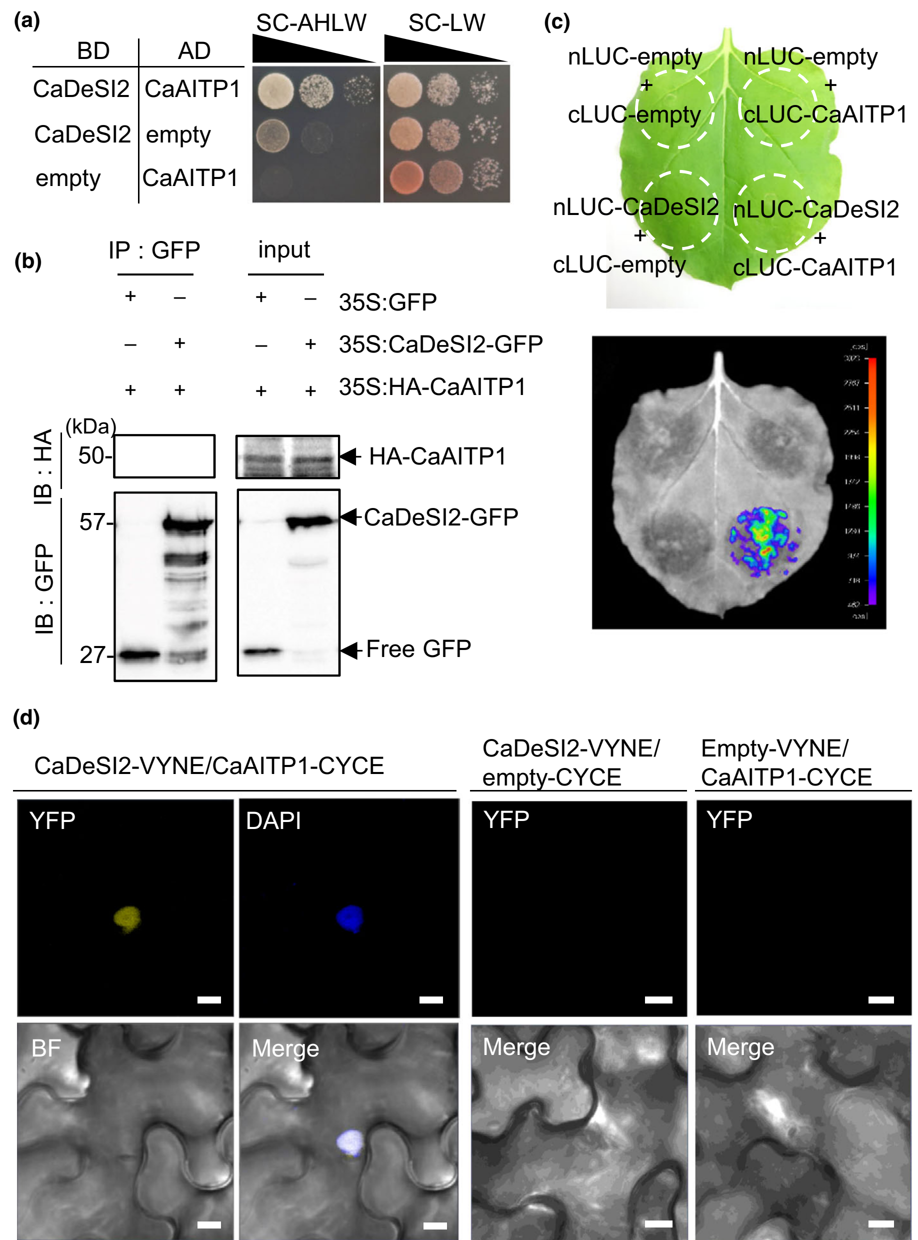

Fig. 2 Interaction of CaDeSI2 with CaAITP1. (a) Yeast two-hybrid assay of CaDeSI2 and CaAITP1. Growth in the selection medium (SC-adenine-histidine-leucine-tryptophan + 10 mM 3′AT) served as an indicator of an interaction between these proteins (left-hand column), whereas growth in SC-leucine-tryptophan medium was used as control (right-hand column). (b) Co-immunoprecipitation assay of CaDeSI2 with CaAITP1. Proteins were extracted from the leaves of *Nicotiana benthamiana* co-expressing *35S:HA-CaAITP1* with *35S:CaDeSI2-GFP* or *35S:GFP* (negative control) and immunoprecipitated with GFP-trap. Immunoblot analysis was performed using anti-HA and anti-GFP antibodies. (c) Split-luciferase complementation imaging assay of nLUC-CaDeSI2 with cLUC-CaAITP1 in *N. benthamiana* leaves (LUC, luciferase). As negative controls, nLUC-CaDeSI2 and cLUC-CaAITP1 were co-expressed with cLUC-empty and nLUC-empty vectors, respectively. (d) Bimolecular fluorescence complementation assay of the interaction between CaDeSI2 and CaAITP1. As negative controls, CaDeSI2-VYNE and CaAITP1-CYCE were co-expressed with empty-CYCE and empty-VYNE vectors, respectively. White bars, 10 μm.

localization of CaDeSI2 based on transient expression of *35S:CaDeSI2-GFP* in the leaves of *N. benthamiana* plants (Fig. 3b), with microscopic analysis revealing that this protein is localized in both the nucleus and cytoplasm, thereby indicating that CaDeSI2 plays roles in both the nuclear and cytoplasmic compartments of plant cells.

DeSUMOylation of CaAITP1 by CaDeSI2

In cysteine proteases belonging to the CP clan, such as DeSI, two conserved residues, histidine and cysteine, are typically found in the active site catalytic dyad (Suh *et al*., 2012). Consistently, CaDeSI2 also possesses active site residues, albeit with a substitution of serine at position 117 instead of cysteine. This amino acid substitution is highly conserved in DeSI homolog proteins from the Solanaceae family of plants (Fig. S1). Given the putative identification of CaDeSI2 as a SUMO protease, we sought to investigate its potential deSUMOylation activity. To this end, we transiently expressed *35S:HA-CaDeSI2* in the leaves of *N. benthamiana* plants. An inactive variant of CaDeSI2 was generated by substituting serine 117 with alanine, resulting in *35S:HA-CaDeSI2*^{S117A}. The leaves of *N. benthamiana* plants expressing *35S:HA-CaDeSI2* or *35S:HA-CaDeSI2*^{S117A} were detached and dried for 6 h to induce global sumoylation. As shown in Fig. 4, immunoblot assay revealed that expression of CaDeSI2, but not CaDeSI2^{S117A}, suppressed a smear of high molecular weight bands observed in nontransgenic samples. This finding suggests that CaDeSI2 may indeed function as a deSUMOylase enzyme.

Next, we examined whether CaAITP1 could be a substrate of the deSUMOylation activity of CaDeSI2. To test this hypothesis,

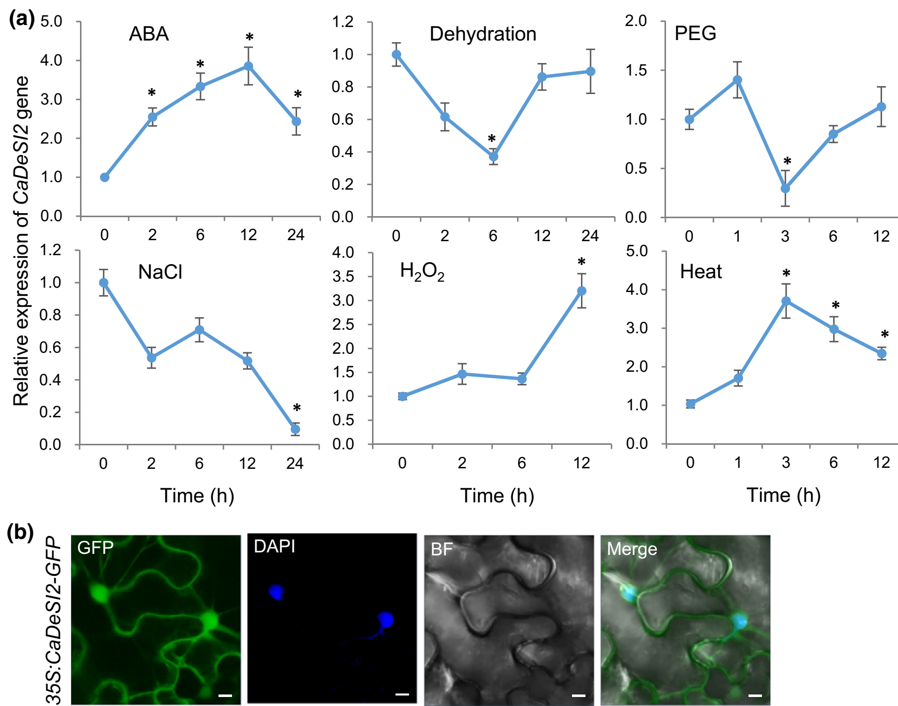

Fig. 3 Molecular characterization of CaDeSI2. (a) Quantitative real-time polymerase chain reaction analysis of *CaDeSI2* gene expression. Patterns of *CaDeSI2* expression were analyzed in the leaves of pepper plants treated with abscisic acid (ABA) (100 μM), NaCl (200 mM), dehydration, H_2O_2 (100 μM), heat stress (45°C), or polyethylene glycol (PEG) 6000 (20%). The pepper *Actin1* (*CaACT1*) gene was used as an internal control. Data are presented as the means ± SE of three independent experiments. Asterisks indicate significant differences (Student's *t*-test; $P < 0.05$). (b) Subcellular localization of CaDeSI2 based on transient expression of a GFP fusion protein in *Nicotiana benthamiana*. White bars, 10 μm.

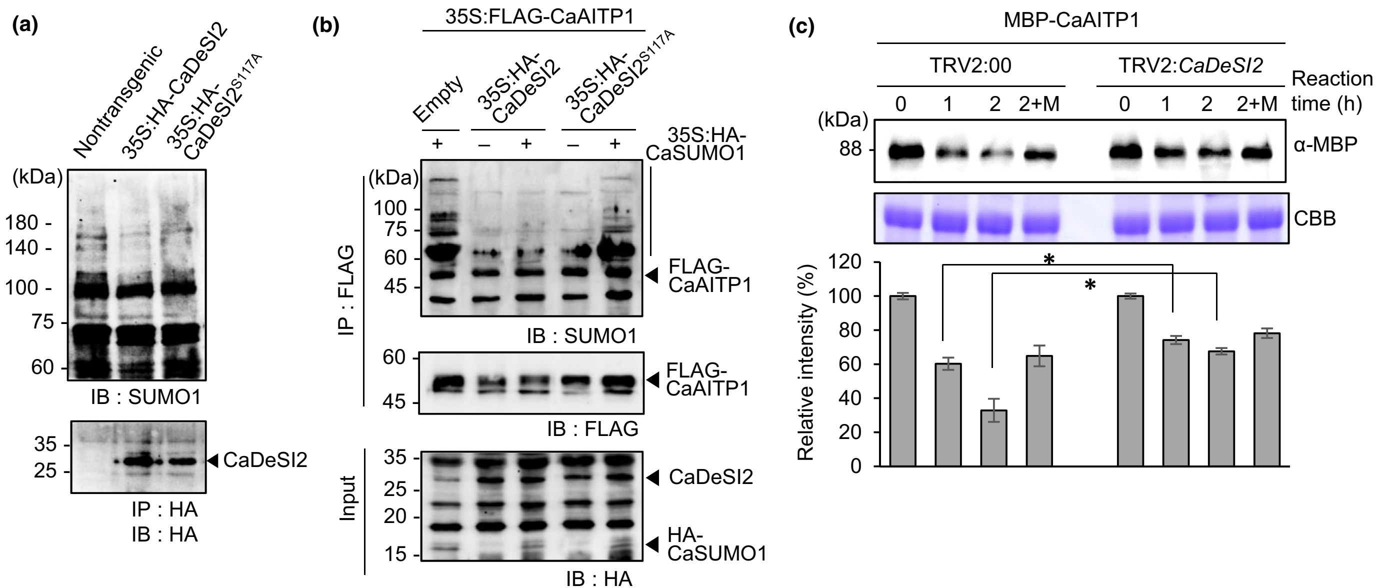

Fig. 4 DeSUMOylation of CaAITP1 by CaDeSI2. (a) Inhibition of global sumoylation in plant cells by CaDeSI2. Proteins were extracted from the dehydrated leaves of *Nicotiana benthamiana* expressing *35S:HA-CaDeSI2* or *35S:HA-CaDeSI2*^{S117A} (negative control). Immunoblot analysis was performed using anti-SUMO1 and anti-HA antibodies. (b) CaAITP1 is DeSUMOylated by CaDeSI2 *in vivo*. Proteins were extracted from the leaves of *N. benthamiana* co-expressing *35S:FLAG-CaAITP1*, *35S:HA-CaSUMO1*, and *35S:HA-CaDeSI2* or *35S:HA-CaDeSI2*^{S117A} and immunoprecipitated with Anti-FLAG Beads. Immunoblot analysis was performed using anti-SUMO1 and anti-FLAG antibodies. (c) Cell-free degradation assay of CaAITP1 using crude protein extracts obtained from *CaDeSI2*-silenced pepper leaves. MBP-CaAITP1 recombinant protein was incubated for 1 and 2 h with the crude extracts obtained from TRV2:00 or TRV2:*CaDeSI2* pepper leaves. Immunoblot analysis was performed using an anti-MBP antibody (upper panel). Coomassie Brilliant Blue (CBB) staining indicates equivalent loading of the crude extracts (lower panel). Error bars represent SE (Student's *t*-test; $P < 0.05$). The relative intensity of the MBP-CaAITP1 protein was measured using ImageJ 1.46r software (http://imagej.nih.gov/ij).

35S:FLAG-CaAITP1 was co-expressed with either *35S:HA-CaDeSI2* or *35S:HA-CaDeSI2*^{S117A} in the leaves of *N. benthamiana* plants (Fig. 4b). Immunoblotting assays revealed a significant reduction in the SUMO conjugation to CaAITP1 when co-expressed with HA-CaDeSI2, even in the presence of CaSUMO1 expression. By contrast, no such reduction was observed with the

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expression of HA-CaDeSI2^{S117A} in the presence of HA-CaSUMO1. Furthermore, we explored whether CaDeSI2 could influence the protein stability of CaAITP1 using a cell-free degradation assay (Fig. 4c). *CaDeSI2*-silenced pepper plants (TRV2:*CaDeSI2*) were generated using virus-induced gene silencing (VIGS), which resulted in a lower expression of *CaDeSI2* than in TRV2:00 control plants (Fig. S2A). Incubatation with crude extracts of the leaves of TRV2:*CaDeSI2* pepper plants showed relatively less degradation of CaAITP1 protein compared to leaf extracts from TRV2:00 plants (Fig. 4c). Collectively, these findings provide convincing evidence in support of the hypothesis that the SUMOylation of CaAITP1 is a reversible process and that CaDeSI2 plays a pivotal role in the deSUMOylation of CaAITP1, leading to a reduction in the stability of the CaAITP1 protein.

Increased drought sensitivity of *CaDeSI2*-silenced pepper plants

In previous studies, we established that the levels of CaAITP1 expression are inversely associated with the resistance of plants to drought stress (Baek *et al.*, 2021). Given the negative role played by CaAITP1 in response to drought stress, we sought to determine the mechanisms whereby *CaDeSI2* influences drought resistance in pepper plants. To this end, 2 wk after agroinfiltration with the respective constructs, we assessed the resistance of TRV2:*CaDeSI2* and TRV2:00 pepper plants that had been subjected to drought stress by withholding water for 11 d (Fig. 5a, upper and middle rows). Compared with TRV2:00 plants, we observed a rapid wilting of TRV2:*CaDeSI2* plants (Fig. 5a, lower row) and at 2 d after re-watering, the survival of TRV2:00 plants (75%) was found to be significantly higher than that of the TRV2:*CaDeSI2* plants (8.3%) (Fig. 5a). Consistently, compared with the leaves of TRV2:00 plants, those of TRV2:*CaDeSI2* plants were characterized by higher transpirational water loss when measured after leaf detachment (Fig. 5b). These findings accordingly indicate that CaDeSI2 may play a positive role in drought resistance.

Having established the heightened drought sensitivity of TRV2:*CaDeSI2* plants, we subsequently sought to ascertain whether this phenotype could be ascribed to alterations in ABA sensitivity. Given that ABA promotes stomatal closure, thereby leading to an elevation in leaf surface temperature by inhibiting evaporative cooling, we measured the surface temperatures of TRV2:*CaDeSI2* and TRV2:00 plant leaves following ABA treatment (Fig. 5c). Whereas before ABA treatment, there were no discernible differences between these two plant lines with respect leaf temperature, at 5 h after ABA treatment, compared with the TRV2:00 plants, those of TRV2:*CaDeSI2* were characterized by a less pronounced elevation in leaf temperature (Fig. 5d). Consistently, we observed a lesser extent of ABA-induced stomatal closure in TRV2:*CaDeSI2* plants (Fig. 5e,f). To complement these findings, we conducted quantitative real-time polymerase chain reaction analyses with specific primers (Table S1) to determine whether the reduced drought resistance of TRV2:*CaDeSI2* plants is accompanied by transcriptional alterations of ABA and/or drought stress-responsive genes (Fig. 5g). In plants subjected to drought stress, we accordingly detected a strong induction of *CaOSR1*, *CaOSM1*, and *CaNCED3* gene expression, although the levels of expression at 3 h after treatment were lower in TRV2:*CaDeSI2* plants than in TRV2:00 plants. Collectively, these findings indicate that *CaDeSI2* positively modulates ABA-induced stomatal closure and drought tolerance in pepper plants.

Enhanced ABA sensitivity and drought tolerance of *CaDeSI2*-OX Arabidopsis

To further elucidate the biological functions of CaDeSI2, we generated *CaDeSI2*-overexpressing (OX) transgenic *Arabidopsis* plants (Figs 6, 7). Semi-quantitative real-time polymerase chain reaction analysis revealed that the levels of *CaDeSI2* in *CaDeSI2*-OX plants were significantly higher than those in wild-type Col-0 plants (Fig. S2B). Using these plants, we initially examined the ABA sensitivity of *CaDeSI2*-OX plants during germination and seedling stages (Fig. 6). In the absence of ABA, we detected no significant differences between the WT and *CaDeSI2*-OX plants with respect to the rate of germination and seedling growth (Fig. 6a–e). Conversely, in the presence of ABA, compared with the WT plants, *CaDeSI2*-OX plants were found to be characterized by a lower rate of germination (Fig. 6a). Moreover, compared with the WT plants, there was a significant reduction in the rate of cotyledon greening rate in the *CaDeSI2*-OX plants (Fig. 6b,c). This enhanced ABA sensitivity in *CaDeSI2*-OX was found to be associated with a notable inhibition of root elongation, with the root lengths of *CaDeSI2*-OX seedlings being significantly shorter than those of WT seedlings when measured at 10 d after plating (Fig. 6d,e). Additionally, *CaDeSI2*-OX plants exhibited enhanced ABA sensitivity during postgerminative stage (Fig. 6f,g): when 3-d-old seedlings of *CaDeSI2*-OX and WT plants were grown on ½MS media supplemented with ABA for 7 d, the root growth of *CaDeSI2*-OX seedlings were notably suppressed compared to that of WT seedlings.

Having characterized the phenotypes of *CaDeSI2*-OX seedlings, we subsequently examined the drought sensitivity of *CaDeSI2*-OX plants during the adult stage (Fig. 7). The *CaDeSI2*-OX and WT plants were treated with drought by withholding watering for 15 d. In contrast to *CaDeSI2*-silenced pepper plants, *CaDeSI2*-OX plants exposed to drought were found to be less severely wilted than the similarly treated WT plants. Moreover, at 2 d after re-watering, we recorded a markedly higher survival among *CaDeSI2*-OX plants (80–98%) compared with that of the WT plants (26.7%). Based on these findings, we speculated as to whether CaDeSI2 enhances drought resistance in *CaDeSI2*-OX *Arabidopsis* by promoting ABA-induced stomatal closure. To verify this conjecture, we measured leaf surface temperatures before and after ABA treatment (Fig. 7b). Although before ABA treatment, we detected no significant difference between *CaDeSI2*-OX and WT, at 5 h posttreatment, leaf temperatures were higher in *CaDeSI2*-OX plants than those recorded for WT plants (Fig. 7c). Consistently, whereas ABA was found to induce stomatal closure in both plant lines, the stomatal apertures were smaller in *CaDeSI2*-OX plants than in WT plants (Fig. 7d,

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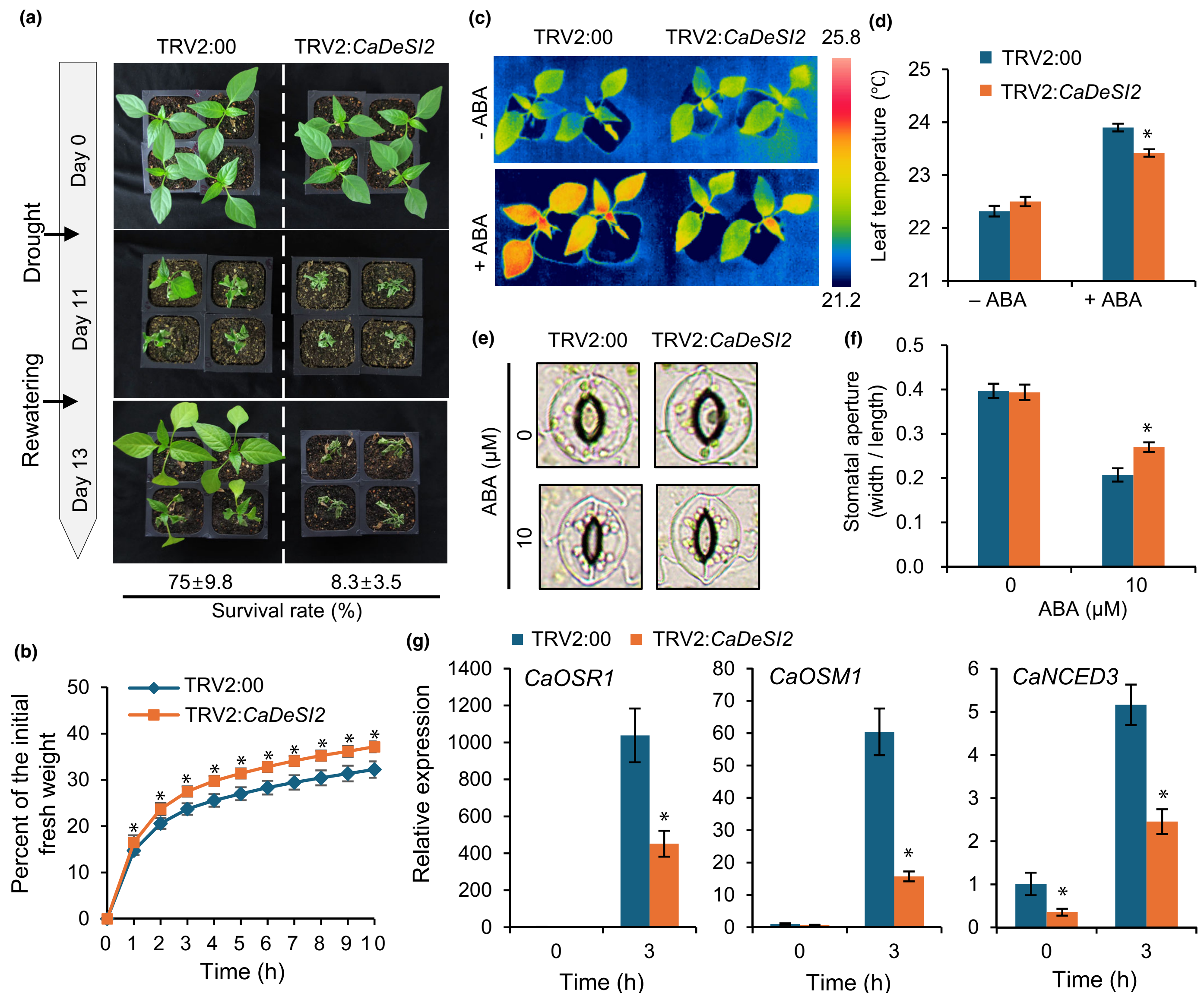

Fig. 5 Enhanced susceptibility of *CaDeSI2*-silenced pepper plants to drought stress. (a) Drought sensitivity of TRV2:*CaDeSI2* and TRV2:00 pepper plants. At 2 wk after agroinfiltration, each plant line was subjected to drought stress by withholding watering for 11 d. Survival of *TRV2:CaDeSI2* and *TRV2:00* pepper plants after 2 d of re-watering. (b) Water loss from the leaves of TRV2:*CaDeSI2* and TRV2:00 pepper plants. The leaf fresh weights of each line were measured at the indicated time points after leaf detachment. (c, d) Temperatures of the leaves of TRV2:*CaDeSI2* and TRV2:00 pepper plants after exposure to ABA. Representative thermographic images of pepper plants before and 5 h after treatment with 50 μM abscisic acid (ABA) (c). Mean leaf temperatures were calculated from 10 plants of each line (d). (e, f) Stomatal apertures in the leaves of TRV2:*CaDeSI2* and TRV2:00 pepper plants treated with ABA. Leaf peels were harvested from each plant line at 2 wk after agroinfiltration and incubated for 2 h in stomatal opening solution (SOS) buffer containing 0 or 10 μM ABA. Representative images were taken (e), and the stomatal apertures were measured under a microscope (f). (g) Quantitative real-time polymerase chain reaction analysis of ABA and/or drought stress-responsive gene expression in dehydrated leaves of TRV2:*CaDeSI2* and TRV2:00 pepper plants. At 2 wk after agroinfiltration, each plant line was subjected to drought stress by removal of their roots. The relative expression of each gene was calculated based on normalization relative to the expression of *CaACT1* used as an internal control gene. Data are presented as the means ± SE of three independent experiments. Asterisks indicate significant differences between *CaDeSI2*-silenced pepper and control pepper plants (Student's *t*-test; $P < 0.05$).

e). These findings would thus tend to indicate that CaDeSI2 plays an important role in ABA-mediated drought tolerance.

Functional relationship between CaAITP1 and CaDeSI2

To elucidate the genetic interaction between CaAITP1 and CaDeSI2 in response to drought stress, we co-silenced these two genes in pepper plants (TRV2:*CaDeSI2*/TRV2:*CaAITP1*), as confirmed by performing semi-quantitative real-time polymerase chain reaction analysis (Fig. S3C). To assess the effects of the drought stress response of gene-silenced pepper plants, at 2 wk after agroinfiltration, TRV2:00, TRV2:*CaDeSI2*, TRV2:*CaAITP1*, and TRV2:*CaDeSI2/CaAITP1* plants, were exposed to drought conditions by withholding watering for 10 d (Fig. 8a, upper and middle rows). Subsequent observations of the treated plants 2 d after re-watering, revealed that TRV2:*CaDeSI2*/

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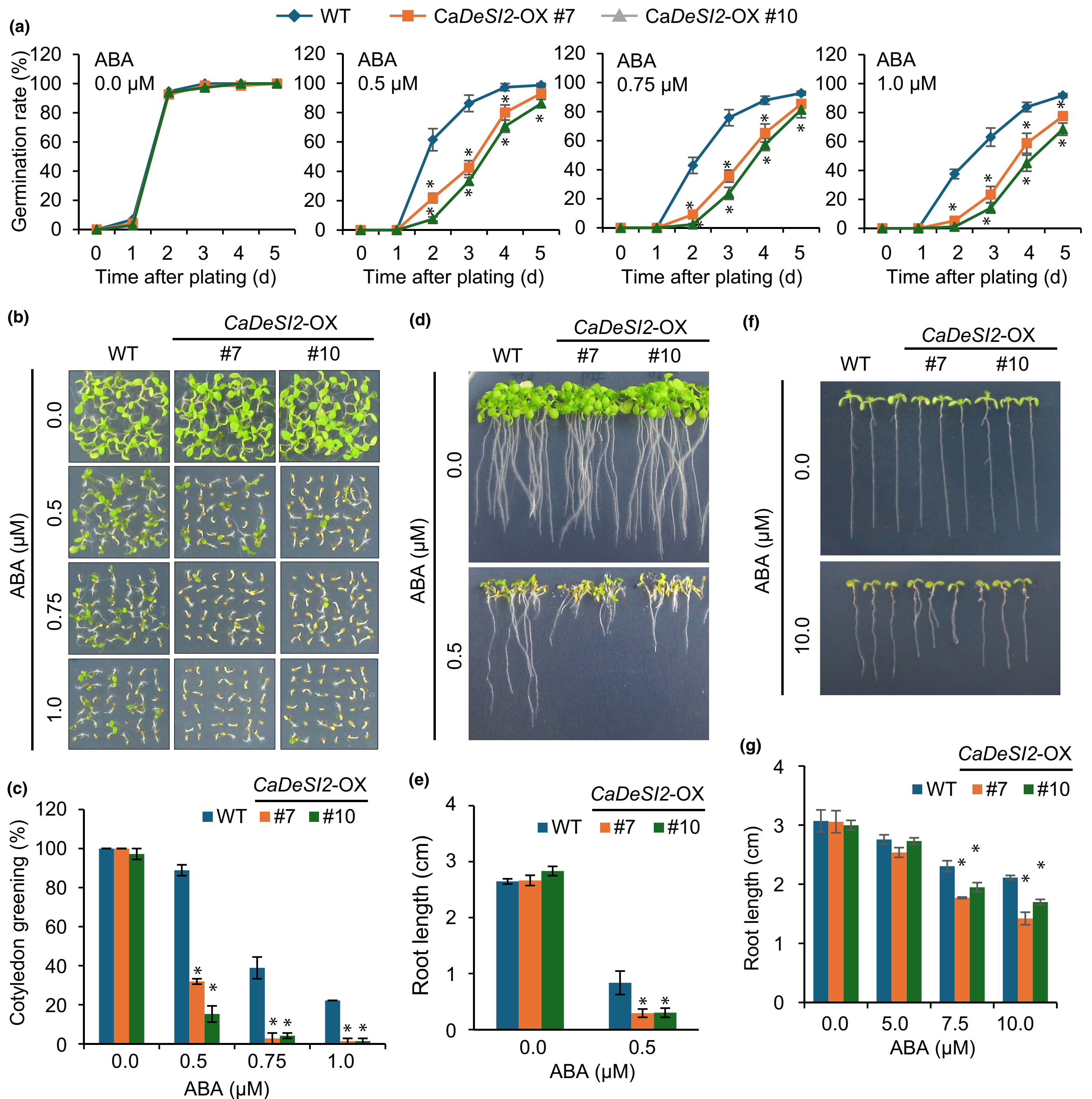

Fig. 6 Enhanced abscisic acid (ABA) sensitivity of *CaDeSI2*-overexpressing (OX) transgenic *Arabidopsis* plants during germination and seedling growth. (a) Seed germination of wild-type (WT) and *CaDeSI2*-OX plants in response to ABA. Seeds were germinated on ½-strength Murashige & Skoog (½MS) agar plates containing different concentrations of ABA. (b, c) Growth of WT and *CaDeSI2*-OX seedlings on ½MS agar plates containing 0, 0.5, 0.75, or 1 μM ABA. Representative photographs were taken 9 d after plating. (d, e) Root elongation of WT and *CaDeSI2*-OX plants in response to ABA. The root length of each plant was measured 10 d after plating. Data are presented as the means ± SE of three independent experiments. Asterisks indicate significant differences between WT and *CaDeSI2*-OX plants (Student's *t*-test; $P < 0.05$).

TRV2:*CaAITP1* plants were characterized by a higher survival than TRV2:00 plants, the level of which was almost as high as that of the surviving TRV2:*CaAITP1* plants (Fig. 8a). Consistently, an assessment of transpirational water loss from detached leaves revealed similar patterns, with the leaves of both TRV2:CaAITP1 and TRV2:*CaDeSI2*/TRV2:*CaAITP1* pepper plants being characterized by notably reduced transpirational water loss relative to that of TRV2:00 plants (Fig. 8b). Co-silencing of *CaDeSI2* and *CaAITP1* was also found to influence drought resistance via the regulation of ABA-induced stomatal closure. In

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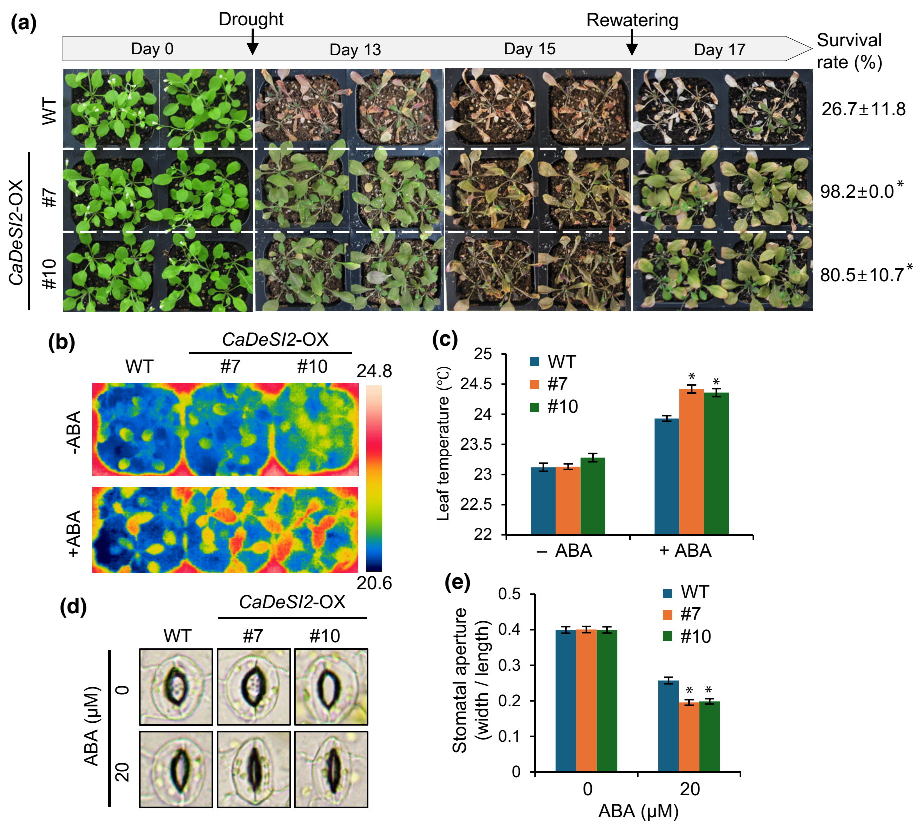

Fig. 7 Enhanced drought stress tolerance of *CaDeSI2*-overexpressing (OX) transgenic *Arabidopsis* plants. (a) Drought tolerance of *CaDeSI2*-OX plants. Four-week-old wild-type (WT) and *CaDeSI2*-OX plants were subjected to drought stress by withholding watering for 15 d followed by re-watering for 2 d, after which plant survival was measured. (b, c) Changes in leaf surface temperature of WT and *CaDeSI2*-OX plants after exposure to ABA. Representative thermographic images were taken before and after treatment with 50 μM ABA (b), and leaf temperature was measured 5 h after treatment. The average leaf temperatures was calculated from 10 plants of each line (c). (d, e) Stomatal apertures in the leaves of WT and *CaDeSI2*-OX 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 or 20 μM ABA. Representative photographs were taken under a microscope (d) and the stomatal apertures were measured (e). Data are presented as the means ± SE of three independent experiments. Asterisks indicate significant differences between WT and *CaDeSI2*-OX plants (Student's *t*-test; $P < 0.05$).

response to treatment with ABA, we detected an elevation of temperature in leaves of the co-silenced TRV2:*CaDeSI2*/TRV2:*CaAITP1* plants (Fig. 8c,d) and reduced stomatal apertures (Fig. 8e,f) compared with TRV2:00 plants. Based on these findings, we thus established that TRV2:*CaDeSI2*/TRV2:*CaAITP1* plants are characterized by ABA-hypersensitive and drought-tolerant phenotypes, which contrasts with the phenotypes of TRV2:*CaDeSI2* plants, although are identical to those observed in TRV2:*CaAITP1* plants (Fig. 8), thereby indicating that CaDeSI2 functions upstream of CaAITP1.

Discussion

In this study, we characterized the SUMOylation/deSUMOylation-mediated modulation of CaAITP1, a pepper group A PP2C protein that negatively regulates ABA signaling and drought tolerance (Baek *et al.*, 2021). In this process, CaDeSI2 was shown to play a pivotal role in the deSUMOylation of CaAITP1, the stability of which was partially reduced under drought stress conditions. Concomitant with the functional interaction with CaAITP1, CaDeSI2 positively modulates ABA-mediated drought resistance. Collectively, our findings thus provide evidence to indicate that CaDeSI2-mediated deSUMOylation modulates ABA signaling and drought stress.

Posttranslational modification plays a pivotal role in the functioning of a range of plant hormone signaling pathways, with the findings of numerous studies indicating that the activity and stability of ABA receptors and coreceptors, such as PYR/PYL/RCAR and PP2Cs, are regulated posttranslationally via phosphorylation and ubiquitination (Bueso *et al.*, 2014; Belda-Palazon *et al.*, 2016; Wu *et al.*, 2016; Yu *et al.*, 2016; Lim *et al.*, 2017; Chen *et al.*, 2018; P. Wang *et al.*, 2018; Zhang *et al.*, 2018, 2019). In contrast to the well-established roles of phosphorylation and ubiquitination in plant hormone signaling pathways, SUMOylation has only relatively recently become a focus of study in this context. Among the important findings in this regard, the potential role of SUMOylation in modulating ABA signaling, along with ubiquitination, has been demonstrated (Lois *et al.*, 2003), with SUMOylation being established to play a dual role, negatively contributing to ABA-mediated growth inhibition, whilst being positively associated with the induction of ABA-responsive genes. Notably, ABA-insensitive 5 (ABI5) has been identified as one of the proteins targeted for SUMOylation, mediated by SUMO E3 ligase AtSIZ1, which results in enhanced protein stability, albeit with reduced activity, thereby negatively modulating ABA signaling (Miura *et al.*, 2009; Yu *et al.*, 2015). Similarly, in pepper plants, the stability of the HD-Zip transcription factor CaDRHB1, which functions as a positive modulator of ABA and drought responses, is enhanced by SUMO E3 ligase CaDSIZ1-mediated SUMOylation (Joo *et al.*, 2022). In the present study, CaAITP1, identified as one of the PP2Cs contributing to ABA signaling in pepper plants (Baek *et al.*, 2021), was found to be SUMOylated (Fig. 1), thereby providing evidence that SUMOylation-mediated posttranslational modification may serve as a potential mechanism underlying the regulation of ABA signaling components. However, the specific SUMO E3 ligases

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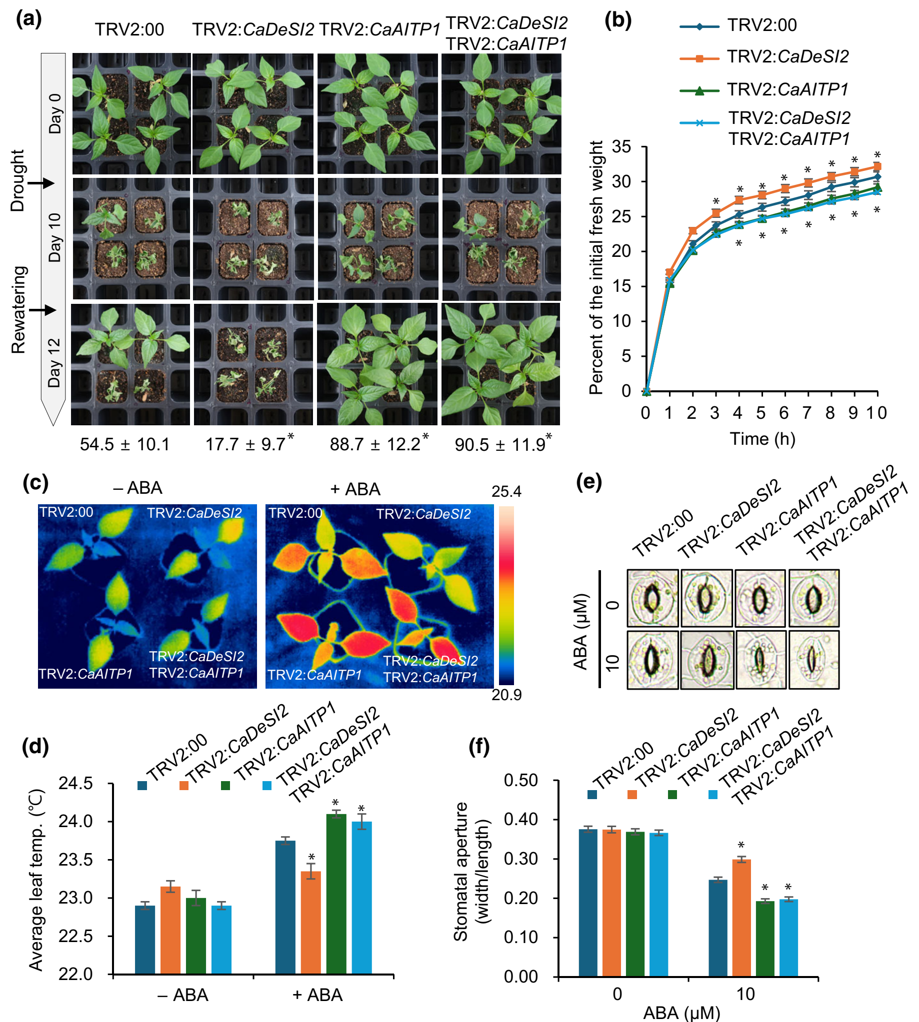

Fig. 8 Enhanced drought tolerance of *CaDeSI2/CaAITP1*-silenced pepper plants. (a) Drought sensitivity of TRV2:00, TRV2:*CaDeSI2*, TRV2:*CaAITP1*, and TRV2:*CaDeSI2*/TRV2:*CaAITP1* pepper plants. At 2 wk after agroinfiltration, each plant line was subjected to drought stress by withholding watering for 10 d followed by re-watering for 1 d. Asterisks indicate significant differences between silenced and control pepper plants (Student's *t*-test; $P < 0.05$). (b) Water loss from the leaves of TRV2:00, TRV2:*CaDeSI2*, TRV2:*CaAITP1*, and TRV2:*CaDeSI2*/TRV2:*CaAITP1* pepper plants. The leaf fresh weights of each line were measured at the indicated time points after leaf detachment. (c, d) Leaf temperatures of TRV2:00, TRV2:*CaDeSI2*, TRV2:*CaAITP1*, and TRV2:*CaDeSI2*/TRV2:*CaAITP1* pepper plants after exposure to ABA. Representative thermographic images of pepper plants before and 5 h after treatment with 50 μM abscisic acid (ABA) (c); mean leaf temperatures were calculated from 10 plants of each line (d). (e, f) Stomatal apertures in the leaves of TRV2:00, TRV2:*CaDeSI2*, TRV2:*CaAITP1*, and TRV2:*CaDeSI2*/TRV2:*CaAITP1* pepper plants treated with ABA. Leaf peels were harvested from each plant line at 2 wk after agroinfiltration and incubated for 2 h in stomatal opening solution (SOS) buffer containing 0 or 10 μM ABA. Representative images were taken (e), and the stomatal apertures were measured under a microscope (f). Data are presented as the means ± SE of three independent experiments. Asterisks indicate significant differences between silenced and control pepper plants (Student's *t*-test; $P < 0.05$).

responsible for this modification remain elusive, and identifying crucial SUMOylation sites within CaAITP1 proved challenging, hampering our ability to determine whether CaDeSI2 interacts with SUMOylated or unSUMOylated forms of CaAITP1 protein in this study. Additionally, the unavailability of specific inhibitors targeting plant SUMOylation pathways limited our experimental approaches. The precise impact of CaAITP1 SUMOylation on drought resistance remains insufficiently clarified. Addressing these gaps will enhance our understanding of the intricate regulatory network governing ABA signaling in plants and its implications for stress responses.

Among the interactions assessed in this study, we established that exposure to drought stress resulted in reductions in both the SUMOylation and stability of the CaAITP1 protein (Fig. 1). Moreover, as a notable discovery, the pepper SUMO protease CaDeSI2 was identified as a potential regulator in this process, as revealed by our findings indicating that this protease interacts directly with, and thereby deSUMOylates, CaAITP1 (Figs 2, 4). Given that CaAITP1 functions as a core component of ABA signaling, these findings provide evidence to indicate the functional role of CaDeSI2 in both drought responses and ABA signaling.

Intriguingly, the expression patterns of the *CaDeSI2* gene exhibited differences in response to dehydration/PEG exposure and ABA treatment. As shown in Fig. 3(a), *CaDeSI2* transcript levels decrease during early dehydration/PEG exposure, followed by an eventual recovery to levels comparable to those before treatment. Considering the known induction of ABA biosynthesis by dehydration/PEG exposure and the significant increase in *CaDeSI2* gene expression upon ABA treatment, it is plausible to infer that the decrease of *CaDeSI2* gene expression may be mediated via an ABA-independent pathway, while the subsequent increase of *CaDeSI2* gene expression may be mediated via an ABA-dependent pathway. Nevertheless, it is important to note that while gene expression levels provide insight into potential regulatory mechanisms, they may not directly correlate with the enzymatic activity of CaDeSI2. Our phenotypic analyses using *CaDeSI2*-silenced pepper and *CaDeSI2*-overexpressing *Arabidopsis* plants revealed that CaDeSI2 positively modulates ABA-mediated drought tolerance (Figs 5–7), and based on additional insights gained from double-VIGS treatment targeting both CaDeSI2 and CaAITP1, we established that CaDeSI2 acts upstream of CaAITP1 (Fig. 8). Collectively, these findings

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indicate that CaDeSI2 acts as a positive regulator of drought tolerance by modifying the stability of the CaAITP1 protein. However, despite this important finding, even though CaDeSI2 may play a significant role in modulating CaAITP1 stability, it is conceivable that this protease is not the sole factor in this regard, as indicated by our observation that the silencing of *CaDeSI2* did not completely inhibit degradation of the CaAITP1 protein (Fig. 4c). In addition, although CaDeSI2 was found to reduce the SUMOylation of CaAITP1, it remains unclear as to whether CaDeSI2 is the sole factor responsible for the deSUMOylation of CaAITP1 under drought stress conditions. Given these, we tentatively speculate there may be as yet undetermined factors contributing to the modulation of CaAITP1 protein stability in response to drought stress.

In our previous study, we established that CaAITP1 interacts with the RING-type E3 ligase CaAIRE1 and that its stability is reduced by CaAIRE1-mediated ubiquitination (Baek *et al*., 2021). Similarly, our findings in the present study have provided evidence to indicate that the CaDeSI2-mediated deSUMOylation of CaAITP1 contributes to reducing its stability (Fig. 4). Given these findings, we speculate that compared with ubiquitination and deSUMOylation, SUMOylation may antagonistically enhance CaAITP1 protein stability. In this regard, it has been established that SUMOylation and ubiquitination can interact either competitively or cooperatively to regulate the function and stability of proteins (Ulrich, 2005; Praefcke *et al*., 2012). For example, in humans, phosphorylation of Flap Endonuclease 1 (FEN1) has been demonstrated to promote SUMOylation, leading to subsequent ubiquitin 26S proteasomal degradation (Guo *et al*., 2012), whereas SUMOylation of IκBα enhances protein stability. Notably, in this regard, it has been reported that the SUMOylation and ubiquitination of IκBα are both mediated via binding to the same lysine residue, thereby leading to the concept that SUMO and ubiquitin function as biological antagonists (Desterro *et al*., 1998). In plants, the Inducer of CBF Expression 1 (ICE1) and MYB30 undergo modification by both SUMOylation and ubiquitination (Miura *et al*., 2007; Zheng *et al*., 2018). SUMOylation of ICE1 reduces its polyubiquitination, thereby increasing ICE1 stability (Miura *et al*., 2007), and similarly, MYB30, a transcription factor involved in the ABA response, is antagonistically SUMOylated and ubiquitinated at the same residue to regulate its stability (Zheng *et al*., 2018). However, the precise mechanisms underlying the cooperative or competitive functions of SUMOylation and ubiquitination in the modulation of CaAITP1 protein stability remain unclear, and accordingly, further studies are needed to gain a more complete understanding of the regulatory network that governs CaAITP1 stability.

In this study, we elucidated the modulation of a core ABA signaling component via reversible SUMOylation–deSUMOylation processes. Our findings reveal that drought stress induces a reduction in the SUMOylation of CaAITP1 and its protein stability. In addition, under conditions in which CaDeSI2 is silenced or the SUMO protease is inactivated, there are increases in CaAITP1 SUMOylation and protein stability. However, despite these persuasive findings, it is important to acknowledge that the evidence accumulated in this study regarding the CaDeSI2-mediated deSUMOylation of CaAITP1 and its subsequent destabilization is essentially indirect. At present, the intricate mechanisms whereby CaDeSI2 modulates the CaAITP1 protein in plant cells during drought stress remain incompletely understood, and accordingly necessitate further comprehensive evaluation. In particular, the identification of CaDeSI2 target proteins would contribute immensely to our understanding of the role of CaDeSI2 in ABA signaling and drought responses. Such studies would provide confirmatory evidence highlighting the significance of SUMOylation as a posttranslational modification process with notable implications for ABA signaling and drought responses.

Acknowledgements

This work was supported by a grant from a National Research Foundation of Korea (NRF) grant funded by the Korean Government (MSIT) (nos. RS-2024-00343006 and 2018R1A5A1 023599, SRC), Republic of Korea.

Competing interests

None declared.

Author contributions

HJ, WB and CWL performed the experiments and analyzed the data; CWL and SCL designed the experiments and wrote the manuscript. HJ, WB and CWL contributed equally to this work.

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Data availability

The data that support the findings of this study are available in the Supporting Information of this article.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Amino acid sequence analysis of the pepper CaDeSI2 protein.

Fig. S2 Expression of the *CaDeSI2* and *CaAITP1* genes.

Table S1 Sequences of the primers used in this study.

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