

The Pepper WPP Domain Protein, CaWDP1, Acts as a Novel Negative Regulator of Drought Stress via ABA Signaling

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Plants are constantly challenged by various environmental stresses, including high salinity and drought, and they have evolved defense mechanisms to counteract the deleterious effects of these stresses. The plant hormone ABA regulates plant growth and developmental processes and mediates abiotic stress responses. Here, we report the identification and characterization of a novel CaWDP1 (*Capsicum annuum*) protein. The expression of CaWDP1 in pepper leaves was induced by ABA, drought and NaCl treatments, suggesting its role in the abiotic stress response. CaWDP1 proteins show conserved sequence homology with other known WDP1 proteins, and they are localized in the nucleus and cytoplasm. We generated CaWDP1-silenced peppers via virus-induced gene silencing (VIGS). We evaluated the responses of these CaWDP1-silenced pepper plants and CaWDP1-overexpressing (OX) transgenic *Arabidopsis* plants to ABA and drought. CaWDP1-silenced pepper plants displayed enhanced tolerance to drought stress, and this was characterized by low levels of leaf water loss in the drought-treated leaves. In contrast to CaWDP1-silenced plants, CaWDP1-OX plants exhibited an ABA-hyposensitive and drought-susceptible phenotype, which was accompanied by high levels of leaf water loss, low leaf temperatures, increased stomatal pore size and low expression levels of stress-responsive genes. Our results indicate that CaWDP1, a novel pepper negative regulator of ABA, regulates the ABA-mediated defense response to drought stress.

Keywords: Abscisic acid • CaWDP1 • Drought stress • Pepper • Virus-induced gene silencing.

Abbreviations: CaMV, *Cauliflower mosaic virus*; DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; MS, Murashige and Skoog; NE, nuclear envelope; OX, overexpressing; PP2C, protein phosphatase 2C; qRT-PCR, quantitative reverse transcription-PCR; RT-PCR, reverse transcription-PCR; SOB, stomatal opening buffer; TRV, *Tobacco rattle virus*; VIGS, virus-induced gene silencing; WDP1, WPP domain protein 1.

Introduction

Environmental stresses such as water deficit and high salinity affect plant growth and development, and have devastating effects on crop production. Drought stress is a common result of exposure to water deficit conditions. Prevention of osmotic stress is dependent on minimizing stomatal water loss from the leaves and maximizing water uptake from the roots (Coca et al. 2000, Apse and Blumwald 2002, Yamaguchi-Shinozaki and Shinozaki 2006). However, the drought stress response in plants is a complex phenomenon, and the precise functional modifications induced by drought stress are poorly understood. To cope with drought stress, plants have evolved adaptive defense mechanisms that result in the expression of stress-responsive genes, thereby leading to plant morphological and physiological alterations.

The phytohormone ABA plays a key role in the defense mechanism leading to adaptation to drought stress, via the induction of several molecular and physiological modifications. Drought stress commonly results in the induction and accumulation of cellular ABA, thereby leading to adaptive stress responses, including stomatal closure and expression of stress-responsive genes (Schroeder et al. 2001, Lee and Luan 2012, Lee et al. 2013). In comparison with other plant hormones, ABA plays an important role in the expression reprogramming of a large number of genes; >10% of *Arabidopsis* genes respond to ABA (Goda et al. 2008, Mizuno and Yamashino 2008). The expression of ABA-responsive genes plays a key role in conferring enhanced drought stress tolerance and protecting plants from drought stress conditions. Characterization of ABA-sensitive or ABA-insensitive mutants—in which ABA-responsive genes are overexpressed or knocked out—has resulted in the identification of various components of ABA signaling in *Arabidopsis* (Ma et al. 2009; Umezawa et al. 2009, Vlad et al. 2009, Lee et al. 2013, Umezawa et al. 2013).

The WPP domain possesses a highly conserved tryptophan–proline–proline motif and is found at the N-terminus of plant Ran GTPase-activating domain proteins (RanGAPs) which establish a functional RanGTP/RanGDP gradient across the

nuclear envelope (NE) (Rose and Meier 2001). The plant-specific WPP domain is required for NE targeting of RanGAP and shares sequence similarity with a small plant protein family, named WPP domain proteins. Tomato LeMAF1 [MFP1 (matrix-associated filament like protein 1)-attachment factor 1] consisting of 16.2 kDa, is the first WPP domain protein identified through a yeast two-hybrid screening with MFP1, a DNA-binding long coiled-coil protein localized in the nucleus and chloroplast (Gindullis et al. 1999). A family of WPP domain proteins is plant specific and its biological function is poorly understood (Meier 2000). LeMAF1 and the Arabidopsis homologs WPP1 and WPP2 proteins showed localization to the NE and cytoplasmic speckles (Gindullis et al. 1999, Patel et al. 2004), suggesting a plant-specific role for WPP domain proteins at the NE. This localization pattern is developmentally regulated; WPP1 and WPP2 proteins target to the nucleus and cytoplasm in cells, such as hypocotyl cells and leaf epidermal cells, which are differentiated and have exited the cell cycles (Patel et al. 2004). The association between the functions of WDP1 (WPP Domain Protein 1) and abiotic stress signaling, including ABA, drought and high salinity, remains to be elucidated. Here, we identified *CaWDP1*, which encodes a pepper MAF protein, as a novel negative regulator of drought stress in *Capsicum annuum*. We showed that the expression of *CaWDP1* was induced by ABA, drought and high salinity. We used knock-down and over-expression of *CaWDP1* in pepper and Arabidopsis, respectively, to elucidate the roles of *CaWDP1* in the defense mechanisms against abiotic stress. We demonstrated that *CaWDP1*-silenced pepper plants exhibited a drought-tolerant phenotype, which was characterized by high leaf temperatures, a low leaf water loss rate and small-sized stomatal apertures. In contrast, *CaWDP1*-overexpressing (OX) plants displayed an ABA-insensitive phenotype during the germinative and post-germinative stages. In addition, the *CaWDP1*-OX plants exhibited a drought-sensitive phenotype, via decreased ABA-induced stomatal closure and low expression levels of stress-responsive marker genes. Our findings indicate that *CaWDP1* negatively regulates drought stress tolerance via ABA signaling.

Results

Isolation and sequence analysis of the *CaWDP1* gene

We subjected total RNA isolated from ABA-treated or non-treated pepper leaves to differential hybridization analysis (Lim et al. 2014). We found that the expression of *CaWDP1* was more strongly induced in the ABA-treated leaves than in the non-treated leaves (data not shown). The putative *CaWDP1* contains a 429 bp open reading frame, and the predicted *CaWDP1* cDNA encodes 142 amino acid residues with a predicted molecular mass of 15.324 kDa and an isoelectric point (pI) of 4.41 (Fig. 1A). The results of encoded amino acid sequence alignments and phylogenetic tree analyses showed that *CaWDP1* homologous proteins are widely conserved in flowering plants (Fig. 1A, B). In particular, *CaWDP1* is clustered in the same clade as WPP domain proteins from the family *Solanaceae*

and the *CaWDP1* protein has a high level of similarity (61.1–80.9%) to them. The results of alignment analysis also revealed that these proteins possess the highly conserved WPP domain in common.

Expression of the *CaWDP1* gene by ABA, drought and high salinity treatment

To analyze organ-specific expression of the *CaWDP1* gene, we measured accumulation of *CaWDP1* transcripts in pepper root, stem, leaf and flower. Quantitative reverse transcription-PCR (qRT-PCR) analysis showed that *CaWDP1* gene was expressed in all examined organs and, in particular, its level was the highest in stem (Fig. 2A). The *CaWDP1* gene was isolated from ABA-treated leaves, and we thus investigated the involvement of *CaWDP1* in abiotic stress signaling. To determine the abiotic stress factors associated with *CaWDP1* expression, we used qRT-PCR analysis to measure the expression levels of *CaWDP1* transcripts in pepper leaves at different time points after treatment with ABA, drought or NaCl (Fig. 2). Consistent with the results of differential hybridization screening, we found that *CaWDP1* transcription gradually increased after 6–24 h of ABA treatment (Fig. 2B). We further showed that the steady-state levels of *CaWDP1* transcripts were slightly up-regulated by drought (Fig. 2C), and transcription of this gene was induced after 24 h, but not after 6 h of NaCl treatment (Fig. 2D).

Subcellular localization of *CaWDP1*

To confirm the subcellular localization of the *CaWDP1* protein in the cells, we fused the *CaWDP1* gene with the green fluorescent protein (GFP) gene under the 35S promoter, to produce *Pro35S:CaWDP1-GFP*. We found that expression of the *CaWDP1*-GFP fusion protein in epidermal cells of *Nicotiana benthamiana* generated GFP signals in the nucleus and cytoplasm (Fig. 3). Using 4',6-diamidino-2-phenylindole (DAPI) staining, we observed that blue signals localized to the nucleus overlapped with the GFP signals. Our results indicate that the *CaWDP1* protein is targeted to the nucleus and cytoplasm and is presumably functional in each of these sites.

Enhanced tolerance of *CaWDP1*-silenced pepper plants to drought stress

The expression level of *CaWDP1* was induced by drought stress (Fig. 2), implying that *CaWDP1* functions in the stress-induced signaling response. We thus examined the role of *CaWDP1* in the drought stress response, by using virus-induced gene silencing (VIGS)-based loss-of-function analysis (Lim and Lee 2014). The expression level of the *CaWDP1* gene is approximately 2.2- to 2.6-fold less in *CaWDP1*-silenced pepper plants [*Tobacco rattle virus* (TRV):*CaWDP1*] than in control plants (TRV:00) treated or not with ABA (Supplementary Fig. S1A). First, we examined the drought phenotype by using TRV:*CaWDP1* and TRV:00 plants (Fig. 4A). Under well-watered conditions, we observed no phenotypic differences between these plants (Fig. 4A, upper panel). In contrast, when we subjected vector control and *CaWDP1*-silenced pepper plants to drought stress by withholding water for 13 d, both types of plant exhibited wilted

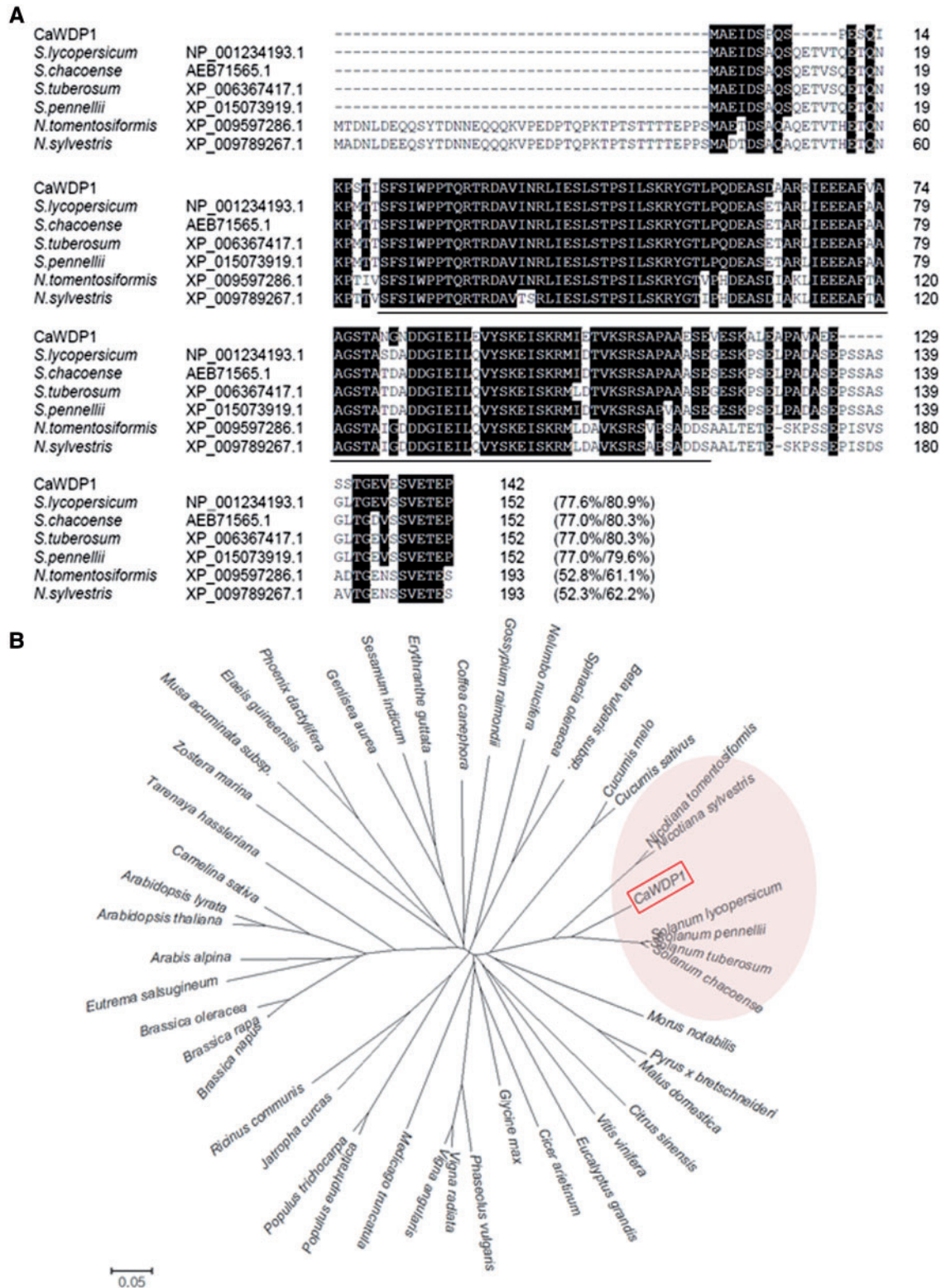


Fig. 1 Amino acid sequence analysis of the pepper CaWDP1 protein. (A) Comparisons of the deduced amino acid sequence of the CaWDP1 protein (accession No. KP644223) with its homologous proteins from members of the *Solanaceae* family including tomato, potato and tobacco. WPP domain sequences are underlined. White letters on black indicate identical amino acid residues, and 'gaps introduced to maximize the alignment of homologous regions are marked by dashes. The numbers in parentheses indicate sequence similarity and identity scores calculated by the pairwise sequence alignment tool EMBOSS Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) at the default settings. (B) Phylogenetic tree analysis of the CaWDP1 protein. After a BLAST search with CaWDP1 as query, protein sequences of the first hit for each species were retrieved and used for phylogenetic tree analysis. Multiple alignment of those amino acid sequences was performed by using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) and the phylogenetic tree was drawn with MEGA software (version 5.2).

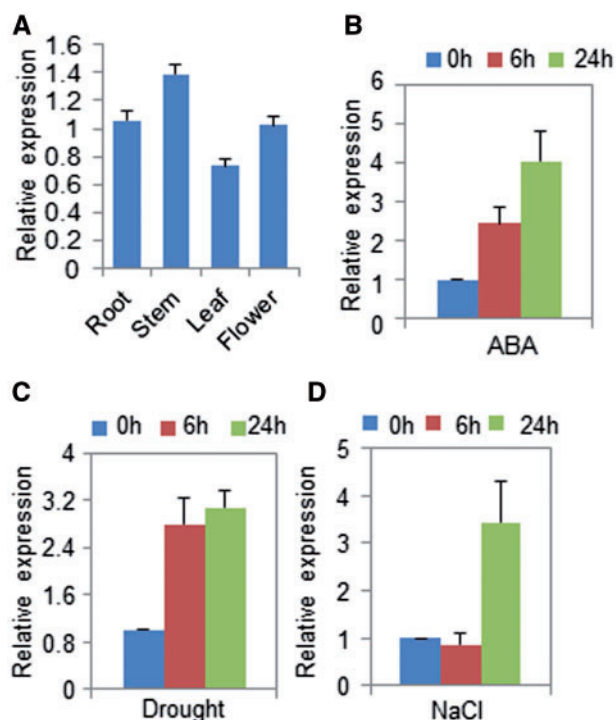


Fig. 2 Expression pattern of the *CaWDP1* gene. (A) Organ-specific expression of the *CaWDP1* gene. (B–D) qRT–PCR analysis of *CaWDP1* gene expression in pepper leaves at 0, 6 and 24 h after treatment with 100 μ M ABA (B), drought (C) or 200 mM NaCl (D). Relative expression levels ($\Delta\Delta$ CT) of each gene were normalized to the geometric mean of *CaACT1* and *CaEF1 α* as internal control genes. Data represent the mean $\pm$ SD of three independent experiments, and different letters indicate significant difference (ANOVA; $P < 0.05$).

phenotypes (Fig. 4A, middle panel). However, most of the *CaWDP1*-silenced pepper plants resumed growth after 3 d of re-watering (Fig. 4A, lower panel). We monitored the survival rate of each plant after 3 d of re-watering. Under these conditions, we found that only 13% of the control plants resumed growth, whereas 63% of the *CaWDP1*-silenced pepper plants were able to resume growth (Fig. 4B). Hence, suppression of *CaWDP1* expression confers enhanced tolerance to drought stress. To determine the correlation between the drought-tolerant phenotype and water retention, we examined the leaf water loss rate by measuring the fresh weights of detached rosette leaves in vector control plants and *CaWDP1*-silenced pepper plants (Fig. 4C). We found that the fresh weight loss of leaves due to leaf water loss was considerably lower in *CaWDP1*-silenced pepper leaves than in control plants, indicating that enhanced drought tolerance is derived from altered rates of leaf water loss. To examine further the drought-tolerant phenotype, we measured the leaf temperatures in control and *CaWDP1*-silenced pepper plants (Fig. 4D). We found that after ABA treatment, *CaWDP1*-silenced pepper plants exhibited significantly higher leaf temperatures than control plants, because of lower leaf water loss rates. The leaf water loss is regulated by stomatal movements; moreover, high sensitivity to ABA leads to decreased stomatal pore size (Lim and Lee 2014, Lim et al. 2015b). To determine whether the low leaf water loss rate in *CaWDP1*-

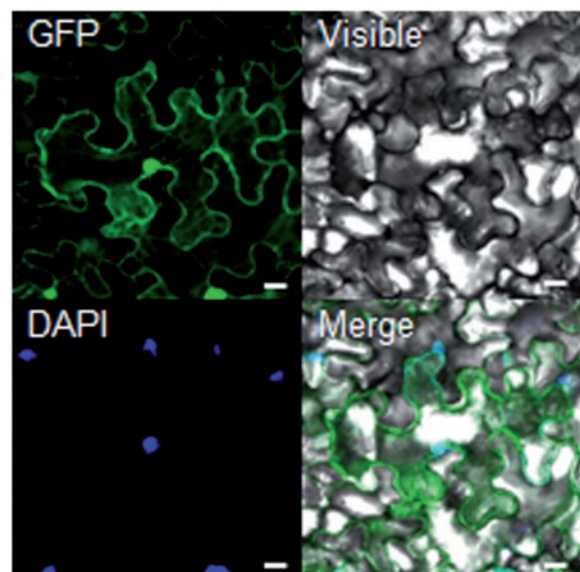


Fig. 3 Subcellular localization of the 35S:CaWDP1–GFP fusion protein in *Nicotiana benthamiana* epidermal cells. The 35S:CaWDP1–GFP construct was expressed by agroinfiltration of *N. benthamiana* leaves and was observed under a confocal laser-scanning microscope. DAPI staining was used as a marker for the nucleus. The scale bar represents 20 μ m.

silenced pepper plants was caused by a decrease in stomatal aperture, we measured stomatal movements after treatment with various concentrations of ABA, which plays a critical role in stomatal closure. In the absence of ABA, we determined that there were no significant differences in stomatal movements between the *CaWDP1*-silenced pepper and control plants. In both types of plant, ABA treatment triggered a reduction in the stomatal pore size; however, *CaWDP1*-silenced pepper plants showed smaller stomatal pores than control plants, indicating that the low leaf water loss rate in *CaWDP1*-silenced pepper plants was derived from enhanced ABA-induced stomatal closure. Previously, we elucidated that the pepper *CaAMP1* and the *AtRD29B* homolog *CaOSR1* were induced by ABA and drought, and these genes play a role as positive regulators of drought tolerance (Lee and Hwang 2009, Park et al. 2016); hence, we measured the expression levels of these genes after drought treatment (Fig. 4F). The drought treatment induced both *CaAMP1* and *CaOSR1* expression; however, the expression levels were higher in *CaWDP1*-silenced pepper than in control plants. Moreover, we measured endogenous ABA contents after drought treatment and re-watering (Fig. 4G). We determined no significant difference in ABA content between control and *CaWDP1*-silenced pepper plants. Our data suggest that the enhanced drought tolerance of *CaWDP1*-silenced pepper plants is caused by ABA hypersensitivity.

Alteration of ABA sensitivity during the germinative and seedling stages of *CaWDP1*-OX plants

To investigate the physiological role of *CaWDP1*, we generated overexpressed *CaWDP1* Arabidopsis plants under the control of

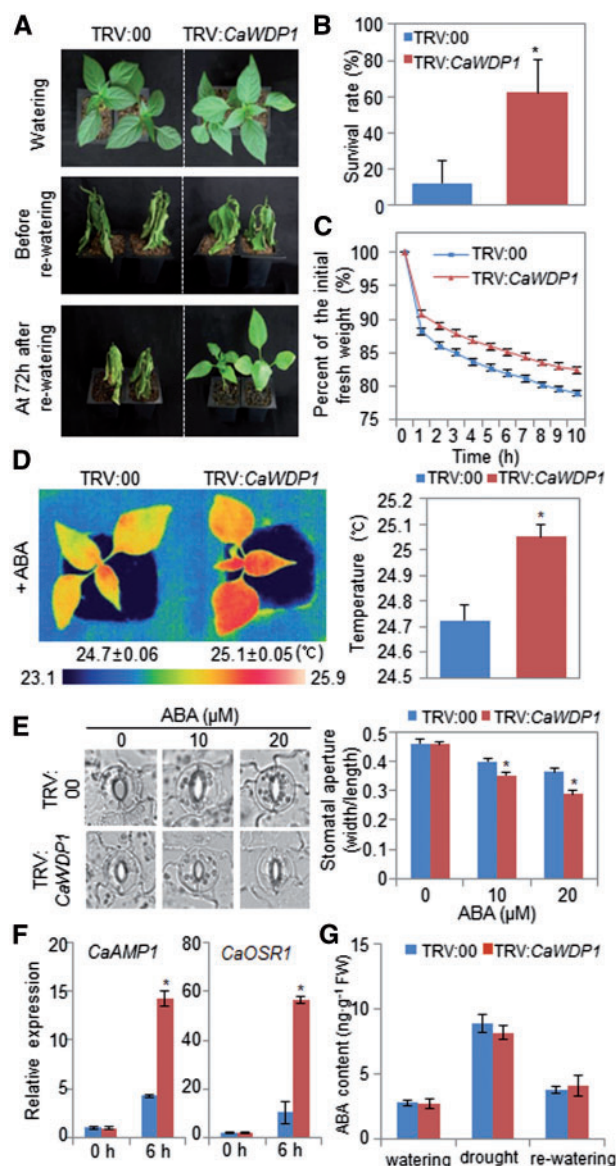


Fig. 4 Enhanced tolerance of *CaWDP1*-silenced pepper plants to drought stress. (A) Empty vector control and *CaWDP1*-silenced pepper were grown in pots for 5 weeks under normal growth conditions. Thereafter, water was withheld for 13 d, followed by re-watering for 2 d. (B) Percentage of surviving plants after 3 d of re-watering. Data represent the mean $\pm$ SE of three independent experiments, each evaluating 25 plants. (C) Leaf water loss from the leaves of empty vector control and *CaWDP1*-silenced pepper plants at various times after detachment of leaves. (D) Increased leaf temperatures of *CaWDP1*-silenced pepper plants treated with ABA. Representative thermographic images of *CaWDP1*-silenced pepper plants and control plants were taken at 3 h after ABA treatment, and the mean leaf temperatures of the three largest leaves were measured. (E) Stomatal apertures in control and *CaWDP1*-silenced pepper plants treated with ABA. Leaf peels were harvested from 4-week-old plants of each line and were incubated in SOB buffer supplemented with 0, 10 or 20 μ M ABA. Data represent the mean $\pm$ SE of three independent experiments, each evaluating 20 plants for 100 stomata. Representative images were taken under a microscope and the stomatal apertures were measured. (F) Relative expression levels of stress-responsive genes in the leaves of control and *CaWDP1*-silenced pepper plants after drought stress treatment. Four-week-old plants

the 35S promoter. Two independent T_3 homozygous lines (*CaWDP1*-OX) showing overexpression of *CaWDP1* (Supplementary Fig. S1B) were used in phenotypic analyses. Under normal growth conditions, we observed no phenotypic differences between wild-type and *CaWDP1*-OX plants during the germinative, seedling and adult plant stages (Figs. 5, 6). ABA signaling shares common signal transduction pathways with drought stress signals, but drought stress signaling is not dependent solely on ABA (Jakab et al. 2005). To determine the in vivo function of *CaWDP1* in response to ABA, we examined seed germination, seedling establishment and root growth after ABA treatment (Fig. 5). First, we germinated *CaWDP1*-OX seeds on Murashige and Skoog (MS) plates supplemented with various concentrations of ABA. We found that in the absence of ABA, the germination rates of wild-type and *CaWDP1*-OX seeds did not differ significantly at 7 d after plating (Fig. 5A). However, after 2–7 d in the presence of 1.0 and 2.0 μ M ABA, the *CaWDP1*-OX seeds were less sensitive to ABA than were the wild-type seeds. In comparison with the ABA response during the germinative stage, the differences between wild-type and *CaWDP1*-OX plants were more distinguishable at the seedling stage (Fig. 5B–E). The *CaWDP1*-OX plants were insensitive to ABA during early seedling establishment and primary root growth. At 10 d after sowing, the establishment of seedlings was higher in *CaWDP1*-OX plants than in wild-type plants (Fig. 5B). In the presence of 1.0 μ M ABA, approximately 12% of the wild-type seedlings exhibited green cotyledons, whereas 30–35% of the *CaWDP1*-OX seedlings developed normal green cotyledons (Fig. 5C). In addition, primary root growth of the wild-type plants was severely impaired after ABA treatment (Fig. 5D). In the absence of ABA, the root morphology and root length did not differ significantly between wild-type and *CaWDP1*-OX plants. In contrast, in the presence of ABA, the root lengths of *CaWDP1*-OX plants were longer than those of wild-type plants (Fig. 5E). Our results indicate that overexpression of the *CaWDP1* gene confers decreased ABA sensitivity in Arabidopsis during the germinative and seedling stages.

Increased susceptibility of *CaWDP1*-OX plants to drought stress

CaWDP1-silenced pepper plants showed a drought-tolerant phenotype (Fig. 4) and *CaWDP1*-OX plants exhibited decreased sensitivity to ABA (Fig. 5). We thus investigated whether *CaWDP1* overexpression alters the drought stress response (Fig. 6). To examine the effect of *CaWDP1* on the drought stress response, we grew wild-type and *CaWDP1*-OX transgenic Arabidopsis lines under well-watered conditions and

Fig. 4 Continued

were subjected to drought stress by removal of their roots. The relative expression level ($\Delta\Delta$ CT) of each gene was normalized to that of *CaACT1* as an internal control gene. (G) ABA content in leaves of control and *CaWDP1*-silenced pepper plants after drought treatment for 8 d and re-watering 2 d. The ABA content of each sample was quantified by using the Phytodetek-ABA kit (Agdia Inc.) according to the manufacturer's instructions. Data represent the mean $\pm$ SE of three independent experiments, and asterisks indicate a significant difference (Student's test; $P < 0.05$).

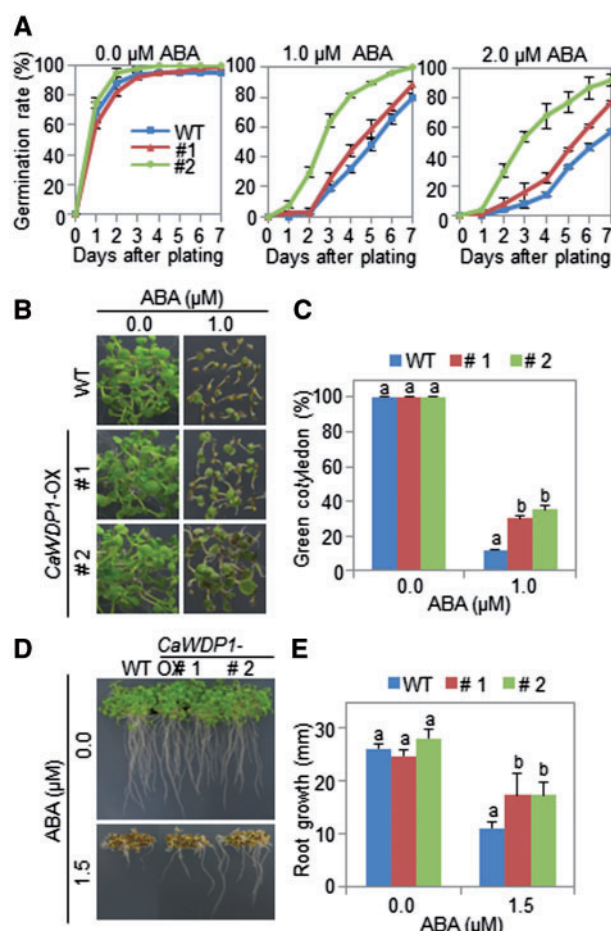


Fig. 5 Reduced sensitivity of *CaWDP1*-OX transgenic *Arabidopsis* lines to ABA. (A) Seed germination of wild-type and transgenic lines in response to ABA after 0–7 d of treatment. Seeds were germinated on $0.5 \times$ MS agar plates supplemented with 0.0, 1.0 or 2.0 μ M ABA. Data represent the mean $\pm$ SE values obtained after evaluating 200 seeds from three independent experiments. (B) Growth of wild-type and *CaWDP1*-OX seedlings on $0.5 \times$ MS agar plates supplemented with 0.0 or 1.0 μ M ABA. Representative photographs were taken 10 d after plating. (C) Quantification of green cotyledons in the wild type and each mutant line was performed at 10 d after plating on $0.5 \times$ MS agar plates supplemented with 0.0 or 1.0 μ M ABA. Data represent the mean $\pm$ SE values obtained after evaluating 72 seeds from three independent experiments. (D) Root elongation of wild-type and transgenic lines in response to ABA. (E) The root length of each plant was measured at 10 d after planting. Data represent the mean $\pm$ SE of three independent experiments, each evaluating 72 plants, and different letters indicate significant difference (ANOVA; $P < 0.05$).

observed no phenotypic differences between these plants (Fig. 6A, upper panel). We subsequently exposed the plants to drought stress by withholding water for 8 d (Fig. 6A, middle panel) and then allowing the plants to recover by re-watering for 2 d (Fig. 6A, lower panel). At the end of this time, we found that a wilted phenotype was present in more transgenic plants than wild-type plants. To confirm that overexpression of *CaWDP1* increases drought susceptibility, we measured the survival rates of wild-type and *CaWDP1*-OX transgenic plants after

2 d of re-watering. We determined survival rates of approximately 15% and 9% for the *CaWDP1*-OX lines #1 and #2, respectively; on the other hand, 50% of wild-type plants survived (Fig. 6B).

We also measured the fresh weight of rosette leaves of both plants to determine the leaf water loss rate and thus evaluate whether the drought-susceptible phenotype exhibited by *CaWDP1*-OX plants was derived from a higher rate of leaf water loss (Fig. 6C). We found that the fresh weight loss of rosette leaves due to leaf water loss was considerably higher in *CaWDP1*-OX plants than in the wild-type plants, suggesting that decreased drought tolerance is derived from altered rates of leaf water loss. Next, we measured the leaf temperatures of wild-type and *CaWDP1*-OX plants after ABA treatment. In contrast to *CaWDP1*-silenced pepper plants, *CaWDP1*-OX plants exhibited significantly lower leaf temperatures than wild-type plants (Fig. 6D). Jeon et al. (2008) showed that the levels of ABA were correlated with stomatal opening and closure. In addition, measurements of stomatal aperture have established that the sensitivity to ABA has determined the level of drought tolerance (Lim and Lee 2014, Lim et al. 2014). To determine whether the high leaf water loss shown by *CaWDP1*-OX plants was derived from an increase in stomatal aperture, we measured the stomatal aperture in the absence and presence of treatment with ABA, which plays a critical role in stomatal closure. We found that ABA treatment triggered stomatal closure; however, *CaWDP1*-OX leaves exhibited larger stomatal pores than wild-type leaves, implying that the observed high leaf water loss rate in *CaWDP1*-OX plants was derived from decreased ABA-induced stomatal closure. Our results suggest that hyposensitivity to ABA in the guard cells of *CaWDP1*-OX plants leads to inability to maintain water under drought conditions, thereby resulting in a drought-sensitive phenotype.

CaWDP1 expression is negatively associated with ABA sensitivity and drought tolerance in pepper and *Arabidopsis*; we thus investigated the correlation between the drought-sensitive phenotype exhibited by *CaWDP1*-OX plants and the induction of ABA contents and the expression of drought tolerance-related genes (Fig. 7) and group A protein phosphatase 2Cs (PP2Cs), which play a role as negative regulators of ABA (Supplementary Fig. S2). The ABA contents of *CaWDP1*-OX plants did not significantly differ in wild-type plants (Fig. 7A). We performed qRT-PCR analysis of several genes, including *NCED3*, *RAB18*, *RD29A* and *RD29B*, in wild-type and *CaWDP1*-OX plants after dehydration treatment with gene-specific primers. Drought stress treatment triggered strong induction of these genes (Fig. 7B). We found that at 6 h after dehydration treatment, accumulation of transcripts of the ABA biosynthesis-related gene *NCED3* was not comparable with that of *CaWDP1*-OX plants. In contrast, the expression levels of drought tolerance-related genes, such as *RAB18*, *RD29A* and *RD29B*, were significantly lower in *CaWDP1*-OX plants than in wild-type plants (Fig. 7B). To examine whether this pattern is associated with alteration of ABA signaling, we measured expression levels of group A PP2C genes, *ABI1*, *HAB2* and *AHG1*, which are the core components of ABA signaling. qRT-PCR analysis revealed that the transcripts

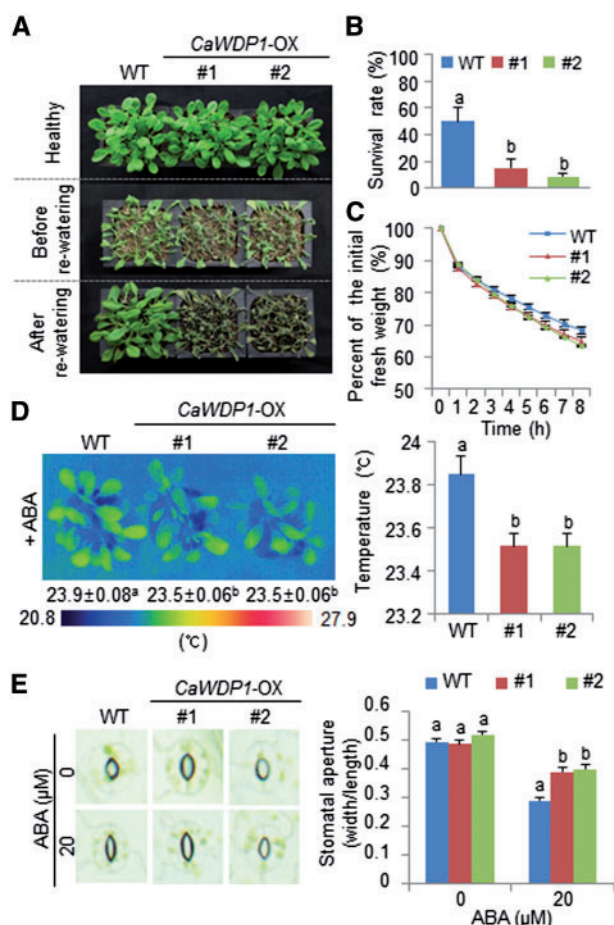


Fig. 6 Increased susceptibility of *CaWDP1*-OX transgenic Arabidopsis lines to drought stress. (A) Drought tolerance of *CaWDP1*-OX transgenic plants. Two-week-old wild-type (WT) and transgenic plants were subjected to drought stress by withholding water for 8 d and re-watering for 2 d. (B) Survival rates of plants after re-watering. Data represent the mean $\pm$ SE of three independent experiments, each evaluating 30 plants. (C) Leaf water loss from the leaves of wild-type and transgenic plants at various times after detachment. (D) Decreased leaf temperatures of *CaWDP1*-OX plants treated with ABA. Representative thermographic images of *CaWDP1*-OX plants and control plants were taken at 5 h after ABA treatment and the mean leaf temperatures of the three largest leaves were measured. (E) Stomatal apertures in *CaWDP1*-OX transgenic lines and wild-type plants treated with 0 and 20 μ M ABA. Leaf peels were harvested from 4-week-old plants and the stomatal apertures were measured under the microscope. Data represent the mean $\pm$ SD of three independent experiments, each evaluating 20 plants for 100 stomata, and different letters indicate significant difference (ANOVA; $P < 0.05$).

of group A *PP2C* genes are more accumulated in *CaWDP1*-OX plants than in wild-type plants (Supplementary Fig. S2). Our data indicate that the conferred expression of *CaWDP1* modulates the response to drought stress and that the decreased expression levels of drought-induced marker genes may further affect the drought sensitivity of *CaWDP1*-OX plants. Taken together, our results suggest that the decreased drought tolerance of *CaWDP1*-OX plants is derived from hyposensitivity to ABA, not a change in the ABA level, and low expression levels of drought-responsive genes.

Discussion

The defense response to biotic and abiotic stresses is generally repressed, in order to prevent loss of energy for growth and development under favorable conditions. The central role of many negative regulators of stress tolerance results in the inhibition of processes related to defense mechanisms, thereby leading to recovery and resumption of normal growth and development under favorable conditions (Gou et al. 2009, Joseph et al. 2014). Recent studies have investigated the ABA signaling pathway, from ABA perception to biotic and abiotic stress responses (Lee and Luan 2012, Lim et al. 2015a). Moreover, the most clearly understood ABA and drought signaling pathway is the RCAR-PP2C-SnRK2-bZIP transcription factor cascade. However, the composition of the downstream region of these factors remains to be elucidated. In the present study, we isolated pepper *CaWDP1* by using differential hybridization screening assays. We demonstrated that *CaWDP1* is a novel negative regulator of ABA and drought signaling. We provide several lines of genetic evidence to support this conclusion. First, *CaWDP1*-silenced pepper plants displayed a drought-tolerant phenotype, which was characterized by increased leaf temperatures and a smaller stomatal pore size. Secondly, *CaWDP1*-OX plants exhibited an ABA-hyposensitive phenotype during the germinative, seedling and adult plant stages. Moreover, in contrast to *CaWDP1*-silenced pepper plants, *CaWDP1*-OX plants exhibited a drought-sensitive phenotype, which was characterized by repression of drought-responsive gene expression.

As there is a lack of evidence for biological function of WPP domain proteins, the role of WDP1 in response to abiotic stresses, including ABA, drought and high salinity, remains unclear. In the present study, the transcripts of *CaWDP1* were induced by osmotic stresses, including drought and high salinity (Fig. 2), indicating that *CaWDP1* functions in response to abiotic stress. The results of our phenotypic analyses revealed that *CaWDP1*-silenced pepper plants exhibited strongly enhanced tolerance to drought stress. Moreover, the data regarding *CaWDP1*-OX plants were consistent with those for *CaWDP1*-silenced pepper plants. We further showed that the transcripts of *CaWDP1* were significantly induced in pepper leaves after treatment with ABA (Fig. 2A). The expression levels of some positive and negative regulators of ABA were previously shown to be decreased and/or induced by ABA exposure (Schweighofer et al. 2004), indicating that *CaWDP1* also participates in ABA signaling. In the present study, we found that *CaWDP1*-OX plants exhibited a phenotype that was markedly insensitive to ABA during the germinative, seedling and adult stages. In particular, *CaWDP1*-OX line #2 exhibited a stronger phenotype than that of line #1, which presumably may be affected by the expression level of *CaWDP1* (Supplementary Fig. S1B). Our data imply that *CaWDP1* participates in the ABA-mediated drought stress response in pepper plants.

To elucidate the role of *CaWDP1* in abiotic stress responses, we examined loss of function via VIGS-based genetic analysis and gain of function by using *CaWDP1*-OX plants. The transformation efficiency in pepper plants was very low, and this hindered genetic analyses. Hence, we used the VIGS system in pepper plants and overexpression in transgenic Arabidopsis

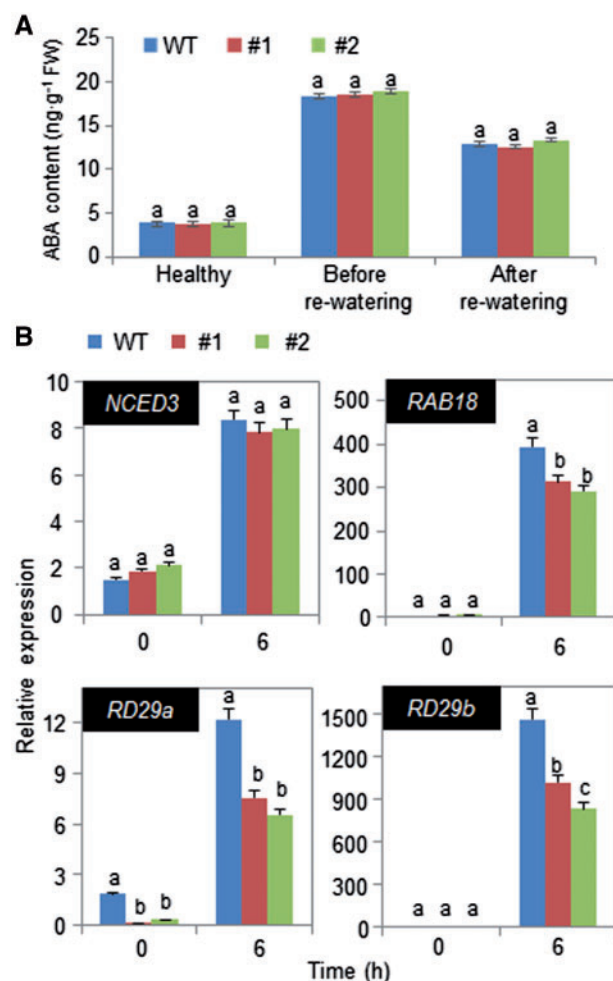


Fig. 7 The levels of endogenous ABA contents and expression of drought-inducible genes in *CaWDP1*-OX plants to response to drought stress. (A) ABA content in leaves of wild-type (WT) and *CaWDP1*-OX plants after drought treatment for 5 d and re-watering for 2 d. The ABA content of each sample was quantified by using the Phytodetek-ABA kit (Agdia Inc.) according to the manufacturer's instructions. (B) qRT-PCR analysis of drought-inducible genes in the leaves of *CaWDP1*-OX mutant plants subjected to drought stress at 6 h after detachment. Relative expression levels ($\Delta\Delta CT$) of each gene were normalized to the geometric mean of *AtACT8* and *AtEF1α* as internal control genes. Data represent the mean $\pm$ SD of three independent experiments, and different letters indicate a significant difference (ANOVA; $P < 0.05$).

plants to examine the *in vivo* functions of *CaWDP1* in the ABA and drought stress responses. Under favorable growth conditions, the phenotypes of *CaWDP1*-silenced pepper plants and *CaWDP1*-OX transgenic Arabidopsis plants were indistinguishable and the expression of the *NCED* gene, which is related to ABA synthesis, was unchanged; hence, the responses of *CaWDP1*-OX plants to drought stress were not a prerequisite for increased ABA production. *CaWDP1* functions as a negative regulator of ABA; however, after ABA and drought treatments, the expression patterns of *CaWDP1* showed steadily increasing trends. Under abiotic stress conditions, negative regulators are generally down-regulated; however, some regulators are up-regulated. In Arabidopsis, contrasting effects of ABA on gene

expression have previously been observed for group A *PP2C* genes (Merlot et al. 2001, Schweighofer et al. 2004). These effects may be explained by negative feedback regulatory mechanisms. The expression level of group A *PP2C* genes is markedly increased by ABA; however, group A *PP2C* genes function as negative regulators of ABA (Schweighofer et al. 2004, Lee and Luan 2012, Lim et al. 2015a). Moreover, ABD1 acts as a negative regulator of ABA signaling and drought stress response, although the expression level of ABD1 is induced by ABA (Seo et al. 2014). The functions of these proteins presumably contribute to resumption of normal growth and development when plant growth conditions change from unfavorable to favorable. Hence, our data imply that, when environmental conditions become favorable for plant growth and development, already expressed *CaWDP1* probably functions in the recovery of stable growth and development. Moreover, based on the expression levels of drought-responsive genes, we propose that *CaWDP1* directly or indirectly regulates the expression of these genes; moreover, *CaWDP1* may function upstream of these genes in response to drought. Under water deficit conditions, plants invoke defense mechanisms such as induction of elevated drought-responsive genes, in order to restrict water consumption and enhance tolerance to drought stress (Urano et al. 2009, Lim et al. 2014, Lim et al. 2015b). In the present study, we showed that after dehydration stress treatment, *CaWDP1*-OX plants exhibited lower expression levels of drought-responsive genes, including *RAB18*, *RD29A*, and *RD29B*, than did wild-type plants, implying that the delivery of drought signaling via *CaWDP1* exerts control over the transcriptional response to drought stress. In addition, *CaWDP1* may function in regulation of stomatal movement in response to drought stress. As shown in Figs. 4E and 6E, the expression level of the *CaWDP1* gene was negatively correlated with stomatal aperture following ABA treatment. ABA-induced stomatal closing is a way to prevent excessive water loss, which helps to enhance tolerance in the plant under drought stress conditions (Lee and Luan 2012, Lim et al. 2015a). The possibility of a function for the *CaWDP1* gene in stomatal regulation may be supported by the expression pattern of *CaWDP1* homologous genes from Arabidopsis, *AtWPP1/2/3*, sharing high sequence conservation of the WPP domain (Supplementary Fig. S3). In particular, expression of *AtWPP1* and *AtWPP3* genes was differentially induced in guard cells and mesophyll cells by ABA. Arabidopsis WPP proteins exhibit different subcellular localizations: WPP1 and WPP2 are targeted to the nuclear envelope, similarly to tomato MAF1, while WPP3 is localized in the nucleus and the cytoplasm, but not in the nuclear envelope (Rose and Meier 2001, Patel et al. 2004). Of Arabidopsis WPP proteins, *CaWDP1* is close to WPP1 (44.2% identity and 55.8% similarity) and WPP2 (40.6% identity and 54.4% similarity). *CaWDP1* showed a similar subcellular localization pattern to WPP3 (28.9% identity and 41.7% similarity). However, the localization pattern of WPP proteins is determined by cell type (Patel et al. 2004). For example, WPP1 and WPP2 proteins accumulated in the nucleus and in the cytoplasm in differentiated cells, such as hypocotyl cells and leaf epidermal cells. In this respect, it is possible that the *CaWDP1* protein can show

different localizations in undifferentiated cells, compared with the data shown in leaf epidermal cells from *N. benthamiana* in Fig. 3.

Taken together, our present findings indicate that CaWDP1 acts as a negative regulator of the ABA signaling and drought stress response in pepper plants. In particular, the involvement of CaWDP1 in the regulation of stomatal opening and/or closure and the induction of drought-responsive genes indicates the requirement for this protein at the early stage of plant stress responses. We have revealed the functional involvement of CaWDP1 in defense mechanisms against drought stress through ABA-mediated signaling; however, the mechanism whereby CaWDP1 functions as a negative regulator of drought stress responses remains unclear. To determine the function of CaWDP1 under water deficit conditions, further studies using molecular analysis to identify the upstream or downstream proteins are required.

Materials and Methods

Plant materials and growth conditions

Arabidopsis ecotype Col-0 plants, pepper (*Capsicum annuum*) cultivar Nockwang and tobacco (*Nicotiana benthamiana*) were planted in a plastic tray containing compost soil mix at $24 \pm 1^\circ\text{C}$ under a 16 h light/8 h dark photoperiod. To perform the genetic analysis, Arabidopsis seeds were sterilized in 70% ethanol followed by three washes with sterilized tap water, then kept at 4°C under dark condition for 4 d. Details of growth conditions are as described previously (Lee et al. 2008).

Subcellular localization analysis

To determine the subcellular localization of the CaWDP1 protein, we produced the CaWDP1–GFP fusion protein by using the GFP-fused vector p326GFP. *Agrobacterium tumefaciens* strain GV3101 carrying this construct was infiltrated into rosette leaves of *N. benthamiana* plants. Subcellular localization was detected by confocal microscopy using a 510 UV/Vis Meta (Zeiss) on the lower side of epidermal cells at 2 d after infiltration. To confirm the site of the nucleus, DAPI staining was used.

Virus-induced gene silencing assay

For the loss-of-function phenotypic assay, pTRV1 and pTRV2 vectors were used for VIGS analysis with generation of CaWDP1 knockdown pepper plants. The 5' region (1–300 bp) of CaWDP1 cDNA was amplified using specific primers and the resulting fragment was cloned into the same site of the TRV2 vector to generate pTRV:CaWDP1. *Agrobacterium tumefaciens* strain GV3101 containing each construct (pTRV1, pTRV2:00 and pTRV:CaWDP1) was resuspended in infiltration buffer (10 mM MgCl_2 , 10 mM MES, pH 5.7) and was infiltrated into the back of cotyledons of pepper seedlings ($\text{OD}_{600} = 0.2$ for each construct). The plants were grown in the same conditions as previously described. To confirm the inhibition of CaWDP1 expression, reverse transcription–PCR (RT–PCR) analysis was used.

Generation of transgenic Arabidopsis plants

To produce CaWDP1-OX transgenic Arabidopsis lines, we used *Agrobacterium*-mediated transformation with the floral dip method (Clough and Bent 1998). For Pro35S:CaWDP1, a full-length cDNA sequence of CaWDP1 (accession No. KT361853.1) was amplified by PCR with specific primers (Supplementary Table S1) and the resulting product was inserted into the plant expression vector harboring a pK2GW7 vector. This plasmid was transformed into *A. tumefaciens* strain GV3101 and then transformed to *Arabidopsis thaliana*. To isolate CaWDP1-OX lines, putative overexpressing plants were selected on

MS agar plates supplemented with $50 \mu\text{g ml}^{-1}$ kanamycin, and T_3 homozygous lines were used for further genetic analysis.

Treatments of ABA and drought stress

For measurement of germination rates and seedling establishment, 36 seeds of wild-type and CaWDP1-OX transgenic plants were stratified at 4°C for 4 d and were then plated on $0.5 \times$ MS agar medium supplemented with various concentrations of ABA.

For the drought treatment, pepper and Arabidopsis were initially grown on compost soil under well-watered condition for 4 and 3 weeks, respectively. Irrigation was then halted until plants showed the lethal phenotype. At that time, irrigation was resumed for 2 d and the survival rates were scored.

Thermal imaging analysis and ABA-induced stomatal closure

Thermal imaging analysis was performed as described previously (Lim and Lee 2016). Two- or four-leaf stage pepper plants and 3-week-old Arabidopsis plants were treated with $100 \mu\text{M}$ ABA; thermal images and leaf temperatures were obtained using an infrared camera (FLIR systems; T420) with FLIR Tools + ver 5.2 software.

To measure stomatal pore size, the epidermal leaves from the pepper and Arabidopsis plants were floated in a stomatal opening buffer (SOB: 10 mM MES-KOH, $10 \mu\text{M}$ CaCl_2 and 50 mM KCl, pH 6.15) for 3 h. Subsequently, various concentrations of ABA were added to the SOB followed by 3 h incubation. The leaf peels of epidermal tissue were stripped by using the blending method and the image was detected by light microscopy using a 510 UV/Vis Meta microscope (Zeiss).

RNA preparation and quantitative real-time RT–PCR analysis

An RNeasy Mini kit (Qiagen) was used to isolate RNA from leaves of pepper and Arabidopsis. To prevent genomic DNA contamination, RNA samples were digested with RNA-free DNase. A $2 \mu\text{g}$ aliquot of total RNA was used to synthesize first-strand cDNA by using a Transcript First Strand cDNA Synthesis kit (Roche), according to the manufacturer's protocol. For qRT–PCR analysis, the cDNA was amplified with SYBR Green Supermix (Bio-Rad) with gene-specific primers (Supplementary Table S1). For normalization, two internal control genes were used (pepper, *CaACT1* and *CaEF1 α* ; Arabidopsis, *AtACT8* and *AtEF1 α*).

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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