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Functional roles of the pepper RING finger protein gene, *CaRING1*, in abscisic acid signaling and dehydration tolerance

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Abstract Plants are constantly exposed to a variety of biotic and abiotic stresses, which include pathogens and conditions of high salinity, low temperature, and drought. Abscisic acid (ABA) is a major plant hormone involved in signal transduction pathways that mediate the defense response of plants to abiotic stress. Previously, we isolated Ring finger protein gene (*CaRING1*) from pepper (*Capsicum annuum*), which is associated with resistance to bacterial pathogens, accompanied by hypersensitive cell death. Here, we report a new function of the *CaRING1* gene product in the ABA-mediated defense responses of plants to dehydration stress. The expression of the *CaRING1* gene was induced in pepper leaves treated with ABA or exposed to dehydration or NaCl. Virus-induced gene silencing of *CaRING1* in pepper plants exhibited low degree of ABA-induced stomatal closure and high levels of transpirational water loss in dehydrated leaves. These led to be more vulnerable to dehydration stress in *CaRING1*-silenced pepper than in the control pepper, accompanied by reduction of ABA-regulated gene expression and low accumulation of ABA and H_2O_2. In contrast, *CaRING1*-overexpressing transgenic plants showed enhanced sensitivity to ABA during the seedling growth and establishment. These plants were also more tolerant to dehydration stress than the wild-type plants because of high ABA accumulation, enhanced stomatal closure and increased expression of stress-responsive genes. Together, these results suggest that the *CaRING1* acts as positive factor for dehydration tolerance in Arabidopsis by modulating ABA biosynthesis and ABA-mediated stomatal closing and gene expression.

Keywords Abscisic acid · CaRING1 · Dehydration · Pepper · Transgenic plant

Abbreviations

ABA	Abscisic acid
Pst	*Pseudomonas syringae* pv. *tomato*
PR	Pathogenesis related
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
ROS	Reactive oxygen species
SA	Salicylic acid
SOS	Stomatal opening solution
VIGS	Virus-induced gene silencing
Xcv	*Xanthomonas campestris* pv. *vesicatoria*

Introduction

Abiotic and biotic stresses, such as pathogenic infections and drought, adversely influence plant growth and crop production. Under these stress conditions, plants activate several defense responses by altering physiological and molecular systems via the synthesis, remodeling, and degradation of proteins (Stone and Callis 2007; Huang et al. 2014; Lim et al. 2014). The ubiquitin-26S proteasome system is important for post-translational modification and regulates crucial eukaryotic cellular processes, including DNA repair, cell signaling, and defense responses (Lee

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et al. 2011; Sadanandom et al. 2012; Callis 2014; Seo et al. 2014). This mechanism has a diverse range of substrates, including transcription factors, hormone receptors, cell cycle regulators, light regulators, and misfolded proteins (Hershko and Ciechanover 1998; Gagne et al. 2004; Zhang et al. 2007; Irigoyen et al. 2014; Seo et al. 2014). Ubiquitin is bound to target proteins by a series of well-characterized reactions that result in the target proteins being marked for degradation (Pickart 2001; Glickman and Adir 2004; Smalle and Vierstra 2004).

Ubiquitin is activated by an E1 ubiquitin-activating enzyme in an ATP-dependent manner and is then transferred to an E2 ubiquitin-conjugating enzyme. Finally, the E2-ubiquitin complex interacts with an E3 ubiquitin-ligase that catalyzes the formation of a bond between the target protein and ubiquitin, leading to autoubiquitination of E3 ligase and ubiquitination of the target protein (Ciechanover 1998; Ciechanover and Schwartz 1998; Jacobson et al. 2009). In vitro ubiquitination analysis have suggested that the combined activities of these three enzymes are necessary and sufficient for autoubiquitination and ubiquitination (Callis 2014). In this system, E3 ubiquitin-ligase has multiple isoforms, indicating that this enzyme selects target proteins for ubiquitination (Callis 2014). The E3 ubiquitin ligases are divided into two groups based on their structure (Pickart 2001). One group functions as a single subunit, including RING (Really Interesting New Gene), U-box, and HECT (Homology to E6-AP Carboxyl Terminus) E3 ligases (Hatakeyama et al. 2001; Miao and Zentgraf 2010; Kim and Kim 2013a, b; Marin 2013). The other group functions as a multisubunit, including APC (Anaphase Promoting Complex), CUL4-DDB1 (CULLIN4-Damaged-specific DNA binding protein 1), and SCF (Skp1, Cullin, F-box) (Zheng et al. 2002; Pazhouhandeh et al. 2011; Chang et al. 2014). Among these, RING type E3 ubiquitin ligases constitute the third largest gene family in Arabidopsis with more than 477 genes (Stone et al. 2005); however, the function of only a few RING type E3 ligases has been elucidated. In plants, several RING type ligases are involved in cellular processes, including development, hormonal signaling, and defense responses to biotic and abiotic stresses (Xie et al. 2002; Stone et al. 2006; Bu et al. 2009; Lee et al. 2011; Kim and Kim 2013a, b).

In particular, many studies have shown that RING type E3 ligases play a crucial role in defense mechanisms and that they are specifically induced by pathogen infection as well as environmental stresses (Zeng et al. 2006; Ryu et al. 2010; Lee et al. 2011). For instance, RING type E3 ligases, such as ACRE132 and ACRE189, act as positive regulators of disease resistance. On the contrary, CaRFP1 is associated with disease susceptibility and abiotic stress tolerance (Hong et al. 2007). Several RING type E3 ligases, which are associated with stress tolerance/susceptibility, are involved in the degradation of ABA signal transduction components. ABA is one of the major plant hormones that play an important role in seed dormancy, plant development, and adaptation to abiotic stress (Bartels and Sunkar 2005; Finkelstein et al. 2002; Leung and Giraudat 1998; Rock 2000). The expression of a number of genes involved in plant defense responses to abiotic stress is regulated by ABA (Jakab et al. 2005). The ABA-induced C3H2C3-type RING E3 ligase AtAIRP1 functions as a positive regulator of drought stress in an ABA dependent manner (Ryu et al. 2010). Moreover, ABI5 binding protein (AFP) acts as a negative regulator of ABA by ubiquitinating and degrading the ABI5 transcription factor (Lopez-Molina et al. 2003). These studies suggest that various RING type ligases are both negative and positive regulators of ABA signaling, which affect tolerance to abiotic stress.

Previously, we reported that the *CaRING1* (*Capsicum annuum* RING finger protein 1) gene is differentially expressed in pepper leaves that are infected by virulent or avirulent strains of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) (Lee et al. 2011). *CaRING1* contains an amino-terminal transmembrane domain and a c-terminal RING domain, which is essential for E3 ubiquitin ligase activity. *CaRING1* is involved in cell death, which is accompanied by changes in reactive oxygen species (ROS) and salicylic acid (SA) accumulation, and PR gene expression. *CaRING1*-silenced pepper plants display enhanced susceptibility to an avirulent strain of *Xcv*, while *CaRING1*-overexpressing (OX) Arabidopsis plants are resistant to *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 and *Hyaloperonospora arabidopsidis* infections. In this study, based on the expression profiles of the *CaRING1* gene in pepper treated with ABA, dehydration, NaCl, and H_2O_2, we evaluated their responses to ABA and dehydration by using *CaRING1*-silenced pepper and overexpressing transgenic Arabidopsis plants. Our data suggest that CaRING1 protein is involved in regulation of ABA biosynthesis and ABA signaling, leading enhanced dehydration tolerance.

Materials and methods

Plant materials

Pepper (*Capsicum annuum* L., cv. Hanbyul) seeds were sown in a steam-sterilized compost soil mix (peat moss, perlite, and vermiculite, 5:3:2, v/v/v), sand, and loam soil (1:1:1, v/v/v). The pepper plants were raised in a growth room at 27 ± 1 °C with 80 μmol photons m^{-2} s^{-1} (white fluorescent light) for 16 h per day as described previously (Lee et al. 2008). Seeds of *35S:CaRING1* Arabidopsis

plants were obtained from individual stable transformants (line #13 and #16) that were used in a previous study (Lee et al. 2011). *35S:CaRING1* transgenic mutants and wild-type plants (ecotype Col-0) were routinely grown in a 9:1:1 ratio of peat moss, perlite, and vermiculite under fluorescent light (130 μmol photons m^{-2} s^{-1}) at 24 °C with 60 % humidity and a 16-h light/8-h dark cycle. Arabidopsis seeds were surface sterilized with 70 % ethanol for 1 min for in vitro culture. After treatment with 2 % sodium hydroxide for 10 min, seeds were washed 10 times in sterile distilled water and were finally sown on Murashige and Skoog (1962) (MS) agar (Sigma, St. Louis, MO, USA) supplemented with 1 % sucrose. The plates were sealed and incubated at 24 °C in a chamber exposed to a 16-h light/8-h dark cycle.

Virus-induced gene silencing (VIGS) of the *CaRING1* gene in pepper

To knockdown the *CaRING1* gene in pepper plants, VIGS was performed as described by Lee et al. (2011). Briefly, *Agrobacterium tumefaciens* strain GV3101 carrying pTRV1 and pTRV2:*CaRING1* or pTRV2:00 as a negative control was co-infiltrated into the fully expanded cotyledons of pepper plants (OD_{600} = 0.2 for each construct). Plants were placed in a growth room at 24 °C with a 16 h light and 8 h dark photoperiod for growth and spread of the virus.

ABA, H_2O_2, NaCl and dehydration treatment

At the six-leaf-stage, pepper plants were used to examine expression pattern of *CaRING1* gene. Pepper plants were sprayed with 100 μM ABA and 100 μM H_2O_2. For treatment with salt stress, plants were irrigated with a salt solution (200 mM). For dehydration stress, plants were carefully removed from the soil to prevent injury and then dried on 3 MM paper (Whatman, Clifton, UK) or only aerial parts of pepper plants were dried after removing their roots.

For the seedling growth test of Arabidopsis plant, 100 seeds of wild-type and *35S:CaRING1* transgenic Arabidopsis lines were sown on plates containing MS agar medium supplemented with various concentrations of ABA, and seedlings with green cotyledons were counted 7 days later. In parallel, seedlings from each line were vertically grown on the MS plates for 7 days and the root lengths of seedlings were measured. For qRT-PCR analysis, 4-week-old *35S:CaRING1* mutants and wild-type plants were treated with 50 μM ABA, or were carefully removed from the soil to be subjected to dehydration stress, and harvested at the given time points after treatment.

Dehydration tolerance assays

Dehydration tolerance assays were carried out as described by Lim and Lee (2014). One-week-old seedlings from the wild-type and *35S:CaRING1* lines were randomly planted in a pot containing soil mix (peat moss, perlite, and vermiculite, 9:1:1) and were grown under normal watering conditions for 2 weeks. To impose dehydration stress, watering was withheld for 2 weeks and the survival rate of the plants with rehydrated leaves was calculated after rewatering for 3 days. Rates of water loss were measured to determine the dehydration tolerance of *35S:CaRING1* mutant plants in a quantitative manner. Ten leaves were detached from 4-week-old plants of each line and placed in petri dishes. The dishes were kept in a growth chamber with 40 % relative humidity, and loss of fresh weight was determined at the indicated times. The experiments were repeated three times.

Stomatal aperture bioassay

A stomatal aperture bioassay was carried out as described previously with the following modifications (Lee et al. 2013). Briefly, leaf peels were harvested from the rosette leaves of 4-week-old plants and floated in stomatal opening solution (SOS: 50 mM KCl and 10 mM MES-KOH, pH 6.15, 10 mM $CaCl_2$) in the light for 2.5 h. To induce stomatal closing, the buffer was replaced with fresh SOS containing various concentrations of ABA and the leaf peels were further incubated for 2.5 h. In each sample, 100 stomata were randomly observed under a Nikon eclipse 80i microscope, and the width and length of individual stomata were recorded using Image J 1.46r (http://imagej.nih.gov/ij). Each experiment was performed three times independently.

RNA isolation and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated from leaves of pepper and Arabidopsis treated with ABA or subjected to dehydration stress using an RNeasy Mini kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. All RNA samples were digested with RNA-free DNase to remove genomic DNA. After quantification using a spectrophotometer, 1 μg of total RNA was used as a template to synthesize cDNA using a Transcript First Strand cDNA Synthesis kit (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions. In parallel, PCR was performed without reverse transcriptase and the products were subjected to qRT-PCR to confirm the absence of genomic DNA contamination in the cDNA samples. The synthesized cDNA was amplified in a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) with iQ™

SYBR Green Supermix and specific primers (Supplemental table S1). Every reaction was performed in triplicate. The PCR was programmed as follows: 95 °C for 5 min, 45 cycles at 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s. The relative expression of each gene was calculated using the ΔΔCt method, as previously described (Livak and Schmittgen 2001). The Arabidopsis *Actin8* gene (*AtACT8*) and pepper *Actin1* (*CaACT1*) was used for normalization.

DAB staining

Staining with 3,3′-diaminobenzidine (DAB) was carried out to visualize H_2O_2 in pepper leaves treated with dehydration stress, according to the method described by Lee et al. (2011). Briefly, leaf samples were collected and submerged into 1 mg ml^{-1} DAB (Sigma) solution (pH 3.8). After incubation for 16 h, the leaf samples were boiled in 100 % ethanol for 10 min to remove the chlorophyll and were then photographed.

Measurement of ABA content

For determination of ABA content, leaves were harvested from pepper and Arabidopsis plants treated with dehydration for 2 h and immediately frozen in liquid nitrogen. Approximately 50 mg of ground tissue were extracted overnight in 1 ml of ABA extraction buffer (methanol, containing 100 mg l^{-1} butylated hydroxyl toluene, 0.5 g l^{-1} citric acid monohydrate) at 4 °C on a rotary shaker. After centrifuged at 1500*g*, the supernatant was transferred to new tube and dried using a speed vac. ABA content of each sample was quantified by using the Phytodetek-ABA kit (Agdia Inc., Elkhart, IN, USA) according to manufacturer's instruction. ABA contents were expressed as pmol mg^{-1} fresh weight of the tissue.

Results and discussion

Induction of the *CaRING1* in pepper leaves by ABA, H_2O_2, dehydration, and high salinity

Many of RING type E3 ligases exhibits specific induction in response to both pathogen attacks and environmental stresses (Zeng et al. 2006; Ryu et al. 2010; Lee et al. 2011). In this study, we investigated involvement of *CaRING1* in abiotic stress. Expression pattern of *CaRING1* was examined in pepper leaves treated with ABA, a phytohormone that acts as a core regulator in abiotic stress responses (Fig. 1a). *CaRING1* transcripts were first detected 2 h after ABA treatment, with maximal levels detected at 12 h. ABA induces H_2O_2 production in guard cells of Arabidopsis (Pei et al. 2000) and *Vicia faba* (Zhang et al. 2007).

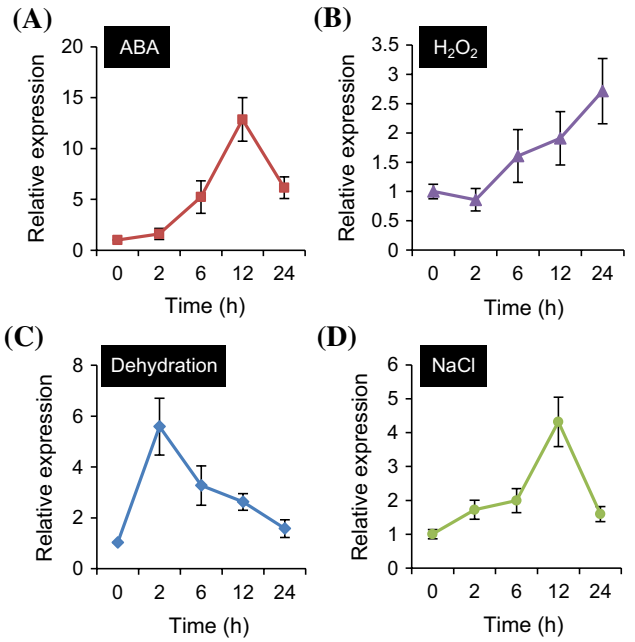

Fig. 1 Expression pattern of the *CaRING1* genes in pepper leaves treated with abscisic acid (100 μM; **a**), H_2O_2 (100 μM; **b**), dehydration (**c**), or NaCl (200 mM; **d**). Relative expression (ΔΔCT) of the *CaRING1* gene was normalized to that of that of *CaACT1* as internal control gene and compared with the value for non-treated leaves. Data are the mean ± standard error from three independent experiments

H_2O_2 levels are also enhanced by ABA in maize embryos and seedlings (Jiang and Zhang 2001). Based on these, we examined whether H_2O_2 promotes expression of *CaRING1* gene. Following treatment with 100 μM of H_2O_2, expression level of *CaRING1* gene gradually increased up to three times in pepper leaves (Fig. 1b). The accumulation of ABA is indispensable for plant defense responses to abiotic stresses, it is synthesized in various tissues, and its levels are increased in plant tissues, particularly the leaves, in response to osmotic stress (Cutler et al. 2010; Hubbard et al. 2010). To ascertain the effect of osmotic stress on the expression of the *CaRING1* gene, pepper plants were subjected to dehydration and high salinity treatments (Fig. 1c, d). As shown in Fig. 1c, dehydration stress rapidly induced *CaRING1* transcription after 2-h exposure and rapidly decreased expression between 6 and 24 h after treatment. Moreover, in pepper leaves treated with 200 mM NaCl, *CaRING1* transcripts started to accumulate 2 h after treatment and reached peak levels at 12 h (Fig. 1d). These findings indicate that *CaRING1* is induced in plants in response to ABA, dehydration, high salinity and H_2O_2. In Arabidopsis genome, there are four genes, At1g20823, At1g76410, At2g17450, and At4g35480, sharing high sequence similarity with CaRING1 (Lee et al. 2011). These genes also exhibit specific expression

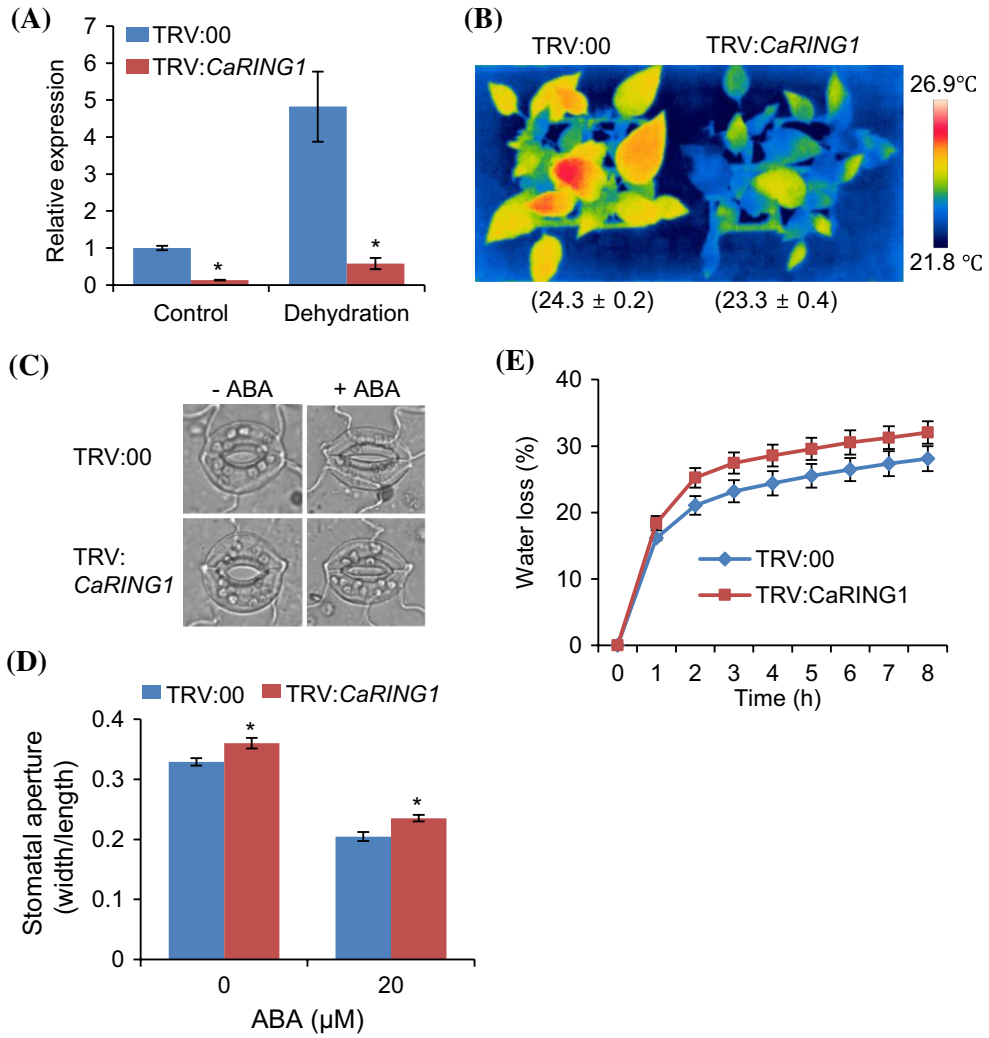

Fig. 2 Reduced stomatal closing in leaves of *CaRING1*-silenced pepper plants. **a** RT-PCR analysis of *CaRING1* expression in leaves of pepper plants transfected with the empty vector (TRV:00) as control or the silencing constructs (TRV:*CaRING1*) 2 h after dehydration. Relative expression level (ΔΔCT) of *CaRING1* gene was normalized to that of *CaACT1* as internal control gene. Four-week-old plants of TRV:00 and TRV:*CaRING1* peppers were subjected to dehydration stress by removing their roots. **b** Representative thermographic image of *CaRING1*-silenced pepper plants 10 days after dehydration treatment and mean leaf temperature was measured from the four upper leaves. **c**, **d** Stomatal apertures in control and *CaRING1*-silenced pepper plants treated with ABA. Stomatal apertures were measured under the microscope after leaf peels harvested from the 4-week-old plants of each line were incubated for 2 h in SOS buffer containing 20 μM ABA (**d**) and representative images were taken. Data are the mean ± standard error from three independent experiments. **e** Water loss from leaves of TRV:00 and TRV:*CaRING1* pepper plants at various times after detachment of leaves. Data are the mean ± standard error from three independent experiments. *Asterisks* indicate significant differences in three independent experiments (Student's *t* test; $P < 0.05$)

under treatment with ABA, drought, osmotic, cold, and high salinity (Supplemental figs. S1 and S2). In particular, ABA treatment leads to high induction of those genes except At1g76410 in leaves and guard cell. Similarly, the three genes At1g20823, At2g17450, and At4g35480 are significantly induced at the early time point under drought condition. Of the four genes, At1g20823 show specific induction in response to all treatments and was chosen for further study. These data provide the possibility that they have similar function in response to those treatments.

Involvement of CaRING1 in regulation of ABA- and dehydration-induced stomatal closure.

To analyze the biological role of the *CaRING1* gene in response to ABA treatment and dehydration stress, we applied the VIGS technique to induce the knockdown gene expression of *CaRING1* in pepper. qRT-PCR analysis was performed to test the efficiency of VIGS using empty vector control (TRV:00) and *CaRING1*-silenced (TRV:*CaRING1*) pepper leaves harvested before and after dehydration treatment for 2 h (Fig. 2a). Under non-treatment, expression

of *CaRING1* gene in TRV:*CaRING1* leaves was hardly detected (>7.4-fold decrease) compared with TRV:00 leaves. Dehydration stress triggered strong induction of *CaRING1* gene in both plant leaves: 3.6 to 6.1-fold in TRV:00 leaves and 3.0 to 6.1-fold in TRV:*CaRING1* leaves compared with that of the non-treated plant leaves. However, the expression level was still lower (>6.6-fold) in TRV:*CaRING1* leaves than that of TRV:00 leaves. Next, 4-week-old plants of TRV:00 and TRV:*CaRING1* pepper grown under normal condition were subjected to dehydration stress for 10 days. We measured leaf surface temperature using an Infrared Thermal Imaging Camera (T420, FLIR system, USA). Leaf temperature can be an indirect indicator for stomatal aperture and transpirational rate, which are controlled by ABA, because stomatal closing induces decreased evaporative cooling and finally increase leaf temperature (Park et al. 2015). As shown in Fig. 2b, *CaRING1*-silenced pepper plants exhibited low leaf temperature relative to that of the control plants. Previous studies have used measurements of stomatal movement to establish that high sensitivity to ABA in leaves leads to increased dehydration tolerance (Cheong et al. 2007; Lim and Lee 2014). In the absence of ABA, TRV:*CaRING1* plants had larger stomatal apertures compared with TRV:00 plants (Fig. 2c, d). This significant difference was still observed after treatment with ABA, indicating silencing of *CaRING1* allowed partial inhibition of ABA-induced stomatal closing. Consistently, rare of transpirational water loss was higher in TRV:*CaRING1* plants than TRV:00 (Fig. 2e). These data suggests that CaRING1 acts as a positive regulator in ABA and dehydration-induced stomatal closing.

Reduced tolerance of *CaRING1*-silenced pepper plants to dehydration stress

Based on the data shown in Fig. 2, we postulated that *CaRING1*-silenced pepper plants are more vulnerable to dehydration stress than the control plants. To prove this hypothesis, 4-week-old plants of TRV:*CaRING1* and TRV:00 were subjected to dehydration stress by withholding watering for 14 days (Fig. 3a). There were no phenotypic differences between the two plant lines under well-watered conditions. However, dehydration stress treatment made TRV:*CaRING1* plants rapidly dried relative to TRV:00 plants. Upon re-watering, over 73 % of the TRV:*CaRING1* plants did not resume growth, while TRV:00 plants exhibited more survival rates approximately 83 %. These data indicated that silencing of *CaRING1* gene confer reduced dehydration tolerance.

Drought sensitivity is correlated with level of ABA and drought-related gene expression (Gonzalez-Guzman et al. 2012; Li et al. 2011; Ryu et al. 2010). The primary function of ABA is the regulation of tolerance to abiotic stress, and ABA signal transduction is associated with defense response to abiotic stress (Zhu 2002). In particular, ABA contributes to plant adaptation to water-deprivation through regulatory circuits that induce gene expression (Wasilewska et al. 2008; Lee and Luan 2012). Based on these, we performed qRT-PCR analysis to examine expression levels of ABA- and drought-responsive gene *CaRD29B* and ABA biosynthesis-related gene *CaNCED3* in the leaves of TRV:00 and TRV:*CaRING1* plants treated with dehydration for 2 h (Fig. 3b; Lim et al. 2015). Before treatment, TRV:*CaRING1* plants exhibited low expression of *CaRD29B* (>2.5-fold decrease) and *CaNCED3* (>2.1-fold decrease) genes, compared with TRV:00 plants. Dehydration stress treatment triggered significant induction of the two genes in the two plant lines: *CaRD29B*, 11.7 to 14.5-fold in TRV:*CaRING1* and 13.2 to 15.4-fold in TRV:00; *CaNCED3*, 1.9 to 3.4-fold in TRV:*CaRING1* and 1.5 to 4.2-fold in TRV:00. However, their expression levels were still lower in TRV:*CaRING1* than in TRV:00, consistent with reduced tolerance of TRV:*CaRING1* plants to dehydration. Especially, *CaNCED3* gene induction by ABA was not surprised because some of ABA biosynthesis-related genes have been induced by endogenous and exogenous ABA (Barrero et al. 2006; Cheng et al. 2002; Xiong et al. 2002). The differential expression of *CaNCED3* gene between TRV:*CaRING1* and TRV:00 plants raised the possibility that CaRING1 functions in regulation of ABA biosynthesis. To test this possibility, we measured ABA level in the leaves of the two plant lines harvested at the same time as qRT-PCR analysis (Fig. 3c). Consistent with the expression level of *CaNCED3* gene, TRV:*CaRING1* plants exhibited low ABA level, compared with TRV:00 plants, and even this pattern maintained after dehydration stress which elevated ABA biosynthesis.

Water stress induces production of H_2O_2 as well as ABA in plant cell and ABA is also essential for H_2O_2 production (Hu et al. 2006). The association of ABA signaling and H_2O_2 production is observed during ABA-induced stomatal closure in Arabidopsis guard cell (Pei et al. 2000; Kwak et al. 2003). As the second messenger in ABA signaling pathway, H_2O_2 is downstream of ABA-INSENSITIVE1 (ABI1), one of the protein phosphates acting as negative regulator in ABA signaling, and upstream of ABI2 (Umezawa et al. 2009; Murata et al. 2001). As shown in Fig. 1b, H_2O_2 treatment triggered gradual accumulation of *CaRING1* transcripts. Based on these, we measured H_2O_2 production in the leaves of TRV:*CaRING1* and TRV:00 plants treated with dehydration stress through DAB staining (Fig. 3d). Dehydration stress led to significant accumulation of H_2O_2 in the both plant line leaves, but its amount were greater in TRV:*CaRING1* plant leaves than in TRV:00 plant leaves. Taken together, these data suggest that

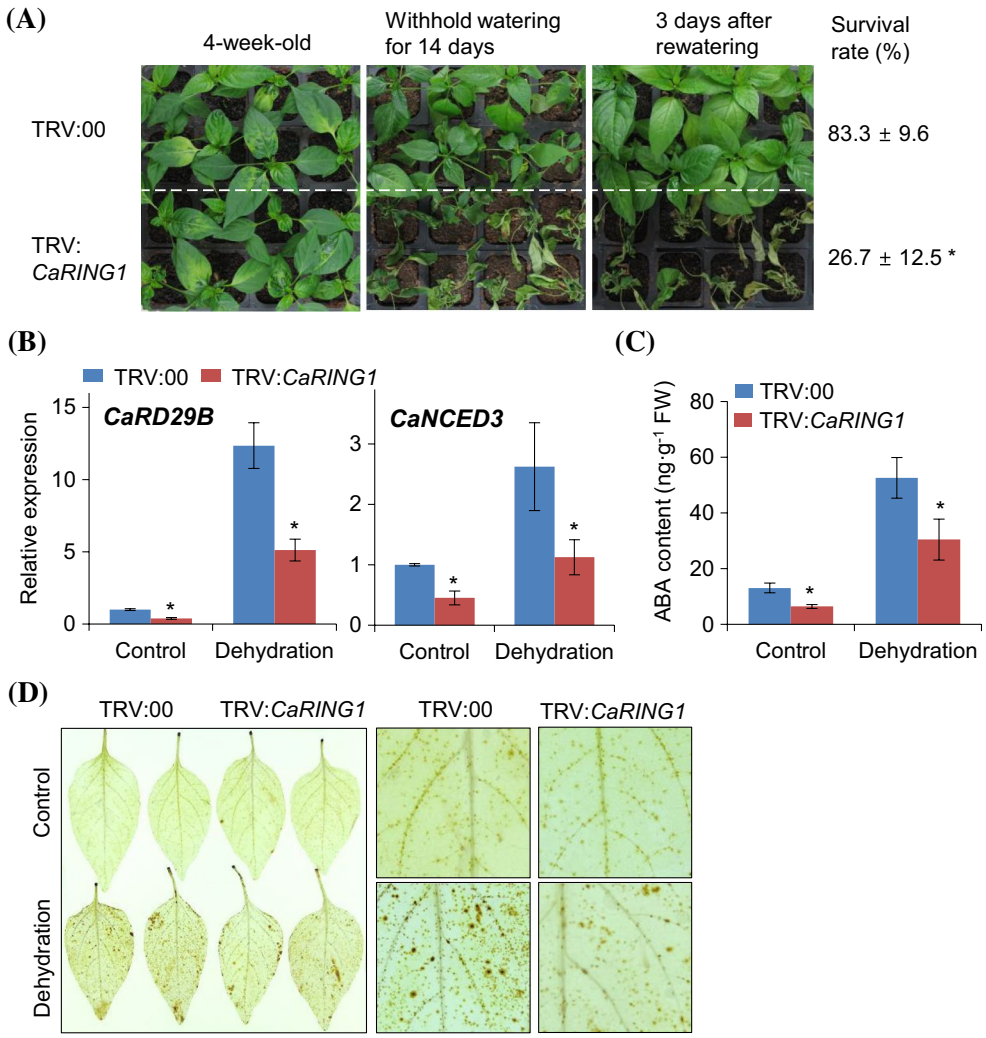

Fig. 3 Reduced tolerance of *CaRING1*-silenced pepper plants to dehydration stress. **a** Dehydration sensitivity of *CaRING1*-silenced pepper plants. Four-week-old CaRING1-silenced pepper plants (TRV:*CaRING1*) and control plants (TRV:00) were subjected to dehydration stress by withholding water for 14 days. Representative images were taken before (*right*) and after (*middle*) dehydration stress and at 3 days after rewatering (*right*). Survival rate was measured by counting plants that have *green* and rehydrated leaves 3 days after rewatering. **b** RT-PCR analysis of ABA-responsive gene and ABA biosynthesis-related gene expression in leaves of TRV:00 and TRV:*CaRING1* pepper plants after treatment with dehydration for 2 h. Relative expression level (ΔΔCT) of each gene was normalized to that of *CaACT1* as internal control gene. Four-week-old pepper plants of TRV:00 and TRV:*CaRING1* were subjected to dehydration stress by drying aerial part of the plants after removing their roots. **c** ABA content in leaves of TRV:00 and TRV:*CaRING1* pepper plants after dehydration treatment for 2 h. Data are the means ± standard error from three independent experiments. **d** Hydrogen peroxide production in the leaves of TRV:00 and TRV:*CaRING1* pepper plants in response to dehydration. Four-week-old pepper plants were treated with dehydration as mentioned above. Leaves harvested 3 h after treatment were stained with DAB solution and representative images were taken

silencing of *CaRING1* reduced dehydration tolerance via downregulation of ABA biosynthesis, ABA-response gene expression, and H_2O_2 production.

Enhanced ABA sensitivity of *35S:CaRING1* Arabidopsis transgenic plants

We previously generated Arabidopsis transgenic plants constitutively expressing *CaRING1* under the control of the strong constitutive 35S promoter (35S: *CaRING1*; Lee et al. 2011). Of the 35S: *CaRING1* plant lines used in the previous study, two lines (#13 and #16) expressing *CaRING1* at high level were selected for use in the present study (Supplemental fig. S3). Under our laboratory conditions, we examined the phenotype of *35S:CaRING1* plants and found them to be indistinguishable from the wild-type plants in terms of the shape of leaf, flower, silique, leaf number, and flowering time (data not shown). To further

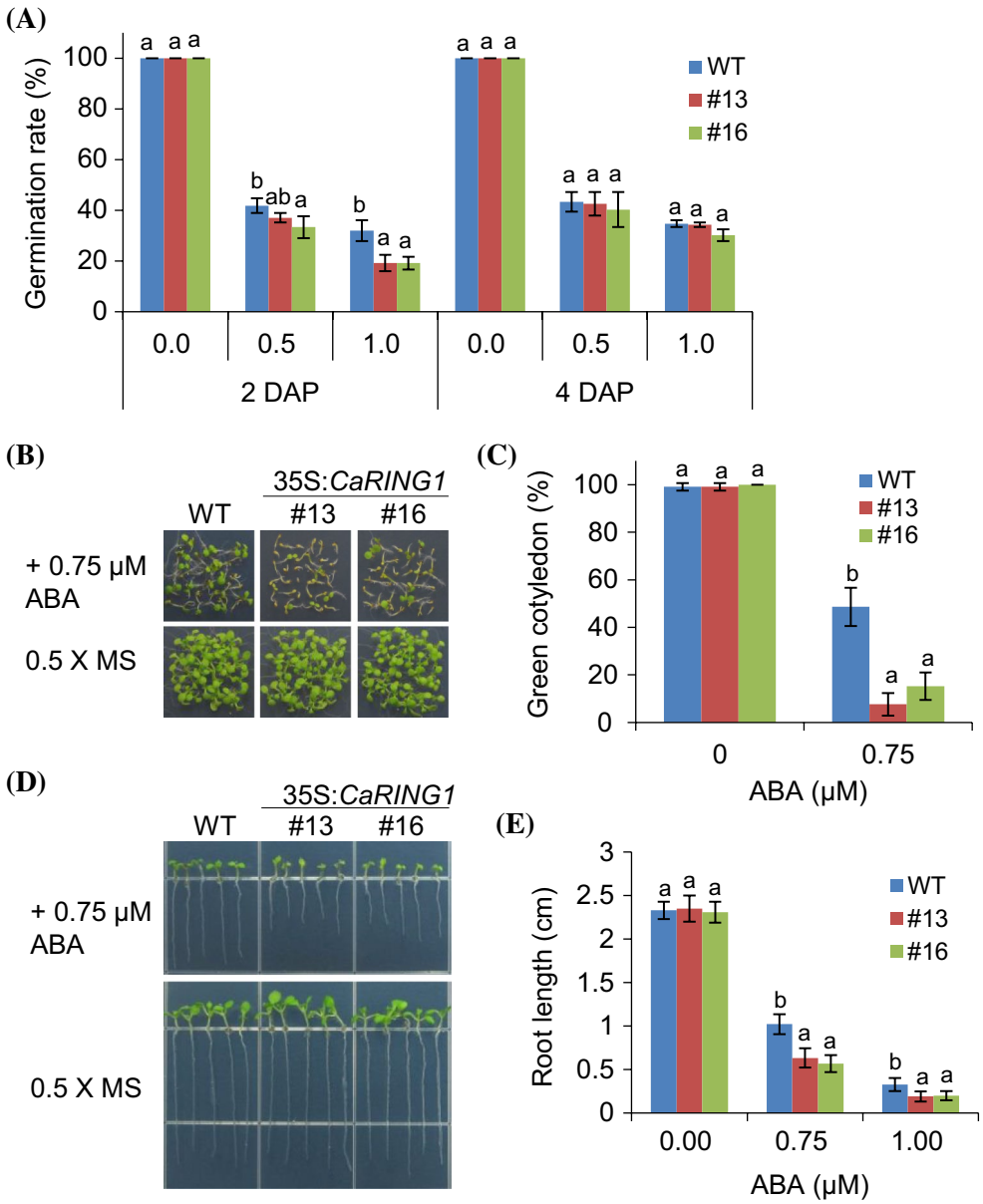

Fig. 4 Phenotypic analysis of the *35S:CaRING1* transgenic Arabidopsis mutants under ABA treatment. **a** Germination rates of wild-type and transgenic plants exposed to norflurazon and ABA. The percentage of seeds with radicle emergence was scored 2 and 4 days after plating on 0.5X MS containing 50 µM norflurazon and various concentration of ABA. **b**, **c** Seedling establishment of wild-type and *35S:CaRING1* plants in 0.5X MS containing 0.75 µM ABA. The number of seedlings with green cotyledons was counted 7 days after sowing and representative images were simultaneously taken. **d**, **e** Seedling growth of wild-type and *35S:CaRING1* plants exposed to ABA. The seedlings were grown vertically in 0.5X MS containing 0.75 and 1.0 µM ABA for 7 days. The representative images were taken (**d**) and root length of each line was measured (**e**). Data are the mean ± standard deviation from three independent experiments each evaluating 50 seeds. *Different letters* indicate significant differences in three independent experiments (ANOVA; $P < 0.05$)

investigate functional involvement of CaRING1 in ABA response using *35S:CaRING1* Arabidopsis plants, we initially analyzed the effect of ABA on seed germination and seedling establishment of *35S:CaRING1* plants. In the germination assay, there were no significant differences in seed germination between *35S:CaRING1* and wild-type plants with or without ABA treatment (data not shown). However, application of norflurazon, an inhibitor of endogenous ABA synthesis (Piskurewicz et al. 2008), allowed observing effect of *CaRING1* constitutive expression during germination. Compared with wild-type plants, germination rate was low in *35S:CaRING1* plants only at 2 days after sowing (Fig. 4a). Although norflurazon prevented root elongation and seedling establishement, the lengths of the radicles

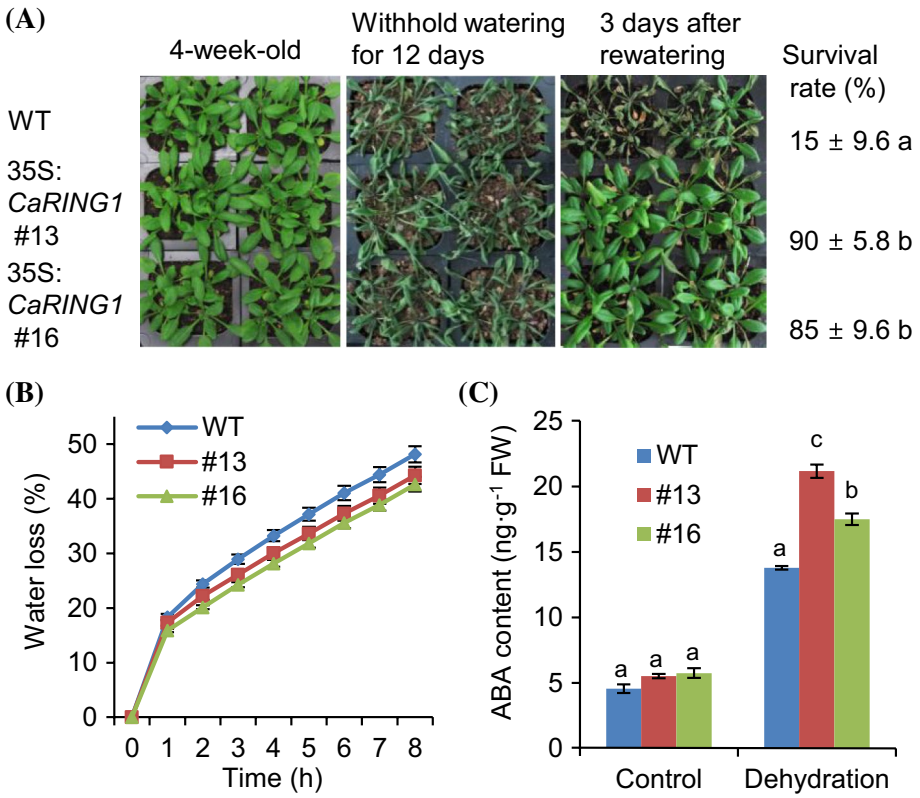

Fig. 5 Enhanced dehydration tolerance of the *35S:CaRING1* transgenic Arabidopsis plants. **a** Dehydration sensitivity of the *35S:CaRING1* plants. Three-week-old wild-type (WT) and transgenic plants were treated with dehydration stress by withholding water for 12 days, followed by rehydration for 3 days. The representative images were taken and percentages of plants that survived were measured. **b** Transpirational water loss of leaves of wild-type and transgenic plants. Rate of water loss from leaves of each line was measured every hour after detachment of leaves for 8 h. Data are the mean ± standard error from three independent experiments each evaluating 12 plants. **c** ABA content in leaves of *35S:CaRING1* and wild-type plants after dehydration treatment for 2 h. Data are the mean ± standard error from three independent experiments. *Different letters* indicate significant differences in three independent experiments (ANOVA; $P < 0.05$)

emerged from *35S:CaRING1* seeds were quite shorter than those of wild-type seeds (Supplemental fig. S4). Consistently, application of 0.75 μM ABA into the media led to strong inhibition of 35S: *CaRING1* seedling growth, relative to that of wild-type plants (Fig. 4b). The development of an expanded green cotyledon in the 35S:*CaRING1* plants was also less than 11–21 % that of the wild-type plants (Fig. 4c). We then analyzed the root lengths of wild-type and *35S:CaRING1* plants treated with various concentrations of ABA (Fig. 4d, e). Seven days after treatment, the inhibitory effects of ABA on root growth were observed in wild-type and 35S:*CaRING1* plants (Fig. 4d). In the presence of 0.75 μM ABA, growth of 35S:*CaRING1* roots was retarded in contrast to that observed in wild-type roots. In plants treated with 0.75 or 1.0 μM ABA, the root length of 35S:*CaRING1* plants was less than 40–60 % of that in the wild-type plants (Fig. 4e). One of the Arabidopsis genes has high sequence homology with *CaRING1*, loss-of-function mutants of *RFH2* gene (At1g20823) exhibited the opposite pattern in root elongation in the presence of 0.75 μM ABA (Supplemental fig. S5a and b). Root lengths of *rfh2* mutants were at least threefold longer than those of WT. These results indicate that constitutive expression of the *CaRING1* gene confers enhanced ABA sensitivity in Arabidopsis during seedling establishment.

Enhanced tolerance of 35S:*CaRING1* Arabidopsis transgenic plants to dehydration stress

Since the *CaRING1* gene was induced in dehydrated pepper leaves (Fig. 1) and silencing of *CaRING1* gene in pepper plants reduced dehydration tolerance (Fig. 3), we investigated the dehydration tolerance of 35S:*CaRING1* plants (Fig. 5). Wild-type and 35S:*CaRING1* plants were grown for 4 weeks under normal growth conditions and were then subjected to dehydration stress when grown under well-watered conditions (Fig. 5a, left panel), and after dehydration treatment in which water was withheld for 12 days (Fig. 5a, middle panel), there was no phenotypic difference between the two plant lines. However, after re-watering for

3 days (Fig. 5a, right panel), the transgenic lines exhibited a phenotype with less wilting compared with that observed in wild-type plants. Only 15 % of wild-type plants survived, whereas 90 and 85 % of 35S:*CaRING1* lines #13 and #16, respectively, were able to resume their growth and survived (Fig. 5a). Interestingly, loss-of-function mutants of *RFH2* gene showed no difference in dehydration tolerance compared with wild-type plants (Supplemental fig. S5c), indicating that *CaRING1* has multiple functions relative to Arabidopsis *RFH2* gene.

To further evaluate the response to dehydration stress, we measured the fresh weight of detached rosette leaves to monitor transpirational water loss and to thus determine whether the dehydration tolerant phenotype of the *35S:CaRING1* plants resulted from a low transpiration rate (Fig. 5b). The weights of rosette leaves detached from 4-week old plants of both wild-type and 35S:*CaRING1* lines were measured over time (0–8 h) and approximately 47 and 42–43 % of their fresh weights were decreased, respectively (Fig. 5b). Since silencing of *CaRING1* partially suppressed ABA biosynthesis (Fig. 3c), we measured ABA levels in leaves of 35S:*CaRING1* plants treated with dehydration stress for 2 h (Fig. 5c). Under normal condition, ABA levels were not significantly differences between 35S:*CaRING1* and wild-type plants. However, upon dehydration stress, 35S:*CaRING1* plants exhibited high ABA accumulation compared with wild-type plants. These data suggest that the level of *CaRING1* expression is positively correlated with stress tolerance. The molecular mechanisms responsible for the positive effects of ABA under dehydration stress are well established (Zhu 2002; Yamaguchi-Shinozaki and Shinozaki 2006). In addition, many studies have reported that ABA sensitivity is associated with dehydration tolerance (Cheong et al. 2007; Lim and Lee 2014; Ryu et al. 2010; Santiago et al. 2009). Based on these, constitutive expression of *CaRING1* gene may lead to increase of ABA-mediated dehydration tolerance. However, we cannot rule out the possibility that ABA-independent responses were also altered, because induction of *CaRING1* gene expression in pepper plant was faster by dehydration stress than by ABA treatment (Fig. 1a, b).

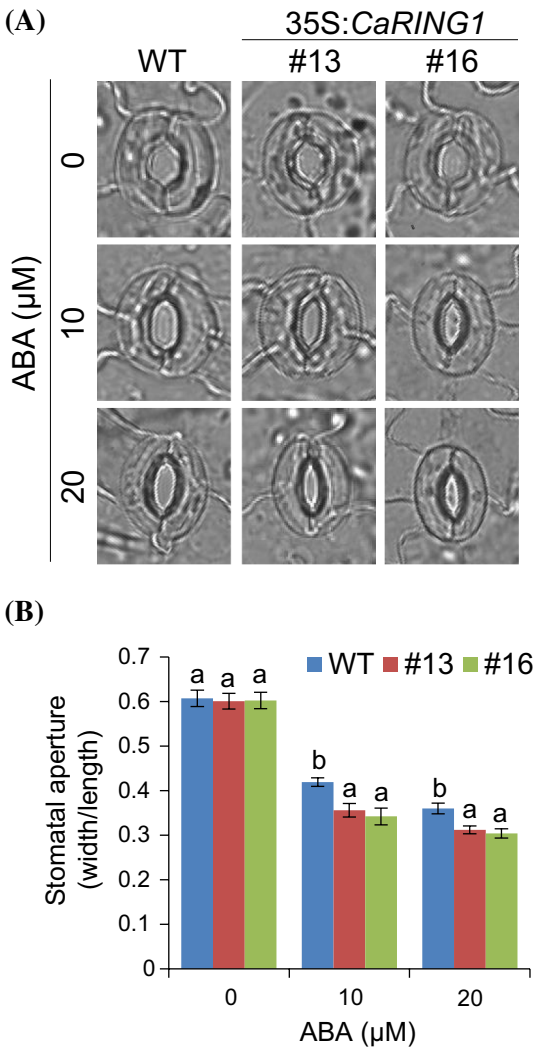

Fig. 6 ABA-induced stomatal closing in leaves of *35S:CaRING1* mutants. Stomatal apertures were measured from leaf peels of wild-type and *35S:CaRING1* mutants plants treated with 10 and 20 μM ABA for 2.5 h and the pictures show representative stomata from each sample (**a**). Average stomatal aperture was calculated as pore width/length (**b**). Data are the means ± standard errors (n = 100). Values are the mean ± SE from three independent experiments. Statistical analysis was performed using ANOVA test ($P < 0.05$) and significant differences between wild-type and mutant plants are indicated by *different letters*

Participation of CaRING1 in ABA-induced stomatal aperture and induction of ABA- or dehydration-related genes in 35S:*CaRING1* Arabidopsis transgenic plants

To determine whether the enhanced dehydration tolerance of 35S:*CaRING1* Arabidopsis transgenic plants is associated with the ABA response, we measured the size of stomatal pores with or without ABA treatment (Fig. 6). There were no significant differences in the size of the stomatal aperture between the wild-type and 35S:*CaRING1* leaves in the absence of ABA. However, the size of stomatal pores decreased more dramatically in leaves of 35S:*CaRING1* plants than in those of wild-type plants after treatment with 10 or 20 μM ABA. These data indicate that ABA hypersensitivity in the guard cells of 35S:*CaRING1* plants may enhance water retention, leading to a dehydration tolerance phenotype.

Since the ectopic expression of *CaRING1* is positively correlated with ABA sensitivity, we examined whether *CaRING1* affects the expression levels of ABA biosynthesis-related or ABA-induced genes including *NCED3*,

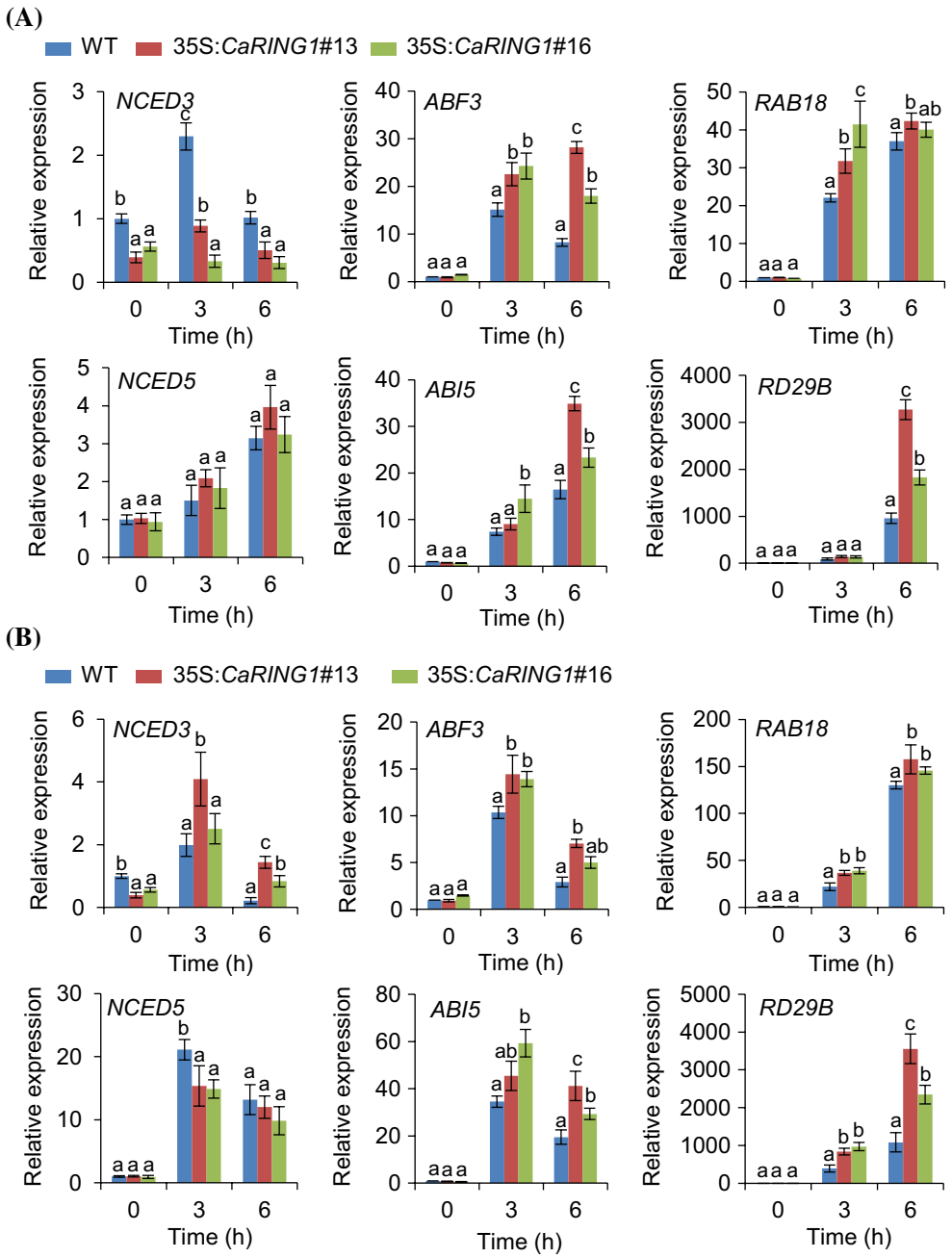

Fig. 7 Expression analysis of ABA biosynthesis-related and ABA-responsive genes in leaves of the *35S:CaRING1* transgenic plants and wild-type plants treated with ABA (50 μM; **a**) and subjected to dehydration (**b**). Relative expression level (ΔΔCT) of *each gene* was normalized to that of *Actin8* as an internal control gene and compared with the value for mock-treated leaves. Data are the means ± standard error from three independent experiments. *Different letters* indicate significant differences in three independent experiments (ANOVA; $P < 0.05$)

NCED5, *ABF3*, *ABI5*, *RAB18,* and *RD29B* (Fig. 7a). Under normal growth conditions, the expression levels of these genes were not significantly different between the wild-type and *35S:CaRING1* plants except for *NCED3*. Compared with the wild-type plant, low expression of *NCED3* in 35S:*CaRING1* did not support the possibility that *CaRING1* promotes *NCED3* gene expression as shown in TRV:*CaRING1* (Fig. 3c). However, in contrast to pepper *NCED3* gene, alteration of Arabidopsis *NCED3* gene did not made difference in ABA content between 35S:*CaRING1* plants and wild-type plants under non-treatment condition (Fig. 5c). This discrepancy may be explained by functional diversity of CaRING1 and different function of NCED3 between two plant species. To

examine effect of ABA on transcriptional alteration of those ABA signaling-related genes, 4-week-old wild-type and 35S:*CaRING1* plants were treated with 50 μM ABA. The level of *NCED3* and *NCED5* transcription, which are associated with ABA synthesis (Frey et al. 2012), was significantly lower in *35S:CaRING1* than in wild-type plants and not different, respectively, (Fig. 7a). However, there was higher accumulation of *ABF3* and *ABI5*, which encode basic leucine zipper (bZIP) transcription factors (Chen et al. 2013), in 35S:*CaRING1* compared with wild-type plants, indicating that *CaRING1* may act upstream of these transcription factors in the ABA signal transduction pathway. Moreover, induction of the ABA-responsive marker genes *RAB18* and *RD29B* was also significantly higher in 35S:*CaRING1* than in the wild-type plants (Fig. 7a). This increased expression of several ABA-induced marker genes may reflect ABA hypersensitivity of 35S:*CaRING1* plants.

In line with these results, to determine that the tolerance phenotype of 35S:*CaRING1* plants to dehydration stress was also influenced by the level of ABA- or dehydration-induced marker gene expression, we performed qRT-PCR analysis in wild-type and 35S:*CaRING1* plants subjected to dehydration conditions (Fig. 5b). There was higher accumulation of *NCED3* transcripts in 35S:*CaRING1* plants compared with wild-type plants after treatment with dehydration stress, whereas the level of *NCED5* transcription was lower in 35S:*CaRING1* plants. This may contribute to high accumulation of ABA in 35S:*CaRING1* plants treated with dehydration stress (Fig. 5c). As shown in Fig. 7b, the expression levels of transcription factors and dehydration -induced marker genes, including *ABF3*, *ABI5*, *RAB18,* and *RD29B*, were significantly higher in the 35S:*CaRING1* plants than in the wild-type plants in response to dehydration-stress conditions, indicating that changes in the expression of stress genes may underlie the altered dehydration tolerance in 35S:*CaRING1* plants. In addition, although stress-related gene expression showed some degree of variation in the two transgenic lines, ectopic expression of *CaRING1* may positively regulate the expression of stress marker genes in plants treated with ABA and exposed to dehydration stress, thereby presumably enhancing dehydration stress tolerance in the 35S:*CaRING1* plants.

In conclusion, these findings suggest that CaRING1 acts as a positive regulator to provide tolerance to dehydration stress through regulation of ABA biosynthesis, ABA-induced stomatal closure, and ABA-mediated stress-responsive gene expression. Although we elucidated the in vivo functions of CaRING1 in plant defense responses to pathogen infection and dehydration, it is still unclear how CaRING1 serves as a positive regulator of biotic and abiotic stress responses. Subsequent molecular and physiological analysis of the downstream target(s) of CaRING1 will improve our understanding of the function of CaRING1 under biotic and abiotic stress conditions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights Human participants and/or animals have not involved in this research.

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