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Research article

CaWRKY27 negatively regulates salt and osmotic stress responses in pepper

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ABSTRACT

WRKY transcription factors are key regulatory components of plant responses to both biotic and abiotic stresses. In pepper (*Capsicum annuum*), CaWRKY27 positively regulates resistance to the pathogenic bacterium *Ralstonia solanacearum* and negatively regulates thermotolerance. Here, we report that CaWRKY27 functions in the response to salinity and osmotic stress. *CaWRKY27* transcription was induced by salinity, osmotic, and abscisic acid (ABA) treatments, as determined using qPCR and GUS assays. Transgenic *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum*) plants heterologously expressing *CaWRKY27* had an increased sensitivity to salinity and osmotic stress, with a higher inhibition of both root elongation and whole plant growth, more severe chlorosis and wilting, lower germination rates, and an enhanced germination sensitivity to ABA than the corresponding wild-type plants. Furthermore, most marker genes associated with reactive oxygen species (ROS) detoxification and polyamine and ABA biosynthesis, as well as stress-responsive genes *NtDREB3*, were downregulated in plants transgenically expressing *CaWRKY27* upon exposure to salinity or osmotic stress. Consistently, silencing of *CaWRKY27* using virus-induced gene silencing conferred tolerance to salinity and osmotic stress in pepper plants. These findings suggest that CaWRKY27 acts as a molecular link in the antagonistic crosstalk regulating the expression of defense-related genes in the responses to both abiotic and biotic stresses by acting either as a transcriptional activator or repressor in pepper.

1. Introduction

In their natural habitats, plants inevitably encounter biotic stresses, such as pathogens, and abiotic stresses, such as salt, drought, heat, and cold. To withstand these stresses, which are often experienced concurrently, plants have evolved sophisticated defense mechanisms that perceive the stress, initiate defense signaling, and translate the signaling into an appropriate defense reaction. Increasing evidence suggests the existence of extensive crosstalk between the plant responses to biotic and abiotic stresses, which is believed to enable plants to fine-tune their responses to stress and ensure they react appropriately (Fujita et al., 2006). However, the detailed mechanisms underlying this crosstalk are yet to be elucidated.

Drought and salt stresses are two important abiotic stresses with similar adverse effects on the growth and development of plants and hence on crop yields. Plants exposed to these stresses generally undergo cellular dehydration and osmotic stress, and exhibit oxidative damage to membranes, proteins, RNA, and DNA caused by reactive oxygen species (ROS) bursts and accumulation (Miller et al., 2008; Møller and Sweetlove, 2010). To adapt to these stresses, plants initiate signaling pathways, including the abscisic acid (ABA)-dependent, ABA-independent, and salt overly sensitive (SOS) signaling pathways, which play crucial roles during the plant adaptive response to salt stress and drought (Huang et al., 2012; Yang and Guo, 2018; Zhu, 2002). These signals accumulate in the nucleus, where they induce transcriptional reprogramming via the action of various transcription factors (TFs) such as CBF/DREB1, DREB2, AREB/ABF, bZIP, MYB/MYC, and WRKY (Ding et al., 2015; Huang et al., 2012; Mondini et al., 2012; Zhu et al., 2019), leading to the reestablishment of cellular ionic, osmotic, and ROS homeostasis and ultimately enhancing plant tolerance to salt stress or drought (Mittler et al., 2004). Despite these insights, the mechanisms underlying plant responses to salt or drought stress have not been fully elucidated.

Members of one of the largest TF families, the WRKYs, are characterized based on the presence of one or two conserved WRKY domains, which bind to the highly conserved cognate W-box (TTGACC/T) (Eulgem et al., 2000; Rushton et al., 2010). Members of this family have been implicated in plant responses to stresses (Jiang et al., 2017),

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including pathogens (Amorim et al., 2017; Sarris et al., 2015), heat (Cai et al., 2015; Dang et al., 2013; Li et al., 2009, 2010, 2011), cold (Marè et al., 2004; Yokotani et al., 2013), salinity (Ding et al., 2015; Hichri et al., 2017), drought (Luo et al., 2013; Marè et al., 2004), and phosphate starvation (Baek et al., 2017). The roles of the WRKY TFs have been intensively investigated in plant immune responses to various pathogens with different lifestyles, and several *WRKY* genes have been found to be transcriptionally modulated by pathogen attack within a given plant species (Bencke-Malato et al., 2014; Jiang et al., 2014). Some of these pathogen responsive WRKYs function in networks as positive or negative regulators of plant immunity (Birkenbihl et al., 2017; Eulgem and Somssich, 2007), and some have been found to be induced by salinity and drought (Jiang et al., 2015; Wei et al., 2018). For example, *FvWRKY42*, a WRKY TF in the diploid woodland strawberry (*Fragaria vesca*), enhances resistance to powdery mildew and improves osmotic stress resistance (Wei et al., 2018), indicating its role in the crosstalk between the response to pathogens and other stresses and a possible function in coordinating the responses to different stresses. However, the roles of different WRKY TFs in terms of coordinating plant immunity and other biological processes have not been fully understood.

Pepper (*Capsicum annuum*) is a commercially important vegetable in the Solanaceae family. The growth and development of pepper plants are frequently challenged by attack from soil-borne pathogens, such as *Ralstonia solanacearum* (RS), and these plants are often exposed to abiotic stresses such as heat, drought, and salt. A subset of WRKY TFs, including *CaWRKY6* (Cai et al., 2015), *CaWRKY22* (Hussain et al., 2018), *CaWRKY27* (Dang et al., 2014), *CaWRKY40* (Dang et al., 2013), and *CaWRKY40b*, have been found to be modulated by RS and act as positive or negative regulators of the pepper response to this pathogen. Among these genes, *CaWRKY6* (Cai et al., 2015), *CaWRKY22* (Hussain et al., 2018), and *CaWRKY40* (Dang et al., 2013) were also found to act as positive regulators of the pepper response to heat stress, while *CaWRKY27* (Dang et al., 2018) acts as a negative regulator of this process, indicating a role of CaWRKY27 as a crucial node in the crosstalk between pepper immunity and thermotolerance. Here, we provide evidence that *CaWRKY27* is also transcriptionally modulated by salt and drought stress and acts as a negative regulator of the response to these two abiotic stresses.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds from *Capsicum annuum* 8# and tobacco (*Nicotiana tabacum*) cultivar K326 were soaked in water overnight at 26 ± 1 °C, and then sown in steam-sterilized soil mix (peat moss, vermiculite, and perlite; 2/1/1, v/v/v) in plastic pots. Plants were grown in a growth room maintained at 26 ± 1 °C with a light intensity of ~100 μmol photons m^{-2} s^{-1} and a relative humidity of 70% under a 16-h-light/8-h-dark cycle.

Wild-type (*Col-0*) and transgenic *Arabidopsis thaliana* seeds were treated at 4 °C for 3 d in darkness and then transferred to ½ MS and 0.8% agar plates, which were incubated in a growth chamber (22 ± 2 °C, ~100 μmol photons m^{-2} s^{-1}, 85% relative humidity, and a 12-h-light/12-h-dark cycle).

2.2. Construction of transgenic plants

To construct vectors for the transgenic expression of *CaWRKY27*, the full-length open reading frame (ORF) of *CaWRKY27* along with the 2000-bp promoter fragment upstream ATG codon of *CaWRKY27* was amplified and cloned into the pK7WG2 (for transgenic expression) and pMDC163 vectors (Thermo Fisher Scientific, USA; for *CaWRKY27* expression assay), respectively. The resulting pK7WG2-*CaWRKY27* and *pCaWRKY27::GUS* constructs were transformed into *Agrobacterium tumefaciens* (strain GV3101) using the freeze-thaw method. *Arabidopsis* was transformed with the constructs using the floral dip method, and the transgenic lines were selected by germinating the seeds on ½ MS medium containing kanamycin (50 mg L^{-1}) or hygromycin (50 mg L^{-1}) to select for plants containing pK7WG2-*CaWRKY27* or *pCaWRKY27::GUS*. The T_4 homozygous lines were employed for further phenotypic effect assays. The T_3 homozygous *CaWRKY27*-expressing tobacco lines were obtained and phenotyped as described previously (Dang et al., 2013).

2.3. Salt, drought, and ABA treatments

For the drought stress treatments, pepper or tobacco seedlings at the six-leaf stage or 21-day-old *Arabidopsis* seedlings were withheld from watering for about 15 days followed by rewatering. For the salt and osmotic stress treatments, *CaWRKY27*-silenced plants germinated and grown in 1/5 Hoagland solution for about one month were treated with or without 100 mM NaCl or 200 mM mannitol for three days and then photographed. In addition, the aerial tissues were harvested at the indicated time points for RNA isolation to assay the transcript levels of *CaWRKY27* and defense-related marker genes.

To assay the effect of the heterologous expression of *CaWRKY27* on the tolerance of *Arabidopsis* to the exogenous application of ABA, salt stress, and mannitol treatments, the seeds were germinated on ½ MS media supplemented with 10 μM ABA, 100 mM NaCl, or 200 mM mannitol. The effect of the stress treatments was detected at the indicated time points. To assess the effect of ABA treatment on pepper plants, seedlings at the six-leaf stage were sprayed with 10 μM ABA in a confined space. Some of these ABA treated plants were harvested at 0, 1, 3, 6, 12, 24, 36, and 48 h post treatment (hpt) and RNA was extracted and used to investigate the transcript levels of the defense-related marker genes and *CaWRKY27*.

2.4. Histochemical staining

To detect GUS expression, the samples were immersed in GUS staining solution [1 mg mL^{-1} X-Gluc, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, 50 mM sodium phosphate buffer (pH 7.0), 10 mM Na_2EDTA, and 0.1% TritonX-100] and incubated overnight at 37 °C. Their chlorophyll was then removed with several washes of 75% ethanol and the phenotypes were observed and documented using a stereomicroscope (Leica Microsystems, Germany).

2.5. Electrolyte leakage measurements

Electrolyte leakage assays in *Arabidopsis* (Clarke et al., 2004) and pepper (Kim et al., 2010) were performed as described previously. Briefly, 4-cm-diameter leaf disks were washed in sterile double-distilled water for 30 min with slight agitation for 1 h at 25 °C. The electrolyte leakage was detected using a conductivity meter (Mettler Toledo, USA).

2.6. Virus-induced gene silencing

Tobacco rattle virus (TRV)-based virus-induced gene silencing was performed to generate *CaWRKY27*-silenced pepper plants (*TRV::CaWRKY27* and *TRV::CaWRKY27-3′UTR*). This process, using highly specific fragments of the *CaWRKY27* conding sequence or the *CaWRKY27-3′UTR*, was described previously (Kim et al., 2010). Fully expanded pepper cotyledons were coinfiltrated with *A. tumefaciens* (strain GV3101) carrying *TRV::00* as well as *TRV::PDS35*, *TRV::CaWRKY27*, and *TRV::CaWRKY27-3′UTR*. About 3 weeks later, a photobleaching phenotype was observed due to phytoene desaturase (PDS) silencing in the positive control pepper plants. The transcript levels of *CaWRKY27* were measured using qRT-PCR in the *TRV::CaWRKY27* and *TRV::CaWRKY27-3′UTR* pepper plants after exposure to salt and drought stress.

2.7. Gene expression analysis

Total RNA was extracted from *Arabidopsis*, pepper, and tobacco plants using the Takara MiniBEST Universal RNA Extraction Kit (Takara Bio., Japan). A 1-μg aliquot of RNA was used to synthesize cDNA with the Takara PrimeScript RT-PCR kit according to the manufacturer's instructions. Transcript levels were measured with a CFX96 real-time PCR instrument (Bio-Rad Laboratories, USA), the SYBR Premix Ex Taq II reagent (Takara Perfect Real Time), and specific primers (Supplementary Table S1). *Arabidopsis UBIQUITIN10* (*AtUBQ10*), tobacco *Elongation factor 1 alpha* (*NtEF1α*), and pepper *Actin1* (*CaActin1*) were used for normalization.

3. Results

3.1. *CaWRKY27* expression is induced by both salt and drought stress

The *CaWRKY27* promoter is enriched with various *cis*-elements, including TCA elements, ABREs, and CGTCA motifs, that are responsive potentially to phytohormones associated with plant immune and stress responses, including salicylic acid, ABA, and jasmonic acid (Narusaka et al., 2003). The presence of these *cis*-elements indicates that *CaWRKY27* might play a role in coordinating the responses of pepper to multiple stresses. We previously demonstrated that *CaWRKY27* is a positive regulator of the pepper response to RS infection (RSI), but a negative regulator of thermotolerance (Dang et al., 2014, 2018).

The presence of the *cis*-elements MBS and ABRE (Supplementary Fig. 1) in the *CaWRKY27* promoter implies that *CaWRKY27* might also be involved in the plant's response to salt and osmotic stress. To test this hypothesis, we examined the transcript levels of *CaWRKY27* in pepper leaves at different time points after exposure to salt stress, mannitol (mimicking drought), and exogenous ABA, a phytohormone associated with the plant's response to abiotic stresses, particularly osmotic stress (Fujita et al., 2011), using qRT-PCR analysis. The abundance of *CaWRKY27* transcripts increased from 1 to 24 h after the ABA treatment, from 1 to 12 h after the mannitol-mimicked drought stress (200 mM mannitol), and from 1 to 48 h after the salt stress (200 mM NaCl) (Fig. 1A, B and C).

We further examined the response of *CaWRKY27* to osmotic stress using transgenic *Arabidopsis thaliana* plants expressing *GUS* driven by the *CaWRKY27* promoter (*pCaWRKY27::GUS*). The expression of *GUS* in both the leaves and roots was extremely low in non-stressed 7-day-old transgenic *Arabidopsis* seedlings, but was significantly upregulated when the plants were treated with ABA (100 μM), mannitol (200 mM), or salt stress (100 mM) for 3 h (Fig. 2). The consistency between the qRT-PCR results (Fig. 1) and *GUS* expression patterns (Fig. 2) implies that *CaWRKY27* functions in the pepper response to salt stress and drought mediated by ABA-dependent signaling.

3.2. *CaWRKY27*-silenced pepper plants have enhanced drought and salt stress tolerance

Next, we examined the role of CaWRKY27 in the plant's response to drought using loss- and gain-of-function assays. A 250-bp fragment of the 3′ untranslated region (UTR) of *CaWRKY27* was used to silence *CaWRKY27* via virus-induced gene silencing. The *CaWRKY27* transcript levels in the *CaWRKY27*-silenced pepper plants were 10–15% those of the control plants (Fig. 3A). After a 15-day drought treatment followed by 3 days of rewatering, the *CaWRKY27*-silenced pepper plants displayed less damage than the control plants (Fig. 3B). This effect was also observed with hydroponically grown plants treated with mannitol to mimic drought (Fig. 3C). The survival rates of the two independent *CaWRKY27*-silenced pepper lines (63.7% and 60.2% of *TRV::CaWRKY27* and *TRV::CaWRKY27-3′UTR*, respectively) were significantly higher than that of the control plants (22.2%) (Fig. 3D). Consistently, the transpirational water loss from the leaves of the control plants was also greater than that of the *CaWRKY27*-silenced plants (Fig. 3E). These data indicate that *CaWRKY27* acts as a negative regulator of the pepper response to drought.

CaWRKY27 expression is known to be induced by salt stress (Fig. 1B). To confirm that *CaWRKY27* plays a role in the response to salt stress, the *CaWRKY27*-silenced and control plants were treated with 200 mM NaCl. The nontreated control and *CaWRKY27*-silenced plants showed no significant phenotypic differences under the control conditions; however, upon exposure to salt stress, the *CaWRKY27*-silenced plants were less affected than the control plants (Fig. 4A). Consistently, the ion leakage levels of the leaves in the control plants were much higher than those of the *CaWRKY27*-silenced plants (Fig. 4B). Thus, *CaWRKY27* acts as a negative regulator of the pepper response to salt stress.

3.3. Heterologous expression of *CaWRKY27* compromises the drought and salt stress tolerance of *Arabidopsis* and tobacco

We further examined the role of CaWRKY27 in the plant's response to salt and drought stress using tobacco (*Nicotiana tabacum*) and *Arabidopsis* lines heterologously expressing *CaWRKY27*, which we had generated previously (Dang et al., 2014, 2018).

The seeds of wild-type tobacco and two independent, homozygous T_2 lines with a single *CaWRKY27* insertion (*CaWRKY27-OE4* and *CaWRKY27-OE9*) were sown on MS media with or without 100 mM NaCl. At 20 days post-treatment (dpt; Supplementary Figs. 2A and B), the *CaWRKY27-OE4* and *CaWRKY27-OE9* tobacco plants exhibited significantly less growth, lower germination rates, shorter primary roots, and lower fresh weights than the control plants under the stress conditions, while no significant differences were observed between the non-stressed transgenic and wild-type plants (Supplementary Figs. 2C

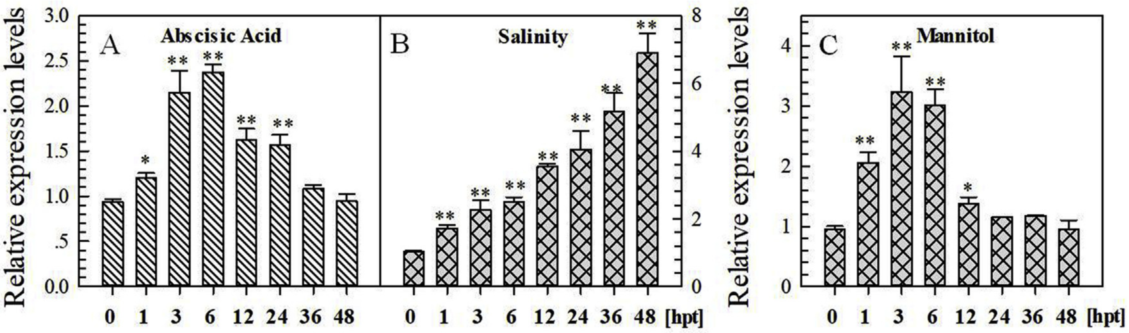

Fig. 1. *CaWRKY27* expression in plants exposed to salt and drought treatments. *CaWRKY27* expression was measured at the indicated time points in the leaves of pepper plants at the six-leaf stage after a challenge with exogenous ABA (10 μM), salt stress (100 mM NaCl), or mannitol (200 mM; mimicking drought stress). Pepper *Actin1* was used as an internal control. Error bars indicate the standard error from experiments repeated three times with at least three independent biological repeats. Asterisks indicate a significant difference from the pretreated expression level (0 hpt; ANOVA Test, **P*-value $<$ 0.05 or ***P*-value $<$ 0.01).

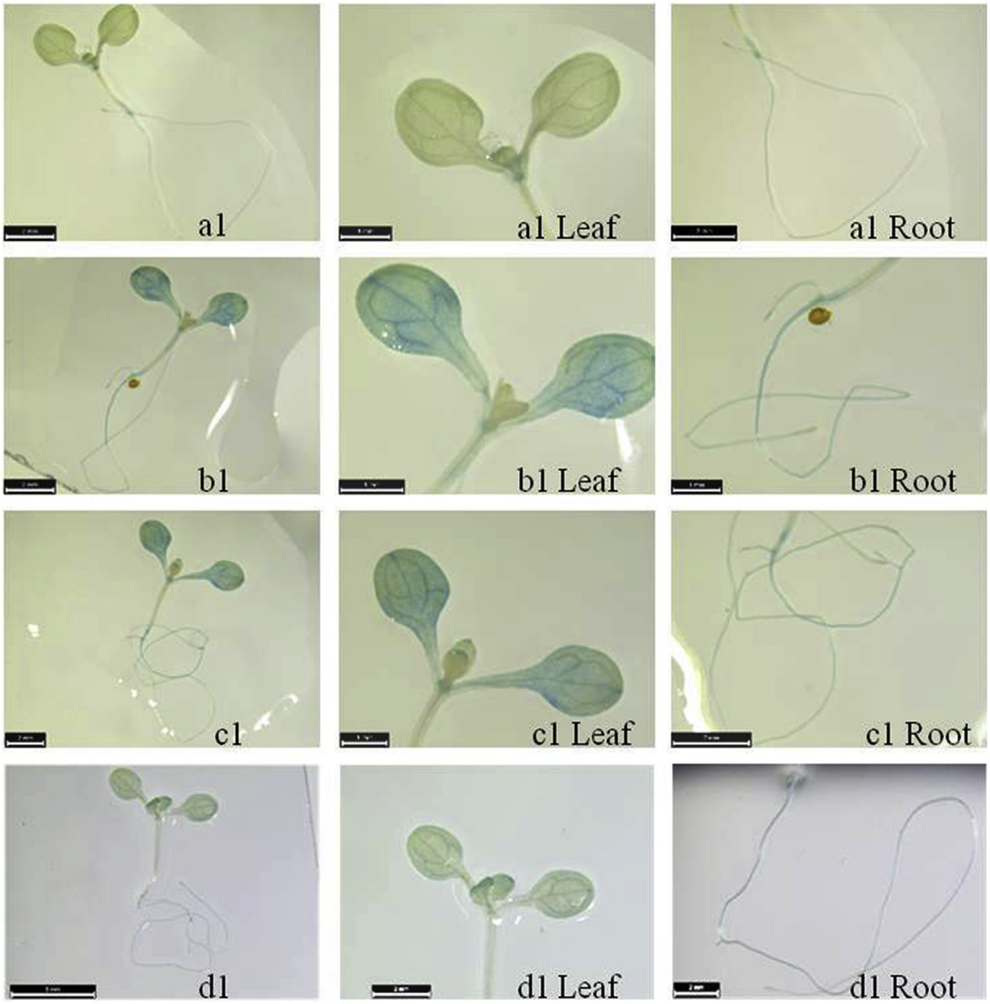

Fig. 2. *pCaWRKY27::GUS* activity is induced by ABA, salt, and mannitol treatments. *GUS* expression in transgenic *Arabidopsis* plants harboring the *pCaWRKY27::GUS* construct. (A) An untreated at 7 days after germination. (B-D). Transgenic *pCaWRKY27::GUS* seedlings treated with an exogenous application of ABA (B), salt stress (C), or mannitol (D).

and D).

In addition, the transgenic *CaWRKY27*-expressing tobacco plants were assayed for their tolerance to osmotic stress. The seeds were sown on MS media with or without 200 mM mannitol, and the seedlings were examined after 20 days of treatment. More severe wilting symptoms were observed in the transgenic plants than in the wild-type plants (Supplementary Figs. 3A–C). Consistently, the transgenic plants had much lower germination rates (Supplementary Fig. 3D) and fresh weights than the corresponding wild-type plants after 20 days of treatment (Supplementary Figs. 3E and F). These data collectively provide further evidence that CaWRKY27 acts as a negative regulator of the plant's response to both salt and osmotic stress.

In parallel, the seeds of wild-type *Arabidopsis* and two independent homozygous T_4 lines harboring a single *CaWRKY27* insertion (*CaWRKY27-OE10* and *CaWRKY27-OE12*) were sown on ½ MS media supplemented with different concentrations of salt or mannitol, and their phenotypes were analyzed after 14 days of treatment. No significant difference was detected in the primary root lengths of the transgenic and wild-type plants under control conditions; however, on the media supplemented with salt or mannitol, the primary root lengths of the two transgenic lines were significantly shorter than those of the wild type (Fig. 5A–D). In addition, the transgenic plants exhibited more serious wilt symptoms than the control plants when challenged with 14 days of drought stress and did not recover to the same extent as the control plants after a 3-day rewatering period (Fig. 5E and F), while no phenotypic differences were detected between these lines under well-watered conditions. These results are consistent with those from the transgenic *CaWRKY27*-expressing tobacco plants and collectively suggest that CaWRKY27 acts as a negative regulator of the plant's response to salt stress and drought.

To further confirm the results from phenotype assays and examine the possible mode of action for CaWRKY27 in the plant's response to salt and drought stress, we analyzed the transcription levels of 11 defense-associated marker genes after 24 and 48 h of treatment with salt stress in the *CaWRKY27-OE4* and wild-type tobacco plants. These genes included those involved in ROS detoxification (*NtSOD*, *NtGST1*, *NtPOX1*, and *NtPOX2*), polyamine biosynthesis (*NtADC1* and *NtSAMDC*), and ABA biosynthesis (*NtNCED1*), as well as *NtDREB3*, a TF involved in the regulation of osmotic tolerance, and the defense-related gene *NaERD10D*. When the plants were subjected to salt stress, the expression levels of most of the tested genes were upregulated in the wild type plants at least at one of the two tested time points, with the exception of *NtAPX1*, which was downregulated after 24 and 48 h of salt stress treatment. When the transgenic *CaWRKY27*-expressing plants were subjected to salt stress, all of the tested genes were downregulated, with the exception of *NtNCED1* and *NtDREB3*, which were upregulated after 48 h of treatment (Supplementary Fig. 4).

The effect of transgenic *CaWRKY27* expression on the expression levels of the above-mentioned genes was also tested in tobacco plants challenged with 250 mM mannitol. Similar to observations in plants

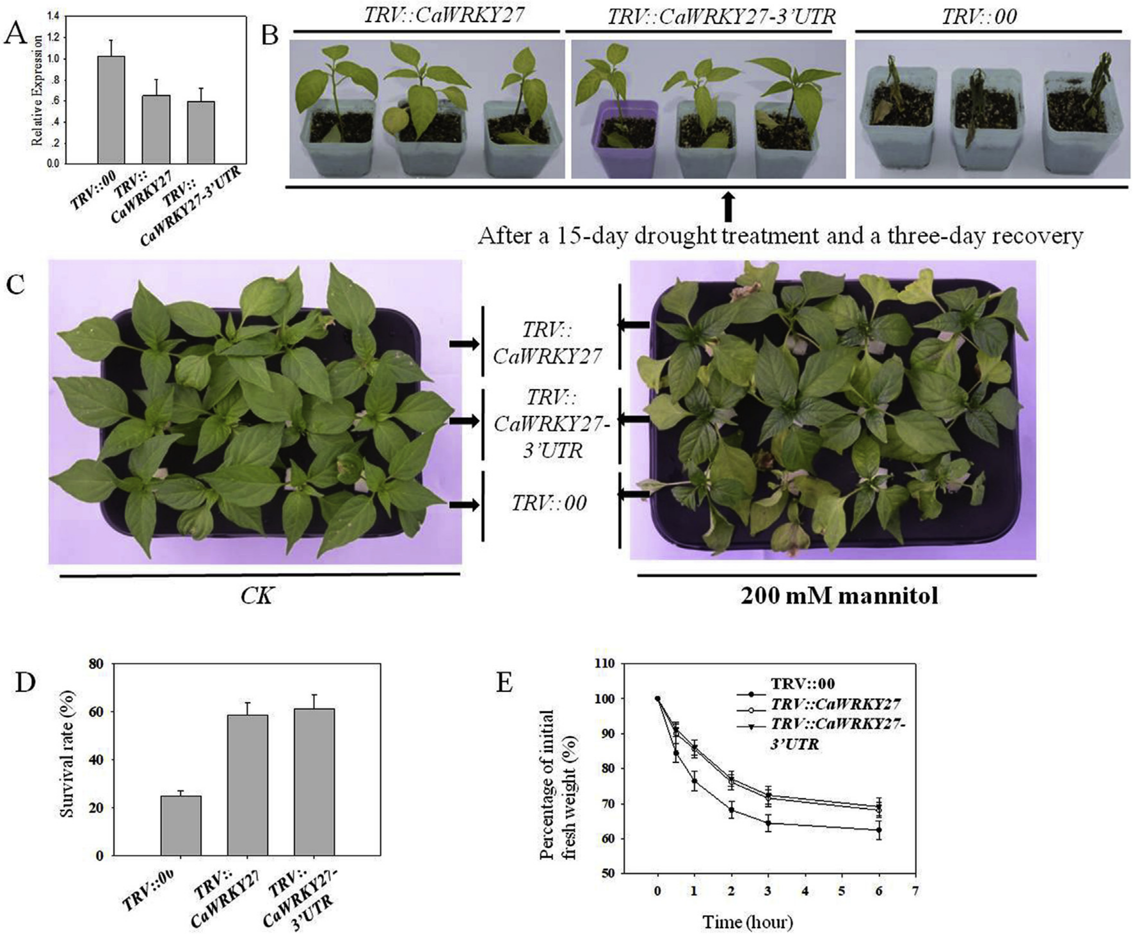

Fig. 3. *CaWRKY27*-silenced plants have enhanced drought tolerance. (A) The transcript levels of *CaWRKY27* in the leaves of the control (*TRV::00*) and *CaWRKY27*-silenced (*TRV::CaWRKY27*) pepper plants determined using qRT-PCR. The data represent the mean ± standard error of three independent experiments. (B) The phenotype of 6-week-old *TRV::00* and *TRV::CaWRKY27* pepper plants after 15 days without watering followed by a 3-day rewatering and recovery period. (C) The phenotype of 20-day-old *TRV::00* and *TRV::CaWRKY27* pepper plants treated with 200 mM mannitol or a control solution (CK) at 5 dpt. (D) Survival rates of *TRV::00* and *TRV::CaWRKY27* pepper plants after a 14-day period of no watering, followed by 3 days of rewatering. (E) Transpirational water loss from the leaves of *TRV::00* and *TRV::CaWRKY27* pepper plants at various time points after detachment. (D) and (E) Data represent the mean ± standard error of three independent experiments, each including 20 plants.

subjected to salt stress, the responses of the tested genes to mannitol differed between the control and *CaWRKY27*-expressing plants (Supplementary Fig. 5). In the control plants, most of the tested genes were upregulated by mannitol at least at one of the two tested time points, with the exception of *NtSAMDC*, which was repressed by mannitol at both time points. Unlike the response to salt stress, in which all of the tested genes except for *NtNCED1* and *NtDREB3* were downregulated by heterologous *CaWRKY27* expression, all of the tested genes were downregulated by *CaWRKY27* in response to mannitol. Noticeably, some of the tested genes exhibited different expression patterns between the two time points under salt stress or mannitol treatment; for example, *NtAPX1*, *NtSOD1*, *NtPOX1*, *NtPOX2*, *NtADC1*, or *NtDREB3* expression was upregulated at one time point and downregulated at the other under the mannitol treatment, whereas *NtPOX1*, *NtPOX2*, *NtGST1*, *NtERD10D*, and *NtDREB3* exhibited a similar pattern under the salt stress conditions.

3.4. Arabidopsis plants heterologously expressing CaWRKY27 have decreased ABA sensitivity

We demonstrated that exogenous ABA induced *CaWRKY27* expression (Fig. 1), indicating that *CaWRKY27* might be functionally related to the ABA-dependent signaling pathway. To test this hypothesis, we assayed the sensitivity of the *CaWRKY27*-expressing *Arabidopsis* plants to exogenously applied ABA by germinating the seeds on ½ MS medium supplemented with 0.0, 0.5, 1.0, or 2.0 μM ABA. No obvious difference in primary root length was observed between the *CaWRKY27-OX* and wild-type seedlings cultured under these conditions (Fig. 6A); however, the transgenic plants produced longer primary roots than the control plants when treated with exogenous ABA (Fig. 6B–E). The fresh weights and germination rates of the transgenic plants were significantly lower than the wild-type plants when treated with exogenous ABA but not in the control conditions (Fig. 6F and G).

To determine whether the heterologous expression of *CaWRKY27* influences the transcription of the ABA-related genes in *Arabidopsis*, we analyzed the relative expression levels of *AtEM6*, *AtAF7*, *AtRD29A*, and *At20* using qRT-PCR. Higher expression levels of these genes were found in the *CaWRKY27*-expressing Arabidopsis plants than in the control plants (Fig. 6H–K), indicating that *CaWRKY27* expression affected ABA-related processes.

4. Discussion

We previously revealed that *CaWRKY27* is a positive regulator of the pepper response to RSI and a negatively regulator of the response to heat stress (Dang et al., 2014, 2018). In this study, we demonstrated that *CaWRKY27* also acts as a negative regulator of the pepper response to salt stress and drought. *CaWRKY27* expression is induced by both salt

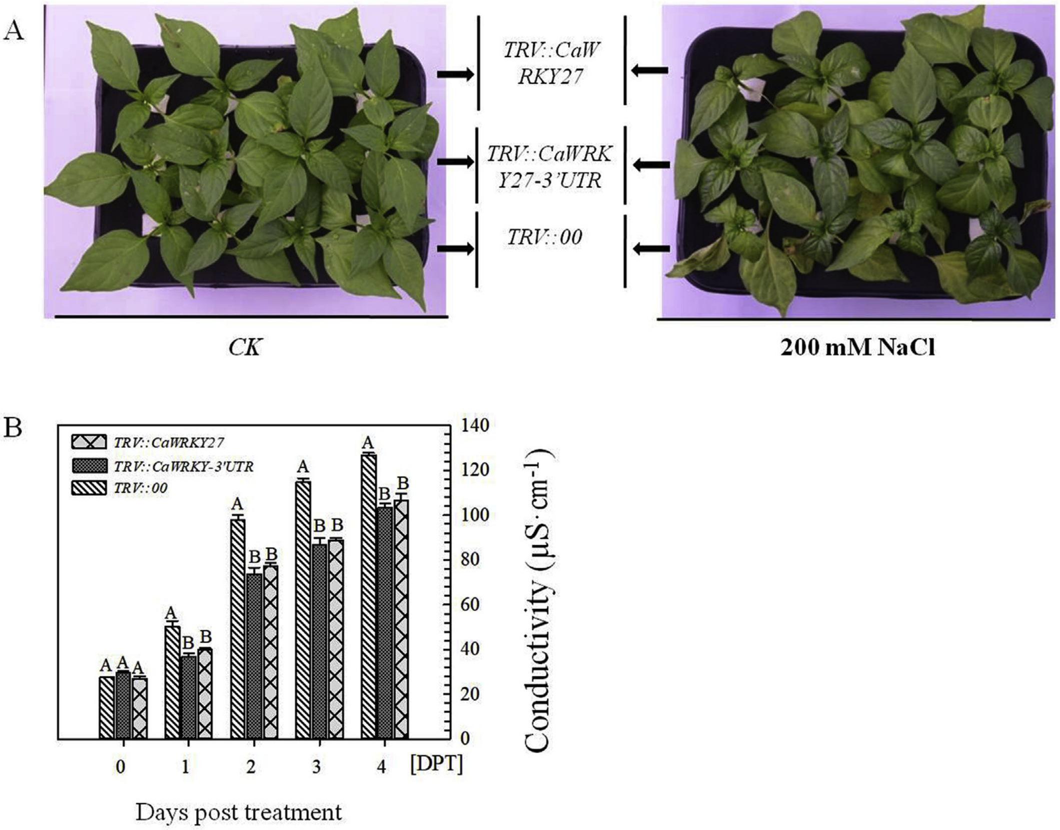

Fig. 4. *CaWRKY27*-silenced plants have enhanced salt tolerance. (A) *CaWRKY27*-silenced (*TRV::CaWRKY27*) pepper plants exhibited a higher level of tolerance to salt stress than the control (*TRV::00*) plants. The phenotypes were observed before (left) and after (right) a 3-day treatment with 200 mM NaCl. (B) Ion leakage (conductivity) was detected in the leaves of *TRV::00* and *TRV::CaWRKY27* plants challenged with salt stress. Data represent the mean ± standard error of three independent experiments, each including 20 plants. Different uppercase letters indicate significant differences (*P*-value < 0.01) as determined by an ANOVA test.

stress and drought, as well as by mannitol, which mimics osmotic stress. By contrast, silencing of *CaWRKY27* significantly enhanced tolerance of pepper plants to salt or drought stress. Heterologous expression of *CaWRKY27* significantly compromised the tolerance of tobacco or *Arabidopsis* plants to salt or drought stress; the transgenic plants had shorter roots and lower fresh weights than the controls under these stress conditions. These data suggest that *CaWRKY27* expression is induced by salt stress or drought, resulting in a compromised tolerance to these conditions.

Plants with an enhanced tolerance of salt stress and drought conditions often have a relatively reduced accumulation of ROS and lower malondialdehyde (MDA) contents (Du et al., 2018; He et al., 2019; Wang et al., 2017). ROS accumulation is modulated by antioxidants, including polyamines (Kumar et al., 2012). In this study, we demonstrated that ROS detoxification genes (*NtAPX1*, *NtSOD*, *NtPOX1*, and *NtPOX2*) and polyamine biosynthesis-associated genes (*NtADC1* and *NtSAMDC*) were repressed in tobacco by the transgenic expression of *CaWRKY27*, indicating a role for CaWRKY27 as a negative regulator of the pepper response to salt stress and drought. In addition, the repression of *NtERD10D* and *NtOSMOTIN*, which both promote plant responses to salt and osmotic stress (Shukla et al., 2015; Gupta et al., 2014; Larosa et al., 1989; Krishna et al., 2019), might represent another mechanism by which CaWRKY27 negatively affects plant tolerance to salt and drought stress. *NtNCED1*, an ABA biosynthesis-related gene (Speirs et al., 2013), and *NtDREB3* are significantly induced by salt stress and regulated by the exogenous application of ABA (Islam and Wang, 2009). These genes were negatively regulated by *CaWRKY27* under the salt stress conditions, which is consistent with the finding that the transgenic expression of *CaWRKY27* substantially decreased sensitivity to the stress tolerance phytohormone ABA in tobacco, indicating that *CaWRKY27* expression might decrease the endogenous concentration of ABA. However, *NtNCED1* and *NtDREB3* were enhanced in plants heterologously expressing *CaWRKY27* and exposed to the mannitol treatment, which appears to contradict the observation that ABA sensitivity was enhanced by the expression of *CaWRKY27* under salt stress conditions, suggesting that CaWRKY27 acts as a negative regulator of drought or osmotic stress tolerance. One explanation for this disparity is that both ABA-dependent and -independent signaling pathways are involved in plant responses to salt stress. While *CaWRKY27* positively regulates ABA-dependent plant tolerance to salt stress, it might negatively regulate ABA-independent signaling, a more intensive effect not compensated for by the ABA-dependent defense reaction (Supplementary Fig. 5).

Intensive crosstalk between plant responses to pathogens and abiotic stresses has been identified, and is believed to provide plants with an enhanced potential to respond appropriately to their environment (Fujita et al., 2006). Among the many members of the WRKY family in different plant species, some are known to regulate several seemingly disparate processes (Rushton et al., 2010); for example, *CaWRKY6* and *CaWRKY40* act as positive regulators of the pepper response to both RSI and heat stress (Cai et al., 2015; Dang et al., 2013). Unlike *CaWRKY6* or *CaWRKY40*, *CaWRKY27* acts as a positive regulator of the pepper response to RSI but a negative regulator of thermotolerance in an H_2O_2-dependent manner, by repressing ROS detoxification (Dang et al., 2014, 2018). Together with the results of this study, we conclude that *CaWRKY27* acts as a positive regulator of pepper immunity but a negative regulator of the pepper response to a series of abiotic stresses via a similar mechanism that represses the expression of genes related to ROS detoxification.

The evolutionary and adaptive significance of this negative

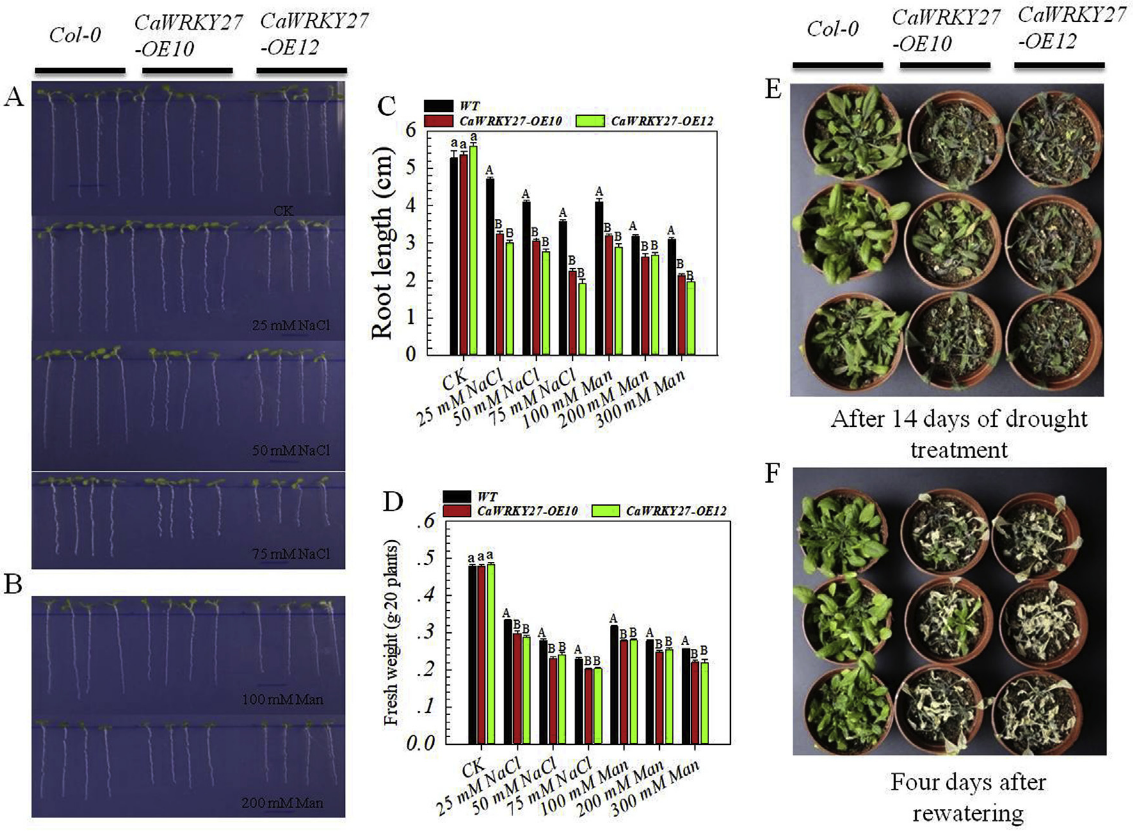

Fig. 5. *CaWRKY27*-expressing *Arabidopsis* plants have decreased tolerance to drought and salt stress. (A-D) Primary root lengths of wild-type (WT; *Col-0*) and *CaWRKY27*-expressing *Arabidopsis* plants challenged with drought and salt stress treatment. The seedlings were grown on ½ MS containing different concentrations of NaCl (A) or mannitol (B; Man) for 14 days. The corresponding root lengths (C) and fresh weights (D) were measured. In (C) and (D), the data represent the mean ± standard error of three independent experiments, each including 20 plants. Different letters indicate a significant difference from the wild type, determined using an ANOVA test (lowercase letters, P-value < 0.05; uppercase letters, P-value < 0.01). (E and F) *Arabidopsis* drought tolerance was decreased by *CaWRKY27* expression. For the drought treatment, three-week-old plants were not watered for 14 days and then rewatered for 4 days.

regulation of plant tolerance to heat, salt stress, and drought is unclear; however, one explanation might be that plants activate the expression of *CaWRKY27*, preventing ROS detoxification when the production of ROS has already stopped and maintaining a balance between ROS production and removal. Similar negative regulators have previously been identified, including *CaWRKY1* (Oh et al., 2008), *OsWRKY62* (Peng et al., 2008), *AtSR1* (Du et al., 2009), and *OsCBT* (Koo et al., 2009), all of which are believed to prevent the inappropriate activation of defense responses at suboptimal concentrations of signaling molecules or to turn off systemic acquired resistance once the pathogen invasion has been dealt with. It is worth noting that the negative regulatory roles of *CaWRKY27* in the pepper responses to heat, salt, and drought stress are closely related to its role as a positive regulator in providing immunity against RSI; the activation of CaWRKY27 by RSI enhances H_2O_2 accumulation by repressing ROS detoxification and therefore activates the plant immune responses, indicating that pepper prioritizes immunity against RSI over tolerance to abiotic stresses when pepper plants are simultaneously or sequentially exposed to these stresses.

The tested ROS detoxification and defense-related marker genes exhibited differential expression levels between the two time points in the tobacco plants challenged with salt or mannitol. In addition, *NtNCED1* and *NtDERB3* were negatively regulated in transgenic plants expressing *CaWRKY27* in the presence of mannitol, while these genes were positively regulated in the transgenic plants when exposed to salt stress. We reported a similar phenomenon in our previous studies, in which *AtHsfA1d*, *AtHsfA2*, and *AtHsfA7a* exhibited different expression patterns in *Arabidopsis* plants under normal or heat stress conditions at 1 and 3 hpt (Higashi et al., 2013; Lin et al., 2018). This was also true for *NtCAT1*, which was activated by RSI in transgenic tobacco plants heterologously expressing *CaWRKY27* but was downregulated in these plants when exposed to heat stress (Dang et al., 2018). A possible explanation for all of these results is that the transcriptional regulation of these genes by *CaWRKY27* might be modulated by other signaling components activated in a time-dependent or stress-specific manner, since the functions of the WRKY TFs can be modulated by physical interaction with a wide range of proteins with roles in signaling, transcription, and chromatin remodeling (Chi et al., 2013; Alves et al., 2014; Shan et al., 2016; Tripathi et al., 2015). Further isolation and functional characterization of the proteins that interact with CaWRKY27 in pepper plants challenged with different stimuli would provide insights into the function of this TF.

Collectively, this study shows that *CaWRKY27* is consistently upregulated by salt stress and drought, which in turn represses the expression of genes related to ROS detoxification, thereby resulting in a decreased tolerance to these abiotic stresses. Together with the results of our previous study, we speculate that CaWRKY27 acts as a positive regulator of the pepper immune response to RSI but as a negative regulator of the responses to salinity, drought, and heat stress via similar mechanisms involving the enhancement of the accumulation of ROS such as H_2O_2.

Author contributions

S. L. H. designed the experiments. J. H. L. and F. F. D. performed most of experiments and analyzed the data. The other authors assisted

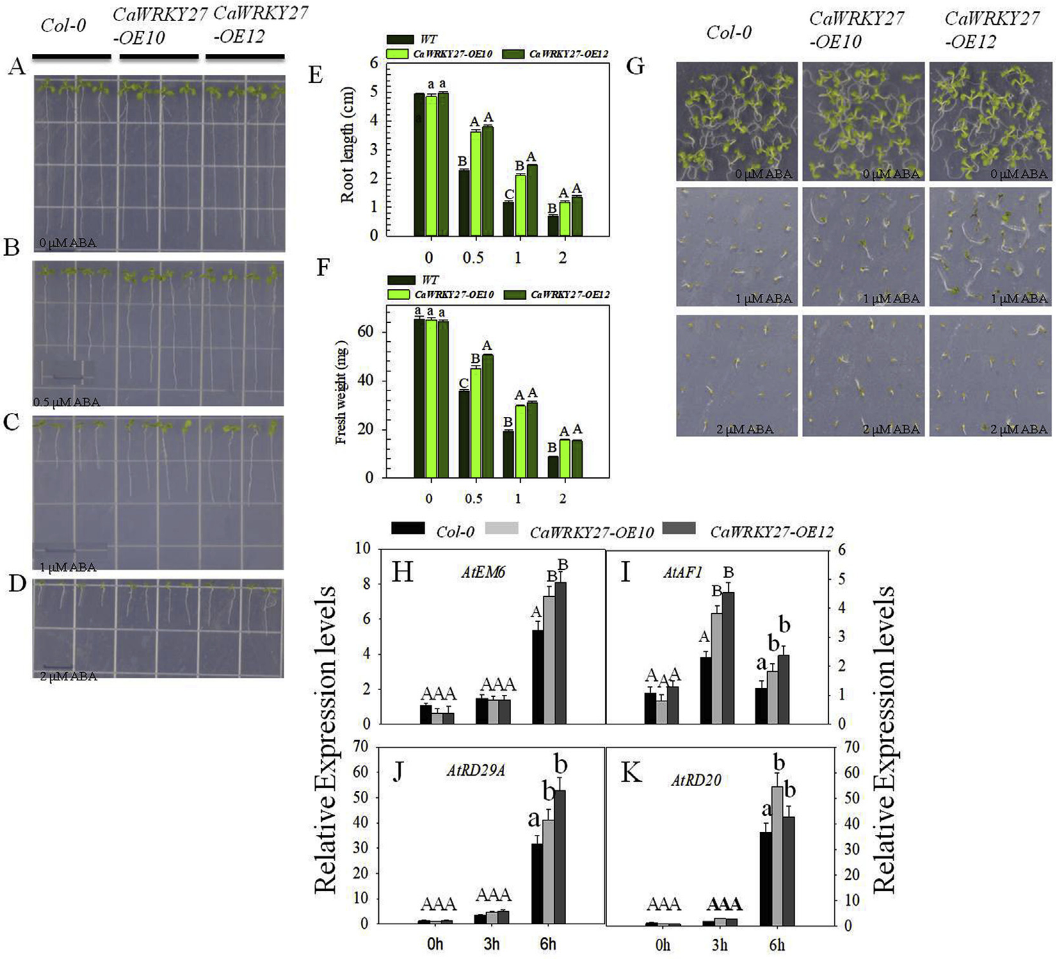

Fig. 6. *CaWRKY27*-expressing *Arabidopsis* plants have decreased sensitivity to exogenous ABA. (A-F) Primary root lengths of wild-type (WT; *Col-0*) and transgenic *CaWRKY27*-expressing plants challenged with an exogenous application of ABA. Seeds were sown on ½ MS containing 0 (A), 0.5 (B), 1 (C), or 2 μM ABA (D). The images were taken at 14 dpt. The root lengths (E) and fresh weights (F) of two transgenic lines and the wild type were measured. Data represent the mean ± standard error of three independent experiments, each including 20 plants. (G) Germination rates of *CaWRKY27-OX* and wild-type plants on ½ MS medium supplemented with different concentrations of ABA. Data represent the mean ± standard error of three independent experiments, each including 50 seeds. (H) to (K) Quantitative reverse transcription polymerase chain reaction analysis of ABA-relative genes in *CaWRKY27-OX* plants exposed to ABA treatment at 3 h and 6 h after detachment. In (E), (F), and (H) to (K), different letters indicate significant differences, as determined using an ANOVA test (lowercase difference *P*-value < 0.05; uppercase difference *P*-value < 0.01).d

in experiments and discussed the results. J. H. L. and S. L. H. wrote the manuscript.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.08.013.

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