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The Pepper Lipoxygenase CaLOX1 Plays a Role in Osmotic, Drought and High Salinity Stress Response

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In plants, lipoxygenases (LOXs) are involved in various physiological processes, including defense responses to biotic and abiotic stresses. Our previous study had shown that the pepper 9-LOX gene, *CaLOX1*, plays a crucial role in cell death due to pathogen infection. Here, the function of CaLOX1 in response to osmotic, drought and high salinity stress was examined using *CaLOX1*-overexpressing (*CaLOX1*-OX) Arabidopsis plants. Changes in the temporal expression pattern of the *CaLOX1* gene were observed when pepper leaves were treated with drought and high salinity, but not when treated with ABA, the primary hormone in response to drought stress. During seed germination and seedling development, *CaLOX1*-OX plants were more tolerant to ABA, mannitol and high salinity than wild-type plants. In contrast, expression of the ABA-responsive marker genes *RAB18* and *RD29B* was higher in *CaLOX1*-OX Arabidopsis plants than in wild-type plants. In response to high salinity, *CaLOX1*-OX plants exhibited enhanced tolerance, compared with the wild type, which was accompanied by decreased accumulation of H_2O_2 and high levels of *RD20, RD29A, RD29B* and *P5CS* gene expression. Similarly, *CaLOX1*-OX plants were also more tolerant than wild-type plants to severe drought stress. H_2O_2 production and the relative increase in lipid peroxidation were lower, and the expression of *COR15A, DREB2A, RD20, RD29A* and *RD29B* was higher in *CaLOX1*-OX plants, relative to wild-type plants. Taken together, our results indicate that CaLOX1 plays a crucial role in plant stress responses by modulating the expression of ABA- and stress-responsive marker genes, lipid peroxidation and H_2O_2 production.

Keywords: Abscisic acid • CaLOX1 • Drought stress • High salinity • 9-Lipoxygenase • Reactive oxygen species.

Abbreviations: CaLOX1, *Capsicum annumm* lipoxygenase 1; DAB, 3,3′-diaminobenzidine; H_2O_2, hydrogen peroxide; JA, jasmonic acid; LOX, lipoxygenase; MDA, malondialdehyde; MS, Murashige and Skoog; PR, pathogenesis related; *Pst*, *Pseudomonas syringae* pv. *tomato*; RD, response to dehydration; ROS, reactive oxygen species; qRT–PCR, quantitative reverse transcription–PCR; SA, salicylic acid; SOS, stomatal opening solution; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; WT, wild type; *Xcv*, *Xanthomonas campestris* pv. *vesicatoria*.

Introduction

As sessile organisms, plants have evolved extraordinary defense mechanisms that enable them to adapt to various environmental stress conditions. High salinity, drought, oxidative stress and pathogens are common environmental stresses that adversely affect plant growth. They present challenges in agriculture by causing severe losses in crop yields (Agarwal et al. 2006). Osmotic stress is a common result of exposure to high salinity and dehydration stress. Prevention of osmotic stress depends 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). The defense mechanisms involved in osmotic stress have been widely studied (Zhu 2002, Yamaguchi-Shinozaki and Shinozaki 2006). However, the osmotic stress response in plants is a complex phenomenon, and the exact structural and functional modifications induced by osmotic stress are poorly understood.

The plant hormone ABA plays a central role in the defense mechanism for adaptation to osmotic stress by inducing many molecular and physiological alterations. Osmotic stress increases the cellular level of ABA, which induces adaptive stress responses, such as stomatal closure via the efflux of anions and K^+ from guard cells, and the expression of many osmotic stress-responsive genes (Schroeder et al. 2001, Lee and Luan 2012, Lee et al. 2013). ABA regulates a larger number of genes than other plant hormones: $>10\%$ of the genes in Arabidopsis respond to ABA (Goda et al. 2008, Mizuno and Yamashino 2008). The expression of ABA-responsive genes plays a key role in conferring enhanced stress tolerance and protecting plants from osmotic stress conditions. Characterization of ABA-sensitive or -insensitive mutants in

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which ABA-responsive genes are overexpressed or knocked out has identified various components of ABA signaling in Arabidopsis (Ma et al. 2009, Umezawa et al. 2009, Vlad et al. 2009, Lee et al. 2013).

Lipoxygenases (LOXs) are ubiquitous in the animal and plant kingdoms and are involved in various physiological processes (Brash 1999). LOXs are non-heme iron-containing dioxygenases that catalyze the conversion of polyunsaturated fatty acids and lipids into hydroperoxy fatty acids, which are degraded into oxylipins, such as traumatin, jasmonic acid (JA) and methyl jasmonate (MeJA) (Blee 2002). Oxylipins play an important role in development, senescence, formation of flavor compounds, and the defense response to biotic or abiotic stresses (Williams et al. 2000, Ye et al. 2000, Vellosillo et al. 2007, Yang et al. 2012). The plant LOXs, classified as 9-LOX and 13-LOX, catalyze the oxygenation of linoleic acid and linolenic acid (Feussner and Wasternack 2002). Many 13-LOX-derived compounds, such as JA, 12-oxophytodienoic acid and 13-hydroxyoctadecatrienoic acid, are well-known regulators of the plant defense response (Almeras et al. 2003). JA is a key defense molecule that regulates resistance to necrotrophic pathogens (Penninckx et al. 1996, Rance et al. 1998, Glazebrook 2005). Moreover, 13-LOX expression and JA accumulation increase in tobacco leaves infected by an avirulent strain of *Pseudomonas syringae* pv. *phaseolicola* (Kenton et al. 1999). However, it is not known whether 9-LOXs are involved in defense mechanisms against biotic and abiotic stresses.

The 9-LOX gene *CaLOX1* is significantly induced in pepper (*Capsicum annuum* L.) leaves infected with an avirulent strain of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) or treated with abiotic elicitors, including ethylene, salicyclic acid (SA), NaCl and methyl viologen (Hwang and Hwang 2010). CaLOX1 is involved in cell death, which is associated with reactive oxygen species (ROS), lipid peroxidation and SA accumulation. Moreover, *CaLOX1*-silenced pepper plants are susceptible to *Xcv* and *Colletotrichum coccodes* infection, whereas *CaLOX1*-overexpressing (*CaLOX1*-OX) transgenic Arabidopsis plants are resistant to bacterial and oomycete pathogens, as well as to other fungal pathogens (Hwang and Hwang 2010). In this study, based on the expression patterns of the *CaLOX1* gene in pepper in response to ABA, drought and high salt stresses, we evaluated their responses to ABA, osmotic, high salt and drought stresses using *CaLOX1*-OX Arabidopsis plants. The *CaLOX1*-OX plants exhibited tolerance phenotypes to drought and high salt stresses via rapid scavenging of ROS and inducing high expression of ABA- and stress-responsive marker genes.

Results

Induction of the *CaLOX1* gene by drought, high salinity and ABA in pepper leaves

Northern blot analysis has shown that expression of the *CaLOX1* gene is triggered in response to biotic and abiotic stresses, such as NaCl, ethylene, methyl viologen and the avirulent strain *Xcv* Bv5-4a (Hwang and Hwang 2010). In this study, the involvement of the *CaLOX1* gene in osmotic, drought and high salinity was investigated. Quantitative reverse transcription–PCR (qRT–PCR) analysis was performed to determine the expression pattern of the *CaLOX1* gene after drought treatment. As shown in **Fig. 1A**, *CaLOX1* transcription in pepper leaves was strongly induced by 12 h of drought treatment. In addition, the induction of *CaLOX1* gene expression in response to high salinity was also investigated using qRT–PCR analysis. *CaLOX1* transcription was induced at 6 h and gradually declined from 12 to 24 h after NaCl treatment (**Fig. 1B**), consistent with previous findings (Hwang and Hwang 2010). The pattern of *CaLOX1* gene expression was also analyzed in pepper leaves treated with ABA. As a stress-related hormone, ABA is a key signaling molecule in the defense responses to abiotic stresses (Zhang et al. 2006). ABA and abiotic stress signals share common signal transduction pathways, although osmotic stress signaling induced by high salinity and drought is not dependent solely on ABA (Jakab et al. 2005). qRT–PCR analysis showed that *CaLOX1* expression was slightly induced by ABA (**Fig. 1C**), but not in a statistically significant manner, as observed previously (Hwang and Hwang 2010).

Alteration of ABA sensitivity in germinating seeds of *CaLOX1*-OX transgenic Arabidopsis

A phenotypic analysis of *CaLOX1*-OX mutants after treatment with ABA, dehydration and high salinity was performed using

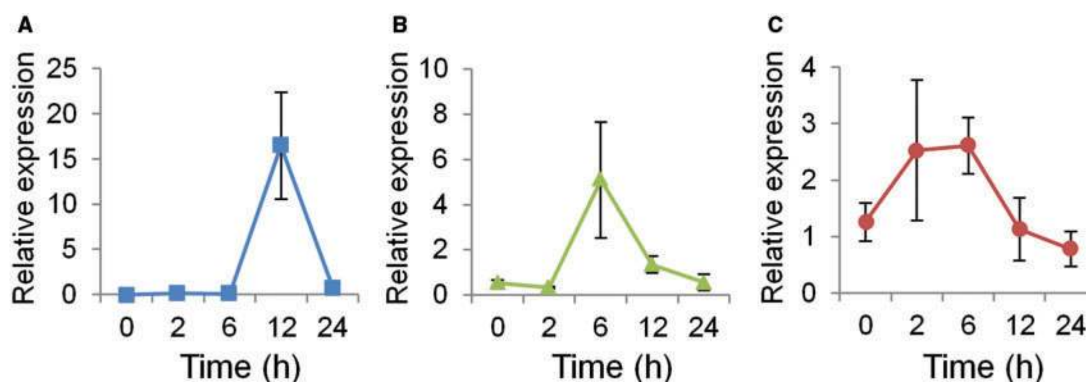

Fig. 1 The *CaLOX1* gene expression pattern in pepper leaves at various times after treatment with drought (A), NaCl (200 mM) (B) and ABA (100 μM) (C). The relative expression (ΔΔCT) of the *CaLOX1* gene was normalized to the geometric mean of *18S rRNA*, *Actin1* and *EF1α* as internal control genes.

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CaLOX1-OX transgenic Arabidopsis, which exhibit higher LOX expression and enzymatic activity than wild-type (WT) plants (**Supplementary Fig. S1**) (Hwang and Hwang 2010). First, *CaLOX1*-OX seeds were germinated on Murashige and Skoog (MS) plates with various concentrations of ABA. After 3 d, the germination rate of *CaLOX1*-OX seeds was slightly higher than that of WT seeds, but the difference was not statistically significant (**Fig. 2A**). At 7 d after sowing, the number of seedlings with a green cotyledon was higher in *CaLOX1*-OX plants than in the WT (**Fig. 2B**) and also the root lengths of *CaLOX1*-OX plants were longer than those of the WT in the presence of ABA (**Fig. 2C**). To determine whether this decreased ABA sensitivity in root growth of *CaLOX1*-OX plants is also retained at the post-germination stage, 4-day-old seedlings of both WT and *CaLOX1*-OX seeds germinated on medium without ABA were transferred to medium with 20 μM ABA (**Fig. 2D, E**) and grown further for 7 d. Root growth was consistently enhanced in the *CaLOX1*-OX plants relative to the WT plants (**Fig. 2D**) and the primary root length of the *CaLOX1*-OX plants was > 10% longer than that of the WT plants (**Fig. 2E**). These results indicated that overexpression of the *CaLOX1* gene confers reduced ABA sensitivity in Arabidopsis during seedling growth.

Next, qRT–PCR analysis was carried out to examine how *CaLOX1* overexpression affects ABA signaling and biosynthesis. Treatment with 50 μM ABA strongly induced the ABA-responsive marker genes *RAB18* and *RD29B* in 3-week-old *CaLOX1*-OX and WT plants, and the expression of these genes was higher in *CaLOX1*-OX plants (**Fig. 2F**). In contrast, the expression of the ABA biosynthesis genes *NCED3* and *NCED5* was not significantly different in *CaLOX1*-OX and WT plants. This suggests that the *CaLOX1* gene plays a positive role in ABA-induced gene expression, but not in ABA biosynthesis.

Enhanced tolerance of *CaLOX1*-OX Arabidopsis plants to osmotic stress

To examine how overexpression of *CaLOX1* alters osmotic stress responses during seed germination and seedling stages, seeds from WT and *CaLOX1*-OX plants were germinated on MS plates with various concentrations of mannitol (**Fig. 3**). In the presence of mannitol, the germination rate of *CaLOX1*-OX seeds was higher than that of WT seeds (**Fig. 3A**). Approximately 25% of the WT seeds germinated within 7 d after the application of 500 mM mannitol, whereas the *CaLOX1*-OX seeds achieved 47–50% germination. Consistently, *CaLOX1*-OX plants exhibited a high number of seedlings with a green cotyledon relative to the WT plants after growth on medium containing 400 mM mannitol for 7 d (**Fig. 3B**). Overexpression of *CaLOX1* also conferred enhanced tolerance of root growth in response to mannitol compared with WT plants (**Fig. 3C, D**). Root growth in all plants was suppressed in a concentration-dependent manner. However, the roots of the *CaLOX1*-OX plants were significantly longer than those of the WT plants at various concentrations of mannitol (**Fig. 3D**). *CaLOX1*-OX plants exhibited root growth even in the presence of 500 mM mannitol, which fully suppressed root growth in WT plants. These findings indicate that overexpression of *CaLOX1* confers enhanced tolerance to osmotic stress.

Enhanced tolerance of *CaLOX1*-OX Arabidopsis plants to high salinity

Since the expression of *CaLOX1* is induced by NaCl treatment (**Fig. 1C**), we speculated that overexpression of *CaLOX1* could alter the response to salt stress. The response of *CaLOX1*-OX plants to high salt stress was examined by evaluating seed germination, greening cotyledons and primary root growth (**Fig. 4**). In the germination assay, *CaLOX1*-OX plants were compared with WT plants under conditions of 200 mM NaCl (**Fig. 4A–C**). In general, the *CaLOX1*-OX plants were more tolerant than WT plants to NaCl. At 3 d after sowing, the germination rates of the *CaLOX1*-OX plants were higher than those of the WT plants (**Fig. 4A**). Moreover, the greening cotyledon rate of the transgenic plants was 2.0- to 2.4-fold higher than that of the WT plants (**Fig. 4B, C**). The reduced sensitivity to NaCl in *CaLOX1*-OX plants was not the result of a developmental defect because neither of the *CaLOX1*-OX lines differed from WT plants in terms of seed germination and seedling establishment in the absence of NaCl (**Fig. 4A–C**). *CaLOX1*-OX transgenic plants also exhibited enhanced tolerance to salt stress at the seedling stage (**Fig. 4D–G**). After growth for 7 d on liquid medium containing 175 mM NaCl, fewer *CaLOX1*-OX seedlings than WT seedlings showed leaf bleaching (**Fig. 4D**), and the Chl content was significantly higher in *CaLOX1*-OX plants than in WT plants (**Fig. 4E**). To examine whether overexpression of *CaLOX1* enhanced root elongation in the presence of NaCl, a root bending assay was performed by transferring 4-day-old seedlings from each line onto MS plates containing 100 mM NaCl. There was no significant difference in hook formation (**Fig. 4F**), but primary root lengths were much longer in *CaLOX1*-OX plants than in WT plants (**Fig. 4F, G**).

We also examined the salt stress response of WT and *CaLOX1*-OX plants in soil (**Fig. 5**). Two-week-old WT and transgenic plants were subjected to high salinity stress (250 mM NaCl) for 12 d. Application of NaCl solution suppressed the growth of both WT and *CaLOX1*-OX plants and led to leaf chlorosis and plant death (**Fig. 5A**). Under salt stress conditions, *CaLOX1*-OX plants were tolerant of high concentrations of NaCl compared with WT plants. Approximately 80% of *CaLOX1*-OX plants survived, whereas WT plants had a much lower survival rate (approximately 25%) (**Fig. 5B**). The fresh weight and Chl contents of leaf tissues were also much higher in *CaLOX1*-OX plants than in WT plants (**Fig. 5C, D**). Several studies have suggested that enhancing antioxidant defense, including reducing the level of oxidative stress, improves tolerance to environmental stress (Oberschall et al. 2000, Sunkar et al. 2003). We determined the effect of salt stress on the oxidative burst by measuring hydrogen peroxide (H_2O_2) with 3,3′-diaminobenzidine (DAB) staining (**Fig. 5E**). A greater number of dark-brownish H_2O_2 sites were detected in WT leaves than in *CaLOX1*-OX leaves, suggesting that CaLOX1 plays a role in diminishing the oxidative burst during salt stress. In addition, qRT–PCR analysis was performed to

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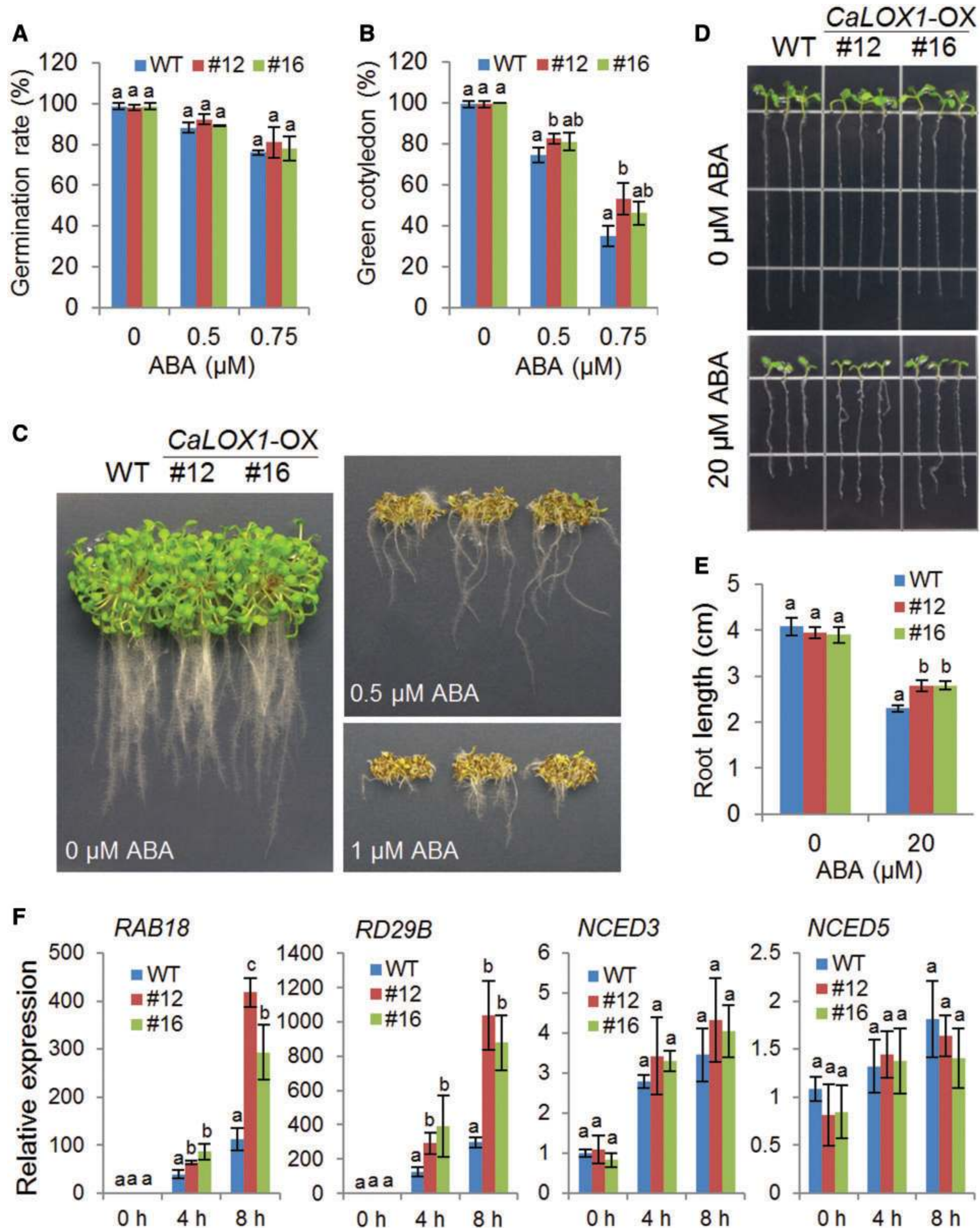

Fig. 2 Reduced sensitivity of the *CaLOX1*-OX transgenic Arabidopsis lines (#12 and #16) to ABA. (A) Germination rates of WT and transgenic plants exposed to ABA. The percentage of seeds with radicle emergence was scored 3 d after sowing. (B) Seedling establishment for WT and transgenic plants exposed to ABA 7 d after sowing. (C) Root growth of WT and transgenic plants exposed to ABA. The seedlings were grown vertically in 0.5 × MS containing 0.5 and 1.0 μM ABA for 7 d. Representative images are shown. (D and E) Root growth of wild-type and transgenic plants exposed to ABA after germination. Four-day-old seedlings grown on 0.5 × MS were transferred to 0.5 × MS containing 0 or 20 μM ABA. After 7 d, the representative images were taken (D), and the root length in each line was measured (E). (F) qRT–PCR analysis of ABA signaling genes and biosynthesis-related genes in the *CaLOX1*-OX mutant after ABA treatment. The relative expression (ΔΔCT) of each gene was normalized to the geometric mean of *Actin2*, *Actin8* and *EF1α* as internal control genes. Data represent the mean ± SD from three independent experiments, each evaluating 20 seeds. The different letters indicate significant differences in three independent experiments (ANOVA; $P < 0.05$).

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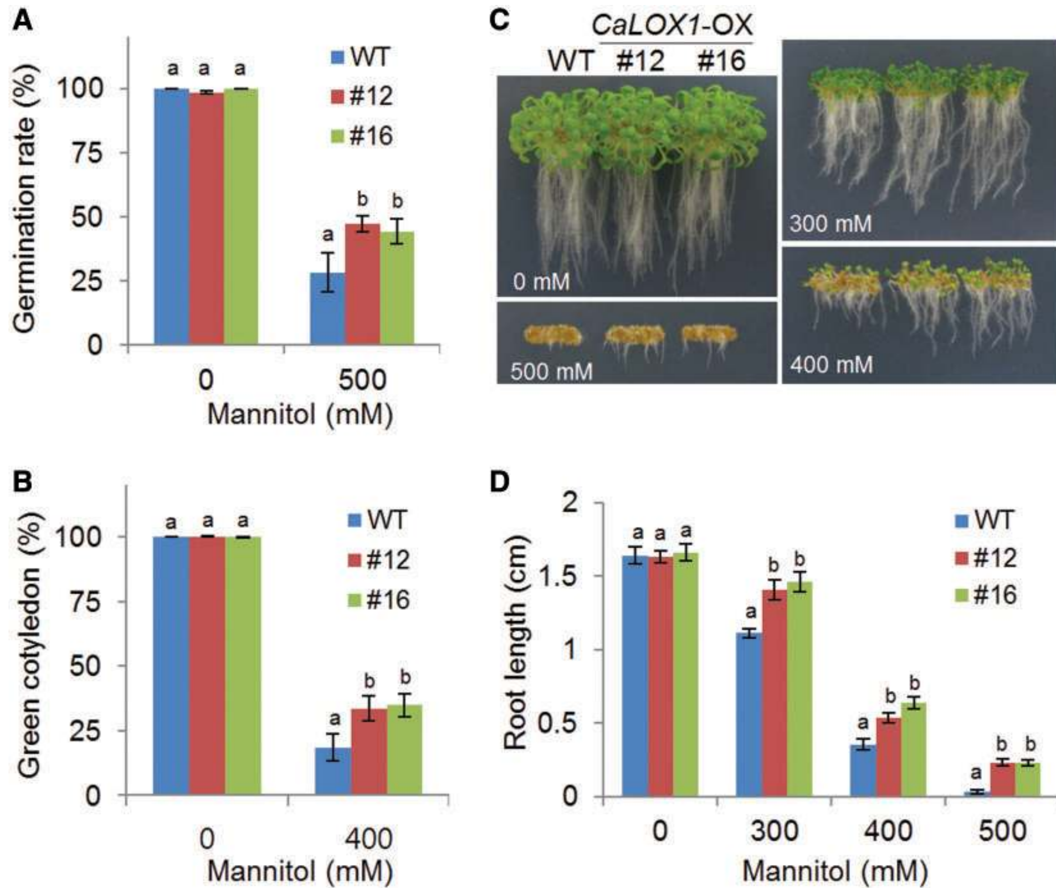

Fig. 3 Reduced sensitivity of the *CaLOX1*-OX transgenic Arabidopsis lines (#12 and #16) to mannitol. (A) Germination rates of WT and transgenic plants exposed to mannitol. The percentage of seeds showing radicle emergence was scored 5 d after plating on 0.5 × MS containing 500 mM mannitol. (B) Seedling establishment of WT and transgenic plants exposed to mannitol 7 d after sowing. (C and D) Root growth of WT and transgenic plants exposed to mannitol. The seedlings were grown vertically in 0.5 × MS containing different concentrations of mannitol. After 7 d, the representative images were taken (C), and the root length in each line was measured (D). The fraction of seedlings with a green cotyledon was determined 7 d after plating. Data represent the mean ± SD from three independent experiments, each evaluating 50 seeds. The different letters indicate significant differences in three independent experiments (ANOVA; $P < 0.05$).

determine whether salt stress tolerance in *CaLOX1*-OX transgenic plants correlated with the expression of salt stress-responsive marker genes, including *DREB2A*, *RD20*, *RD29A*, *RD29B* and *P5CS*. Treatment with salt stress induced the expression of these genes in both WT and *CaLOX1*-OX plants, relative to expression in non-treated plants (**Fig. 5F**). The marker genes, except for *DREB2A*, were expressed at higher levels in the leaves of *CaLOX1*-OX transgenic plants than in those of WT plants, especially at 4 h after treatment with salt stress. These data suggest that overexpression of *CaLOX1* enhances tolerance to salt stress in *CaLOX1*-OX plants.

Enhanced tolerance of *CaLOX1*-OX Arabidopsis plants to drought stress

Because *CaLOX1*-OX plants exhibit enhanced tolerance to mannitol and salt-induced osmotic stress in both seed germination and seedling states, it was of interest to determine whether *CaLOX1*-OX plants also exhibit increased drought tolerance. When the soil was allowed to dry by withholding water for 12 d and then the plants were allowed to recover after rewatering for 2 d, more WT plants than transgenic plants appeared withered (**Fig. 6A**). To confirm that overexpression of *CaLOX1* enhances drought tolerance, the survival rates of WT and *CaLOX1*-OX plants were assayed after drought treatment. Approximately 80% of the *CaLOX1*-OX plants survived, whereas just 35% of WT plants survived (**Fig. 6A**).

To examine drought tolerance at the cellular and molecular levels, transpiration rate, stomatal aperture, lipid peroxidation, oxidative burst, and ABA- and drought-related gene expression were examined. The rates of transpirational water loss in WT and transgenic plants were compared by measuring the fresh weight from detached rosette leaves (**Fig. 6B**). The fresh weight loss of the leaf tissues due to leaf water loss was much lower in the *CaLOX1*-OX plants than that in the WT plants, suggesting that enhanced drought tolerance results from altered rates of leaf transpiration. To determine whether a low transpiration rate in *CaLOX1*-OX plants was caused by an increase in stomatal closure, stomatal aperture was measured after treatment with ABA, which plays a critical role in stomatal closure. In the absence of ABA, *CaLOX1*-OX plants exhibited decreased stomatal aperture relative to WT plants (**Fig. 6C**).

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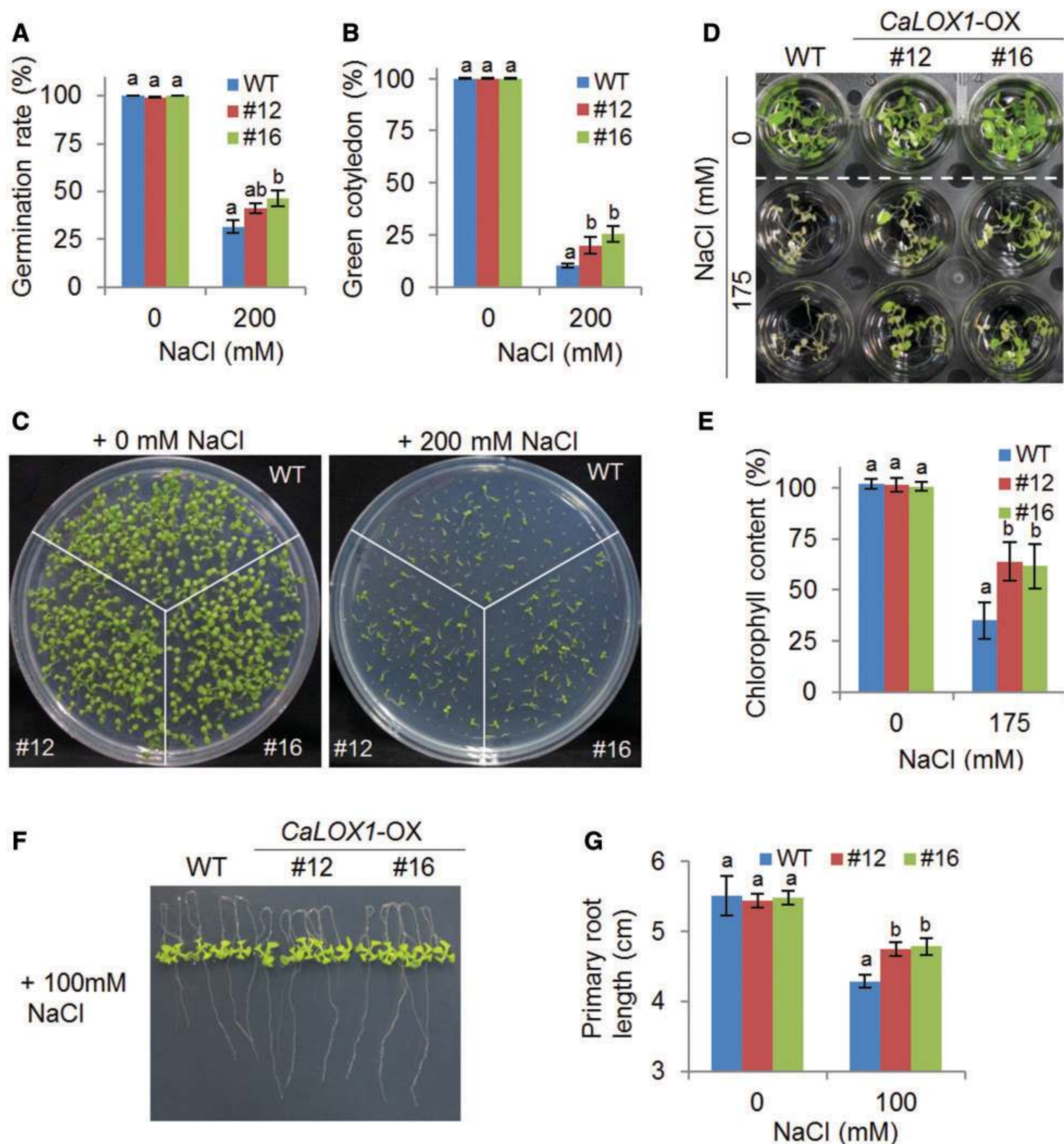

Fig. 4 Enhanced salt tolerance of the *CaLOX1*-OX transgenic Arabidopsis lines (#12 and #16) at the seedling stage. (A) Germination rates of WT and transgenic plants exposed to NaCl. The percentage of seeds with radicle emergence was scored 7 d after plating on 0.5 × MS containing 200 mM NaCl. (B and C) Seedling establishment of WT and transgenic plants exposed to NaCl. The fraction of seedlings with a green cotyledon in each line was determined 7 d after plating and the representative images were taken. (D and E) Seedling growth for WT and transgenic plants exposed to NaCl. Four-day-old seedlings grown on 0.5 × MS plates were transferred to 0.5 × MS liquid medium containing 175 mM NaCl. After 7 d, the representative images were taken (D), and the Chl content in each line was measured (E). (F and G) Root elongation of WT and transgenic plants exposed to NaCl. Four-day-old seedlings grown on 0.5 × MS were transferred to 0.5 × MS containing 100 mM NaCl. After 7 d, the representative images were taken (F), and the primary root length in each line was measured (G). Data represent the mean ± SD from three independent experiments, each evaluating 20 seeds. The different letters indicate significant differences in three independent experiments (ANOVA; $P < 0.05$).

This difference was also observed when leaf peels of the WT and *CaLOX1*-OX plants were incubated with ABA (**Supplementary Fig. S2**). However, there was no obvious difference in the average stomatal aperture after normalization from that of non-treated plants, suggesting that the low transpiration rate of *CaLOX1*-OX plants was not caused by enhanced ABA-induced stomatal closure. Next, we analyzed lipid peroxidation by measuring the accumulation of

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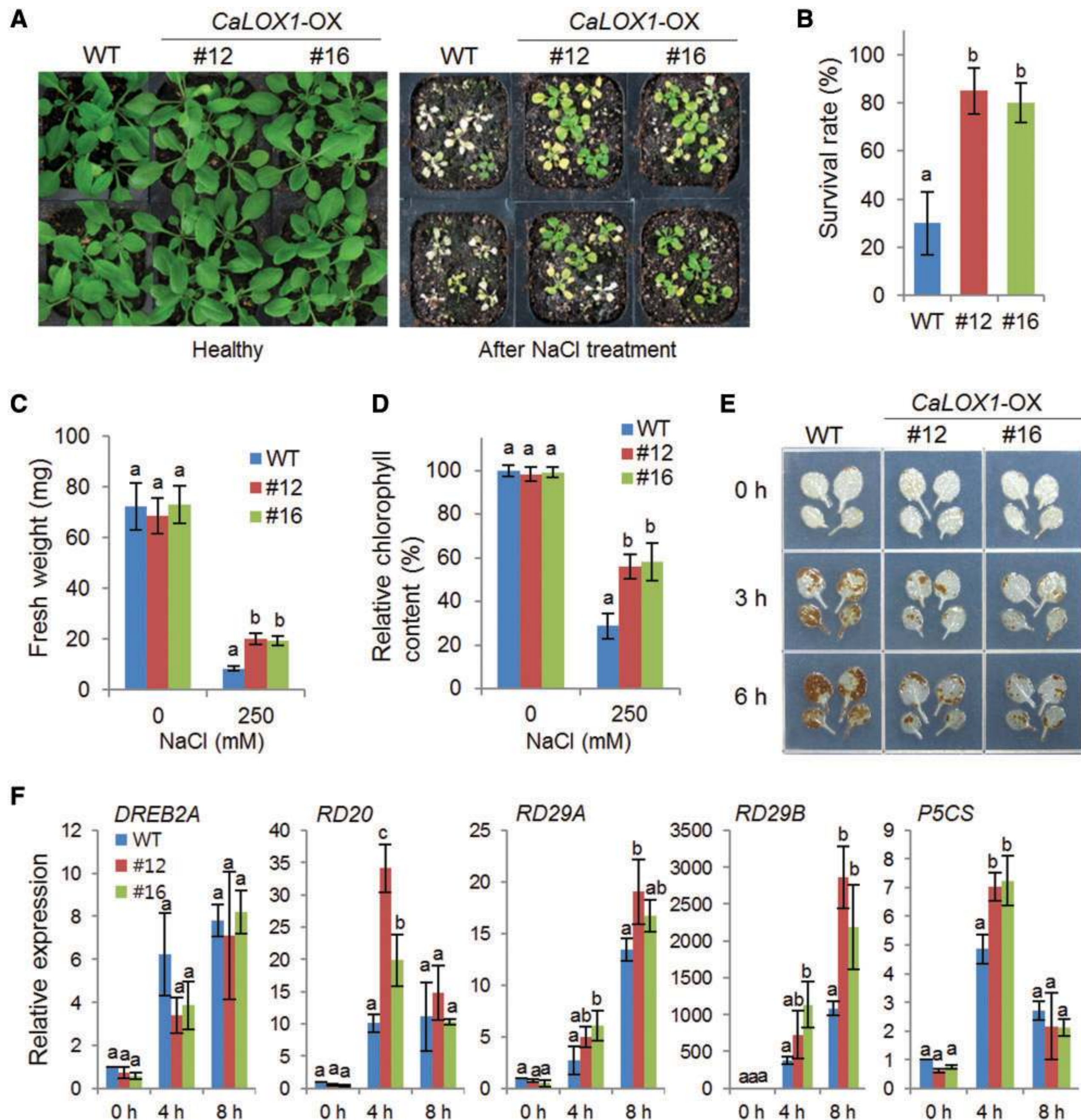

Fig. 5 Enhanced salt tolerance of the *CaLOX1*-OX transgenic Arabidopsis plant lines (#12 and #16). (A and B) Enhanced tolerance of *CaLOX1*-OX transgenic plants in response to high salinity. Two-week-old WT and transgenic plants were watered with salt solution (250 mM) to induce salt stress. After 12 d, the representative images were taken (A), and the percentage of surviving plants (B), fresh weight (C) and relative Chl content in each line were measured. (E) Hydrogen peroxide production in the leaves of WT and transgenic plants. Two-week-old plants were treated with 250 mM NaCl. The leaves were harvested 3 and 6 h after treatment and stained with DAB solution. (F) qRT–PCR analysis of salt-inducible genes in the *CaLOX1*-OX mutant in response to high salinity. The relative expression (ΔΔCT) of each gene was normalized to the geometric mean of *Actin2*, *Actin8* and *EF1α* as internal control genes. Data represent the mean ± SD from three independent experiments, each evaluating 50 seeds. The different letters indicate significant differences in three independent experiments (ANOVA; $P < 0.05$).

malondialdehyde (MDA) in the leaves of plants treated with drought stress. Previous studies have shown that drought stress induces MDA production and there is a negative correlation between MDA content and drought stress resistance in plant (Lima et al. 2002, DaCosta and Huang 2007, Lim et al. 2014). Drought stress treatment led to significant accumulation of MDA in leaves of *CaLOX1*-OX and WT plants, and the MDA content was slightly elevated in *CaLOX1*-OX plants at all time points tested relative to that of the WT plants (**Fig. 6D**). However, when normalized with that of non-treated plants, MDA content after drought stress increased 1.5- to 1.8-fold in WT plants, whereas it increased 1.2- to 1.5- (#12) or 1.0- to 1.2- (#16) fold in Ca*LOX1*-OX plants. Oberschall et al. (2000) have suggested that the oxidative burst affects the response to

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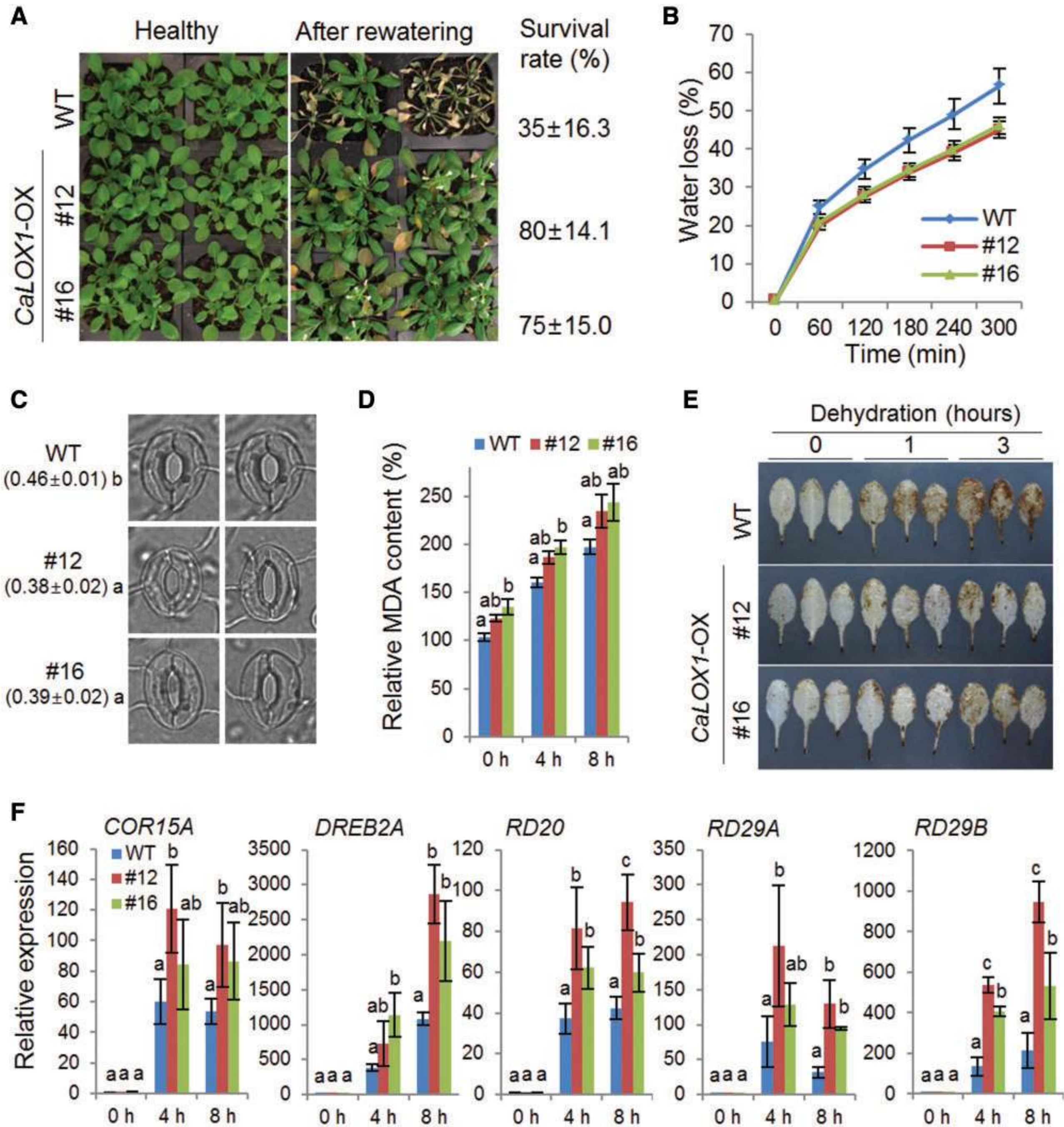

Fig. 6 Enhanced drought tolerance of the *CaLOX1*-OX transgenic Arabidopsis plant lines (#12 and #16). (A) Drought sensitivity of *CaLOX1*-OX transgenic plants. Water was withheld from 3-week-old WT and transgenic plants for 13 d to induce dehydration. After rehydration for 3 d, the representative images were taken (A), and the percentage of plants that survived was determined. (B) Water loss from the leaves of WT and transgenic plants at various times after leaf detachment. (C) Stomatal apertures in *CaLOX1*-OX transgenic and WT plants treated with ABA. Stomatal apertures were measured using leaf peels harvested from 4-week-old plants. The representative images were taken under a microscope. (D) Quantification of lipid peroxidation, expressed as the percentage increase in TBARS in the leaves 4 or 8 h after removal of the root to induce dehydration. The values were normalized to those of non-treated samples in each line. Data represent the mean ± SE from three independent experiments. (E) Hydrogen peroxide production in the leaves of WT and transgenic plants. The roots of 3-week-old plants were removed to induce dehydration. Leaves harvested 1 and 3 h after treatment were stained with DAB solution. (F) qRT–PCR analysis of drought-inducible genes in the *CaLOX1*-OX mutant in response to high salinity. The relative expression ($\Delta\Delta$CT) of each gene was normalized to the geometric mean of *Actin2*, *Actin8* and *EF1α* as internal control genes. Data represent the mean ± SD from three independent experiments. The different letters indicate significant differences in three independent experiments (ANOVA; $P < 0.05$).

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drought stress. Therefore, H_2O_2 was measured with DAB staining (**Fig. 6E**). Before dehydration treatment, dark-brownish H_2O_2 sites were rarely observed in the tested leaves, whereas, upon dehydration, a greater amount of H_2O_2 accumulated in WT leaves than in *CaLOX1*-OX leaves. In addition, to determine whether drought tolerance in *CaLOX1*-OX transgenic plants correlated with the expression of ABA- and drought-related genes, including *COR15A*, *DREB2A*, *RD20*, *RD29A* and *RD29B*, qRT–PCR assays were performed (**Fig. 6F**). The expression of these genes was significantly higher in *CaLOX1*-OX (#12) plants than in WT plants at 4 and 8 h after dehydration treatment. Moreover, the expression of ABA- and drought-related genes was also moderately higher in *CaLOX1*-OX (#16) plants. Taken together, these results suggest that overexpression of *CaLOX1* enhances drought tolerance.

Discussion

In plants, LOXs are present in multiple isoforms and have active roles in various processes, such as seed development, germination, vegetative growth and stress responses (Porta and Rocha-Sosa 2002). The LOX pathway has several branches and produces many signaling molecules, called oxylipins, which play pivotal roles in the defense responses to a variety of pathogens and stress factors. In this regard, LOXs are important enzymes for stress response signaling. The pepper 9-LOX gene *CaLOX1* plays a positive role in broad-spectrum resistance and the cell death response during pathogen infection by regulating ROS accumulation, lipid peroxidation, SA accumulation and defense-related gene expression (Hwang and Hwang 2010). In this study, investigation of the role of CaLOX1 in the response to abiotic stresses revealed that CaLOX1 also plays a positive role in plant defense responses to drought, high salinity and osmotic stresses.

Information on the functional involvement of LOXs in the response to abiotic stress is limited. In contrast, much attention has been paid to the role of LOX in defense response against pathogens, such as the hypersensitive response (Jalloul et al. 2002, Montillet et al. 2002, Cacas et al. 2005). Many researches have also focused on the biosynthetic pathway initiated by 13-LOX rather than 9-LOX, and on its main product, JA, which plays an important role in resistance to wounding, insect attack and establishment of systemic immunity (Truman et al. 2007, Koo and Howe 2009, Onkokesung et al. 2010). However, alterations in *LOX* gene expression in response to abiotic stresses have been reported. For example, the *LOX* family genes in cucumber are differentially expressed in response to abiotic stress and hormone treatment, indicating that they have diverse functions in the responses to abiotic stress and hormone response (Yang et al. 2012). The transcriptional regulation of a 9-LOX gene in olive fruit mesocarp was shown in response to different abiotic stresses including low and high temperature, darkness and wounding (Padilla et al. 2012). The expression of the *CaLOX1* gene is modulated by drought, NaCl and well-known modulators of defense responses in plants, such as ethylene, SA and methyl viologen (Hwang and Hwang 2010). These data suggest that the *CaLOX1* gene has functional roles in the responses to abiotic stresses as well as biotic stresses. Here, this possibility was tested using *CaLOX1*-OX transgenic plants, which were found to be tolerant to drought, high salinity and osmotic stress, compared with WT plants.

Intriguingly, the increased tolerance of *CaLOX1*-OX plants to drought and high salinity was accompanied by a relatively small increase in lipid peroxidation and down-regulation of H_2O_2 production, when compared with that in WT plants. In plants, LOXs catalyze the oxygenation of membrane lipids (Feussner et al. 1995) and the regio- and stereo-specific dioxygenation of polyunsaturated fatty acids that have a *cis*-1,4-pentadiene moiety, such as linoleic (18:2) and linolenic (18:3) acids, to produce the corresponding hydroperoxides (Liavonchanka and Feussner 2006). The LOX-dependent peroxidative pathway is associated with the defense response, especially hypersensitive cell death in response to pathogen attack in tobacco (Montillet et al. 2002). Similar findings have been reported in several plant species, including cotton (Marmey et al. 2007, Sayegh-Alhamdia et al. 2008), potato (Göbel et al. 2003) and pepper (Buonaurio and Servili 1999). CaLOX1 also plays a crucial role in cell death, and the level of lipid peroxidation positively correlates with CaLOX1 activity in pepper plants infected with virulent and avirulent strains of *Xcv* or in Arabidopsis plants infected with the avirulent strain *Pseudomonas syringae* pv. *tomato* (*Pst*) *avrRpm1* (Hwang and Hwang 2010). Based on these findings, it was expected that lipid peroxidation after drought treatment would be higher in *CaLOX1*-OX plants than in WT plants. The MDA concentration was somewhat higher in *CaLOX1*-OX plants than in WT plants after drought stress as well as under non-treatment conditions. This is not consistent with a drought-resistant phenotype of *CaLOX1*-OX plants, since MDA content is negatively correlated with drought stress resistance in plants (Lima et al. 2002, S. Lim et al. 2014). However, it was found that the rate of relative increase in MDA content was lower in *CaLOX1*-OX plants than in WT plants, when normalized with that of a non-treated sample of each plant line, indicating that overexpression of *CaLOX1* hardly contributes to lipid peroxidation induced by drought stress. This also raises the possibility that the lipid peroxidation occurs via different mechanisms, e.g. via a non-enzymatic reaction, after drought stress. Non-enzymatic lipid peroxidation is a natural and continuosly occurring process in living organisms and its level can dramatically increase under oxidative stress, which promotes ROS and radical production (Sattler et al. 2006). Drought stress and high salinity increase oxidative stress by promoting the production of ROS (Miao et al. 2006, Zhu et al. 2007, Miller et al. 2010), hence enhancing the level of non-enzymatic lipid peroxidation in plants (Baryla et al. 2000). In this regard, it is possible that the increase of MDA content after drought stress is largely induced by non-enzymatic lipid peroxidation rather than *CaLOX1*-mediated lipid peroxidation.

In addition, enhanced tolerance of *CaLOX1*-OX plants in response to drought and high salinity was accompanied by a low level of H_2O_2 production relative to that of WT plants.

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Drought and high salinity as major abiotic stresses impose osmotic stress and provoke excess production of ROS, such as H_2O_2, leading to oxidative damage to biomolecules such as lipids, proteins and nucleic acids, or even cell death (Mittler 2002, Apel and Hirt 2004). Therefore, down-regulation of the H_2O_2 level is essential to avoid ROS-induced injury for cell survival and may be associated with a small increase in relative MDA content of *CaLOX1*-OX plants subjected to drought. These findings are comparable with previous data showing a positive relationship between CaLOX1 activity and the accumulation of H_2O_2 after pathogen infection, although *CaLOX1*-OX plants exhibited a high level of H_2O_2 after infection only with a virulent strain of *Pst*, compared with WT plants (Hwang and Hwang 2010). The discrepancy in the change of H_2O_2 level and MDA content after stress treatment between the two studies may be due to differences between defense mechanisms against biotic and abiotic stresses and the numerous biological roles of the 9-LOX pathway in response to various environmental stimuli. A recent study has shown that the *lox1lox5* double mutant, which is deficient in 9-LOX activity, and the *eto1-14* mutant, which is insensitive to the 9-LOX product 9(*S*)-hydroxy-10,12,15-octadecatrienoic acid (9-HOT), exhibit high accumulation of H_2O_2 and MDA after infection with *Pst* DC3000 and *Pst* DC3000 *avrRpm1* (Lopez et al. 2011). This might reflect the complexity of the 9-LOX pathway, since the 9-LOX pathway plays a positive role in plant defense response to *Pst* DC3000, but a different role in accumulation of H_2O_2 and MDA. Although the detailed molecular mechanism underlying the 9-LOX pathway in response to abiotic stress still remains unclear, these data suggest that the 9-LOX pathway is involved in modulating oxidative stress, lipid peroxidation and plant defense.

In response to drought stress and high salinity, *CaLOX1*-OX plants showed the up-regulation of stress marker genes, including *DREB2A*, *RD20*, *RD29A* and *RD29B*, compared with the WT. In particular, the expression of *RD20* and *RD29B* is predominantly dependent on ABA (Shinozaki and Yamaguchi-Shinozaki 2007, Aubert et al. 2010). This suggests that CaLOX1 plays a role in expression of ABA-responsive genes under drought stress and high salinity, which trigger an increase in the ABA concentration, especially in leaf tissues (Hubbard et al. 2010, Raghavendra et al. 2010). Consistently, expression of the ABA-responsive genes *RAB18* and *RD29B* was higher in *CaLOX1*-OX plants than in WT plants following treatment with ABA. ABA acts as the primary phytohormone in plant responses to abiotic stress, and several studies have shown that ABA sensitivity and the levels of ABA-inducible stress marker genes positively correlate with stress tolerance in plants (Zhang et al. 2006, Hu et al. 2008, Aubert et al. 2010, Fujita et al. 2011, Lim and Lee 2014, C.W. Lim et al. 2014). The enhanced ABA response in *CaLOX1*-OX plant may not affect stomatal aperture. This provides the possibility of a role for CaLOX1 or CaLOX1-derived oxylipins in ABA-independent stomatal closure, since *CaLOX1*-OX plants exhibited smaller stomatal aperture than the WT under ABA treatment as well as normal conditions. Furthermore, ABA plays a multifaceted and pivotal role in plant–pathogen interactions (Cao et al. 2011). In particular, ABA negatively affects Arabidopsis immunity against *P. syringae* (de Torres-Zabala et al. 2007, Goritschnig et al. 2008, C.W. Lim et al. 2014). In this regard, the enhanced ABA response in *CaLOX1*-OX plants could provide a clue to understanding the high susceptibility of *CaLOX1*-OX plants to *Pst* DC3000.

In conclusion, this study provides evidence that CaLOX1 plays a positive role in plant defense response to abiotic stress, such as osmotic, drought and high salinity stress, as well as biotic stress. Involvement of CaLOX1 in modulation of ROS accumulation, lipid peroxidation and ABA-induced gene expression contributes to increased tolerance of *CaLOX1*-OX plants to those abiotic stresses. In addition, we did not rule out the possibility that ABA-independent stomatal closure and gene expression partially enhanced the stress tolerance of *CaLOX1*-OX plants. The molecular mechanism of the CaLOX1-mediated pathway still remains unclear in plant responses to osmotic, drought and high salinity stress, and the biological roles of 9-LOX pathway in response to various environmental stimuli are complex. Further studies to determine the relationship between CaLOX1, ABA, ROS and MDA are needed to clarify the role of CaLOX1 in the plant defense response to abiotic stress. Identification and functional analysis of CaLOX1-derived products are also required to better understand the molecular mechanism underlying the CaLOX1-mediated pathway in plant stress response.

Materials and Methods

Plant material and growth conditions

Seeds of *CaLOX1*-OX Arabidopsis plants were obtained from individual stable transformants (lines #12 and #16) showing significantly higher LOX activity than the WT in a previous study (Hwang and Hwang 2010). *Arabidopsis thaliana* 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. For in vitro culture, Arabidopsis seeds were surface sterilized with 70% ethanol for 1 min and 2% sodium hydroxide for 10 min. Seeds were sown on MS agar (Sigma) supplemented with 1% sucrose. Following stratification at 4°C for 2 d, seeds were incubated at 24 °C in a chamber exposed to a 16 h light/8 h dark cycle.

ABA, mannitol, NaCl and drought treatments and phenotypic analyses

For germination tests, 100 seeds per genotype were sown on plates containing MS agar medium supplemented with various concentrations of ABA, mannitol or NaCl, and seeds with radicle emergence were counted 5 or 7 d later. For root growth assays during the post-germinative stage, 4-day-old seedlings from WT and *CaLOX1*-OX transgenic Arabidopsis lines were transferred into MS agar medium supplemented with 20 µM ABA or 100 mM NaCl. After 7 d, the root lengths of the seedlings were measured. Dehydration treatment was performed as described previously (Lim and Lee 2014). Arabidopsis plants were carefully removed from the soil to avoid injury and harvested at the given time points after drying. For dehydration and salt stress treatment of the Arabidopsis plants grown in soil, five seedlings from each line per pot were grown for 2 or 3 weeks. The pots were randomly placed in a tray before treatment. Dehydration stress was imposed on 3-week-old-plants by withholding water for 12 d. Plants were then watered for 3 d to promote recovery, and the survival rate of rehydrated plants with green leaves was calculated. To assess drought tolerance in a quantitative manner, Arabidopsis leaves were detached from each plant and placed in Petri dishes. The dishes were kept in a growth chamber with 40% relative humidity, and the loss of fresh weight was measured at the indicated times.

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For salt stress, 2-week-old plants were treated with 250 mM NaCl solution for 12 d. The Chl content was measured as described previously (Lee and Hwang 2009). Briefly, leaf samples from WT and *CaLOX1*-OX transgenic plants subject to salt stress were ground in liquid nitrogen and extracted overnight with 2 ml of 95% ethanol. The Chl content of the samples was determined spectrophotometrically according to the formula (Ch $a + b = 5.24 \times A_{664} + 22.24 \times A_{648}$). The experiments were repeated three times.

Stomatal aperture bioassay

The stomatal aperture bioassay was performed as described previously (Lim and Lee 2014). Leaf peels were harvested from the rosette leaves of 4-week-old plants and floated in stomatal opening solution (SOS: 50 mM KCl, 10 mM MES-KOH, pH 6.15, 10 mM $CaCl_2$) with light exposure. After 2.5 h, the buffer was replaced with new SOS. After further incubation for 2.5 h, 100 stomata were measured per sample. Each experiment was performed three times.

Lipid peroxidation

Lipid peroxidation levels were monitored by measuring the concentration of thiobarbituric acid-reactive substances (TBARS), which is formed by the reaction of MDA, a secondary product of lipid peroxidation, with thiobarbituric acid (TBA), as described previously (Heath and Packer 1968). Leaf samples (100 mg) collected from Arabidopsis were homogenized in a solution of 0.5% (w/v) TBA in 20% trichloroacetic acid (w/v). The mixture was boiled at 95 °C for 30 min and cooled on ice for 5 min. After centrifugation at $13,000 \times g$ for 10 min, the absorbance of the resulting supernatant was determined at 532 and 600 nm. The non-specific absorbance at 600 nm was subtracted from the absorbance at 532 nm, and the MDA concentration was calculated using an extinction coefficient of 155 $mM^{-1} cm^{-1}$. Data represent the mean of at least three independent experiments.

DAB staining

Staining with DAB was carried out to visualize H_2O_2 in Arabidopsis leaves treated with NaCl and drought stress, according to the method described by Kim and Hwang (2012). Briefly, leaf samples were collected and submerged in 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 Chl, and were then photographed.

RNA isolation and qRT–PCR

Total RNA was isolated from the leaf tissues of Arabidopsis and pepper plants using an RNeasy Mini kit (Qiagen). All RNA samples were digested with RNase-free DNase to remove genomic DNA. After quantification using a spectrophotometer, 1 µg of total RNA was used to synthesize cDNA. A Transcriptor First Strand cDNA Synthesis kit (Roche) was used 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 resulting cDNA was amplified in a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad) using iQ™ SYBR Green Supermix and specific primers (**Supplementary Table S1**). Each reaction was performed in triplicate. The PCR was programmed as follows: 95 °C for 5 min, 45 cycles at 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s. qRT–PCR analysis was carried out with at least two biological and two technical replicas. The relative expression value of each gene was calculated by the $\Delta\Delta$Ct method (Livak and Schmittgen 2001). To ensure accurate measurement of target gene expression, three internal control genes were used for normalization: *Actin 1*, *EF1α* and *18s rRNA* for pepper, and *Actin 2*, *Actin8* and *EF1α* for Arabidopsis.

Supplementary material

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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