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Characterization of a Homeodomain-Leucine Zipper Gene 12: Gene Silencing in Pepper and Arabidopsis-Based Overexpression During Abiotic Stress

Ruixing Zhang[1] · Quanhui Li[1] · Jingjing Xiao[1] · Saeed ul Haq[1] · Abid Khan[2] · Guoxin Cheng[1,3] · Huiling Yan[4] · Zhenhui Gong[1]

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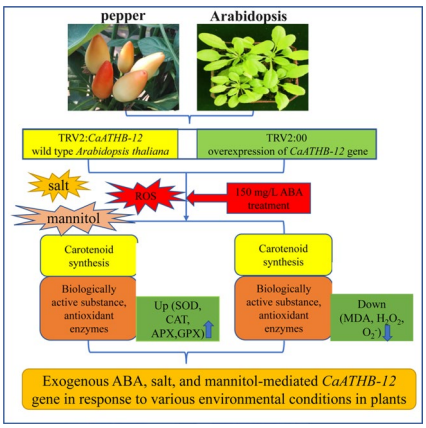
Abstract

Homeodomain-Leucine Zipper (HD-Zip) proteins are important ubiquitous and diverse molecular chaperones in plants. We characterized a gene *CaATHB-12* derived from HD-Zip I subfamily which was intensively induced by exogenous abscisic acid (ABA), salt, and mannitol applications in a pepper cultivar. Efficient gene silencing lines were created from pepper, and stable heterologous overexpression lines were created from Arabidopsis to achieve a comprehensive exploration of gene function. The functional study of *CaATHB-12* in pepper increased plant sensitivity to ABA stress, while the over-expressing *CaATHB-12* in Arabidopsis lines revealed that tolerance to ABA, salt, and mannitol stresses was decreased. Furthermore, *CaATHB-12* plays a fundamental role in elevating the tolerance to these stresses through the increased expression of other stress related genes, increasing the activities of antioxidant enzymes and scavenging the reactive oxygen species. The studied functions of the *CaATHB-12* gene may provide some insights in exquisite molecular detail by pursuing signal transduction mechanisms that converge on gene expression patterns.

Graphical Abstract



Keywords *CaATHB-12* · Abiotic stress · Carotenoids · Transgenic *Arabidopsis*

Introduction

Plants produce substantial amount reactive oxygen species (ROS) to carry out key cellular functions in normal conditions as well as in response to stresses caused by biotic and abiotic factors (Foyer 2020; Khan and Khan 2017).

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Environmental stresses affect the plant growth, development and reduce the yield, nutrition, and quality of the crops. To cope up with these environmental stresses, plants have developed sophisticated defense mechanisms, including antioxidant enzyme systems (glutathione peroxidase, GPX; superoxide dismutase, SOD; catalase, CAT; peroxidase, POD; ascorbate peroxidase, APX) and bioactive substances (phenolic compounds, flavonoids, carotenoids, tocopherols) (Gill and Tuteja 2010; Nafees et al. 2011). The homeodomain-leucine zipper (HD-Zip) proteins with a highly conserved and unique sequence which plays an extremely key role in the growth and development of plants (Ariel et al. 2007; Ré et al. 2014). The homeobox gene acts as a major regulator of all aspects of plant development, while some HD-Zip proteins participate in fruit development and also responds to different abiotic stimuli (Ariel et al. 2007). The HD-Zip proteins (I–IV) are pressure-sensitive HD-Zip I proteins and are studied in recent years (Ariel et al. 2007; Henriksson et al. 2005; Hjellstrom et al. 2003). The *ATHB6*, *ATHB7*, and *ATHB12* showed an up-regulation to externally applied ABA and water-deficit treatments, suggesting their important roles in regulating crops responses to drought stress (Olsson et al. 2004; Soderman et al. 1996). Similar findings were observed in the model plant *Arabidopsis*, where overexpression of *ATHB-7* facilitated photosynthesis, increased chlorophyll content and leaf growth (Hjellstrom et al. 2003; Soderman et al. 1996). Studies on *ATHB-12* gene in response to stress in the *Arabidopsis* (Olsson et al. 2004; Ré et al. 2014), and many of the studies focused on salt stress and water deficit induction (Henriksson et al. 2005). In the *Craterostigma plantagineum*, *CpHB4–CpHB7* genes are reported to show response to drought stress. The expression *CpHB6* and *CpHB7* were induced by ABA treatment and drought stress, while *CpHB4* have showed down-regulation to ABA treatment (Deng et al. 2002; Frank et al. 1998). In rice, the HD-Zip I gene *Oshox22* mediated drought and salt stresses following the ABA-mediated signal transduction pathway (Zhang et al. 2012). The sunflower HD-Zip I protein Hahb-4 was reported for the improved drought tolerance in *Arabidopsis* (Manavella et al. 2006). Recently, *MtHB1*, a *Medicago truncatula* HD-Zip I protein has shown an induced expression in response to salinity stress (Ariel et al. 2010). Although many HD-Zip I genes have been studied, no systematic study of HD-Zip I proteins has been conducted in pepper.

Abscisic acid (ABA), as a stress signal, enhances the plants tolerance to several environmental stresses (Yu et al. 2006), including extreme temperatures (Verslues and Zhu 2005), drought (Bartels and Sunkar 2005), and salinity (Ahuja et al. 2010). Exogenous application of ABA can regulate the accumulation of secondary metabolites in fruits (Satoru et al. 2009; Zhu et al. 2012). It has also been shown that ABA treatment increased carotenoid and chlorophyll concentrations in tomato leaves and fruit (Barickman et al. 2014). Previous studies have shown that the stress tolerance of plants against adverse environmental conditions is affected by ABA, which is the key regulator in response to environmental stresses (Taylor et al. 1988). It can greatly improve the resistance of higher plants to adversities (Bartels and Sunkar 2005). It has been reported that ABA treatment can lead to a sharp increase in POD activity, while POD can neutralize part of H_2O_2 and reduce the damage of H_2O_2 in plants (Bueno et al. 1998). Under drought stress, ABA treatment can reduce the active oxygen species in maize and further improve its antioxidant capacity (Jiang and Zhang 2002). ABA treatment can also affect the accumulation of carotenoids in tomato (Barickman et al. 2014; Zhu et al. 2012). Furthermore, due of its function as a signaling molecule, ABA can stimulate the change of fruit color. Exogenous ABA treatment of grapes resulted in quicker carotenoid production in the peel (Coombe and Hale 1973). Recently, Tian et al. (2016) reported that exogenously ABA (150 mgL^{-1}) treated fruits of pepper, resulted in a significant increase the *Capsaicin* synthesis. A 150 mg L^{-1} of ABA treatment was used by Xiao (2014) to treat pepper leaves, resulting in a rapid decrease in chlorophyll and yellowing of leaves. It has also been found that treating tomato fruits with ABA (100 mg L^{-1}) during ripening stimulates an increase in lycopene content, which is mainly affected by the negative effect of ABA on the GA_3 content (Yu et al. 2016).

In higher plants, carotenoids are composed of the skeleton C_{40}, which is cleaved to form ABA (Zhang et al. 2009). ABA regulates the expression of some chlorophyll degradation-related genes and accelerates the decomposition of chlorophyll (Li et al. 2015). Similar investigations have been conducted in apples and tomatoes (Sun et al. 2012; Yu et al. 2016). Simultaneously,, the endogenous ABA treatment was also up-regulated the carotenoid synthesis pathway genes in grapevine (Enoki et al. 2017). There are currently very few research findings on whether ABA can prevent the formation of carotenoids in pepper fruits or not.

Our previous studies have shown that *CaATHB-12* gene could regulates carotenoid content under cold stress, and potential associated with oxygen scavenging mechanism (Zhang et al. 2020). Here, our aim to explored the function of *CaATHB-12* using overexpression (OE) and virus-induced gene silencing (VIGS) in both *Arabidopsis* and pepper. Our results provide further insights into the function of *CaATHB-12* in plant ABA, salt, and osmotic stresses response.

Materials and Methods

Plant Materials and Growth Conditions

The gene silencing lines were created from *Capsicum annuum* and the overexpressed lines were created from

Arabidopsis thaliana to explore the *CaATHB-12* gene function from both positive and negative aspects. Two plant materials were used. *Capsicum annuum* cv. R24 was obtained from the pepper research group, College of Horticulture, Northwest A&F University, P.R. China. Seedlings of pepper were maintained in a growth chamber under 16 h light at 25 °C and 8 h dark cycles at 20 °C as following in (Ul Haq et al. (2019). Fruit was harvested at 25, 35, and 50 days post full blooming of flowers. Fruit samples were stored in frozen form in liquid nitrogen for later chemical analysis and gene expression analysis through quantitative real-time PCR (RT-qPCR). *Arabidopsis thaliana* ecotypes Columbia-0 was procured from College of Horticulture, Northwest A&F University, P.R. China, and maintained temperatures at 22 °C to light and 18 °C at night and a 65% of relative humidity (Wang et al. 2017).

Sequence and Phylogenetic Analysis of *CaATHB-12* in Pepper

The *CaATHB-12* sequence analysis was performed using the NCBI BLASTp program (Available online: http://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein sequences were analyzed for finding the HD-Zip domain with other plant species by using the CLUSTALW following the methods of Guo et al. (2016). The alignment of CaATHB-12 proteins with other plant species were done through the DNAMAN (Version 5.0) software and the phylogenetic tree was generated by the MEGA 6.0 program with the default parameters as described by Benson (Benson et al. 2000). Other physico-chemical properties such as the molecular weight (MW) and isoelectric point (*PI*) of the CaATHB-12 were determined by EXPASY program (Available online: https://www.expasy.org/) according to the method of He et al. (2018).

Expression of *CaATHB-12* Gene in Different Tissues of Pepper Using RNA-Seq Data

The RNA-seq database (Version 1.5) of CM334 was used for analyzing the tissue-specific expression (http://pepperhub.hzau.edu.cn/index.php, Kim et al. 2014). Data regarding RPKM (reads per kilo base per million mapped reads) of the *CaATHB-12* gene for different organs including leaves, stems, roots, as well as for the placenta and pericarp at 6 days post anthesis, 16 days post anthesis, and 25 days post anthesis was recorded and normalized at $\log_2$ while a the heatmap was constructed by program ImageGP (Available online: http://www.ehbio.com/ImageGP/) as described by Huo et al. (2019).

VIGS Assay of *CaATHB-12* in Pepper Fruits

The tobacco rattle virus (TRV) based silencing method was used to knockdown the *CaATHB-12* gene in pepper fruits (cv. 'R24'). Tobacco rattle virus RNA1 (TRV1) and RNA2 (TRV2) sequences were used as vectors in the pepper plant (Macfarlane 1999). A 307 bp portion of the *CaATHB-12* ORF was sequenced confirmed from pepper cDNA using the specific primer pair (Supplementary Table S1) with the restriction enzymes sites *Xba*I and *Kpn*I. Special primers of *CaATHB-12* and *CaPDS* were designed and then the target genes were injected into the TRV vector to generate TRV2:*CaATHB-12* and TRV2:Ca*PDS* (phytoene desaturase gene, the positive control) (Tian et al. 2014). The TRV:00 was acted as a negative control. The TRV1, TRV2 and TRV2:*CaATHB-12* vectors were injected into the target fruits by using the *Agrobacterium tumefaciens* strain GV3101. The suspensions with *Agrobacterium* and TRV1, TRV2, and TRV2:*CaATHB-12* (OD600 = 1.0) were injected into the green mature stage of pepper fruits (25 days after full bloom). The fruits were packed in sterilized filter papers in a clean and sterilized container and were placed in a growth chamber in dark condition for 48 h maintain the temperature at 18 °C with 35% of relative humidity. After 2 days in the dark, the treated pepper fruits were shifted to 16 h/23°C on light day and 8 h/20 °C a dark day, following the methods of Tian et al. (2014). The control and silenced fruits were used for further analysis after 15 days of treatment as described by Tian et al. (2014). To reduce experimental error, all the experiments were independently repeated three times.

CaATHB-12 Transgenic *Arabidopsis* Lines

The full-length ORF of the *CaATHB-12* was cloned from pepper cDNA with specific primer pairs having restriction enzymes site *Xba*I and *Kpn*I (Supplementary Table S1). The transgenic plants were grown on Murashige and Skoog (MS) medium supplemented with 50 mmol/L kanamycin and their screening was done through PCR. For further experiments, the third generation (T_3) *Arabidopsis* seeds were used following the method of Zhang et al. (2020).

Stress Treatments and Sample Collection

To investigate the response of *CaATHB-12* to abiotic stresses, leaf discs of 0.5 cm in diameter were collected from the *CaATHB-12*-overexpressed *Arabidopsis* leaves and floated in different concentrations of ABA (0, 50, 100, 150, 200 and 250 mg L^{-1}). The excised leaf discs of the *CaATHB-12* overexpressed plants exposed to ABA stresses were put in a control environment at 26 °C with continuous fluorescent light for 3 days, and the method of Xiao et al.

(2014) was followed to conduct the treatments with a little modification.

The selected TRV2:*CaATHB-12* and TRV2:00 detached fruits were sprayed with 150 mg L^{-1} ABA following the methods of Zhang (2016) and Tian et al. (2016) with a little modification. Fruit samples collection was conducted at 0, 6, 12, 24 and 48-hours post treatment. Three independent biological replicates were conducted for each treatment experiments.

Additionally, the 3-week-old *CaATHB-12* overexpressed (OE1 and OE2) and WT lines of *A. thaliana* were selected to further analyze their ABA, salt, and mannitol stress tolerance. For ABA stress, the seedlings (raised under normal growing conditions) were sprayed with 150 mg L^{-1} ABA and leaf samples were collected at 48 h. And then, the MDA, superoxide anion free radical, antioxidant enzymes activities, chlorophyll and carotenoid contents were measured. For salt stress, seedlings were watered 150 mM NaCl every two days for 7 days. For mannitol stress, seedlings were watered 200 mM mannitol every two days for 7 days. *Arabidopsis* seedlings incubated under normal conditions were calculated as the following the control. Three separate seedlings samples were collected randomly, immediately kept in liquid nitrogen and stored at − 80 °C. Three independent biological replicates were used in experiments.

Fruit Color Measurement

Color measurement of the fruit samples were conducted according to the method of Michael et al. (1997), and the colorimetric system (CR-400, KONICA MINOLTA, Japan) was used to record the L, a, b, and C values of the fruits (Hunter 1987). The above-mentioned parameters represent respectively the "luminance", "degree of red/green", "degree of yellow/blue", and "Chroma (saturation or vividness of color); Chroma= $(a^2+b^2)^{1/2}$". After measurement of each fruit, the instrument was recalibrated and for reading the fruits were directly put on the diam aperture. These experiments were conducted in three biological replicates.

RNA Extraction and Quantitative Real-Time PCR (RT-qPCR) Analysis

The total RNA extraction, synthesis of cDNA and RT-qPCR was done according to the methods of (Guo et al. 2014; Khan et al. 2018). The ubiquitin binding gene (*CaUBI3*) of pepper (Wan et al. 2011), and *AtActin2* gene of *Arabidopsis* were correspondingly used as reference genes. NCBI Primer BLAST was used to design all the primer pairs (Supplementary Table S1) for RT-qPCR. Relative gene expression levels were determined following the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008).

Measurement of the Contributed Parameters in Pepper Silenced Fruits and Transgenic *Arabidopsis*

The lipid peroxidation in cell plasma membranes of pepper fruits and *Arabidopsis* lines were assessed by measuring the antioxidant system and antioxidant substances. The malonaldehyde (MDA) content was measured using 0.5% 2-thiobarbituric acid (TBA) containing 5% (w/v) trichloroacetic acid reaction following the method of Buege and Aust (1978). The chlorophyll and carotenoid contents were quantified and calculated by the method described by Porra et al. (1989). Anthocyanins content was measured according to the method of Christie et al. (1994). The determination of total phenols and flavonoids were measured according to the method of Rodov et al. (2010) with slight modification, we used (OD_{280}/g) and (OD_{325}/g) to indicate the relative amounts of total phenols and flavonoids, respectively, while the catalase (CAT) activity was determined using the method of Aebi (1984), superoxide dismutase (SOD) and peroxidase (POD) activities were measured following the method of Stewart and Bewley (1980), glutathione peroxidase (GPX) activity was measured according to Flohé and Günzler (1984), ascorbate peroxidase (APX) activity was determined the protocol of Nakano and Asada (1987), superoxide anion free radical ($O_2^{-}\cdot$) accumulation was determined as described previously by Zweier (1988), hydrogen peroxide (H_2O_2) content was measured by the method of Brennan and Frenkel (1977).

Statistical Analysis

Statistical analysis was executed through Statistical Analysis System software (IBM SPSS Statistics 19.0, USA) for analysis of variance (ANOVA). A least significant difference ($P \leq 0.05$) test was used to identify significant differences among the treatments. All experiments were performed and analyzed with three independently biological replicates.

Results

Expression of *CaATHB-12* at Different Developmental Stages and Analysis of Color Parameters of Pepper Fruit

To illuminate the function of *CaATHB-12*, with tissue-specific analysis of the vegetative (roots, stems, and leaves) and reproductive parts (three different developmental stages of pericarp and placenta) were conducted using the Pepper Hub from the pepper CM334 (http://pepperhub.hzau.edu.cn/index.php, Kim et al. 2014). As revealed in Supplementary Fig. S1, at 6 days post-anthesis (DPA) of the pericarp (PC) displayed the highest

expression of *CaATHB-12* gene, followed by stems, PC-16 DPA, roots, and PL-6 DPA, while leaves had the lowest expression at PL-25 DPA. However, there were no obvious changes in the expression profile of carotenoid-biosynthetic genes. The transcriptomic results indicated that the expression of *CaATHB-12* was higher in the pericarp development process. Furthermore, the transcript levels of *CaATHB-12* under normal condition in three continuous developmental stages of 'R24' pepper fruit (Fig. 1A) was investigated by RT-qPCR. Results obtained showed that "L" (represented "luminance"), "b" and "C" values initially decreased, followed by an increase at the final stage of fruit ripening, while the "a" value increased in the whole period of fruit ripening (Fig. 1B). *CaATHB-12* transcripts were detectable in all stages, with Stage 3 (50 days after full bloom) and Stage 2 (35 days after full bloom) having the highest expression level, and the least expression at stage 1 (25 after full bloom) (Fig. 1C). These dynamic changes in the above-mentioned parameters proposed that fruit color change follows the rule: green, bottle green, light-colored, and red.

Virus-Induced Gene Silencing (VIGS) of *CaATHB-12* in Pepper Detached Fruit

TRV2 vector carrying the *CaATHB-12* gene was vaccinated into the *Capsicum annuum* cv. R24 detached fruits. 15 days post-inoculation, different colors were noted in the *CaATHB-12*-silenced fruits as compared to the control (Fig. 2A). The color of the *CaATHB-12*-slienced fruits changed from green to yellow, while that of the control fruits were from green to red color. Simultaneously, the TRV2:*CaPDS* (positive control) detached fruits were green to orange-yellow color. Furthermore, silencing efficiency measured through RT-qPCR affirmed that *CaATHB-12* transcript level in the silenced fruits were 86% lower as compared to the control (Fig. 2B). Similarly, the carotenoids content in the silenced fruit was also significantly lower than the control fruit (Fig. 2C).

Effect of ABA Stress on CaATHB-12 Silenced Pepper Fruit

Further investigated the function of *CaATHB-12* under ABA treatment, the *CaATHB-12*-silenced and control fruits were treated with ABA solution (150 mg L^{-1}). To study the silencing effect of *CaATHB-12* in pepper fruits, the ROS,

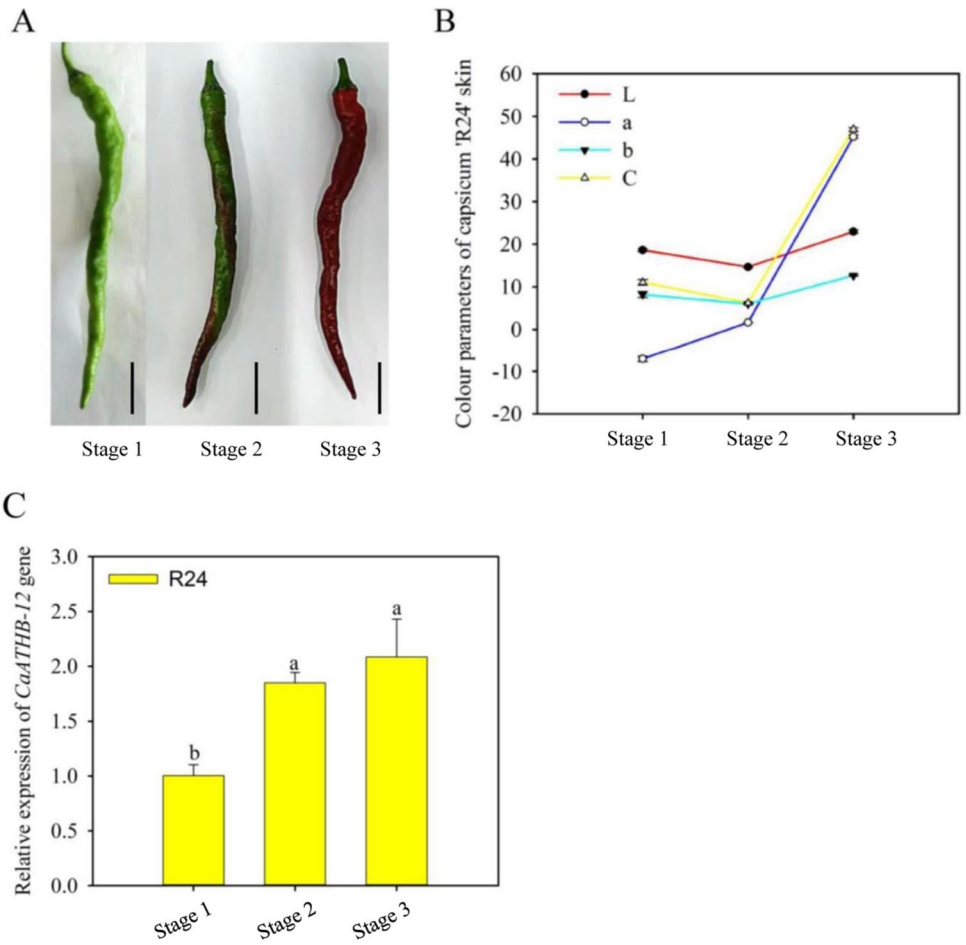

Fig. 1 Dynamics of change of color parameters in pepper R 24 fruits at three stages 25, 35, and 45 days after full bloom denoted as Stage 1, Stage 2 and stage 3, respectively. **A** Fruit phenotypes. Scale bar represents 1 cm **B** Colorimetric L value (luminescence), a value (degree of red/green), b value (degree of yellow/blue) and C value (saturation or vividness of color) at the three different stages of fruit development. **C** Expression of *CaATHB-12* gene in the three development stages. Error bars represent standard error for three replicates, and the different letters indicate the significant level at the $P > 0.05$

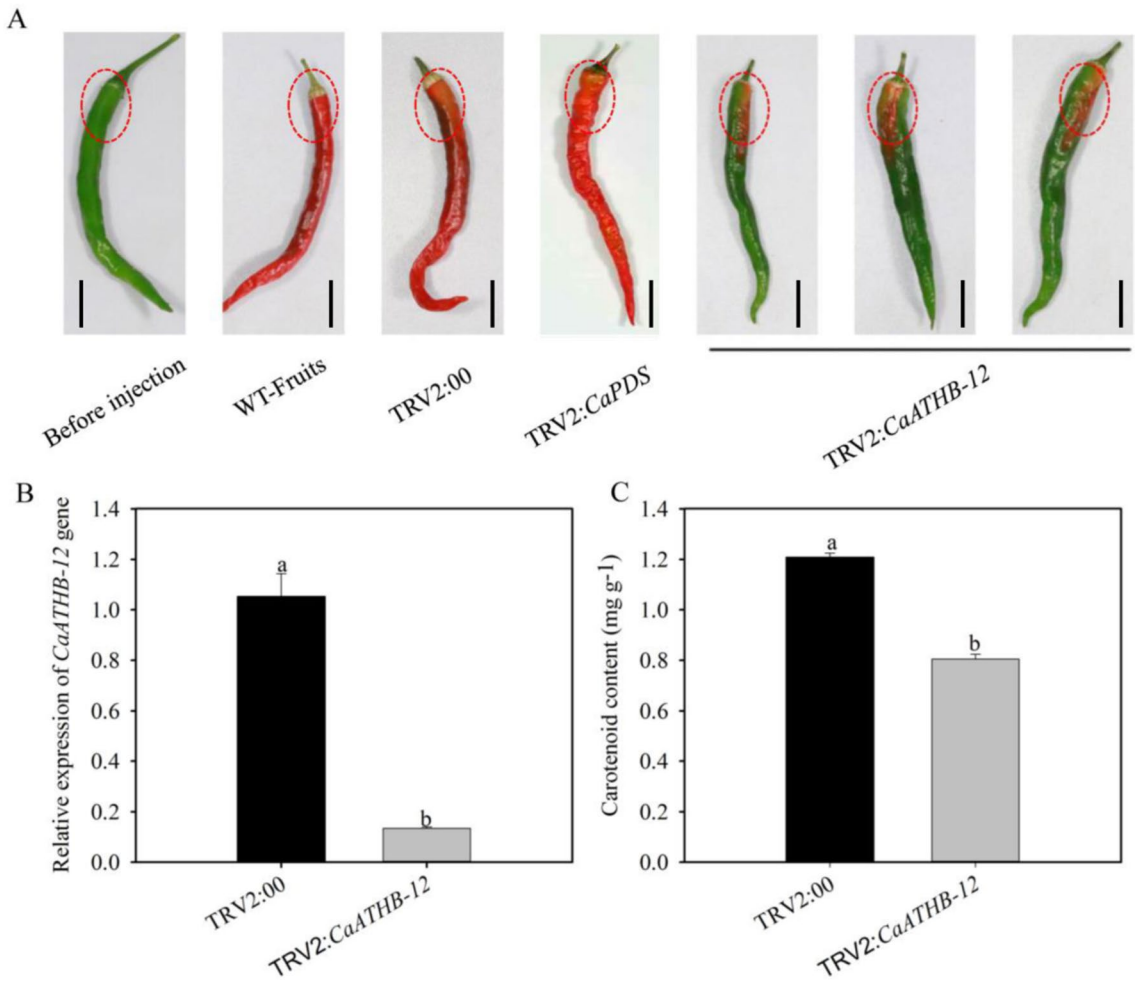

Fig. 2 Effect of *CaATHB-12* gene silencing on the pepper fruit color. **A** Phenotypical changes of pepper fruits. WT-fruit, no injection treatment in pepper fruits; TRV2:00, pepper fruits injected with the TRV empty vector; TRV2:*CaATHB-12*, pepper fruits injected with the TRV vector carrying *CaATHB-12* gene; TRV2:*CaPDS*, pepper fruits injected with the TRV vector carrying *CaPDS* gene, used as positive control. **B** The relative expression of *CaATHB-12* in fruits of *CaATHB-12*-silenced and control (TRV2:00) fruits. **C** Carotenoid content in the fruits of *CaATHB-12*-silenced and control (TRV2:00). Error bars denote standard deviation for three replicates. The letters in lowercase indicate significantly different levels at $P > 0.05$

MDA contents, chlorophyll contents, and ROS scavenging antioxidants enzymes were measured at different time points (0, 6, 12, 24 and 48 h) post treatment in pepper fruits. The H_2O_2 content of the empty vector (control) fruits were significantly higher (4 folds) at 48 h than the *CaATHB-12*-silenced fruits (Fig. 3A). Correspondingly, the malondialdehyde (MDA) and $O_2^{-}\cdot$ levels followed similar trend of increment after ABA treatment in both the control and silenced fruits (Fig. 3B, C).

The antioxidant enzyme system of the pepper plants was stimulated in response to stress in order to mitigate the ROS associated damage. The activities of CAT, POD, SOD and APX gradually increased at each time point (Fig. 4). However, the antioxidant enzyme activities of the above-mentioned enzymes were significantly higher in the *CaATHB-12*-silenced fruits than the control. The POD activity increased at all time points and reached their peaks in both TRV2:00 and TRV2:*CaATHB-12* at 48 h, which was about 4-fold and 5.8-fold respectively (Fig. 4A). Both the SOD and POD activities levels followed the same trends of enhancement after ABA treatment in both control and silenced pepper fruits, but their respective peaks were higher than control fruits (Fig. 4B, C). Though the GPX activity significantly increased up to 24 h post stress in the *CaATHB-12*-silenced fruit and then decreased at 48 h (Fig. 4D).

Thus, we measured the total carotenoid, anthocyanin, flavonoid and total phenolic contents from both *CaATHB-12*-silencedand control pepper fruits at different time points. In control fruits, a slight increase in total carotenoid contents was noted until 48 h post-treatment, whereas in the *CaATHB-12*-silenced fruits, the ABA treatment caused a significant and dynamic increase in the total carotenoid

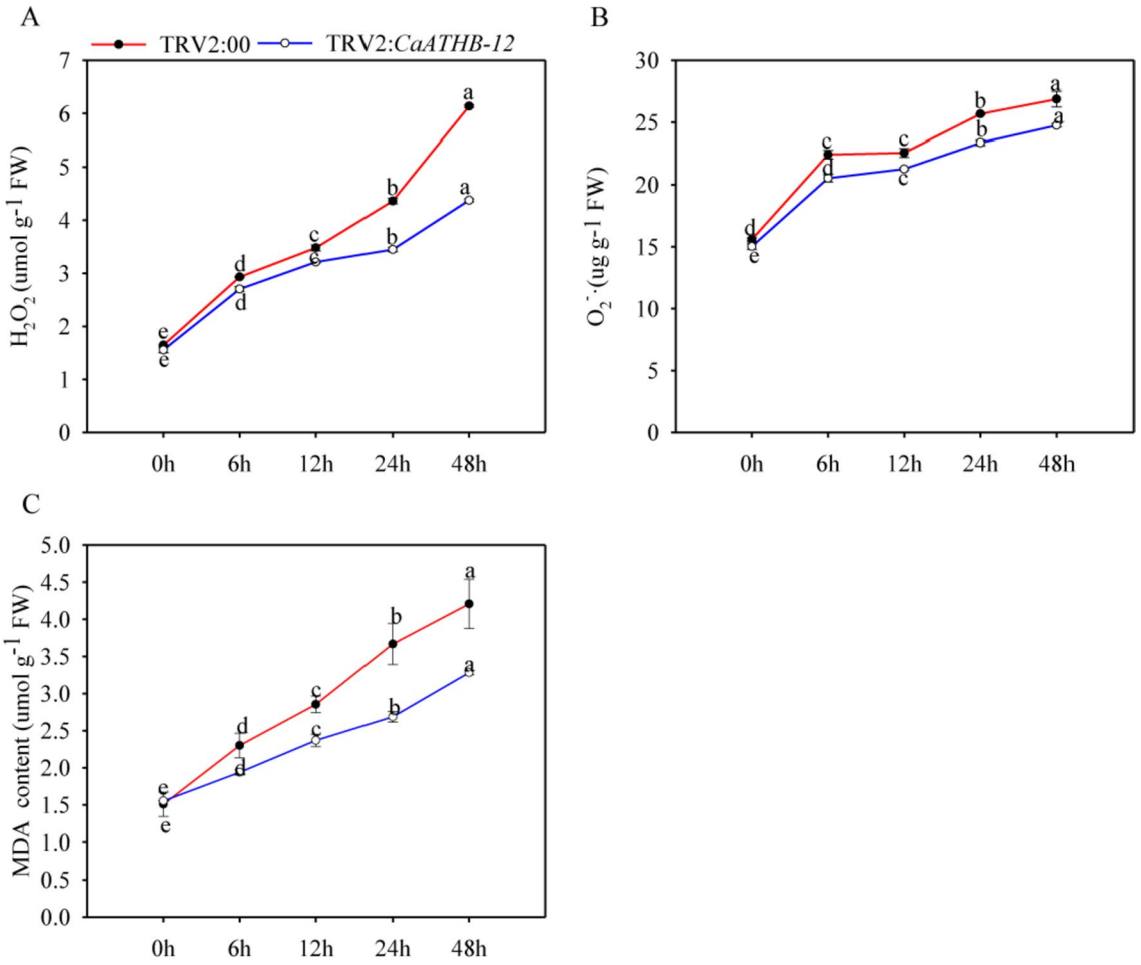

Fig. 3 Effect of *CaATHB-12* silencing on pepper tolerance to ABA stress. **A** H_2O_2 Content **B** Level of O2-· **C** MDA content under ABA stress. Error bars denote standard deviation for three replicates. The letters in lowercase indicate significantly different levels at $P > 0.05$

contents (Fig. 5A). Similarly, the measured anthocyanin contents at 48 h were also almost significantly higher (7 folds) in the silenced fruits as compared to the control fruit (4.5 folds) (Fig. 5B). Flavonoid and total phenolic contents followed the similar trend of increment after ABA stress in both silenced and control pepper fruits. In addition, after ABA stress the flavonoid at 24 h and total phenolic contents at 12 h were significantly higher in the silenced fruits compared to control, and then a bit decrease was recorded in the silenced fruits, but they were still significantly higher than control fruits (Fig. 5C, D).

Additionally, exogenous application of ABA (150 mg L^{-1}) induced the transcript level of *CaATHB-12* in the both silenced and control fruits, but the expression level of the silenced fruits was lesser than that in the control fruits at 0 and 6 h, while its expression abruptly increased at 12 h, reached to maximum at 48 h, and was significantly higher than the control (Fig. 6A). The carotenoid synthesis related genes (*CaPSY*, *CaZEP*, *CaBCH*, *CaLCYB*) were also differentially induced, and their expression levels were significantly higher in the *CaATHB-12*-silenced fruits than that in the control at 48 h, expect *CaLCYB* gene (Fig. 6B, E). Furthermore, the transcript levels of the defense-related genes (*CaPOD*, *CaSOD* and *CaMYB44*) were significantly higher at 24 and 48 h in the *CaATHB-12*-silenced fruits as compared to the control except *CaSOD* which reached to peak at 12 h (Fig. 6F, H). These results partially revealed that *CaATHB-12* played a negative role in the plant defense response against ABA osmosis stress.

Effect of *CaATHB-12* Overexpressing on Transgenic *Arabidopsis*

To further explore the function of *CaATHB-12*, the *CaATHB-12*-overexpressed transgenic lines of *Arabidopsis* were generated. Under normal growth conditions, there are no discernible differences between *CaATHB-12*-overexpressed lines (*CaATHB-12*-OE) and wild type *Arabidopsis* plants (Fig. 7A). Furthermore, the yellowing symptoms in leaf discs of both transgenic and WT lines aggravated

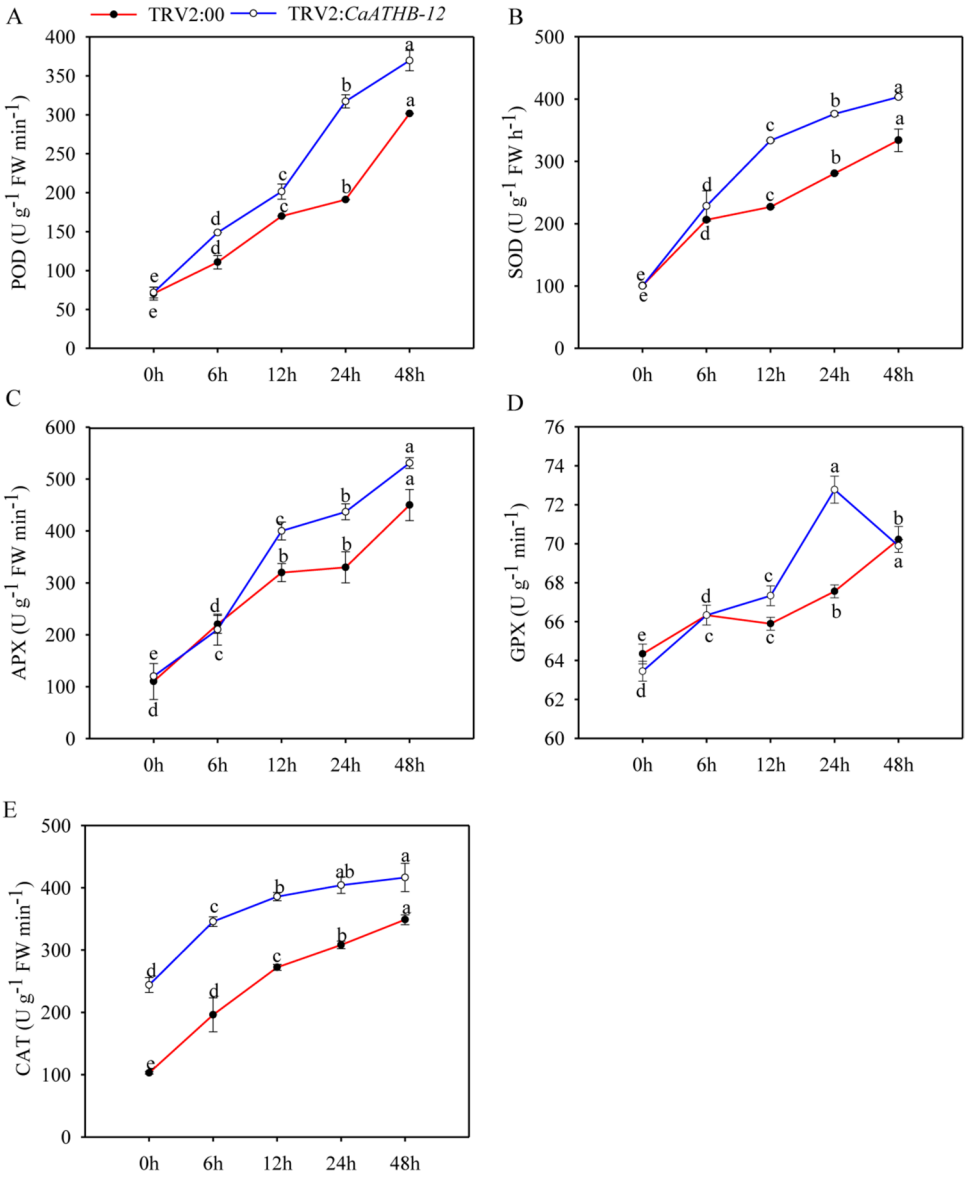

Fig. 4 The effect of *CaATHB-12* silencing on antioxidant enzymes under ABA stress in pepper. **A** POD activity; **B** SOD activity; **C** APX activity; **D** GPX activity; **E** CAT activity. Error bars denote standard deviation for three replicates. The letters in lowercase indicate significantly different levels at $P > 0.05$

as the ABA concentration increases (Supplementary Fig. S2A). Though, as compared to the transgenic lines, the carotenoid content was significantly reduced in WT lines when treated with ABA, while the chlorophyll content of the transgenic lines displayed lower levels than that in WT, and the highest variation was noted with 150 mg L^{-1} ABA solution, where transgenic lines provided approximately 0.2 mg/g and WT lines provided 0.83 mg/g chlorophyll content, the former being 75% lower than the latter (Supplementary Fig. S2B-C). After treated with exogenous ABA (150 mg L^{-1}) treatment at 48 h, no difference was detected in the withering symptom of both transgenic and WT lines (Fig. 7A). Interestingly, the carotenoid and total chlorophyll contents in OE lines were lower than that of the wild type (Fig. 7B-C). However, the ROS included H_2O_2 contents and $O_2^{-}\cdot$ levels in OE lines were higher than that of the wide type (Fig. 7D-E). Furthermore, the MDA content of the WT lines was slightly increased and was higher than transgenic seedlings (Fig. 7F). Furthermore, the CAT, POD, SOD, APX and GPX activities of the transgenic seedlings were significantly lower than the WT (Fig. 8), and a significant difference was found in the above-mentioned antioxidant enzymes activities of the transgenic lines and wild type *Arabidopsis*. Meanwhile, the transgenic *Arabidopsis thaliana* showed dehydration and wilting with weak growth under salt and mannitol treatment (Fig. 9A). However, the WT plants showed a slight yellowing phenotype with lower MDA, H_2O_2, $O_2^{-}\cdot$content and higher CAT, POD activities than OE lines (Fig. 9B–F).

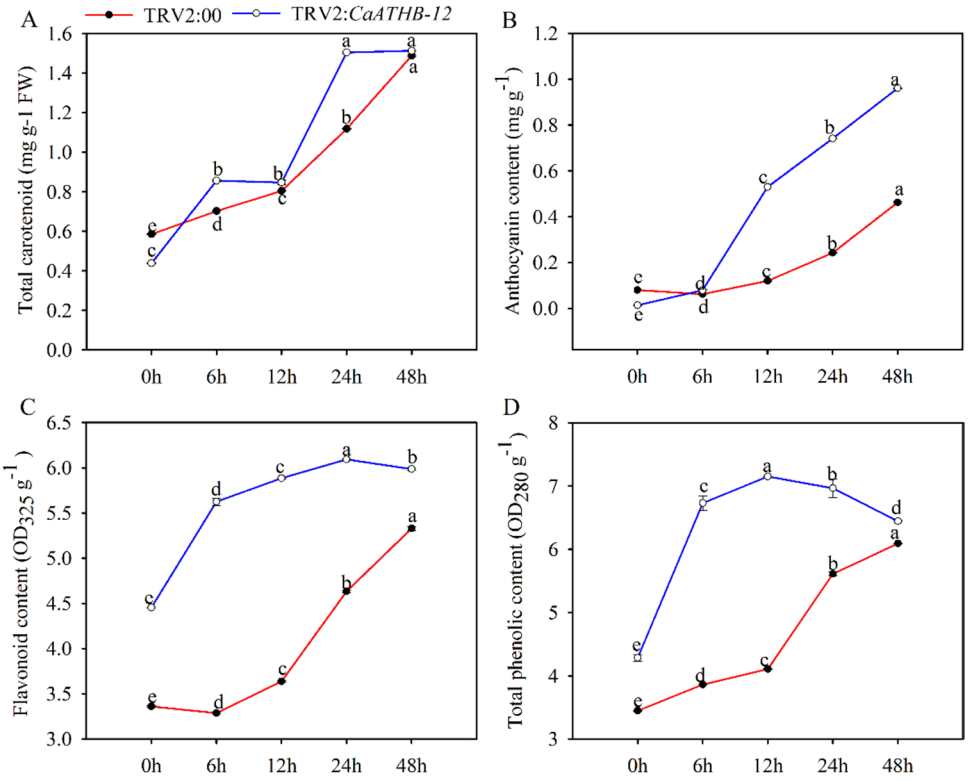

Fig. 5 The effect of *CaATHB-12* silencing on pigment content under ABA stress in pepper. **A** total carotenoid content, **B** anthocyanin content, **C** flavonoid content and **D** total phenolic content under ABA stress. Error bars denote standard deviation for three replicates. The letters in lowercase indicate significantly different levels at $P > 0.05$

Discussion

As sessile organisms, plants are exposed to various environmental stresses during growth and development. If subjected to adversity environments such as drought, high salt and abscisic acid, the membrane integrity of the plants will be disrupted, resulting in adverse effects such as low photosynthetic rate and cell dysfunction, which will eventually lead to crop yield reduction (Wei et al. 2016), it will also lead to the formation of ROS, cell damage, metabolic disorders, and aging processes (Jaleel et al. 2009). The ROS in low concentrations are important signaling molecules, but the increased quantities of ROS result in the generation of oxidative secondary stress (Bailey-Serres and Mittler 2006). In higher plants, abiotic stressors (extremes heat/cold, salt) are due to the imbalance between pro-oxidants and antioxidants resulting in oxidative stress (Sreenivasulu et al. 2007). HD-Zip I transcription factors were involved in response to various environmental stresses such as low temperature, salt, and ABA in regulating fruit development (Jiang et al. 2017; Zhang et al. 2020), by regulating the expression of downstream related genes to promote plant oxidation stress response (Ariel et al. 2010; Harris et al. 2011). On the other hand, the HD-Zip transcription factors also help in the synthesis of color pigments such as chlorophyll and carotenoids (Lu et al. 2014; Manavella et al. 2008). In our previous studies, we identified and cloned the *CaATHB-12* gene of the HD-Zip I subfamily which includes corresponding conserved HD and Zip motifs (Zhang et al. 2020), interact in vitro with the pseudo-palindromic sequence CAAT(A/T)ATTG (Ariel et al. 2010), and were involved in the tolerance to exogenous ABA application (Ribichich et al. 2013).

Previously, it was reported that the expression of photosynthesis-related gene was regulated by the HD-Zip transcription factor *HAHB4* in sunflower, which further regulated the synthesis of carotenoids in transgenic *Arabidopsis* (Manavella et al. 2008). This gene interacted with MYB, bHLH and WD40 partners in the cytoplasm and participates in the regulation of related pigment accumulation (Jiang et al. 2017). The stability and integrity of the cell membrane is an important foundation for plant to grow (Rui et al. 2010). The production of MDA in plants has an important impact on the normal function of the cell membrane. The chlorophyll content is reduced by the influence of excess reactive oxygen, which approximately measures the degree of stress damage in the plant (Choudhury et al. 2017). Under normal growth conditions, ROS such as H_2O_2 and $O_2^{-}\cdot$ are in dynamic balance, but under abiotic stress, the balance is broken, and excess reactive oxygen will damage the cell structure and corresponding functional proteins in the cell. In this experiment, the control fruit accumulated more H_2O_2 and $O_2^{-}\cdot$ than *CaATHB-12*-silenced fruit, and the amount of H_2O_2 and $O_2^{-}\cdot$ of the OE plants were found significantly higher than that of WT. Our results revealed that the pepper *CaATHB-12* gene plays a regulatory role in improving

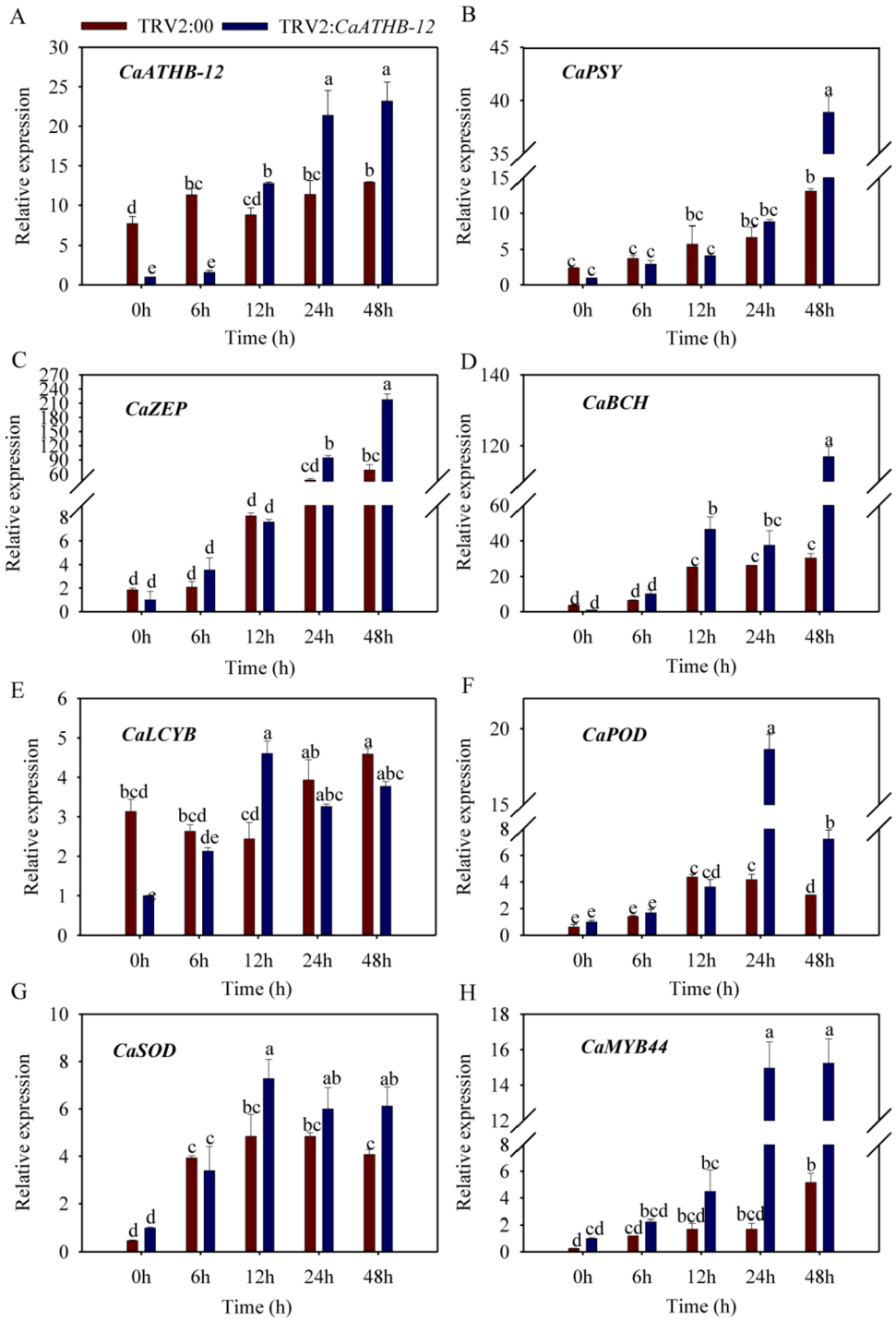

Fig. 6 Expression profiles of carotenoid synthesis regulatory genes and antioxidant enzyme related genes in response to ABA stress. The expression levels of **A** *CaATHB-12*, **B** *CaPSY*, **C** *CaZEP*, **D** *CaBCH*, **E** *CaLCYB*, **F** *CaPOD*, **G** *CaSOD*, **H** *CaMYB44* were investigated by RT-qPCR. Error bars denote standard deviation for three replicates. The letters in lowercase indicate significantly different levels at $P > 0.05$

the scavenging capacity of ROS and lowering the oxidative stress.

Abscisic acid (ABA) being a "stress hormones" (Brandt et al. 2014), while the induction of exogenous ABA tolerance could be due to elevating the activities of the antioxidant enzymes system, which helped to reduce the ROS accumulation and protect the membrane structure from oxidative damage (Yu et al. 2019). Higher plants possess a sophisticated and complex system of antioxidant enzymes and non-enzymatic systems in response to various stresses (Ali et al. 2008), in which SOD acts as a large number of antioxidants (SOD, CAT, POD, APX, and GPX) can decompose $O_2^{-}\cdot$ into H_2O_2, which is further removed by POD and APX (Choudhury et al. 2017). On the other hand, non-enzymatic active oxygen scavenging systems mainly contain bioactive substances such as carotenoids, anthocyanins,

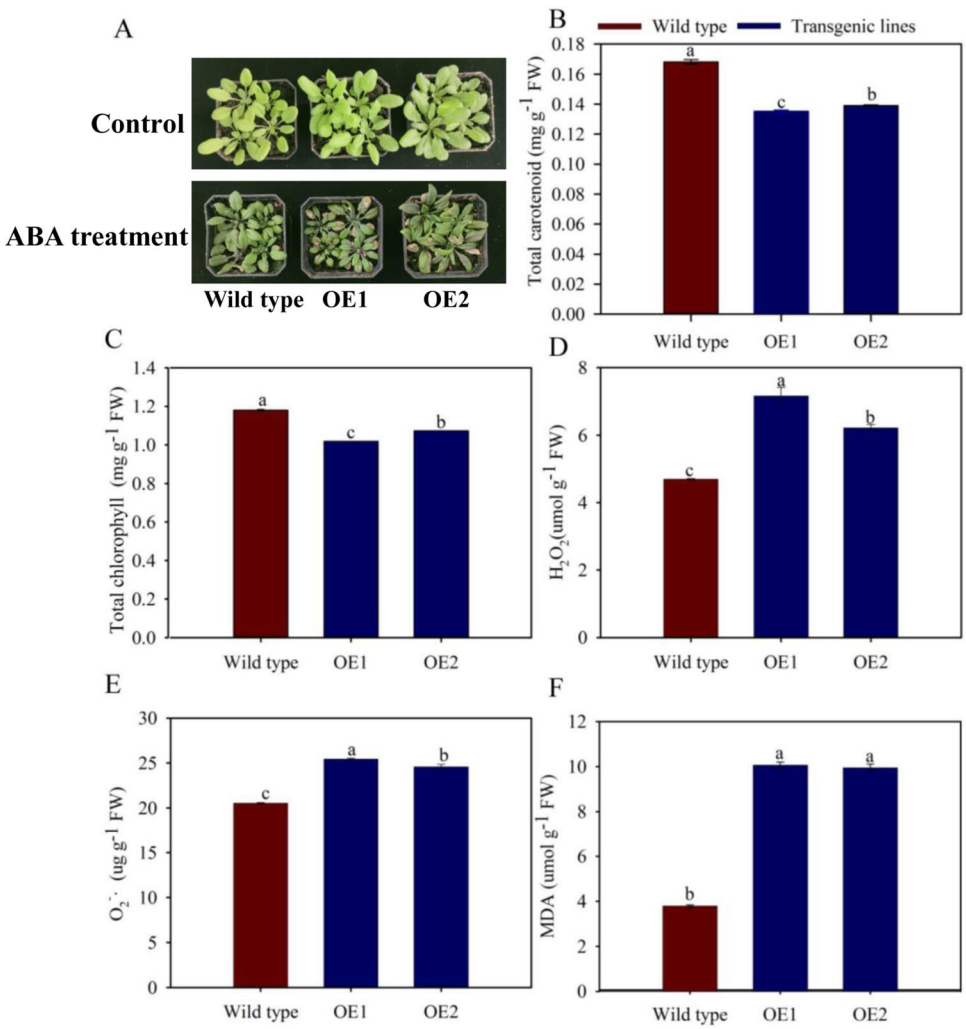

Fig. 7 Over-expression of the *CaATHB-12* reduced ABA stress tolerance in *Arabidopsis*. **A** Phenotypes of the *Arabidopsis thaliana* (wild type OE1 and OE2) after ABA (150 mg L^{-1}) treatments; **B** total carotenoid content of *Arabidopsis* transgenic lines; **C** total chlorophyll content; **D** H_2O_2 content; **E** Level of $O_2^{-}\cdot$ and **F** MDA content under ABA stress. Scale bar represents 1 cm. Error bars denote standard deviation for three replicates. The letters in lowercase indicate significantly different levels at $P > 0.05$

polyphenols and flavonoids (Gill and Tuteja 2010). Relevant research reported that the *ATHB-12* gene in *Arabidopsis* negatively regulates the elongation of plant stems and is induced by NaCl and ABA treatments (Olsson et al. 2004). Previously, we found that the expression of *CaATHB-12* could be modulated significantly during cold stress (Zhang et al. 2020). In this study, the *CaATHB-12* gene was heterogeneously expressed in *Arabidopsis thaliana*. After ABA (150 mg L^{-1}) treatment, the activities of GPX, SOD, APX and POD in the transgenic lines were lower than that of WT, while the activity of CAT was contrary to the law. We speculate that overexpression of *A. thaliana* leads to a reduction in the activity of most antioxidant enzymes in plants, and the MDA content is higher than that of WT, indicated that the extent of cell membrane damage in transgenic plants caused by abiotic stress was higher than in WT plants. Although CAT is an important enzyme for removing H_2O_2 (Karpinski and Muhlenbock 2007; Lee et al. 2007), due to the reduction in the activity of other important antioxidant enzymes, it is not enough to remove excess $O_2^{-}\cdot$, which in turn reduced the ability of the expression strain to clear ROS reduces the stress resistance of the plant.

The antioxidant capacity of plants is manifested through the synergistic effect of various antioxidants. Bioactive substances (such as total phenols, flavonoids, carotenoids, and anthocyanins) as antioxidants have a crucial role in improving plant resistance (Chang et al. 2019; Singh et al. 2017). Wang et al. (2020) reported that exogenous application of ABA (150 mg L^{-1}) had a forceful inhibitory effect on the nitrogen accumulation of fruit, resulting in disorders of nitrogen metabolism, so affecting pigmentation in 'Red Fuji' apple fruit. At the same time participate in the construction of photosynthetic complex protein PSI and maintain the stability of thylakoid membrane (Gill et al. 2011; Niyogi et al. 2001). Havaux (2014) reported that carotenoids function as an oxidative stress signaling molecule. β-carotene is the main component of carotenoids, that can interact with hydrogen peroxide (H_2O_2), superoxide radicals ($O_2^{-}\cdot$) accumulation, free radical reaction (Mahapatra et al. 2013), at sufficiently high concentrations, carotenoids are

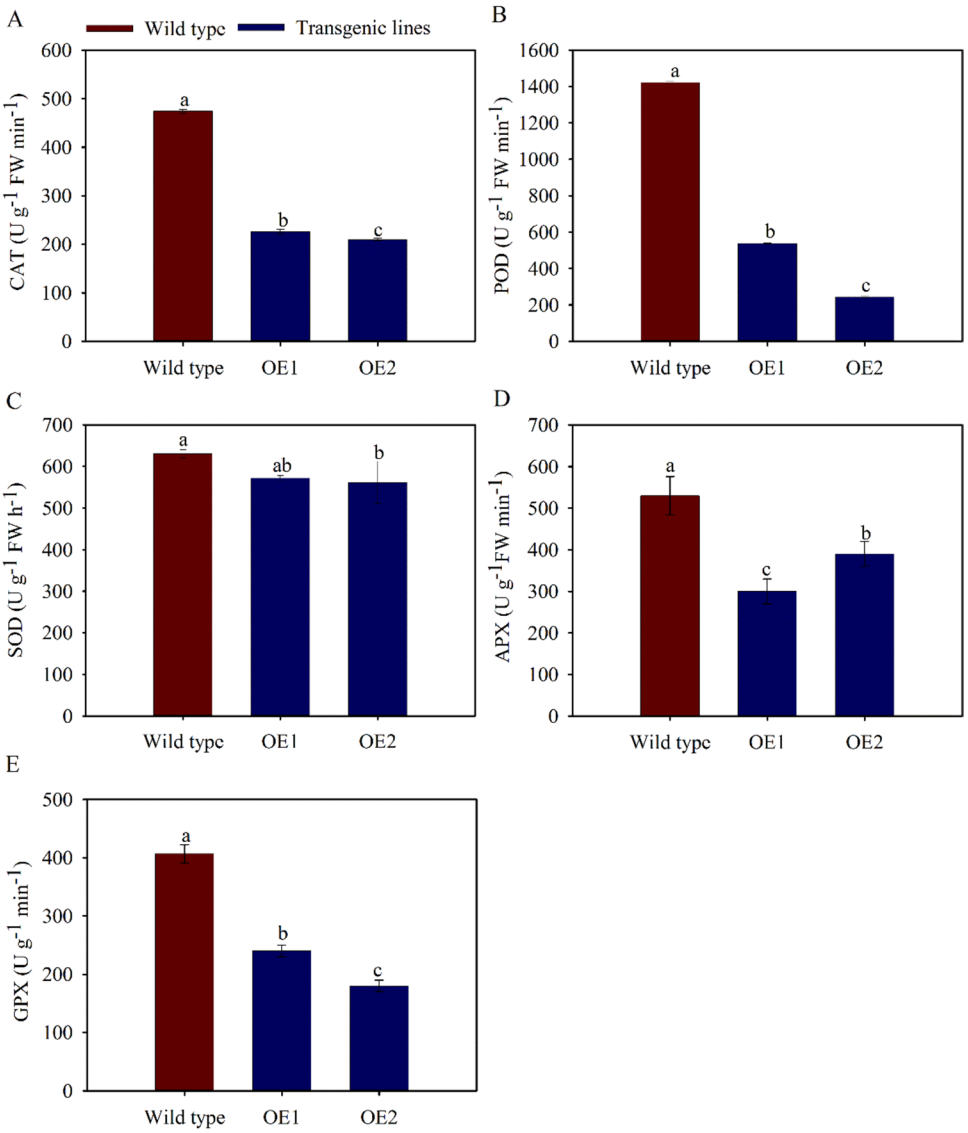

Fig. 8 The levels of antioxidant enzymes in WT and *CaATHB-12*-OE lines under ABA stress. **A** CAT activity; **B** POD activity; **C** SOD activity; **D** APX activity; **E** GPX activity. Error bars denote standard deviation for three replicates. The letters in lowercase indicate significantly different levels at $P > 0.05$

more effective in protecting lipids from peroxidative damage (Ahmad et al. 2010). Because of their unique structure, flavonoids can locate free radical molecules in cells and at the same time scavenge free radicals, making them important for plants under harsh environmental conditions (Løvdal et al. 2010). Studies have reported that the concentration of flavonoids will increase under conditions of cold damage, low temperature, and lack of hormones (Winkel-Shirley 2002). At the same time, it was found that flavonoids have good nitrogen tolerance under the condition of nitrogen deficiency (Peng et al. 2008). Interestingly, polyphenols are directly involved in the process of plant antioxidant stress response, and has the function of metal chelating agent (Ksouri et al. 2008). Studies have shown that the antioxidant activity of blueberries is closely related to the total phenolic content and anthocyanin content (Ehlenfeldt and Prior 2001), and the high content of total phenol and anthocyanin content improves the antioxidant activity of plants (Kalt et al. 2000). In our study, silencing the *CaATHB-12* reduced the carotenoid content, and the expression levels of the related gene involved in carotenoid regulation were also reduced compared to the control fruit (Fig. 6). Previous studies also showed that *RhHB1* (HD-Zip I) impacted the flower color of rose (*Rosa hybrida*) (Lu et al. 2014). Similarly, Jiang et al. (2017) showed that silencing of *MdHB1* (HD-Zip I) caused the accumulation of anthocyanin in 'Granny Smith' flesh apple, whereas its overexpression reduced the flesh content of pigment in 'Ballerina' (red-fleshed apple). After exogenous ABA treatment, the carotenoid synthesis rate of the silenced fruits was significantly higher than that of the control fruits, while the contents of total phenol and flavonoids were higher than that of the control fruit (Fig. 5). On the other hand, after ABA treatment, the carotenoid content of the *CaATHB-12*-overexpressed lines was slightly lower than

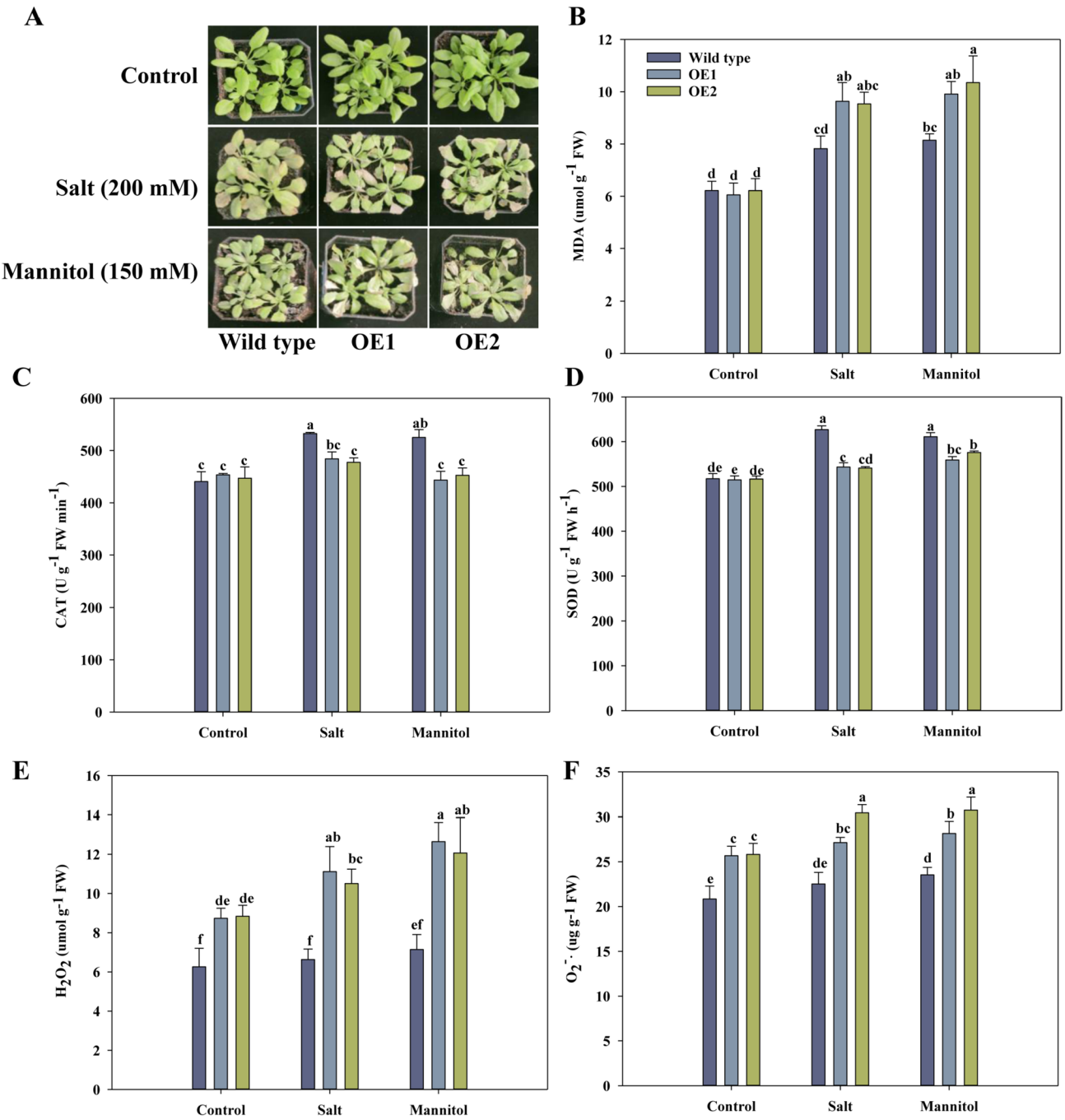

Fig. 9 The salt, and drought resistance of *CaATHB-12*-OE and control *Arabidopsis* plants. **A** Phenotypes of wild-type (WT) and *CaATHB-12*-OE *Arabidopsis*, **B** MDA content, **C** CAT activity, **D** SOD activity, **E** H_2O_2 content, and **F** the O_2^- · of WT and *CaATHB-12*-OE *Arabidopsis* plants for 7 days with containing 150 mM NaCl and 200 mM mannitol, respectively. Error bars denote standard deviation for three replicates. The letters in lowercase indicate significantly different levels at $P > 0.05$

that of WT (Fig. 7). We speculated that the *CaATHB-12* gene of pepper is involved in inducing the production of related bioactive substances and regulating the resistance of plants to abiotic stress.

Exogenous ABA also modulates the expression of gene networks that control other ameliorative and adaptive stress responses in plants (Lim et al. 2015). In previous studies, ABA responses have a wide and various range of downstream effects, and their network of the hormonal pathways is further complicated by interactions with ROS (Pinheiro and Chaves 2011). So, *CaATHB-12* could become fundamental part of the ROS-mediated ABA signaling cascade in

plants. On the other hand, loss-of-function *ATHB12* mutants have illustrated that the gene activates clade a protein phosphatases 2 C (PP2C) genes to interact with proteins of the basal transcriptional machinery and repress AHG3 (Protein Phosphatase 2CA), ABI2 (ABA Insensitive 2), and ABI1 (ABA Insensitive 1) (Ma et al. 2009; Rubio et al. 2009), thus acting as negative regulators of ABA signaling networks, meanwhile the binding of some of these targets is ABA-dependent for *ATHB12* (Valdés et al. 2012). In addition, *ATHB20* and *ATHB5* act as negative regulators of ABA sensitivity in germinating plants (Barrero et al. 2010; Johannesson et al. 2003) and *ATHB6* has also been proposed as negative modulator of the ABA response (Himmelbach et al. 2002; Reyes et al. 2006). Moreover, over-expression of *CpHB-7* isolated from *Craterostigma plantagineum* in *Arabidopsis* resulted in reduced sensitivity towards ABA treatment (Deng et al. 2006). Many stress-related genes in plants generally mediate the response of plants to stress. For example, *AtMYB44* gene can regulate ABA signal-mediated plant response to NaCl and drought stress (Nguyen et al. 2019), *AtDREB2A* as an ABA signal response gene can be induced by low temperature expression (Nakashima et al. 2000), *Mn-SOD* and *POD*, as marker genes related to the antioxidant system, are involved in responding to various stresses (Guo et al. 2012). Rai et al. (2013) study revealed that under the control of stress-inducing factor (*RD29A*), the overexpression of *AtDREB1A* in tomatoes showed enhanced levels of antioxidant enzymes and antioxidant substances, and the ability to drought-induced oxidative stress greatly enhanced. Our research shows that after ABA treatment, the content of *POD* and *SOD* in the *CaATHB-12*-silenced fruits are higher than that of the control fruits. Altogether, our study indicating that the *CaATHB-12* gene is involved in the regulation of ABA-mediated oxidative stress response, which is further induced by exogenous ABA, but the exact molecular regulatory mechanisms need further study. Therefore, reduced tolerance to ABA stresses of the *CaATHB-12*-overexpressed plants may be due to partially impeded expression of these genes. Furthermore, this research will closely focus on fundamental insights for future studies to precisely explore the role of the *CaATHB-12* gene in regulatory pathways.

Conclusions

Taken together, we characterized a gene *CaATHB-12* derived from HD-Zip I subfamily which was intensively induced by exogenous ABA, salt, and mannitol applications. Efficient gene silencing lines were created from pepper, and stable heterologous overexpression lines were created from *Arabidopsis* to achieve a comprehensive exploration of gene function. The functional study of *CaATHB-12* in pepper increased plant sensitivity to ABA stress, while the over-expressing *CaATHB-12* in *Arabidopsis* lines revealed that tolerance to ABA, salt, and mannitol stresses was decreased. Furthermore, *CaATHB-12* plays a fundamental role in elevating the tolerance to these stresses through the increased expression of other stress related genes, increasing the activities of anti-oxidant enzymes and scavenging the ROS. The studied functions of the *CaATHB-12* gene may provide some insights in exquisite molecular detail by pursuing signal transduction mechanisms that converge on gene expression patterns.

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Author Contributions RXZ and ZHG: Conceptualization, Methodology, Investigation, Data curation, Writing—original draft. RXZ, QHL, and GXC: Writing—review and editing. JJX: Conceptualization, Visualization, Investigation, Writing—review and editing. AK and SH: Methodology, Writing—review and editing. HLY and ZHG: Supervision, Visualization, Resources, Writing—review and editing. All authors have read and approved the final manuscript.

Data Availability All data generated or analysed during this study are included in this manuscript and its supplementary information files.

Declarations

Competing interests The authors declare that they have no conflicts of interest in this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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Authors and Affiliations

Ruixing Zhang[1] · Quanhui Li[1] · Jingjing Xiao[1] · Saeed ul Haq[1] · Abid Khan[2] · Guoxin Cheng[1,3] · Huiling Yan[4] · Zhenhui Gong[1]

✉ Huiling Yan
hiede@163.com

✉ Zhenhui Gong
zhgong@nwafu.edu.cn

Ruixing Zhang
xingqing@nwafu.edu.cn

Quanhui Li
lqh2016@nwafu.edu.cn

Jingjing Xiao
jingjingxiao136@163.com

Saeed ul Haq
saeed_ulhaq@nwafu.edu.cn

Abid Khan
abidagriculturist@gmail.com

Guoxin Cheng
lvge2011@126.com

[1] College of Horticulture, Northwest A and F University, Yangling 712100, Shaanxi, China

[2] Department of Agricultural Sciences, The University of Haripur, Haripur, Pakistan

[3] College of Agriculture, Ningxia University, Yinchuan 750002, Ningxia, China

[4] Institute of Cash Crops, Hebei Academy of Agricultural and Forestry Sciences, Shijiazhuang 050051, Hebei, China