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Research article

A small heat shock protein *CaHsp25.9* positively regulates heat, salt, and drought stress tolerance in pepper (*Capsicum annuum* L.)

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ABSTRACT

Extreme environmental conditions seriously affect crop growth and development, resulting in a decrease in crop yield and quality. However, small heat shock proteins (Hsp20s) play an important role in helping plants to avoid these negative impacts. In this study, we identified the expression pattern of the *CaHsp25.9* gene in a thermotolerance pepper line R9 and thermo-sensitive line B6. The transcription of *CaHsp25.9* was strongly induced by heat stress in both R9 and B6. The expression of *CaHsp25.9* was induced by salt and drought stress in R9. Additionally, the CaHsp25.9 protein was localized in the cell membrane and cytoplasm. When silencing the *CaHsp25.9* gene in the R9 line, the accumulation of malonaldehyde (MDA), relative electrolytic leakage, hydrogen peroxide, superoxide anion were increased, while total chlorophyll decreased under heat, salt, and drought stress. Over-expression of *CaHsp25.9* in *Arabidopsis* resulted in decreased MDA, while proline, superoxide dismutase activity, germination, and root length increased under heat, salt, and drought stress. However, peroxidase activity was higher in drought stress but lower in heat and salt stress in transgenic *Arabidopsis* compared to the wild type (WT). Furthermore, the transcription of stress related genes was more highly induced in transgenic lines than WT. Our results indicated that *CaHsp25.9* confers heat, salt, and drought stress tolerance to plants by reducing the accumulation of reactive oxygen species, enhancing the activity of antioxidant enzymes, and regulating the expression of stress-related genes. Therefore, these results may provide insight into plant adaption mechanisms developed in variable environments.

1. Introduction

In the changing environment, plants suffer from various biotic and abiotic stress such as pests, fungus infection, high temperature, high salinity, and drought (Zhai et al., 2017). However, abiotic stresses such as high temperature, salt, and drought, have become the primary factors that can affect crop growth and development (Zhai et al., 2017). Besides, when plants suffer from these stresses, many proteins will be denatured and form aggregations, losing their native functions and resulting in the serious reduction of crop yield and quality (Wang et al., 2003; Khurana et al., 2013). For avoiding the injuries resulting from denatured protein aggregations, plants have developed some mechanisms (Shan et al., 2007), including the most effective mechanism: when plants suffer from adverse conditions, heat shock proteins (HSPs), which are regulated by heat shock transcription factors (HSFs), can bind and disassemble the aggregations, then refold the aggregations to form native proteins, thus avoiding the damages caused by aggregations (Wang et al., 2004; Lambert et al., 2011; Ruibal et al., 2013; Li et al., 2016b). Accordingly, HSPs play a significant role in protecting plants from adverse environments (Kotak et al., 2007; Jiang et al., 2009), therefore, it is necessary to understand the functions of HSPs.

HSPs are an important molecular chaperone. To date, five major HSPs have been identified: small heat shock proteins (sHsps/Hsp20s), Hsp60s, Hsp70s, Hsp90 and Hsp100s (Hu et al., 2009; Li et al., 2016a). Among these HSPs, Hsp20s, which are ATP-independent heat shock proteins, contribute to the first step of protecting plants from aggregations (Haslbeck et al., 2005). Hsp20s have a strong capacity to bind non-native proteins and change their physical properties, assisting other HSPs, such as Hsp70-Hsp100 bi-chaperone systems, to effectively disassemble and refold the aggregations (Wang et al., 2004; Matuszewska et al., 2005; Liberek et al., 2008). Hsp20s play an important role in protecting plant from aggregations and helping other HSPs work effectively (Stengel et al., 2010; Rutsdottir et al., 2017).

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Therefore, it is important to study the functions of Hsp20s under adverse environmental conditions.

Previous studies have reported that, Hsp20s are extremely diverse and variable in plants, and many of them are involved in responding to adverse environmental conditions (Yu et al., 2016). The monomeric molecular masses of most Hsp20s are approximately 15–42 KDa (Ré et al., 2017). The functions of Hsp20s are related to their structures, which contain three major functional parts: 1) a conserved C-terminal domain called the α-crystallin domain (ACD) or HSP20 domain, which can form a compact β-sheet sandwich structure, helping oligomers dissociate to dimers and bind to non-native proteins; 2) a C-terminal extension region, which may be involved in the stabilization and solubilization of the oligomeric assemblies; and 3) a variable N-terminal region, which plays a role in transiting, leading, or signaling (Kotak et al., 2007; Waters et al., 2008; Poulain et al., 2010; Haslbeck et al., 2015).

Furthermore, plant Hsp20s can group into many subfamilies, such as cytoplasmic/nuclear, mitochondria, chloroplast, peroxisome, and endoplasmic reticulum (Waters and Vierling, 1999a, b; Waters, 2013). Different Hsp20s have different functions, but most of them can be induced by heat, salt, and drought stress. Moreover, previous studies have suggested that Hsp20s play a positive role in enhancing plant tolerance to adverse environments. For example, in *Arabidopsis*, *AtHsp21* plays a positive role in the thermo-tolerance of plants and the extension of the thermo-memory phase (Sedaghatmehr et al., 2016). The *TaHSP26* gene in *Triticum aestivum* and the *CsHSP17.2* gene in *Camellia sinensis* are crucial in enhancing the thermo-tolerance of plants (Khurana et al., 2013; Wang et al., 2017b). In addition, over-expression of *Malus sieversii MsHsp16.9* gene in *Arabidopsis* increased plant tolerance to heat stress by alleviating the damages of reactive oxygen species (ROS), and regulating the expression level of stress-related genes (Song et al., 2017). Transient over-expression of the *JrsHSP17.3* gene in *Juglans regia* displayed higher tolerance to cold, heat, and salt stress by producing less hydrogen (H_2O_2) and MDA, while accumulating more antioxidant enzyme and proline contents (Zhai et al., 2016). In *Oryza sativa*, small heat shock protein *OsHSP18.2* positively regulate the germination and cotyledon emergence under adverse environments (Kaur et al., 2015). Additionally, the *Arabidopsis* class I and class II cytoplasmic Hsp20s, such as *AtHSP17.4* and *AtHSP17.6*, accumulated in maturing seed, and played a protective role in seed development, indicating that Hsp20s are also involved in plant growth and development (Wehmeyer et al., 1996; Wehmeyer and Vierling, 2000; Sun et al., 2016). These studies demonstrate that Hsp20s are of great concern in plant tolerance to abiotic stress. Therefore, it is significant to study the functions of Hsp20s under heat, salt, and drought stress.

Pepper (*Capsicum annuum* L.) which is cultivated worldwide, is economically and medicinally important (Guo et al., 2016). In addition, it is a thermophilic crop; the growth and development of pepper are sensitive to high temperature, salt, and drought stress (Guo et al., 2014b). Hsp20s play a major role in pepper tolerance against these stresses. The Hsp20s family in pepper has 35 putative genes, among which the expression level of *CaHsp25.9* gene was highly induced by heat stress in both R9 and B6 lines (Guo et al., 2015). Additionally, the analysis of the *CaHsp25.9* promoter sequences exhibited that it contains a heat shock element (HSE) and some other stress related elements (Guo et al., 2015). Therefore, we speculated that the *CaHsp25.9* gene may be involved in plants responding to abiotic stress. However, no previous studies have provided details evidence regarding whether or not the *CaHsp25.9* gene is involved in the tolerance of pepper to abiotic stress.

In this study, to identify the functions of the *CaHsp25.9* gene in abiotic stress, we identified the localizations of CaHsp25.9 protein; and the expression pattern of *CaHsp25.9* under heat, salt, and drought stress conditions. In addition, we silenced the *CaHsp25.9* gene in the R9 pepper line and over-expressed the target gene in *Arabidopsis* to investigate its major functions. Our results indicated that, the *CaHsp25.9* gene positively regulates heat, salt, and drought stress tolerance in pepper. Therefore, our results may provide insight into the function of Hsp20s in the plant adaption to variable environments.

2. Materials and methods

2.1. Plant materials and growth conditions

Thermo-tolerance pepper line R9 (introduced from the World-Asia Vegetable Research and Development Center, PP0042-51), thermo-sensitive line B6 (selected by the Pepper Research Group, College of Horticulture, Northwest A&F University, Yangling, China), and *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) were used in this study. Pepper seedlings were grown in a growth chamber with a photoperiod of 16 h light/8 h dark cycle, and a relative humidity of 65%. The temperature was varied depending on the experiment. To analyze the gene transcript level in R9 and B6 lines, pepper was grown at 25/20 °C day/night (Guo et al., 2016; Wang et al., 2017a; Zhai et al., 2017). Moreover, the R9 pepper line was grown at 22/18 °C day/night for virus-induced gene silencing (VIGS) experiment (Wang et al., 2013). *Arabidopsis* seedlings were grown at 22/18 °C with a photoperiod of 16 h/8 h (day/night) and a relative humidity of 65% (Wang et al., 2017a; Zhai et al., 2017).

2.2. RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated following the methods described by Guo et al. (2014a). The first chain was synthesized by the Primer Script™ Kit (TaKaRa, Dalian, China). The iQ5.0 Bio-Rad iCycler thermocycler (Bio-Rad, Hercules, CA, USA) was used for qRT-PCR, and SYBR Green Supermix (TaKaRa, Dalian, China) was used for the reaction system. The pepper ubiquitin binding gene *CaUBI3* was used as reference in pepper, and *Arabidopsis Atactin2* was used as reference in *Arabidopsis*. All primer pairs (Supplementary Table 1) used for qRT-PCR were designed using NCBI Primer BLAST. Relative gene expression levels were determined following the $2^{-\triangle\triangle CT}$ method (Schmittgen and Livak, 2008).

2.3. Subcellular localization

The open reading frame (ORF) of *CaHsp25.9* without a termination codon was cloned from pepper cDNA using the specific primer pair (Supplementary Table S1) with the restriction enzymes sites *Xba*I and *Kpn*I. Then, the PCR-amplified *CaHsp25.9* fragment was cloned into a pVBG2307:GFP vector, and a pVBG2307:GFP vector without the *CaHsp25.9* gene was used as a control. All recombinant fusion vectors were transient over-expressed in *Nicotiana benthamiana* (*N. benthamiana*) leaves using the transformation of *Agrobacterium tumefaciens* strain CV3101 (Yu et al., 2017). Besides, fluorescent lipophilic tracer DiI (Solarbio, Peking, China) was used to dye cell membrane. Leaves were socked in 5 μM DiI for 15–30 min before we observed fluorescence. Fluorescence of green fluorescent protein (GFP) and DiI (red flurecence) were observed using an automatic ortho-fluorescence microscope BX63 (Olympus Corporation, Tokyo, Japan).

2.4. Virus-induced gene silencing (VIGS) of CaHsp25.9 in pepper

A 264 bp fragment of the *CaHsp25.9* ORF was cloned from pepper cDNA using the specific primer pair (Supplementary Table S1) with the restriction enzymes sites *Xba*I and *Bam*HI. *CaHsp25.9* was cloned into TRV2:00, the empty vector TRV2:00 was used as a negative control, and TRV2:*CaPDS* (phytoene desaturase gene) was used as positive control. Then, TRV2:*CaHsp25.9*, TRV2:00, and TRV2:*CaPDS* were respectively injected into R9 leaves (at the two true leaves stage) using *Agrobacterium tumefaciens* strain CV3101 for transformation (An et al., 2008; Zhang et al., 2013). After 40–45 d, when most of the TRV2:*CaPDS* injected leaves exhibited the photo-bleaching phenotype, the silencing efficiency of TRV2:*CaHsp25.9* and TRV2:00 were detected by qRT-PCR.

2.5. Generation of CaHsp25.9 transgenic arabidopsis lines

The full-length of the *CaHsp25.9* ORF was cloned from pepper cDNA using the specific primer pair (Supplementary Table S1) with the restriction enzymes site *Xba*I and *Kpn*I. The PCR-amplified products were cloned into the plant expression vector pVBG2307. The recombinant fusion vector was transformed into *A. thaliana* using the *Agrobacterium tumefaciens* strain CV3101 for transformation (Clough and Bent, 1998; Guo et al., 2014b). Transgenic plants were grown on Murashige and Skoog (MS) medium containing 50 mmol/L kanamycin. T3 seeds were used for further experiments.

Furthermore, five transgenic lines (OE1, OE2, OE3, OE10, and OE15) were used to select the homozygous T3 lines. First, the expression level of the *CaHsp25.9* gene was detected by qRT-PCR. Second, 7-day-old seedlings of WT and the five transgenic lines, which were grown on MS medium, were exposed to 45 °C for 2 h, and the survival rate was measured after seedlings were allowed to recover at 22 °C for 4–5 d. Based on the overall expression level of the *CaHsp25.9* gene and the survival rate of the five transgenic lines, three of the transgenic lines were selected downstream experiments.

2.6. Stress treatments and sample collection

To analyze the expression pattern of *CaHsp25.9*, both pepper line R9 and B6 (grown at 25/20 °C day/night normal conditions) at the six to eight leaf stage were used for heat, salt, and drought stress treatments (Guo et al., 2015, 2016; Wang et al., 2017a). For the basic thermo-tolerance treatment, seedlings were incubated at 45 °C, then roots and leaves were collected at 0, 0.5, 1, 2, 4, 12, and 24 h post treatment. For the acquired thermo-tolerance treatment, the time course of heat stress treatment is described as Supplementary Fig. S1. First, seedlings were incubated at 38 °C for 4 h, roots and leaves were collected at 0, 2, and 4 h. Second, after treatment at 38 °C, seedlings were allowed to recover at 22 °C for 48 h, and then leaves and roots were collected. Finally, after recovery at 22 °C, seedlings were incubated at 45 °C for 6 h, roots and leaves were collected at 2 h and 6 h. For salt stress treatment, R9 roots were soaked in 0, 50, 100, 200, 400, or 600 mM NaCl, respectively. For drought stress treatment, R9 roots were soaked in 0, 50, 100, 200, 400, or 600 mM mannitol, respectively. After 6 h of the salt and drought stress treatment, leaves and roots were collected. All leaves and roots samples were collected from six separate seedlings and the experiment were conducted with three biological replicates. Samples were immediately frozen in liquid nitrogen and kept at −80 °C for RNA extraction.

TRV2:*CaHsp25.9* and TRV2:00 plants were used to analyze the functions of *CaHsp25.9* under heat, salt, and drought stress. For heat stress, pepper seedlings were exposed to 45 °C for 16 h. For salt stress, seedlings were soaked in 300 mM NaCl for 24 h. For drought stress, seedlings were soaked in 300 mM mannitol for 36 h. Pepper leaves were collected for the determination of MDA, relative electrolytic leakage (REL), total chlorophyll content, H_2O_2, and superoxide anion (O_2^-) radical accumulation.

To analyze the tolerance to salt stress of the transgenic seeds during germination period, seeds were grown on MS medium containing 0, 50, 100, or 200 mM NaCl, respectively. To analyze the drought stress tolerance of transgenic seeds during germination period, seeds were grown on MS medium containing 0, 50, 100, 200, or 300 mM mannitol, respectively. After salt and drought treatments, the percentage of germination was measured at 6 d, the percentage of green cotyledon rate was measured at 4 d, and the root length was measured at 8 d.

Additionally, to further analyze heat, salt, and drought stress tolerance of the transgenic lines during the seedling growth period, 3-week-old *CaHsp25.9*-overexpressed and WT *Arabidopsis* lines were used. For heat stress, seedlings were incubated at 40 °C for 16 h. For salt stress, seedlings were watered 200 mM NaCl every two days for 7 d. For drought stress, seedlings were not watered for 5 d. *Arabidopsis* seedlings incubated under normal conditions were used as the control. Leaves were collected to determine MDA, proline, superoxide anion radical accumulation, analysis of antioxidant enzyme activity, and extraction of RNA.

2.7. Measurement of physiological indicators

The MDA content and REL were determined using the thiobarbituric acid reaction (Stewart and Bewley, 1980). The total chlorophyll content was measured following the method described by Arkus et al. (2005). The H_2O_2 level was analyzed by DAB staining and the superoxide anion radical level was analyzed by NBT staining (Thordal-Christensen et al., 1997; Kim et al., 2012). The quantification of ROS (DAB and NBT stained area) was obtained following the method described by Sekulska-nalewajko et al. (2016). Proline contents was measured following the method described by Naser et al. (2010). Superoxide dismutase (SOD) and peroxidase (POD) activity was measured following the methods previously described by Stewart and Bewley (1980) and Jariteh et al. (2015).

2.8. Statistical analysis

Statistical analysis was performed using Statistical Analysis System software (IBM SPSS Statistics 22, USA) for one-way analysis of variance (ANOVA). Treatments were considered significantly different at $p \leq 0.05$. All experiments were performed and analyzed separately with at least three biological replicates.

3. Results

3.1. Expression of the CaHsp25.9 gene in pepper under abiotic stress

To identify whether *CaHsp25.9* is involved in the response to heat, salt, and drought stress, the expression pattern of *CaHsp25.9* in the R9 and B6 lines were investigated. For the basic thermo-tolerance analysis (Fig. 1A and B), *CaHsp25.9* was strongly up-regulated after the 45 °C treatment, and was highest at 2 h in the leaves of both R9 and B6. However, the *CaHsp25.9* expression level was higher in B6 than that in R9 at 2 h. In roots, the expression level of *CaHsp25.9* was increased in R9 at 2 h. However, the expression level of *CaHp25.9* was higher in leaves than that in roots. Likewise, for the acquired thermo-tolerance analysis (Fig. 1C and D), the transcriptional expression of *CaHsp25.9* was only highly induced after the 45 °C treatment in the leaves of both R9 and B6. While in roots, the *CaHsp25.9* expression was strongly increased at the 38 °C treatment stage, decreased at the 22 °C recovery stage, and highly increased at 45 °C in both R9 and B6. For salt and drought stress analysis, after 6 h treatment, the expression level of *CaHsp25.9* was highest at 50 and 100 mM NaCl treatment in leaves and roots, respectively (Fig. 1E). In addition, the expression level of *CaHsp25.9* was highest at 600 and 50 mM mannitol treatment in the leaves and roots, respectively (Fig. 1F).

3.2. Subcellular localization of the CaHsp25.9 protein

To identify the subcellular localization of *CaHsp25.9*, the pVBG2307:GFP and pVBG2307:CaHsp25.9:GFP fusion plasmids were transiently expressed in *N. benthamiana* leaves. As shown in Supplementary Fig. S2 and Fig. 2, the green fluorescence from pVBG2307:CaHsp25.9:GFP was observed in the cell membrane and cytoplasm, while pVBG2307:GFP was observed throughout the whole cell (Fig. 2, GFP and Merged#2). Besides, as shown in Fig. 2, the cell membrane exhibited red fluorescence after DiI-labeled (DiI). Yellow fluorescence was observed in the merged cell membrane area (Merged#1), while green fluorescence was observed in the merged cytoplasm area (Merged#1), indicated that the CaHsp25.9 protein localized in the cell membrane and cytoplasm (Fig. 2).

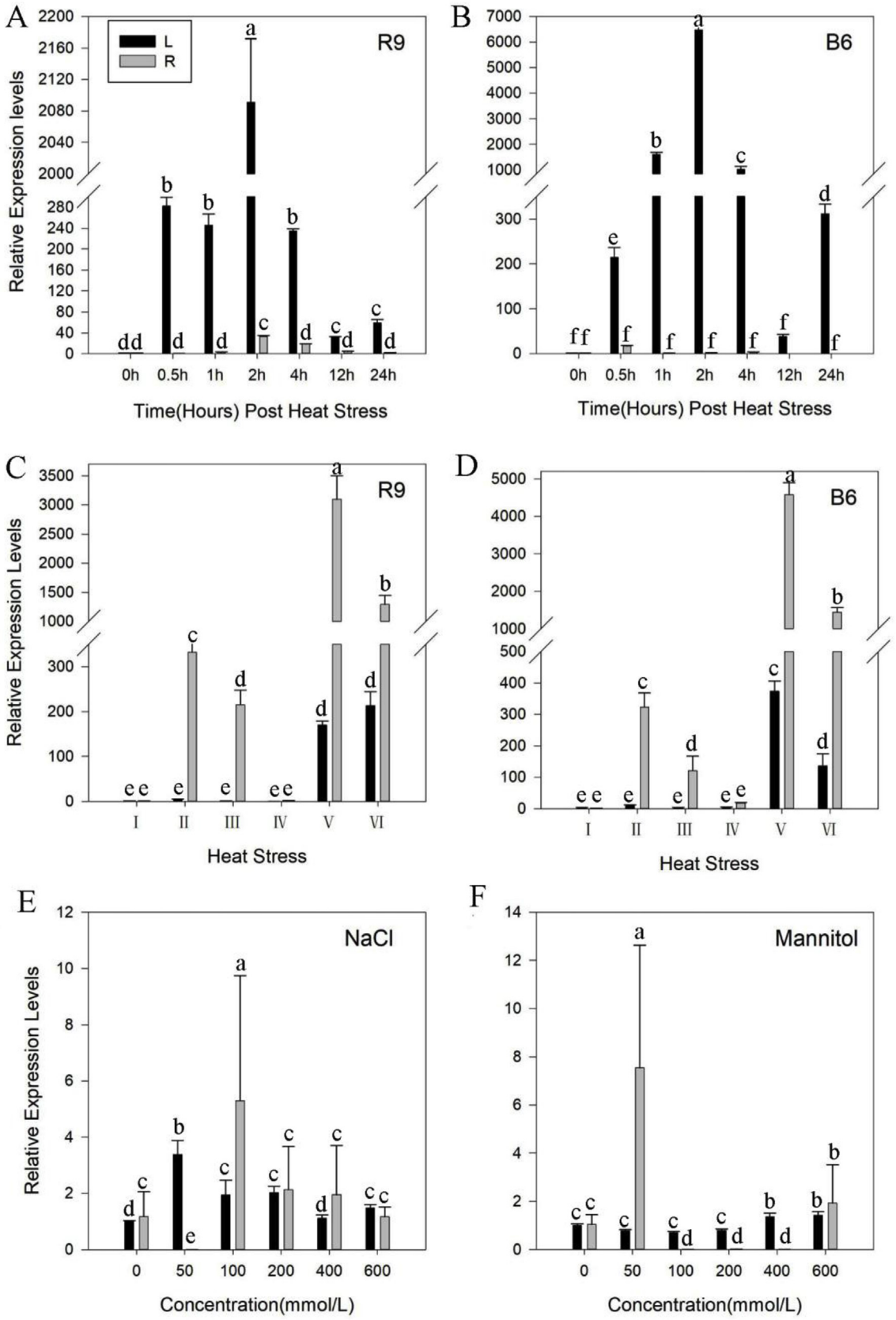

Fig. 1. Expression pattern of *CaHsp25.9* under heat, salt and drought stress in pepper leaves (L) and roots (R). (A–B) Expression levels of *CaHsp25.9* following heat stress (45 °C) in R9 and B6. (C–D) Expression levels of *CaHsp25.9* following different temperature treatments in R9 and B6: 38 °C treatment for 0 h (I), 2 h (II), and 4 h (III); 22 °C recovery for 48 h (IV); 45 °C treatment for 2 h (V), and 6 h (VI). (E–F) Expression levels of *CaHsp25.9* following salt and drought treatment in R9 leaves and roots. Data are means with standard deviations of three biological replicates. Different letters denote statistical significance at $p \leq 0.05$.

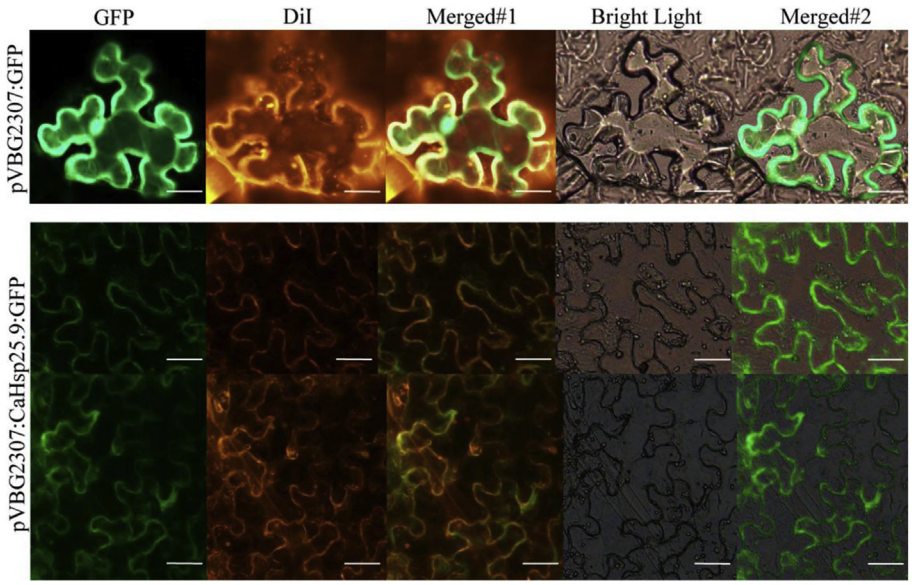

Fig. 2. Subcellular localization of the pVBG2307:CaHsp25.9:GFP fusion protein in *N. benthamiana* leaves, pVBG2307:GFP was used as control. The fluorescence was observed under bright and fluorescence field. GFP: green fluorescence of Green fluorescence protein (GFP). DiI: DiI-labeled fluorescence under red fluorescence. Merged#1: GFP and DiI, Merged#2: GFP and Bright Light. Bar = 80 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. CaHsp25.9-silenced plants sensitive to abiotic stress

The leaves of TRV2:*CaPDS* (positive control) plants exhibited the photo-bleaching phenotype (Supplementary Fig. S3A), thus the silencing efficiency of TRV2:*CaHsp25.9* and TRV2:00 were detected. As shown in Supplementary Fig. S3A, there were no visual differences between *CaHsp25.9*-silenced (TRV2:*CaHsp25.9*) and control (TRV2:00) plants under normal conditions, and the silencing efficiency was over 80% (Supplementary Fig. S3B). Therefore, the *CaHsp25.9*-silenced and control plants were used for further studies.

After heat stress treatment at the 45 °C for 16 h, the growing point of *CaHsp25.9*-silenced plants were wilted, while no obvious wilted symptom was observed in the control plants (Fig. 3A). In addition, the MDA content in *CaHsp25.9*-silenced plants were higher than that in the control (Fig. 3B and C). For salt stress, after socking in 300 mM NaCl for 24 h, wilted and yellowish symptoms were observed in *CaHsp25.9*-silenced plants, while control plants were only yellowish (Fig. 3D). Moreover, the MDA content in *CaHsp25.9*-silenced plants was significantly higher than that in the control (Fig. 3E), whereas the total chlorophyll content in *CaHsp25.9*-silenced plants was lower than that in the control (Fig. 3F). For drought stress, after socking in 300 mM mannitol for 36 h, leaves of *CaHsp25.9*-silenced pepper wilted and eventually fell off, while these symptoms were mild in the control plants (Fig. 3G). Furthermore, the MDA content was significantly higher and the total chlorophyll content was significantly lower in *CaHsp25.9*-silenced plants than that in the control (Fig. 3H and I).

Additionally, in order to analyze the accumulation of ROS in the *CaHsp25.9*-silenced and control plants. DAB and NBT staining were used to detect peppers H_2O_2 and O_2^- levels. As shown in Fig. 4A and Fig. 4B, after heat, salt, and drought stress treatments, the DAB stained area was significantly increased in the silenced pepper line than in the control line (Fig. 4A and B). Additionally, as shown in Fig. 4C and D, the NBT stained area was significantly increased in the silenced pepper line than that in the control line after stress treatment (Fig. 4C and D). These results indicated that higher accumulation of H_2O_2 and O_2^- was detected in leaves of *CaHsp25.9*-scilenced plants compared with control plants.

3.4. Overexpression of CaHsp25.9 enhances plant tolerance to heat stress

To select the thermo-tolerant homozygous T3 seeds for further study, we first detected the relative expression level of *CaHsp25.9* in WT and five different *CaHsp25.9*-overexpressed (*CaHsp25.9*-OE) *Arabidopsis* lines (OE1, OE2, OE3, OE10, and OE15) under normal conditions. As shown in Supplementary Fig. S4A, the expression level of *CaHsp25.9* in OE2, OE10, and OE15 lines were significantly higher than that in the WT (Supplementary Fig. S4A). Second, WT and the five transgenic lines were treated at 45 °C for 2 h, and their survival rates were measured after 5 d of recovery at 22 °C. The results showed that the survival rates of OE2, OE10, and OE15 seedlings were obviously greater than that of the WT (Supplementary Figs. S4B and C). These results implied that OE2, OE10, and OE15 were thermo-tolerant homozygous T3 transgenic lines. Therefore, OE2, OE10 and OE15 were selected for further studies.

To identify the heat stress tolerance of the transgenic seedlings, 3-week-old seedlings of WT and OE2, OE10, and OE15 were treated at 40 °C for 16 h. After heat stress treatment, the leaves of the WT were severely wilted, while the leaves of the OE2, OE10, and OE15 were only slightly wilted (Fig. 5A). Besides, the MDA content was highly increased in both WT and transgenic lines after heat treatment, whereas the MDA content was significantly higher in WT than that in the transgenic lines (Fig. 5B). Additionally, the proline content and SOD activity of *CaHsp25.9*-OE seedlings significantly increased after heat treatment compared with that of the WT (Fig. 5C and D). The POD activity also increased after heat treatment, but was slightly higher in the WT than that in the transgenic lines (Fig. 5E). In addition, the expression level of heat stress-related genes *AtHSA32* (*Arabidopsis* heat-stress-associated 32-kD protein), *AtHSP101* (*Arabidopsis* Hsp100s gene), *AtMYB* (*Arabidopsis* MYB transcription factor), and *AtP5CS* (pyrroline-5-carboxylate synthase of proline) were strongly induced in both WT and transgenic *Arabidopsis* after heat treatment. However, the expression level of the *AtHSA32*, *AtHSP101* and *AtP5CS* genes were higher in transgenic seedlings than that in WT seedlings, whereas the expression level of the *AtMYB* gene was lower in transgenic seedlings than that in the WT (Fig. 5F).

3.5. Overexpression of CaHsp25.9 enhances plant tolerance to salt stress

For investigating the tolerance of transgenic seeds to salt stress during germination period, transgenic and WT seeds were grown on MS medium containing NaCl to determine the germination percentage, green cotyledon rate and root length. After growth on MS medium containing 200 mM NaCl for 6 d, the germination rate of transgenic and WT lines was reduced compared with seeds growth on MS medium containing 0 mM NaCl. However, the germination rate of the transgenic lines was significantly higher than that of the WT (Supplementary Figs. S5A and B). Moreover, the green cotyledon rate of the transgenic lines

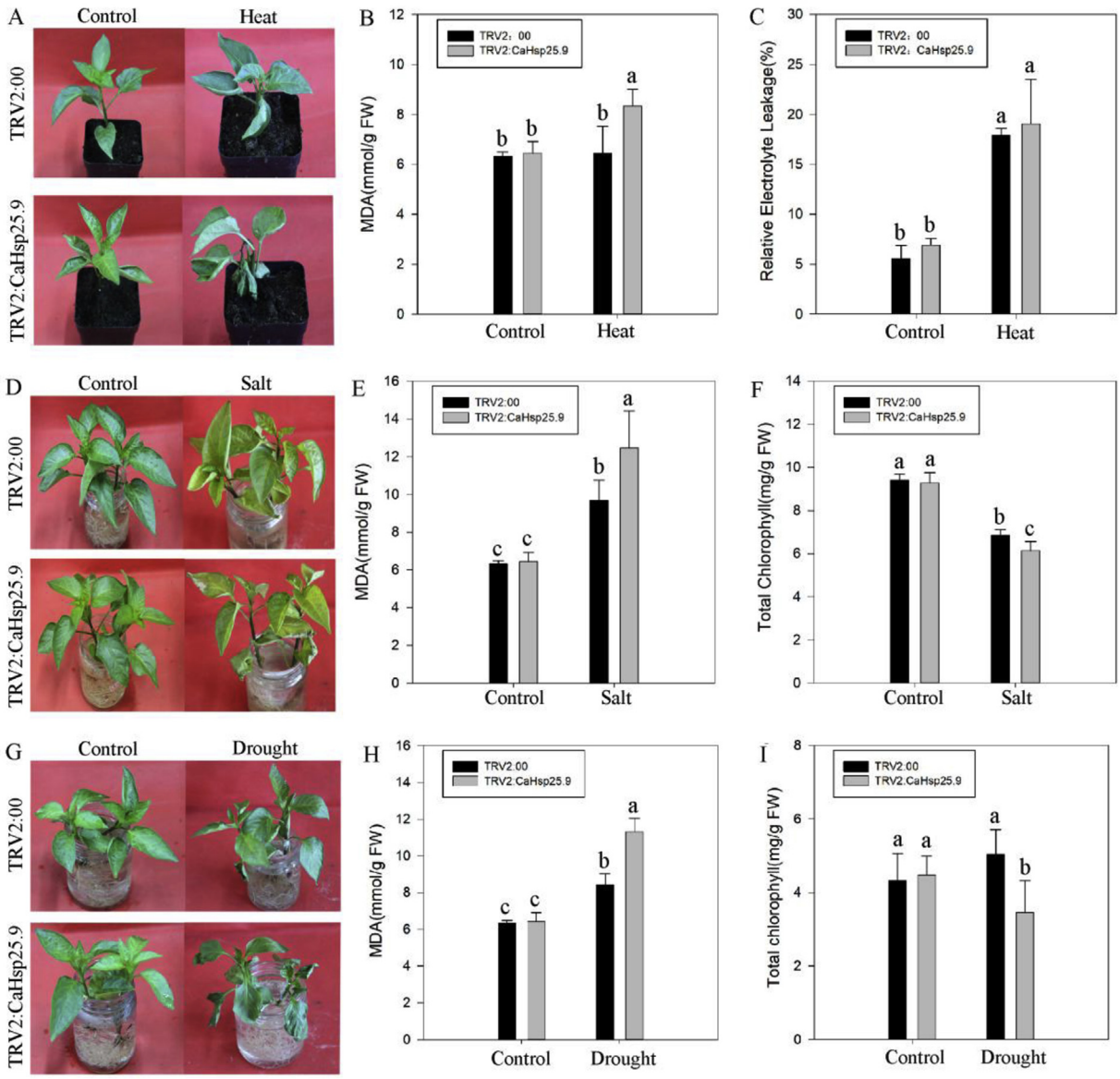

Fig. 3. Heat, salt and drought stress tolerance are decreased in *CaHsp25.9*-silenced peppers. (A–C) Phenotype, MDA content and REL of TRV2:00 and TRV2:*CaHsp25.9* plants following heat stress at 45 °C for 16 h. (D–F) Phenotype, MDA content and total chlorophyll content of TRV2:00 and TRV2:*CaHsp25.9* plants following salt stress after socking in 300 mM NaCl for 24 h. (G–I) Phenotype, MDA content and total chlorophyll content of TRV2:00 and TRV2:*CaHsp25.9* plants following drought stress after socking in 300 mM mannitol for 36 h. Data are means with standard deviations of three biological replicates. Different letters denote statistical significance at $p \leq 0.05$.

and the WT considerable decreased after growth on MS medium containing 100 mM NaCl for 4 d; however, the green cotyledon rate was obviously higher in transgenic lines than in the WT (Supplementary Figs. S6A and B). Besides, when *Arabidopsis* seeds were grown on MS medium containing 50, 100, or 150 mM NaCl for 8 d, the roots length of the transgenic lines and WT was reduced; the root length was significantly longer in transgenic lines than in the WT (Supplementary Figs. S7A and B).

To further detect the salt tolerance of transgenic seedlings, 3-week-old transgenic and WT seedlings were used. After watering the 3-week-old seedlings with 200 mM NaCl for 7 d, leaves of WT seedlings showed dehydration and wilting, while no apparent symptoms were observed in transgenic seedlings (Fig. 6A). The MDA content was highly increased in the WT line and the MDA was higher in WT than that in the transgenic seedlings. In addition, the proline content, SOD, and POD activity of transgenic seedlings significantly increased after salt treatment. However, the proline content and SOD activity were significantly higher, while the POD activity was lower in transgenic seedlings than that in the WT (Fig. 6B–E). Likewise, the expression level of the salt stress related genes *AtDREB2A* (*Arabidopsis* DRE/CRT-binding 2A proteins), *AtSOS1* (*Arabidopsis salt overly sensitive 1* gene), *AtMYB*, and *AtP5CS* were all induced in transgenic and WT *Arabidopsis*, but their expression levels were higher in the transgenic seedlings than that in WT (Fig. 6F).

3.6. Overexpression of CaHsp25.9 enhances plant tolerance to drought stress

When the WT and transgenic seeds were grown on MS medium containing 100, 200, or 300 mM mannitol for 5 d, no apparent difference was observed in the germination rate between transgenic and WT lines (Supplementary Figs. S8A and B). However, the root length of WT and transgenic seeds was decreased when they were grown on MS medium containing 200 and 300 mM mannitol for 8 d. Besides, the root

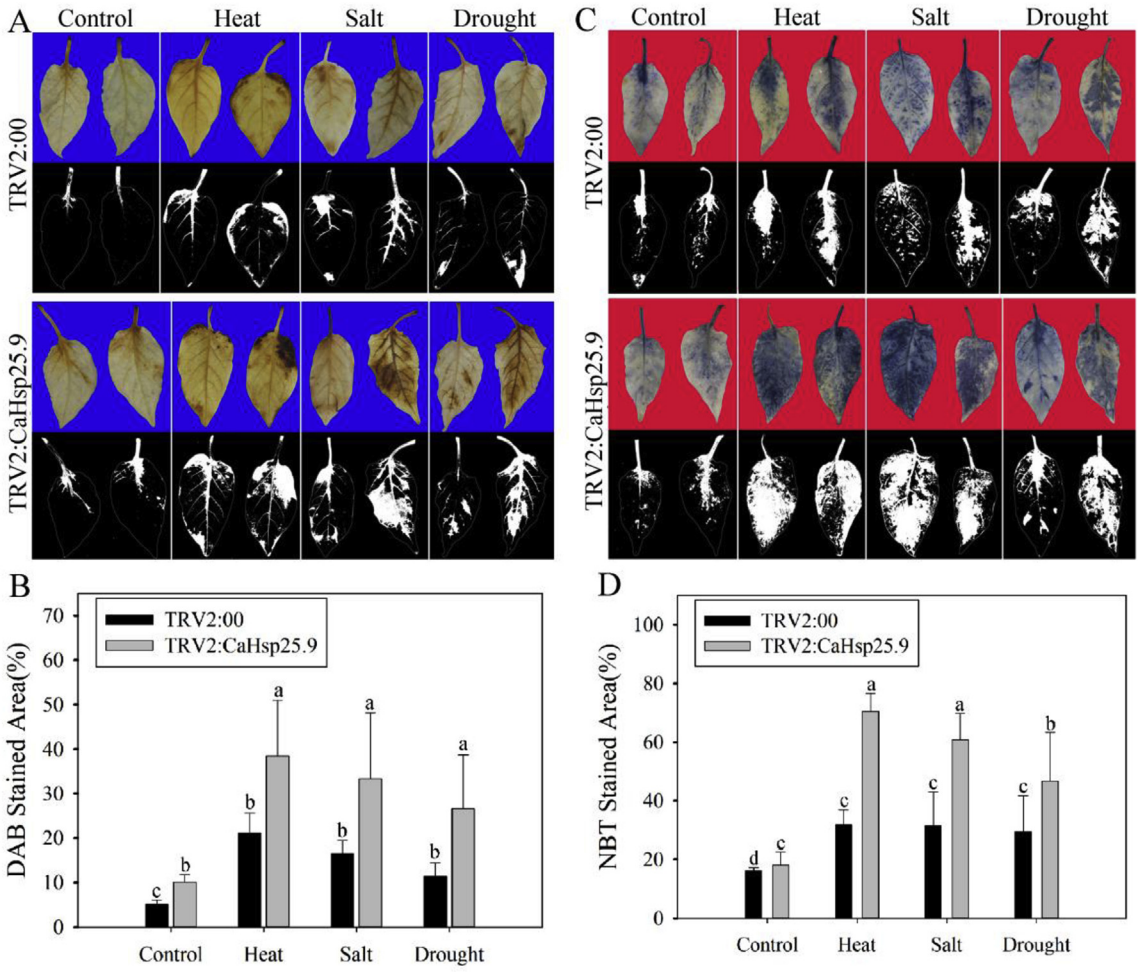

Fig. 4. DAB and NBT staining in TRV2:00 and TRV2:*CaHsp25.9* plants under heat, salt, and drought stress. (A) DAB staining in leaves of control and *CaHsp25.9*-silenced plant at 45 °C for 16 h (heat stress), 300 mM NaCl for 24 h (slat stress), 300 mM mannitol for 36 h (drought stress). (B) DAB stained area in silenced and control plants. (C) NBT staining in leaves of control and *CaHsp25.9*-silenced plants leaves at heat, salt, and drought stress treatments. (D) NBT stained area in silenced and control plants. Data are means with standard deviations of three biological replicates. Different letters denote statistical significance at $p \leq 0.05$.

length was longer in the *CaHsp25.9*-OE lines than that in the WT (Supplementary Figs. S9A and B).

To further analyze the drought stress tolerance of transgenic seedlings, 3-week-old transgenic and WT seedlings were used. After withholding water from 3-week-old seedlings for 5 d, WT seedlings became severe wilted, while only slight wilting was observed in transgenic seedlings (Fig. 7A). Moreover, the MDA content increased in both transgenic and WT lines, while the MDA was significantly lower in transgenic seedlings than in the WT (Fig. 7B). In addition, the proline content, SOD and POD activity increased in both transgenic and WT lines, and were significantly higher in transgenic seedlings than that in WT (Fig. 7C–E). The expression level of the drought stress related genes *AtRD29A* (*Arabidopsis* drought responsive 2A gene), *AtSOD1* (*Arabidopsis* SOD synthase gene), *AtHSA32*, and *AtP5CS* were all induced in transgenic and WT seedlings, but their expression levels were higher in transgenic lines compared to the WT (Fig. 7F).

Additionally, to analyze the accumulation of ROS in the transgenic and WT lines, NBT staining was used to detect the superoxide anion radical level. As shown in Fig. 8A and B, the NBT stained area was strongly increased in both transgenic and WT lines after drought treatment; however, the stained area was significantly lower in the transgenic seedlings than that in WT (Fig. 8A and B), indicating that the WT accumulated more O_2^- than transgenic plants.

4. Discussion

Extreme environmental conditions, such as high temperature, can lead to the accumulation of misfolded and denatured proteins, ultimately affecting physiology (Wang et al., 2017b). Therefore, to functionally normalize and avoid the risk of misfolding, the Hsp20s have the ability to bind denatured proteins and regulate other related proteins to participate in responding to heat stress (Wang et al., 2017b; Huang et al., 2018). A previous study showed that the expression level of *CaHsp25.9* was highly induced by heat stress, but there was no evidence regarding the involvement of the *CaHsp25.9* gene in heat stress, salt, and drought stress (Guo et al., 2015). In this study, we identified that *CaHsp25.9* plays a positive role in enhancing plant tolerance to heat, salt, and drought stress.

Most Hsp20s can be strongly induced under extreme environments. For example, the expression level of the *CaHsp16.4* gene was strongly induced by heat and drought stress in *C. annuum* (Huang et al., 2018), and an increased transcript level of the *CaHsp26* was observed in *C. annuum* under heat stress (Guo et al., 2007). Additionally, the expression of the *Medicago sativa MsHSP17.7* was induced by heat, NaCl, H_2O_2, and osmotic stress (Li et al., 2016b), and the expression of the *Rosa chinensis RcHSP17.8* was strongly induced by heat, cold, salt, and drought stress (Jiang et al., 2009). The dynamic expression analysis of *CaHsp20s* in pepper under the heat stress treatment showed that, the *CaHsp25.9* gene can be highly induced in both the R9 and B6 pepper

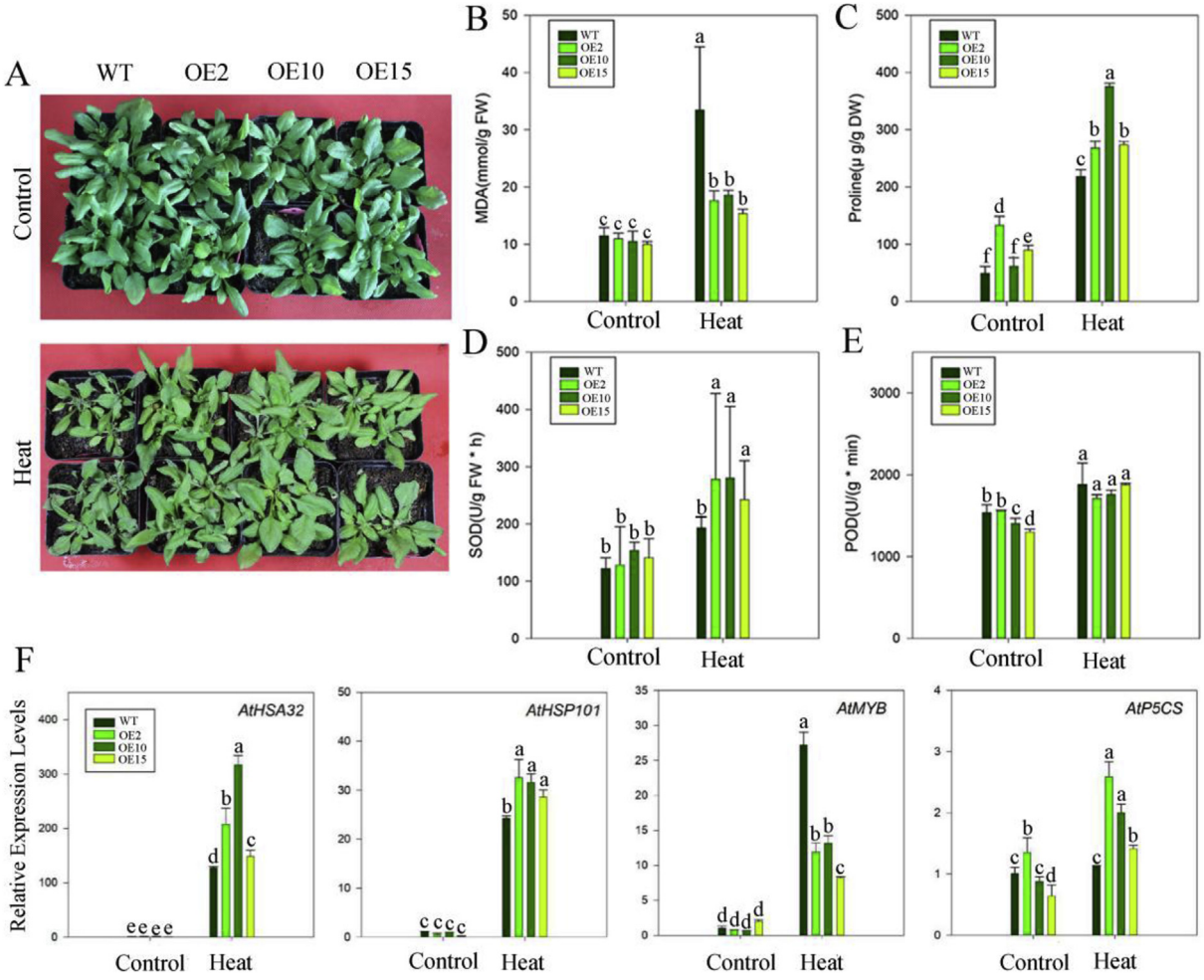

Fig. 5. Over-expression of the *CaHsp25.9* gene enhanced tolerance to heat stress. (A–E) Phenotype, MDA content, proline content, SOD and POD activity of WT and *CaHsp25.9*-OE *Arabidopsis* lines (OE2, OE10, and OE15) at 40 °C for 16 h. Seedlings grown at 22 °C were used as the control. (F) Relative expression levels of heat stress-related genes in WT and transgenic lines under heat stress. Data are means with standard deviations of three biological replicates. Different letters denote statistical significance at $p \leq 0.05$.

lines (Guo et al., 2015). In agreement with this result, in our thermo-tolerance study, the expression level of *CaHsp25.9* was strongly induced in both the R9 and B6 lines after heat stress treatment. Otherwise, the transcription level of *CaHsp25.9* was higher in B6 than in R9 after the 45 °C treatment for 2 h. It has been reported that the expression level of HSFs in the heat-tolerance lines and heat-sensitive lines were related to their dynamic expression levels during heat stress (Schramm et al., 2006; Guo et al., 2014a). For instance, the expression level of *CaHsp25.8* and *CaHsp30.1* in the heat-tolerance line was higher than in the heat-sensitive line at the early stage (0.5–1 h) of heat stress treatment. However, with elongating the heat treatment time, their expression in the heat-sensitive line was higher than in the heat-tolerance line (Guo et al., 2015). Besides, the expression level of *CaHSF2A* was higher in the pepper B6 line than in the R9 line after 40 °C treatment for 2 h (Guo et al., 2014a). In addition, the expression level of *VvHSF01* was higher in the thermo-sensitive line than in the thermo-tolerance line after heat treatment in *Vitis Vinifera* (Liu et al., 2018). Therefore, the expression level of *CaHsp25.9* in the heat-sensitive line was higher than in the heat-tolerance line may be due to this reason, while this needed further study.

Moreover, the expression pattern of the *CaHsp25.9* gene was different between the basic thermo-tolerance and acquired thermo-tolerance analysis. This may bedue to the HSFs, which were upstream regulators of HSPs, play different roles in the two different heat stress treatments (Schramm et al., 2008; Peng et al., 2013). For example, the expression of *VdHSF01*, *VdHSF04* and *VdHSF07* was strongly induced after the 38 °C treatment (acquired thermo-tolerance), while there was no changes after the 47 °C treatment (basic thermo-tolerance) in *Vitis davidii* (Liu et al., 2018). Besides, *VdHSF06* and *VdHSF10* were up regulated after the 38 °C treatment, while down regulated after the 47 °C treatment in *Vitis davidii* (Liu et al., 2018). In addition, the heat shock transcription factor *FaHSFC1* had a lower expression level in the 45 °C (1–24 h) treatment in the roots of *Festuca arundinacea*, while reached a peak 1 h in the leaves (Zhuang et al., 2018).

In summarized, our results demonstrate that, *CaHsp25.9* gene deeply involved in plants response to heat stress. The heat shock response pattern of *CaHsp25.9* may significantly difference and complicated in different thermo-tolerance lines and different plant tissues (Guo et al., 2015). Accordingly, these findings may provide a window for further study of plant heat shock responses and mechanisms developed in variable environments. Additionally, the expression level of *CaHsp25.9* was induced by NaCl-induced salt stress and mannitol-induced drought stress (Fig. 1). These results suggested that, the *CaHsp25.9* gene is deeply involved in plant response to salt and drought stress.

Hsp20s are localized in a wide range, such as *Oryza sativa OsHsp18.0* gene localized in the cytoplasm and the nuclear envelope (Kuang et al., 2017), *Pyropia tenera PtsHSP19.3* localized in cytosol (Jin et al., 2017). Otherwise, the subcellular localizations of Hsp20s may associate with their functions in plants. In *Arabidopsis thaliana*, *AtHsp21* is located in

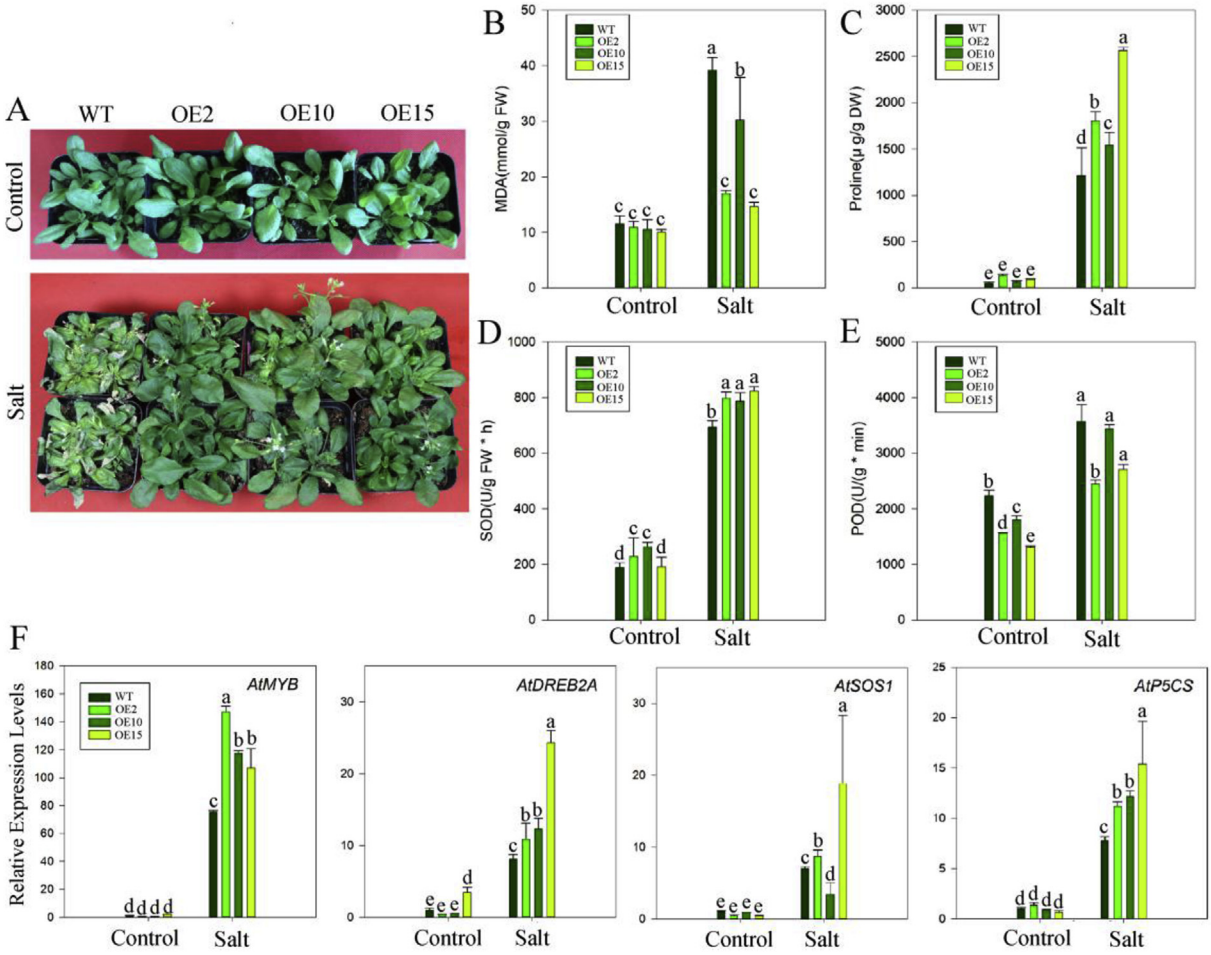

Fig. 6. Over-expression of the *CaHsp25.9* gene enhanced tolerance to salt stress. (A–E) Phenotype, MDA content, proline content, SOD and POD activity of WT and *CaHsp25.9*-OE *Arabidopsis* seedlings following watered with 200 mM NaCl for 7 d. (F) Relative expression levels of salt stress-related genes in WT and transgenic seedlings under salt stress. Data are means with standard deviations of three biological replicates. Different letters denote statistical significance at $p \leq 0.05$.

chloroplast, and it may be associates with the thylakoid membrane under heat stress (Bernfur et al., 2017). In this study, when we transiently expressed *CaHsp25.9* in *N. benthamiana*, we observed that CaHsp25.9 distributed in the cell membrane and cytoplasm (Fig. 2), suggesting that CaHsp25.9 protein may play a role in the cell membrane and cytoplasm.

VIGS experiment in peppers and over-expression experiment in *Arabidopsis* are common methods to reveal gene function under adverse environments. For example, *Lycopersicon esculentum LeHSP21*-overexpressing tobacco exhibited increased tolerance to heat and oxidative stress by showing higher physiologic indicators than in the WT (Zhang et al., 2016). *Populus trichocarpa PtHSP17.8*-overexpressing plants showed higher survival rates and root length than control plants under heat and salt stress (Li et al., 2016a). In this study, the silencing of *CaHsp25.9* in pepper increased plant sensitivity to heat, salt, and drought stress by exhibiting severe injury symptoms, increased MDA, REL, H_2O_2, and O_2^- content, and decreased total chlorophyll content compared with control plant. This demonstrates that silencing of *CaHsp25.9* negatively regulates pepper stress tolerance (Figs. 3–4). Over-expression of *CaHsp25.9* in *Arabidopsis* decreased plant sensitivity to heat, salt, and drought stress by exhibiting minimal injury symptoms, increased survival rate, germination, root length, and physiologic indicators, as well as decreased accumulation of O_2^- compared with WT (Figs. 5–8). This indicates that over-expressing the *CaHsp25.9* gene positively regulates plant stress tolerance. These results indicated that *CaHsp25.9* plays a positive role in enhancing plant tolerance to heat, salt, and drought stress.

ROS, such as H_2O_2 and O_2^-, are of great importance for plant response to adverse environments; however, the excess accumulation of H_2O_2 and O_2^- under stress resulted in damage to the intracellular environment, cell structure, and photosynthesis (Uzilday et al., 2012; Tuteja et al., 2014; Srivastava et al., 2016). Therefore, the content of H_2O_2 and O_2^- were often used to measure the injury level of plant cells (Li et al., 2018). In this study, the accumulation of H_2O_2 and superoxide anion radical in *CaHsp25.9*-silenced plant was higher than in control plants under heat, salt, and drought stresses. However, *CaHsp25.9* transgenic *Arabidopsis* displayed lower accumulation of O_2^- than that in the WT under drought stress (Fig 4; Fig 8). These results indicate that *CaHsp25.9* may play a positive role in reducing the accumulation of ROS, thus we speculated that *CaHsp25.9* confers plant an ability to remove excess H_2O_2 and O_2^- to avoid damage from ROS under heat, salt, and drought stress. However, the exact mechanisms need further study.

To relieve the damages from ROS under adverse environments, plants have developed some mechanisms to scavenge excess ROS, such as ROS-scavenging antioxidative enzymes (SOD, POD, catalase and ascorbate peroxidase) and non-enzymatic antioxidants (vitamin C and proline). During the function of the ROS-scavenging system, SOD first decomposes O_2^- to H_2O_2. Then the H_2O_2 is scavenged by POD in the cytosol and extracellular space, or scavenged by catalase (CAT) in the peroxisomes or by ascorbate peroxidase (APX) in different cell compartments (Gill and Tuteja, 2010; Uzilday et al., 2012). Therefore, the activities of antioxidative enzymes were highly induced by adverse environments. For example, transgenic *Arabidopsis* with the *Zea mays ZmHsp16.9* gene exhibited higher tolerance to heat stress by

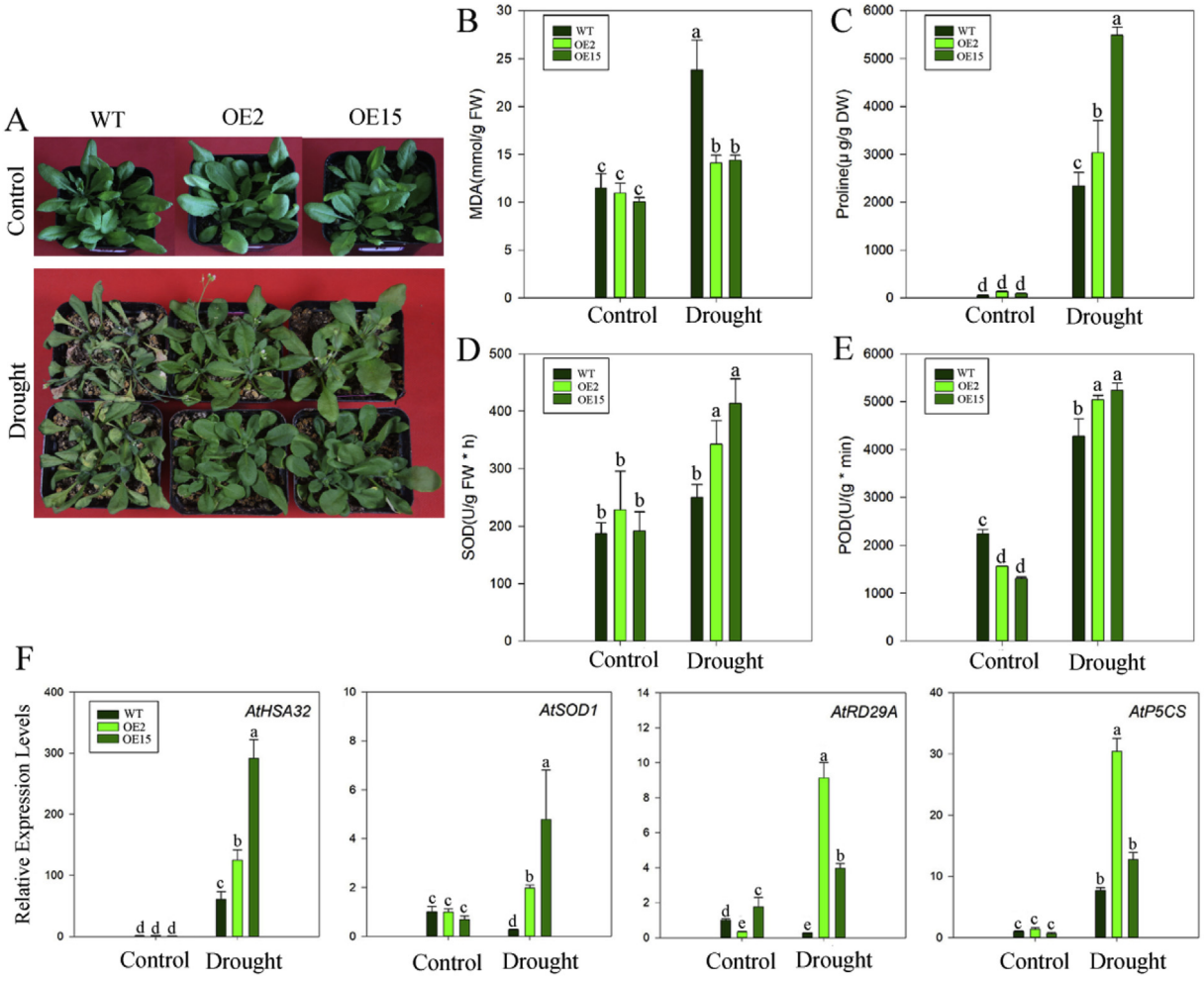

Fig. 7. Over-expression of the *CaHsp25.9* gene enhanced tolerance to drought stress. (A–F) Phenotype, MDA content, proline content, SOD and POD activity of WT and *CaHsp25.9*-OE *Arabidopsis* seedlings under drought stress performed by withholding water for 5 d, seedlings watered every 2 d were used as the control. (F) Relative expression levels of drought stress-related genes in WT and transgenic seedlings under drought stress. Data are means with standard deviations of three biological replicates. Different letters denote statistical significance at $p \leq 0.05$.

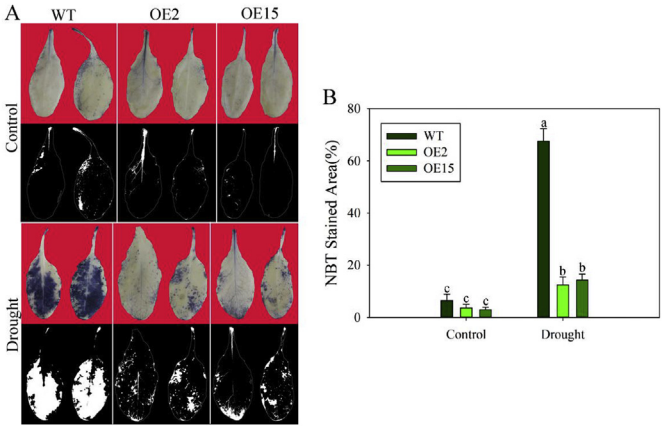

Fig. 8. Over-expression of the *CaHsp25.9* gene enhanced tolerance to drought stress. (A) NBT staining in WT and *CaHsp25.9*-OE *Arabidopsis* seedlings under drought stress. (B) NBT stained area in WT and transgenic *Arabidopsis* seedlings under drought stress. Data are means with standard deviations of three biological replicates. Different letters denote statistical significance at $p \leq 0.05$.

accumulating more SOD, CAT and POD activity than WT (Sun et al., 2012). Similarly, in this study, *CaHsp25.9*-overexpressing *Arabidopsis* exhibited significantly improved SOD and POD activity, and proline content, compared with the WT, under drought stress. Under heat and salt stress, the transgenic lines showed higher SOD activity and proline content, while lower activity of POD than WT. It has been reported that CAT, APX, and proline, which can effectively scavenge H_2O_2 together with POD, were also induced by heat and salt stress in the transgenic lines (Mittler et al., 2004; Temple et al., 2005; Verbruggen and Hermans, 2008; Choudhury et al., 2016). Therefore, in this study, the transgenic line produced lower POD than WT under heat and salt stress. Consequently, these results suggested that *CaHsp25.9* enhances plants tolerance to heat, salt, and drought stress though enhancing the activity of ROS-scavenging related antioxidant enzymes.

Plants contain many stress related genes, which may be involved in the response to the heat, salt, and drought stress. For instance, acquired thermo-tolerance related genes *AtHSA32* and *AtHSP101* can be induced by heat stress (Charng et al., 2006; Burke and Chen, 2015). ABA-responsive genes *AtDREB2A* and *AtMYB* can be considerably induced under chilling stress (Nakashima et al., 2000; Huang et al., 2018). The salt stress related gene *AtSOS1* was up-regulated under NaCl stress (Shi et al., 2000). Besides, the drought responsive gene *AtRD29A*, the P5C synthase gene *AtP5CS*, and the SOD synthase gene *AtSOD1* can be induced by heat, salt, and drought stress (Chen and Dickman, 2005; Verbruggen and Hermans, 2008; Huang et al., 2018). Interestingly, many of these genes were regulated by Hsp20s, suggesting that Hsp20s have the ability to regulate these related proteins to participate in plant response to heat stress (Wang et al., 2017b; Huang et al., 2018). For

example, the *OsMSR-4* (*Multi-Stress-Responsive 4*) in *Oryza sativa*, and *CaHsp16.4* could increase plant drought tolerance by regulating the expression of stress-related genes under drought stress (Huang et al., 2018; Yin et al., 2015). In this study, the expression level of the *AtHSA32*, *AtHSP101*, *AtDREB2A*, *AtSOS1*, *AtRD29A*, *AtSOD1*, and *AtP5CS* genes were strongly induced in the *CaHsp25.9* transgenic *Arabidopsis* than in WT under heat, salt, and drought stress. Therefore, these results suggested that *CaHsp25.9* may be involved in plant heat, salt, and drought stress tolerance by modulating the expression level of stress related genes, but the exact mechanisms need further study.

5. Conclusion

Our results demonstrated that the transcription of *CaHsp25.9* was induced by heat, salt, and drought stress, while its protein localized in the cell membrane and cytoplasm. The silencing of *CaHsp25.9* increased plant sensitivity to heat, salt and drought stress, while the over-expressing *CaHsp25.9 Arabidopsis* lines showed decreased sensitivity to heat, salt, and drought stress. These results indicate that *CaHsp25.9* positively regulates plant heat, salt, and drought tolerance. Furthermore, *CaHsp25.9* may play a positive role in tolerance to these forms of stress by reducing the accumulation of ROS and modulating the expression of stress related genes. Therefore, it can be assumed that *CaHsp25.9* plays a crucial role in plant adaption to extreme environments. The identified functions of the *CaHsp25.9* gene may provide some evidence for the further study of adaption mechanisms developed by plants in variable environments.

Author contribution statement

XHF and ZHG conceived the idea and designed the research. XHF, HXZ, WXG, and GXC conducted experiments. XHF: Formal analyzed the data. XHF: Writing – original draft, and wrote the manuscript. MA helped in improvement of text. ZHG, QGY, SBY and XXL contributed reagents/materials/an analysis tools. ZHG: Writing - review & editing. All authors read and approved the manuscript.

Compliance with ethical standards

Study presented in the manuscript did not involve in human or animal subjects.

Conflicts of interest

The authors declare that they have no conflict of interest.

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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.2019.07.001.

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