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Heat stress transcription factor *DcHsfA1d* isolated from *Dianthus caryophyllus* enhances thermotolerance and salt tolerance of transgenic *Arabidopsis*

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Abstract

Heat shock transcription factors (Hsfs) participate in a variety of plant physiological processes including the regulation of transcription factors associated with thermotolerance. Here, a *Hsf* gene *DcHsfA1d* was identified from carnation (*Dianthus caryophyllus* L.). The open reading frame (ORF) of *DcHsfA1d* was 1 368 bp and encoded a protein of 455 amino acids with a molecular mass of 51.039 kDa and an isoelectric point of 4.94. Sequence domain prediction revealed that DcHsfA1d protein exhibited five typical functional features and motifs. The transcription of *DcHsfA1d* was significantly up-regulated under heat stress or ABA treatment. Yeast two-hybrid experiment indicated that DcHsfA1d and DcHsp70 physically interact with each other. Overexpression of *DcHsfA1d* in *Arabidopsis* ecotype Columbia enhanced seedling thermotolerance by increasing the activities of catalase, peroxidase, and superoxide dismutase while reducing relative electrolyte leakage, malondialdehyde content, accumulation of O_2^- and H_2O_2 and by initiating transcriptional regulation of thermal protective gene expression under heat stress. Furthermore, under salt stress, the root length and fresh mass of *Arabidopsis* ectopically expressing *DcHsfA1d* were significantly higher than those of wild type, which indicated that the salt tolerance of transgenic *Arabidopsis* was improved to a certain extent. In summary, *DcHsfA1d* was demonstrated to play a positive regulatory role in heat stress response and it might be a candidate gene for salt tolerance using genetic modification.

Keywords: carnation, *Dianthus caryophyllus* L., heat shock transcription factors, salt tolerance, thermotolerance, transgenic plants.

Introduction

Higher plants may encounter a variety of environmental stresses, such as extreme temperatures, drought and salinity, and therefore have formed an effective defence mechanism to adapt to the unfavourable conditions (Scharf *et al.* 2012). High temperatures (heat stress) negatively influence plant growth and development as well as compromise crop yield (Mittler *et al.* 2012, Ding *et al.* 2020). Plants express heat shock proteins (Hsps) in response to various abiotic stresses. Heat shock transcription factors (Hsfs) act as a terminal component of the signal transduction pathway and regulate the expression of Hsps and other heat-responsive transcripts (Ohama *et al.* 2017).

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Abbreviations: ABA - abscisic acid; CAT - catalase; DBD - DNA-binding domain; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; HR-A/B - hydrophobic repeat regions A/B; Hsfs - heat shock transcription factors; Hsps - heat shock proteins; HSR - heat shock response; MDA - malondialdehyde; NES - nuclear export signal; NLS - nuclear localization signal; OD - oligomerization domain; ORF - open reading frame; POD - peroxidase; qPCR - quantitative PCR; ROS - reactive oxygen species; SOD - superoxide dismutase; Tail-PCR - thermal asymmetric interlaced PCR.
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In comparison with *Drosophila melanogaster*, *Caenorhabditis elegans* and yeast, plants express a wide range of Hsfs. There are 21 Hsfs in *Arabidopsis*, 25 Hsfs in *Oryza sativa*, and 27 Hsf members in *Populus trichocarpa* (Scharf *et al.* 2012). Plant Hsfs contain an N-terminal DNA-binding domain (DBD), an oligomerization domain (OD), and a nuclear localization signal (NLS) (Scharf *et al.* 2012). Some Hsfs also contain nuclear export signal (NES) and a characteristic pattern of aromatic amino acid residues embedded in short acidic motifs (AHA motifs) which are critical for the activator function (Nover *et al.* 2001, Scharf *et al.* 2012). While DBD is relatively conserved among different species, other functional domains are variable (Scharf *et al.* 2012). Plant Hsfs are allocated into three classes, A, B, and C based on their sequence homology and structural characteristics (Nover *et al.* 2001). As transcriptional activators, HsfAs are mainly responsible for regulating the transcription of heat shock genes while HsfBs act as repressors *per se*. For example, tomato HsfB1 has no activation domain but can act as a co-activator of HsfA. Class C Hsfs such as those found in *Festuca arundinacea FaHsfC1b* or *Triticum aestivum TaHsfC2a-B* have been reported to confer heat tolerance in transgenic plants (Hu *et al.* 2018, Zhuang *et al.* 2018).

Amongst the Hsf family, HsfA1s play the primary role as positive regulators of heat shock response (Yoshida *et al.* 2011). Tomato *HsfA1a* is a major regulator of heat stress (Mishra *et al.* 2002). HsfA1a together with HsfA2, HsfB1, and other chaperones, such as Hsp70 and Hsp90, form a complex network and thus regulate thermal response (Chan-Schaminet *et al.* 2009). In contrast with tomato, none of the four *AtHsfA1* (*HsfA1a*, *A1b*, *A1d* and *A1e*) members of *Arabidopsis* seems to act as a master regulator (Lohmann *et al.* 2004, Nishizawa-Yokoi *et al.* 2011). Transcriptome analysis of *AtHsfA1a* and *AtHsfA1b* double-knockout mutants indicate a series of genes related to thermotolerance. These genes (such as *Hsp101*, *Hsp70*, and *sHsps*) mainly encode for a range of Hsps; few *Hsfs*, such as *GolS1* and *Ips2*, encode metabolism-related enzymes (Busch *et al.* 2005). Moreover, some studies suggest that plant HsfA1s also mediate responses to other abiotic stresses. For example, *Arabidopsis HsfA1s* confer tolerance to salt, osmotic and oxidative stresses by mediating the induction of *Hsp* genes (Liu *et al.* 2011, 2013, Bechtold *et al.* 2013).

Very few studies have focused on the regulation of heat tolerance of ornamental plants by *Hsfs vis a vis* model plant. Carnation (*Dianthus caryophyllus* L.) is one of the leading flowers amongst ornamental plants. The growth and development of carnations are severely impaired due to high temperature, resulting in an adverse effect on the quality of cut-flowers. The genome sequence information of carnation (Yagi *et al.* 2014) provides valuable genetic resources for follow-up studies. To date, very little data is available regarding the heat shock response (HSR) mechanism of carnation. In the present study, we identified a carnation *DcHsfA1d* gene, which was not previously reported in the carnation genome database (Yagi *et al.* 2014). This gene was cloned and studied for expression as well as regulation of responses to heat and salinity. The overall goal of the study was to improve our understanding of the functional roles of *DcHsfA1d* and provide ideas for breeding novel carnation cultivars with enhanced thermotolerance by genetic engineering.

Materials and methods

Plants and growth conditions: Seedlings of carnation (*Dianthus caryophyllus* L.) cv. Fancy were propagated *in vitro* in a growth chamber at a temperature of 24 °C, a 14-h photoperiod, and an irradiance of 120 µmol m^{-2} s^{-1}. Seeds of *Arabidopsis thaliana* L. Columbia (Col-0) ecotype were sterilized and sown on Murashige and Skoog (MS) medium and cultivated at a temperature of 22 °C, a 16-h photoperiod, and an irradiance of 100 µmol m^{-2} s^{-1}.

Cloning and sequence analysis of *DcHsfA1d*: DNA extraction from carnation leaves was done by the cetyltrimethylammonium bromide (CTAB) method according to Lu *et al.* (2012). After incubation at 42 °C for 1 h, total RNA was extracted and first-strand cDNA was synthesized using *EasySpin* plant RNA extraction kit (*Aidlab*, Beijing, China) and *PrimeScript*® reverse transcription (RT) reagent kit (*TaKaRa*, Tokyo, Japan), respectively. A partial sequence of *DcHsfA1d* was amplified using degenerate primers (DcHsfA1dF1 and DcHsfA1dR1) based on the conserved sequences of variant *HsfA1s*, and the resulting fragment was cloned into pMD18-T (*TaKaRa*) for sequencing. Thermal asymmetric interlaced PCR (Tail-PCR) was used for the amplification of 3'-terminal and 5'-terminal sequences. Based on the *DcHsfA1d* fragment sequence, specific primers (DcHsfA1dSPF1, DcHsfA1dSPF2, and DcHsfA1dSPF3) and random degenerate primers (AD1 - AD8) were designed and employed for the amplification of the 3'-terminal sequence. Following three rounds of PCR, the product was cloned to pMD18-T for sequencing. Subsequently, a degenerate primer (DcHsfA1dF2) and a specific primer (DcHsfA1dR2) were designed according to conserved sequences of *HsfA1s* and the new fragment sequence, respectively, for DNA sequence amplification. Per the obtained sequence, another three specific primers (DcHsfA1dSPR1, DcHsfA1dSPR2, and DcHsfA1dSPR3), were designed for the amplification of the 5'-terminal sequence. Then the full-length gDNA sequence was assembled, and two specific primers (DcHsfA1dF and DcHsfA1dR), were synthesized to amplify the coding sequence of *DcHsfA1*. The thermal cycling parameters for Tail-PCR are listed in Table 1 Suppl. All primers used for cloning the *DcHsfA1d* gene have been listed in Table 2 Suppl.

The relative molecular mass and theoretical isoelectric point of DcHsfA1d protein were analyzed using *Prot Param* software (http://web.expasy.org/protparam/). To analyze the conserved functional domain of DcHsfA1d, *Clustal X 1.83* was used to compare the amino acid sequences of DcHsfA1d and *Arabidopsis* HsfA1 members. The phylogenetic tree was constructed by neighbor-joining method using *MEGA 5* software.

Stress treatments and gene expression analyses: For induction of heat stress, 40-d-old carnations of uniform size were transferred into a chamber set at 42 °C for 0, 1, 2, 4, and 8 h. For abscisic acid (ABA) treatment, seedlings were sprayed with distilled water containing 100 µM ABA and leaves were collected after 0, 1, 3, 6, 12, and 24 h. All treatment groups were independently replicated thrice. Samples were frozen immediately in liquid nitrogen and stored at -80 °C until RNA extraction.

Real-time quantitative PCR (qPCR) analysis was performed using *SYBR® Premix Ex TaqTM II* kit (*TaKaRa*) and the reaction was performed in the *7500 fast* real-time PCR system (*Applied Biosystems*, Foster City, CA, USA). The reaction conditions were 40 cycles at 95 °C for 3 s, 60 °C for 30 s, and 95 °C for 30 s (Wan *et al.* 2016). The $2^{-\Delta\Delta CT}$ method was used to calculate gene relative expressions. Each sample had three biological replicates. The carnation *DcGAPDH* (*glyceraldehyde-3-phosphate dehydrogenase*) or *Arabidopsis AtACTIN2* was used as an internal control gene. Primers used for the real-time qPCR analysis are listed in Table 3 Suppl.

Plasmid construction and *Arabidopsis* transformation: The coding region of *DcHsfA1d* was amplified into the modified vector pCAMBIA2300s, using *Sal*I and *Sac*I sites. The recombinant plasmid, *35S*::*DcHsfA1d*, was then introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed to *Arabidopsis thaliana* ecotype Columbia (Clough and Bent 1998). Positive seeds were screened and T3 homozygous seeds were analyzed thereafter.

The relative expressions of *DcHsfA1d* were analyzed by semi-quantitative reverse transcription PCR. The *AtACTIN2* gene was used as an internal reference gene. Primers for reverse transcription PCR analysis of transgenic *Arabidopsis* can be found in Table 4 Suppl.

Yeast two-hybrid interaction: Primers for yeast fusion expression vector construction are listed in Table 5 Suppl. The coding region of *DcHsfA1d* was amplified to prey vector pGADT7, using *NdeI* and *BamHI* sites. Similarly, the coding region of *DcHsp70* was cloned between *NdeI* and *BamHI* sites of the bait vector pGBKT7. The generated plasmid combinations pGADT7-DcHsfA1d and pGBKT7-DcHsp70 were co-transformed into the strain AH109, according to the manual of *Matchmaker™ Library Construction & Screening* kit (*Clontech*, Palo Alto, USA). Meanwhile, pGADT7-DcHsfA1d and pGBKT7, pGBKT7-DcHsp70 and pGADT7 were also co-transformed as control combinations. The co-transformation solution (0.1 cm^3) was uniformly coated on synthetic dextrose (SD)/-Leu/-Trp medium and cultured in an incubator at 30 °C. For protein interaction assay, single colonies on SD/-Trp/-Leu plates were picked for PCR detection. The positive clones of the target band were diluted for 0, 10, 100, 1 000, and 10 000 times. Then, 5 mm^3 of those solutions was added on the selective medium SD/-Trp/-Leu/-His/-Ade in the presence of X-α-gal and cultured in an incubator at 30 °C to observe the growth and colour rendering.

Stress treatments of transgenic *Arabidopsis*: For heat stress treatment, each line was repeated in 3 Petri dishes, and 30 seeds were sown in each dish. Each experiment was repeated 3 times. 12-d-old seedlings were transferred to an incubator at 44 °C for 2 h. After a 5-d recovery period, the survival rates were recorded. The physiological measurement, reactive oxygen species (ROS) detection, and heat-protective related gene expression analysis were also performed. For this, four-week-old plants were shifted to 42 °C for 6 h, after which leaves were collected from both control as well as stressed *Arabidopsis* plants and analyzed further.

To study the effect of salinity on root growth, 7-d-old seedlings were shifted onto MS media containing 0 or 150 mM NaCl. Root length and fresh mass of the seedlings were recorded after 5 d. In each Petri dish, 5 seedlings of each line were grown and 6 dishes were used. The root lengths were analyzed based on the average root length of 10 seedlings, and the fresh mass analyzed was based on the total fresh mass of 10 seedlings. All experiments were repeated three times.

Physiological measurement and ROS detection: Sample (0.3 g) was homogenized on ice with 1 cm^3 of 50 mM potassium phosphate buffer (pH of 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 % polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 12 000 *g* and 4 °C for 20 min, and the supernatant was used to determine the activities of catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD). CAT activity was measured by detecting the degradation rate of H_2O_2 at 240 nm, as per Aebi (1984). One unit of CAT activity was defined as the amount of enzyme that reduced absorbance by 0.1 per minute. POD activity was determined according to the change in absorbance at 470 nm due to the oxidation of guaiacol according to Maehly and Chance (1954). One unit of POD activity was defined as the amount of enzyme that increased absorbance by 0.01 per minute. SOD activity was measured according to the inhibition of photochemical reduction of nitrogen blue tetrazole (NBT), with reference to Giannopolitis and Ries (1977). One unit of SOD activity was defined as the amount of enzyme causing 50 % inhibition of NBT reduction. To evaluate the relative electrolyte leakage, a 0.1 g fresh sample was added into a centrifuge tube containing distilled deionized H_2O, and the conductivity value S1 was measured after 2 h. Then, the centrifuge tube was bathed in boiling water at 100 °C for 10 min and cooled to room temperature, and the conductivity value S2 was measured (Sairam and Srivastava 2002). The conductivity value of distilled deionized H_2O was S0. Calculation formula was: electrolyte leakage [%] = 100 × (S1 - S0)/(S2 - S0). To measure the malondialdehyde (MDA) content, a 0.3 g fresh sample was homogenized and used for subsequent determination according to Heath and Packer (1968). The superoxide and hydrogen peroxide accumulation were determined as described by Fukao *et al.* (2011) and Wan *et al.* (2016). The NBT staining method was used for O_2^- staining and the diaminobenzidine (DAB) staining method was used for H_2O_2 staining.

Statistical analysis: Each experiment was repeated in at least three different plants and conducted in triplicate. Data are represented as means ± standard deviation. All data were analyzed using Fisher's LSD test. *SAS* statistical software was used for analysis of variance (*SAS 8.1*, *SAS Institute Inc.*, Cary, NC, USA), and the *P*-value thresholds are < 0.05 (*), < 0.01 (**), and < 0.001 (***).

Results

A 4 852 bp gDNA sequence of *DcHsfA1d* was assembled by homologous cloning and Tail-PCR technology. The sequence consisted of two exons of 282 bp and 1 086 bp, respectively, with a 3 484 bp intron. Based on the gDNA sequence, a 1 368 bp length open reading frame (ORF)

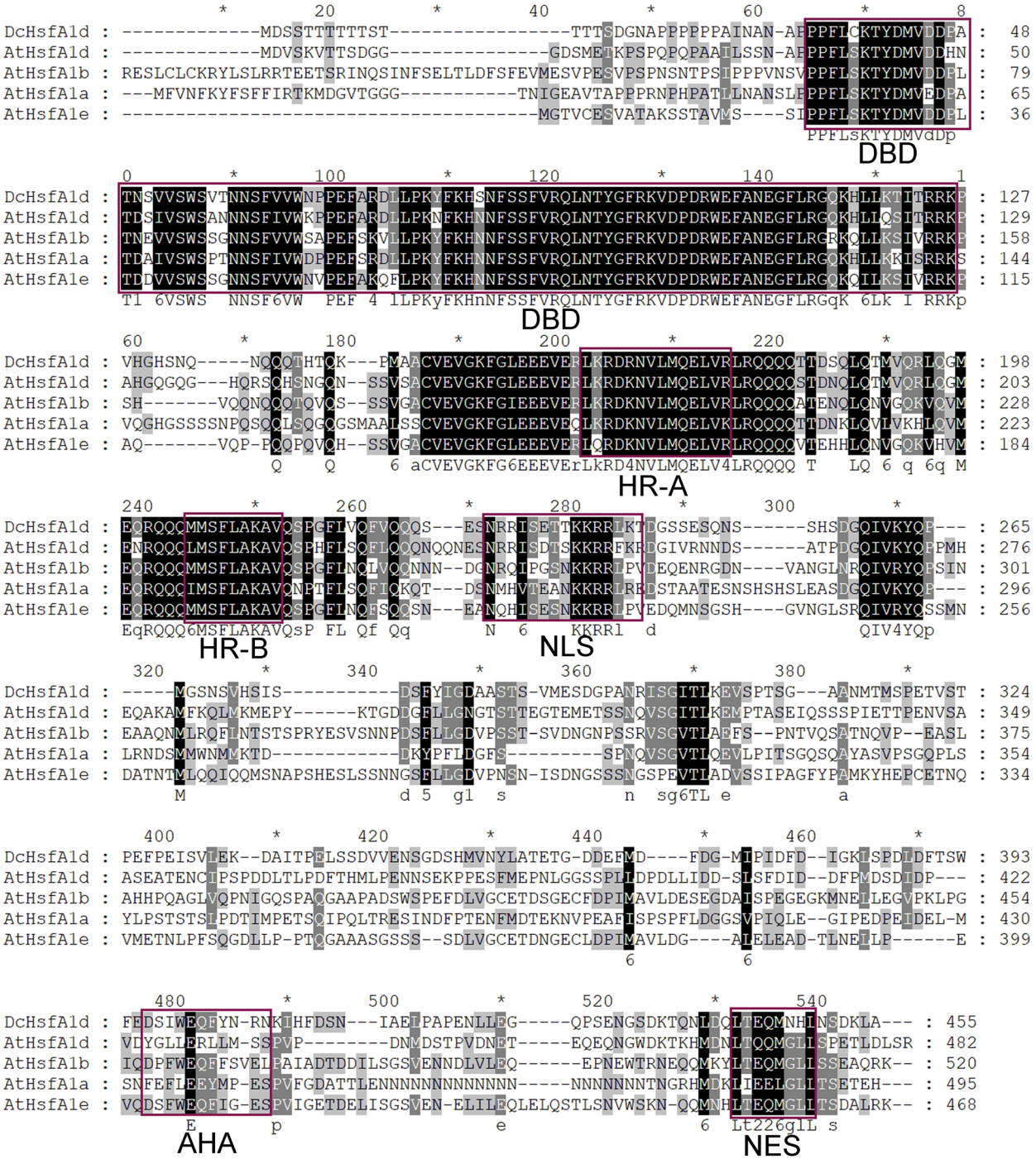

Fig. 1. Alignment of amino acid sequences of DcHsfA1d and AtHSFA1s. Five typical domains and motifs are shown in frames. The GenBank accession numbers of AtHsfA1 for sequence alignment are as follows: AtHSFAla, CAB10555; AtHSFAlb, CAA74397; AtHSFAld, AAF81328; AtHSFAle, AAF26960. DBD - DNA-binding domain, HR-A and HR-B - hydrophobic repeat regions A/B, NLS - nuclear localization signal, AHA - aromatic hydrophobic acidic, NES - nuclear export signal.

was cloned. It encoded a putative protein of 455 amino acids, with a molecular mass of 51.039 kDa and an isoelectric point of 4.94. Sequence domain prediction revealed that DcHsfA1d protein exhibited five typical functional features and motifs, possessing a DBD domain, hydrophobic repeat regions A/B (HR-A/B), NLS, AHA, and a leucine-rich NES region (Fig. 1). The phylogenetic analysis revealed that identified protein belonged to the HsfA1 family so we name it DcHsfA1d (Fig. 1 Suppl., Fig. 2 Suppl.).

To examine the transcriptional characteristics of *DcHsfA1d* under heat stress, carnation seedlings were exposed to a temperature of 42 °C. The expression of *DcHsfA1d* was induced and peaked at 1 h, following which the accumulation of *DcHsfA1d* decreased (Fig. 3*A* Suppl.). After ABA treatment, the expression of *DcHsfA1d* rapidly triggered and peaked at 6 h with a 120-fold increase, after which the expression decreased sharply. However, even then the expression was higher than that of control (Fig. 3*B* Suppl.).

Previous studies have shown that Hsp70 can repress the activity of HsfA1 by direct interaction. Therefore, we studied the interaction between DcHsfA1d and DcHsp70 in carnation by yeast two-hybrid system. The coding region of *DcHsfA1d* was amplified in prey vector pGADT7 while the coding region of *DcHsp70* was cloned to bait vector pGBKT7. The plasmid combinations pGADT7-DcHsfA1d and pGBKT7-DcHsp70 were co-transformed into the yeast strain AH109. Results indicated that DcHsfA1d and DcHsp70 physically interact with each other (Fig. 4 Suppl.).

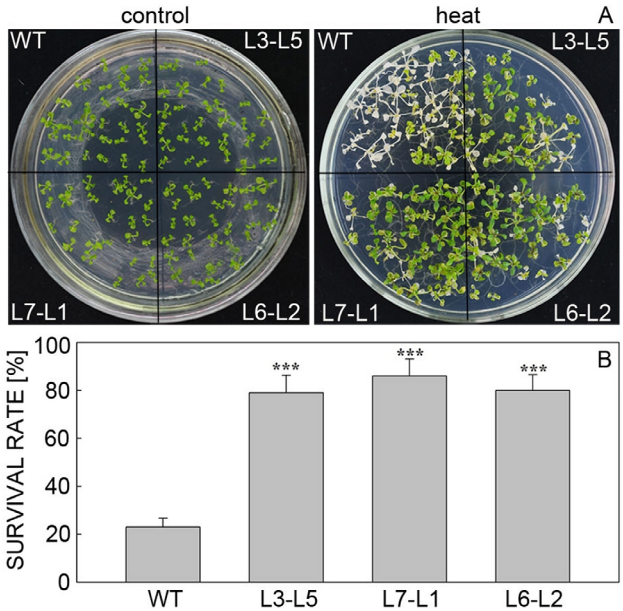

Fig. 2. Phenotypes of *DcHsfA1d* transgenic *Arabidopsis* plants under heat stress. *A* - Phenotypes of 12-d-old WT and transgenic seedlings under normal (control) and 5-d-recovery period (after heat). *B* - Survival rates of the WT and transgenic lines. Survival rates were calculated at day 5 after exposure to 44 °C for 2 h. Data represent the mean of three independent replicates. *Asterisks* indicate significant differences between the WT and transgenic plants ($P < 0.001$).

To investigate the biological functions of *DcHsfA1d*, three independent T3 transgenic *Arabidopsis* lines (L3-L5, L7-L1, and L6-L2) that showed relatively different expression levels of *DcHsfA1d* were screened for subsequent experiments (Fig. 5 Suppl.). Thermotolerance of transgenic plants was evaluated upon exposure of 12-d-old seedlings to 44 °C and the survival rates were measured after a recovery period of 5 d. Significantly increased survival rates, better and more robust growth state were detected in *DcHsfA1d* over-expressing lines (Fig. 2). These results demonstrated that overexpression of *DcHsfA1d* improved seedling thermotolerance.

Plants have an effective system of scavenging free radicals to protect cells from abiotic stress. To further analyze the acquired thermotolerance of transgenic plants, antioxidant enzyme activities were monitored. Results showed higher activities of CAT, POD, and SOD in transgenic *Arabidopsis* (Fig. 3*A-C*) as compared to controls. Besides, we also measured the relative electrolyte leakage, which reflected membrane permeability during stress. MDA is an important indicator to measure the degree of membrane lipid peroxidation. Transgenic lines showed significantly lower electrolyte leakage and lower MDA content than WT after HS (Fig. 3*D*,*E*). These data indicated that overexpression of *DcHsfA1d* conferred a higher amount of CAT, POD, and SOD activities to protect the plant by lowering cell membrane damage and lipid peroxidation.

Upon exposure to heat stress, plants produce a large amount of reactive oxygen species (ROS). The accumulation of superoxide and hydrogen peroxide was visualized *via* NBT and DAB tissue staining methods. As shown in Fig. 3*F*, comparable or slightly lower accumulation of O_2^- and H_2O_2 was detected with respect to WT under control conditions. Upon exposure to heat stress, the content of these ROS increased as indicated by deeper staining in the WT plants, indicating that cell death could be lowered by *DcHsfA1d* under heat stress.

To further understand the underlying mechanisms of positive effects of *DcHsfA1d* on thermotolerance, we analyzed the relative transcription of *AtHsp101*, *AtHsp90*, *AtHsp70*, *AtHsp18.2*, *AtHsfA2*, and *AtMBF1c* in *DcHsfA1d*-OE lines and wild type plants using qPCR (Fig. 4). Under normal conditions, the amount of transcripts of *AtHsp90*, *AtHsp70*, *AtHsfA2*, and *AtMBF1c* in transgenic lines was significantly higher than in the wild type. After heat stress, the transcription of these genes was up-regulated and it was significantly higher in one or two transgenic lines than in the wild plants.

To analyze the function of *DcHsfA1d* in salt stress, 7-d-old seedlings of WT *Arabidopsis* and three *DcHsfA1d*-OE lines were transferred on MS media containing 0 (control) or 150 mM NaCl (salt). Root length and fresh mass of the seedlings were recorded after 5 d. Under normal conditions, no significant differences in root length were observed between transgenic lines and WT, however, there was a significant increase in the fresh mass as compared to the WT. After salt treatment, the growth of seedlings was seriously hindered, but the leaves of *DcHsfA1d-OE* lines looked better than those of WT, and the root length and

fresh mass were also significantly higher in comparison with WT (Fig. 5).

Discussion

Hsf is an important member of the signal transduction pathway under abiotic stress conditions and a core regulator of Hsps and heat stress-related genes, which plays a vital role in plant thermotolerance (Nover *et al.* 2001). Among plant *Hsf* family members, *HsfA1* subclass genes participate in plant growth and development and play a key role in HSR (Scharf *et al.* 2012). *Arabidopsis HsfA1d* gene enhanced heat tolerance of transgenic potato (Shah *et al.* 2020). Although *HsfA1s* have been extensively studied in model plants, very few studies have focused on their role in ornamental plants (Wu *et al.* 2018).

In the present study, we identified a *Hsf* gene from carnation, an ornamental plant. Based on the sequence domain predictions and phylogenetic analysis *DcHsfA1d* might be homologous to the *HsfA1d* gene. The transcription of *DcHsfA1d* was induced by HS at 1 h and then reduced (Fig. 3*A* Suppl.). The transcription pattern was similar to *HsfA1* studied in other plants (such as *Lilium longiflorum* and *Brassica campestris*), and implied that *DcHsfA1d* might be involved in the early phase of the heat stress process (Gong *et al.* 2014, Zhu *et al.* 2018). Moreover, the expression of *DcHsfA1d* was rapidly triggered and peaked at 6 h after ABA treatment, followed by a sharp decline (Fig. 3*B* Suppl.). This pattern is similar to the one observed

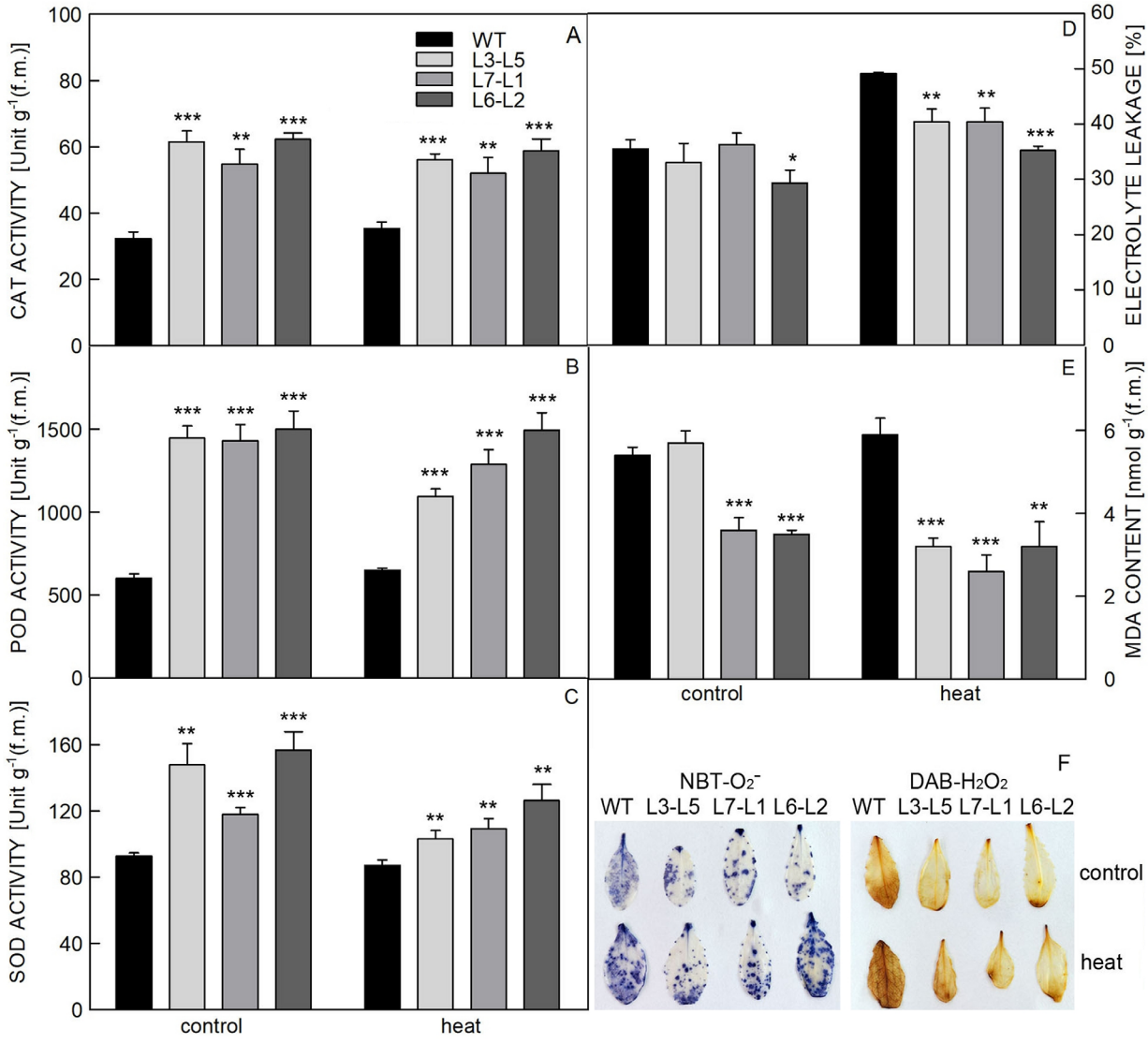

Fig. 3. Changes in antioxidant enzyme activities, electrolyte leakage, MDA content and ROS accumulation in WT and transgenic plants after heat stress treatment. *A* - Catalase (CAT) activity. *B* - Peroxidase (POD) activity. *C* - Superoxide dismutase (SOD) activity. *D* - Relative electrolyte leakage. *E* - Malondialdehyde (MDA) content. *F* - ROS accumulation. NBT staining of O_2^- accumulation; DAB staining of H_2O_2 accumulation. The 4-week-old seedlings were exposed to 42 °C for 6 h. Means ± SDs, $n = 3$. All comparisons were statistically analyzed using Fisher's LSD test. *Asterisks* indicate significant differences between the WT and transgenic lines (* - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$).

with *Festuca arundinacea* where ABA treatment induced the transcription of *FaHsfA2c*. Spraying ABA enhanced the heat tolerance of tall fescue and reduced the damage of the membrane system caused by heat stress (Wang *et al.* 2017a).

In tomato, *LpHsfA1a* acts as a "master" regulator of HSR (Mishra *et al.* 2002). Overexpression of *HsfA1* from *Lilium longiflorum* or *Brassica campestris* has also been reported to increase the heat resistance of transgenic lines (Gong *et al.* 2014, Zhu *et al.* 2018). Our results also demonstrated that alternation of the *DcHsfA1d* expression resulted in an enhancement of thermotolerance in *DcHsfA1d*-OE lines (Fig. 2). Therefore, we conclude that the role of *DcHsfA1d* in improving the heat resistance was consistent with the *HsfA1s* of other species.

Although, overexpression of *DcHsfA1d* can increase plant resistance to high-temperature stress, however, the physiological mechanism of its resistance is still unclear. O_2^- and H_2O_2 are toxic ROS that can cause damage to proteins and nucleic acids. Plants express low levels of ROS under normal growth conditions and the levels rapidly increase when being subjected to abiotic stress (Miller *et al.* 2010). ROS accumulation greatly relies on the balance between formation and scavenging (Mittler *et al.* 2004). Thus, the measurement of ROS accumulation may be used as an indicator of oxidative stress. Our results indicated a lower amount of O_2^- and H_2O_2 in the *DcHsfA1d*-OE lines (Fig. 3*F*) than in WT, suggesting the modulatory effect of *DcHsfA1d* in response to HS.

To adapt to the unfavourable environment and protect themselves from oxidative damage, plants have established an effective system to remove reactive oxygen species (Gill and Tuteja 2010). Our study indicated higher activity of CAT, POD, and SOD in the *DcHsfA1d* overexpression transgenic lines following high-temperature treatment (Fig. 3*A-C*). These results hint at a possible role of

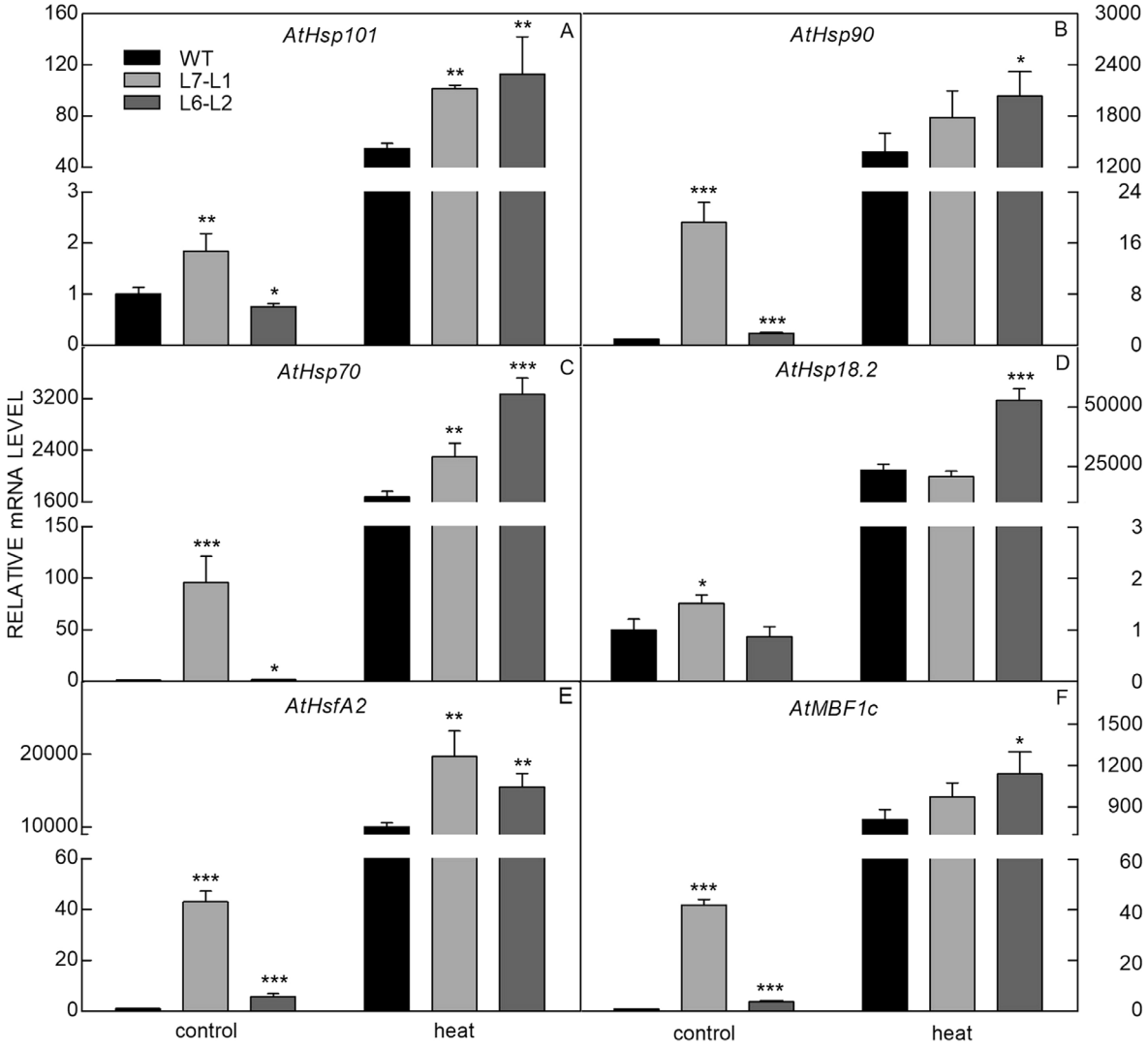

Fig. 4. Real-time qPCR analysis of some target genes of *AtHsfA1s*. 4-week-old seedlings were treated at 42 °C for 6 h and leaves were sampled for RT-qPCR analysis. The raw data were normalized using the *AtACTIN2* gene. The data represent means ± SD of three replicates. Significant differences between wild type and transgenic lines are indicated using Fisher's LSD test (* - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$).

DcHsfA1d in improving the ROS scavenging ability by creating a more effective antioxidant system, thereby reducing the damage to the cell membrane. A similar increase in POD and SOD activities in the *Brassica campestris BcHsfA1* overexpression in transgenic tobacco has been previously reported (Zhu *et al.* 2018). To assess the extent of membrane damage, electrolyte leakage was analyzed. Reduced electrolyte leakage was detected in *DcHsfA1d*-OE plants under heat stress (Fig. 3*D*), which was consistent with previous studies on *Festuca arundinacea* and *Brassica campestris* (Wang *et al.* 2017b, Zhu *et al.* 2018). MDA content estimation, an indicator for the degree of membrane lipid peroxidation, also substantiated the results obtained so far. The transformants showed lower MDA content under heat stress (Fig. 3*E*). All these results indicated that *DcHsfA1d* could enhance heat tolerance by reducing the degree of lipid peroxidation and increasing antioxidant enzyme activities and so ROS scavenging ability under heat stress conditions.

Plant adaptation to HS requires transcriptional regulation of a number of genes involved (Singh *et al.* 2012, Xue *et al.* 2014). Hsps act as molecular chaperones under heat stress to prevent irreversible protein aggregation and participate in protein refolding (Tripp *et al.* 2009). In our study, the expression of *AtHsp90*, *AtHsp70*, and *AtHsp18.2* was induced to varying degrees by the introduction of *DcHsfA1d* (Fig. 4). Tomato Hsp70 is known to repress the activity of HsfA1 by direct interaction (Hahn *et al.* 2011). Tomato Hsp70 interacts with HsfA1 and regulates its function. Hsp70 inhibits the activity of HsfA1 and, together with Hsp90, HsfA2, and HsfB1 to form a versatile regulatory regime to control HSR (Hahn *et al.* 2011). In the present work, we observed physical interaction between DcHsfA1d and DcHsp70 (Fig. 4 Suppl.). Previous studies have also reported that *HsfA2* is downstream to *HsfA1* and could, therefore, be activated by *HsfA1* (Yoshida *et al.* 2011, Gong *et al.* 2014). The expression of *AtHsfA2* in the present study was significantly higher in *DcHsfA1d*-OE lines (Fig. 4). In addition, *HsfA1* may also regulate the expression of *MBF1c* (*multiprotein bridging factor1c*) which is related to HSR (Suzuki *et al.* 2005, Bechtold *et al.* 2013). We saw the induction of *AtMBF1c* at varying degrees in transgenic lines (Fig. 4). Taken together, these results indicated that the involvement of *DcHsfA1d* in thermotolerance may be related to the inducement of these stress-related genes.

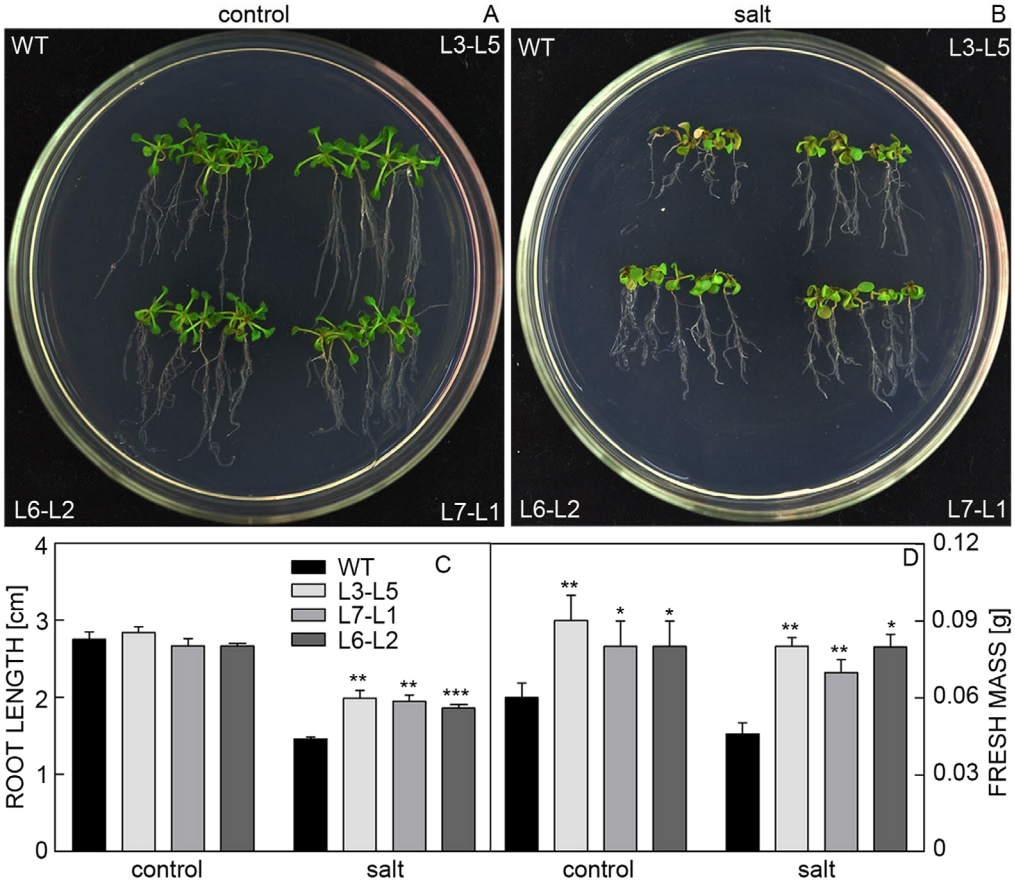

Fig. 5. Root length and fresh mass of *Arabidopsis* transgenic lines and wild type. *A* - Growth of transgenic lines and wild type *Arabidopsis* under normal conditions. *B* - Growth of transgenic lines and wild type *Arabidopsis* under salt stress (7-d-old seedlings on MS medium with 150 mM NaCl for 5 d). *C* - Comparison of root length of transgenic lines and wild type *Arabidopsis* under control and salt stress conditions. *D* - Comparison of the fresh mass of transgenic lines and wild type *Arabidopsis* under control and salt stress conditions. Means ± SDs of three replicates. Significant differences between wild type and transgenic lines are indicated using Fisher's LSD test (* - $P < 0.05$, ** - $P < 0.01$, *** - $P < 0.001$).

Different kinds of TFs are involved in root growth and architecture upon salt stress conditions (Gruber *et al.* 2009). Previous studies showed that *Arabidopsis AtHsfA2* and *AtHsfA4a*, rice *OsHsfA2e*, and *Chrysanthemum CmHsfA4* conferred salt tolerance (Ogawa *et al.* 2007, Yokotani *et al.* 2008, Pérez-Salamó *et al.* 2014, Li *et al.* 2018). In this study, overexpression of *DcHsfA1d* in *Arabidopsis* caused the development of significantly higher root lengths and fresh mass of transgenic plants than it was in the WT under NaCl treatment (Fig. 5). These results are similar to those of studies on salt tolerance of maize *ZmHsf04* (Jiang *et al.* 2018). These results indicated that *DcHsfA1d* played a positive role in promoting the growth of seedlings under salt stress.

Conclusions

Our data demonstrated that *DcHsfA1d* could be induced by heat stress or ABA treatment. Overexpression of *DcHsfA1d* in *Arabidopsis* enhanced seedling thermotolerance by increasing the activities of antioxidant enzymes while reducing membrane damage and initiating transcriptional regulation of thermal protective gene expression under heat stress. Furthermore, under salt stress, the root length and fresh mass of *Arabidopsis* overexpressing *DcHsfA1d* were significantly higher than those of wild type. Taken together, *DcHsfA1d* was demonstrated to play a positive regulatory role in heat stress response and might be a candidate gene for salt tolerance using genetic modification.

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