Chrysanthemum CmHSFA4 gene positively regulates salt stress tolerance in transgenic chrysanthemum

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Introduction

Soil salinization is one of the major issues threatening crop productivity worldwide as it affects plant growth by causing osmotic imbalance, mineral deficiency and overall toxicity (Parvaiz and Satyawati, 2014). To cope with salinity stress, plants tend to re-establish ionic and ROS homeostasis. Regarding ionic homeostasis, it is important to maintain a low level of Na⁺ while maintaining a high concentration of K⁺ in the cytosol (Guan et al., 2002). The salt overly sensitive (SOS) signalling pathway is involved in the transport of toxic ions. SOS1 encodes for a plasma membrane antiporter Na⁺/H⁺ which removes Na⁺ from cells (Rahman et al., 2016). HKT1, a member of the high-affinity K⁺ transporters gene family, plays important roles as a Na⁺-selective uniporter, under normal K⁺ concentration, HKT1 is mainly involved in Na⁺ unloading, and the major function of SOS1 is Na⁺ exclusion (Wang et al., 2014). They consequently play a crucial role in maintaining cellular ion homeostasis under salt stress by reducing the accumulation of Na⁺ and maintaining stable levels of K⁺ under salt stress (Ashraf and Sharif, 2008).

Except for ionic toxicity, salt stress leads to the accumulation of high levels of reactive oxygen species (ROS). ROS, including superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radicals (OH⁻), are continuously produced by aerobic metabolism in mitochondria, chloroplasts and peroxisomes in plants and can cause oxidative damage to proteins, DNA and lipids under stress (Akter, 2015). The balance of oxidative stress levels and ROS-scavenging enzymes are directly related to ROS cellular toxicity (Mittler et al., 2011). Plant cells utilize antioxidant mechanisms to defend from the damage of ROS (Wrzaczek et al., 2013). Nonsymmetrical ROS-scavenging mechanisms include the major cellular redox buffers such as ascorbate, glutathione (GSH), ascorbic acid and carotenoids (Mittler, 2002). The enzymatic mechanisms mainly include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) (Sevvelam et al., 2016). SOD converts hydrogen superoxide into hydrogen peroxide, which acts as the first line of defence to degrade the accumulating H₂O₂. APX and CAT convert hydrogen peroxide into water and subsequently detoxifies H₂O₂ by the ascorbate–glutathione cycle (Schmitt et al., 2014).

Heat-shock factors (HSFs) are important regulators of cellular stress. HSF gene families are large, including 21 genes in Arabidopsis, 24 in tomato, 52 in soybean and more than 56 in wheat (Xue, 2013). HSFs can be subdivided into three classes, A, B or C, depending on their domains. They all share a conserved N-terminal DNA-binding domain, which is responsible for heat-shock response element (HSE) recognition in the promoters of HSF target genes (Akerfelt et al., 2010). The hydrophobic heptad repeat region for oligomerization (HR-A/B), which is located close to the DNA-binding domain and mediates trimmerization, is a prerequisite for their transcription factor activity (Lutz Nover et al., 2001). In response to biotic and abiotic stresses, HSF proteins have various roles as positive or negative regulators. Most of them are regulated by heat shock, especially HSFA1, HSFA2 and HSFA6 in tomato and wheat (Mishra et al., 2002).

Furthermore, it was reported that ATHSFA3 is regulated by DREB2A and enhances drought stress tolerance in Arabidopsis (Scharf et al., 2012); FaHSFA2c acted as a positive regulator conferring thermotolerance through the regulation of heat-protective gene transcriptional expression in Arabidopsis and tall fescue (Wang et al., 2017). LIHSAF1, which interacts with LIHSF2A, enhanced thermotolerance in transgenic Arabidopsis overexpressing LIHSAF1 (Gong et al., 2014); HSBs have no transcription activity and usually act as negative regulator. AthSFB1 repressed expression of AthHSF2, AthHSFA7a and AthHSFB2b under moderate heat conditions (28 °C) in transgenic Arabidopsis (Ikeda and Ohme-Takagi, 2011). VpHSF1’s
overexpression lines reduced basal tolerancelerance, increased acquired thermotolerance and reduced tolerance to osmotic stress in transgenic tobacco (Peng et al., 2012). However, HsfB1 represents a novel type of coactivator cooperating with class A HSFs (e.g. with tomato HsfA1; Bharti et al., 2004). Although HSFs have a wide array of members and complex responses to stress, the function of the HSFA4 group is not well known (Perezsalamó et al., 2014). TaHSFA4a and OsHSFA4a enhanced Cd tolerance by up-regulating metallothionein gene expression in rice plants (Shim et al., 2009); Co-overexpression of Helianthus annuus HaHSFA4a and HaHSFA9 enhanced the tolerance to dehydration and drastic oxidative stress, and the improved tolerance is accompanied with the accumulation of small heat shock proteins (sHSP) which are activated by HaHSFA9 in transgenic tobacco (Personat, 2014). Arabidopsis HSFA4A was implicated in the regulation of responses to high light and oxidative stress by regulating the transcription of the APX1 and ZAT12 genes (Daveloal et al., 2005). These data indicated that the HSFA4 group contributes to Cd stress, high light stress and oxidative stress. In addition to this, HSFA4 confers salt tolerance. Knockout plants of Athsfa4a are hypersensitive to salt stress because of the elevated hydrogen peroxide accumulation and lipid peroxidation under salinity (Perezsalamó et al., 2014). HSFA4A’s function results in the enhanced expression of stress–response transcripts and regulates plant ROS homeostasis under stress.

Chrysanthemum, a major commercial ornamental plant, is readily subjected to salinity stress, which causes leaf chlorosis and causes serious damages to the plant’s health. Improving salt stress tolerance of chrysanthemum is a single copy integration line (Figure S1b,c) were selected for further salt tolerance assay. The expression level of CmHSFA4 in ox lines H4 and H5 was much higher that in wild-type plant under the salinity treatment (Figure 5a). Salt tolerance of CmHSFA4 overexpressing plants was assessed upon 200 mmol/L NaCl treatment for 7 days. CmHSFA4 overexpressing plants H4 and H5 showed less stress damage compared with WT chrysanthemum plants. The top of CmHSFA4 overexpressing plants remained green compared with WT plants and only the base leaves turned yellow after 7-day salinity treatment, while the wild type became severely wilted, withered and some plants died after salinity treatment for 7 days (Figure 5b). The survival rate of H4 and H5 plants was 55.6% and 48.3%, while that of WT plants was only 28.0% (Figure 5c), indicating that overexpression of CmHSFA4 enhanced salt tolerance of chrysanthemum.

CmHSFA4 overexpression enhanced salt tolerance of chrysanthemum

CmHSFA4 transgenic chrysanthemum was successfully generated and validated by PCR (Figure S1a). The expression levels of CmHSFA4 were higher in CmHSFA4 ox (overexpressing) plants than that of WT (wild type) (Figure S1b). Two independent ox lines H4 and H5 exhibiting high transcript levels of CmHSFA4 with a single copy of T-DNA integration (Figure S1b,c) were selected for further salinity tolerance assay. The expression level of CmHSFA4 in ox lines H4 and H5 was much higher than that in wild-type plant under the salinity treatment (Figure 5a). Salinity tolerance of CmHSFA4 overexpressing plants was assessed upon 200 mmol/L NaCl treatment for 7 days. CmHSFA4 overexpressing plants H4 and H5 showed less stress damage compared with WT chrysanthemum plants. The top of CmHSFA4 overexpressing plants remained green compared with WT plants and only the base leaves turned yellow after 7-day salinity treatment, while the wild type became severely wilted, withered and some plants died after salinity treatment for 7 days (Figure 5b). The survival rate of H4 and H5 plants was 55.6% and 48.3%, while that of WT plants was only 28.0% (Figure 5c), indicating that overexpression of CmHSFA4 enhanced salt tolerance of chrysanthemum.

CmHSFA4 overexpression retarded chlorophyll contents decrease under salinity stress

Under normal growth conditions, the chlorophyll contents in ox lines H4 and H5 were almost comparable to those in the WT plants (Figure 6). Total chlorophyll contents in ox lines and WT plants both decreased upon 200 mmol/L NaCl treatment for 7 days. The total chlorophyll content reduced by 45.9% in WT plants but 32.4% and 39.6% in ox lines H4 and H5 on day 7 (Figure 6a). Consistently, chlorophyll a content in ox lines H4 and H5 showed 43.2% and 51.6% reduction, while 57.3% in wild-type plants under salinity (Figure 6b). Similarly, chlorophyll b content in WT plants reduced by 52.7%, while 29.7% and 34.0% reduction in H4 and H5 plants (Figure 6c), indicating that overexpression of CmHSFA4 protected chlorophyll from degradation by salinity stress.

CmHSFA4 overexpression balanced ion homeostasis and changed salinity stress-related gene expression

To assess the effect of CmHSFA4’s overexpression on ion homeostasis, six to eight leaf-stage transgenic and wild-type plants were subjected to 200 mmol/L NaCl for 7 days. Under nonstress growing conditions, there was little variation in the Na+ and K+ contents between the transgenic and wild-type plants. On
day 7, the Na⁺ contents of H4, H5 were significantly lower than those of WT plants, with 50.4% and 45.9% of WT plants in roots, 58.2% and 53.3% of WT plants in stems and 73.3% and 65.7% of WT plants in leaves (Figure 7a). Under salinity stress conditions, H4, H5 plants had a higher K⁺ content than that found in the WT plants. The K⁺ content in the roots of H4 was 48.3%, and H5 was 74.7% higher than the level in WT plants, the stem K⁺ contents, respectively, 59.2% and 72.2%, 55.2% and 74.2% in leaves (Figure 7b). At the same time, the transcript levels of ion homeostasis genes CmSOS1 and CmHKT2 in CmHSFA4 overexpression reduced ROS levels and activated ROS scavenger activities

For in vivo localization and quantification of H₂O₂ and O₂⁻⁻, WT and transgenic leaves were stained with diaminobenzidine (DAB) (dark brown) and nitrotetrazolium blue chloride (NBT) (dark blue). CmHSFA4 overexpressing exhibited clearly lower intensities of DAB and NBT staining in leaves compared to WT plants,
reflecting a low level of H$_2$O$_2$ and O$_2$\textsuperscript{−} accumulation (Figure 8a, b). In contrast, under normal growth conditions, H$_2$O$_2$ levels were comparable in WT and CmHSFA4\textsuperscript{ox} plants, treatment with 200 mmol/L NaCl for 7 days increased the amount of H$_2$O$_2$ by 88% in the wild type but only by 59% and 63% in HSFA4\textsuperscript{ox} plants. Consistently, O$_2$\textsuperscript{−} contents in H4 and H5 plants increased by 80.2% and 92.7% but 107% in WT under salinity (Figure 8c,d).

To further elucidate the role of CmHSFA4 in ROS homeostasis, we examined the activities of ROS scavengers and found SOD, APX, CAT in OX plants were higher compared with WT at day 0 and day 7 of the salinity stress treatment (Figure 9a–c). The expression levels of ROS homeostasis-associated genes including CmSOD, CmAPX and CmCAT (ROS scavenger encoding genes), CmHSP70 and CmHSP90 (heat-shock protein genes) were examined by real-time PCR analysis. Small differences between WT and OX plants under nonstress conditions were observed. Under salinity stress, ROS scavenger genes and CmHSPs were upregulated in both WT and OX plants, and the expression levels in OX plants were always higher than those in WT plants (Figure 9d–h).

**Discussion**

*CmHSFA4* confers salinity tolerance in chrysanthemum

Members of the HSF family are involved in various stresses such as heat stress, drought, anoxia, cold, high light and pathogen systemic acquired resistance (Guo et al., 2016). A few members of class A HSFs have been reported to play a role in salinity tolerance. For example, *AtHSFA2* increased salt/osmotic stress tolerance of Arabidopsis (Ogawa, 2007); *OsHSFA2e* enhanced tolerance to high-salinity stress in transgenic Arabidopsis (Yokotani and Oda, 2008). In addition, *AtHSFA4a* conferred salt tolerance and oxidative stress (Pérez-salamanca et al., 2014). In the present study, overexpressing the class A HSFA4 homolog *CmHSFA4*, probably a single copy gene in chrysanthemum (Figure S3), enhanced tolerance to salinity in chrysanthemum, indicating *CmHSFA4* is functionally conserved for salinity tolerance. The expression level of *CmHSFA4* in OX lines was much higher than that in WT plant under the salinity treatment (Figure 5a), which might be a consequence of the posttranscriptional stabilization of *CmHSFA4* transcripts by salinity stress. Similarly, posttranscriptional stabilization of *SOS1* in 35S:SOS1 overexpressing plants under salt stress has been previously described (Chung et al., 2008). We supposed that *CmHSFA4* overexpression contributed to the acquired tolerance of OX lines H4, H5 plants to salinity, while an induction of *CmHSFA4* by salinity in WT plants might confer to a basal tolerance of WT plants to salinity.

We also testified whether *CmHSFA4* contributed to osmotic adjustment; however, no significant differences in wilting, relative water contents and osmotic potential between WT and OX lines H4, H5 subjected to PEG6000 treatment have been observed (Figure S4), inferring that *CmHSFA4* might not contribute to osmotic adjustment.
CMHSFA4 enhanced tolerance to salinity in chrysanthemum is a consequence of ion homeostasis

Ion transport is the basic factor determining salinity tolerance. Along with ion uptake and transport, sequestration and extrusion, Na⁺-K⁺ homeostasis governs the principal mechanisms of salt tolerance in plants (Vinod et al., 2013). Transcript accumulation of ion homeostasis-associated genes served as the main regulators under salinity (Parvaiz and Satyawati, 2014). SOS1-overexpressing transgenic tobacco and Arabidopsis accumulated less Na⁺ than WT plants under salt stress by limiting loading Na⁺ into the xylem and controlling long-distance Na⁺ transport from xylem stream (Yue et al., 2012). SOS1 retrieves Na⁺ from the xylem stream under severe salt stress (Shi et al., 2002). Under salinity, HKT1 primarily prevents Na⁺ overaccumulation in shoots via a downward stream of phloem in Arabidopsis (Horie et al., 2009). Under the low K⁺ with salt stress, AtSOS1 functions in loading Na⁺ into the xylem to keep a relatively low level in surrounding parenchyma cells (Wang et al., 2014). OsHKT2 acts as a Na⁺-K⁺ symporter in tobacco cells and mediates a large Na⁺ influx into K⁺-starved roots for growth (Jabnoune et al., 2009). Under salinity, HKT1 primarily prevents Na⁺ overaccumulation in shoots via a downward stream of phloem in Arabidopsis (Horie et al., 2009). Under the low K⁺ with salt stress, AtSOS1 functions in loading Na⁺ into the xylem to keep a relatively low level in surrounding parenchyma cells (Wang et al., 2014). OsHKT2 acts as a Na⁺-K⁺ symporter in tobacco cells and mediates a large Na⁺ influx into K⁺-starved roots for growth (Jabnoune et al., 2009). Under salinity, HKT1 primarily prevents Na⁺ overaccumulation in shoots via a downward stream of phloem in Arabidopsis (Horie et al., 2009). Under the low K⁺ with salt stress, AtSOS1 functions in loading Na⁺ into the xylem to keep a relatively low level in surrounding parenchyma cells (Wang et al., 2014). OsHKT2 acts as a Na⁺-K⁺ symporter in tobacco cells and mediates a large Na⁺ influx into K⁺-starved roots for growth (Jabnoune et al., 2009).

Figure 5 CmHSFA4 overexpression enhanced salinity tolerance in chrysanthemum. (a) The expression level of CmHSFA4 in WT and CmHSFA4 OX lines H4, H5 plants under salinity treatment, (b) The phenotypic effect of watering with 200 mmol/L NaCl for 2-weeks, followed by 2-week recovery period, (c) Plant survival rate measured at the end of the recovery period. WT, wild-type, H4 and H5, transgenic plants overexpressing CmHSFA4. Bars indicate standard error.

CmHSFA4 enhanced tolerance to salinity in chrysanthemum is a consequence of ion homeostasis

In this present study, we found that CmHSFA4 enhanced salt tolerance is possibly associated with the re-establishment of ionic homeostasis. Compared to wild type, CmHSFA4 overexpressing plants exhibited a lower accumulation of Na⁺ under salinity conditions, which resulted in a lower accumulation of Na⁺ in the roots, stems and leaves of OX plants. In contrast, Na⁺ accumulation was distinctly enhanced in the roots, stems and leaves of the CmHSFA4 overexpressing plants (Figure 7a,b). Consistently with the ion contents, CmSOS1 and CmHKT2 were up-regulated in OX plants compared to the WT plants (Figure 7c,d). These data indicated that the overexpression of CmHSFA4 worked to restrain the accumulation of Na⁺ and facilitate the absorption of K⁺ to detoxify the ionic toxicity caused by salinity. To our knowledge, PehSF overexpressing tobacco enhanced salinity tolerance, while the re-establishment of ionic homeostasis has not been affected in transgenic plants during the period of salt stress (Shen et al., 2013). This infers that members of HSF of different species may employ different strategies to cope with salinity stress. The mechanism through which HSFs regulate the Na⁺/K⁺ balance directly remains to be elucidated in further studies.

CmHSFA4 enhanced tolerance to salinity in chrysanthemum by regulating ROS homeostasis

ROS is another hampering factor for plant growth caused by high salinity. Salinity stress causes a significant accumulation of ROS in sos1-1 and rcd1-1 mutants leading to serious damage to Arabidopsis (Katiyaragarwal et al., 2006). Compared with wild type, CmHSFA4 overexpressing plants suffered less ROS toxicity. Similarly, in Athsfa4a mutant plants, the H₂O₂ content was much higher than WT, which can be restored by AtHSHA4a, and the accumulation of H₂O₂ in AthSHA4a overexpressing plants under salinity is less than that of WT (Guo et al., 2016). In response to stress-triggered ROS production, it is important to keep a steady ROS level in the cells (Azarabadi et al., 2017). One strategy adopted by plants involves ROS scavengers such as SOD, APX and CAT to minimize ROS damage under salinity stress (Pan et al., 2006). Previous studies demonstrated the relationship between...
Improved CAT activities under the salt stress (Shen et al., 2013). In the present study, the data showed that the activities of CAT in OX plants were more affected by chlorosis and contained less chlorophyll than the CcSOS1 transgenic plants (Gao et al., 2016). Similarly, in present study, Chl a, Chl b and total chlorophyll contents were decreased under saline conditions, while CmHSFA4 overexpressing plants maintained higher contents of chlorophyll compared to the WT plants under saline stress (Figure 6), we supposed that CmHSFA4 overexpression might prevent chlorophyll from the ROS damage to some extent.

A relationship between ROS and HSF has been identified (Timperio et al., 2008). Transcriptional reprogramming, the binding of HSF to HSE in the promoters of HSP genes, is essential for the induction of expression of plant HSP genes (Hua, 2009). AthHSFA4a enhanced salinity tolerance of Arabidopsis by regulating the MPK3 and MPK6 mitogen-activated protein kinase pathway, led to the transcriptional activation of the HSP 17.6A gene. Class A HSF harboured a conserved DBD domain which specifically binds to heat stress elements (HSEs: 5’-AGAAANNCT-3’)) (Guo et al., 2016). Here, CmHSF4A also has a conserved DBD domain, and the Y1H assay showed that it could bind to the HSE element (Figure 3b). In addition, CmHSFA4 has transcription activity in yeast cells depending on the AHA motif (Figure 3a), which is similar with that of AthHSFA4a, suggesting CmHSFA4 could activate the expression of those genes whose promoter regions harbour the HSE element. Transcriptional induction of CmHSF70 and CmHSP90 in OX plants was observed in nonstressed and stressed conditions, suggesting these two genes might be the direct target gene of CmHSFA4; however, in vivo evidence should be provided before we can make a conclusion. Despite this hypothesis, an elevation of CmHSP70 and CmHSP90 in OX plants should also contribute to limiting ROS damage.

In addition, SOS1 not only takes part in ion homeostasis but also reduces ROS level under salinity stress. The durum wheat TdSOS1 improves oxidative stress tolerance of overexpressing Arabidopsis plants (Feki et al., 2017). Cross-talk between ion homeostasis and oxidative stress pathways has been previously described, where AtSOS1 up-regulated the oxidative stress tolerance gene Fe-SOD expression through oxidative tolerance gene RCD1 (Katiyaragarwal et al., 2006). We have previously shown that CmSOS1, CcSOS1 conferred salinity tolerance in chrysanthemum via balancing the Na+/K+ ratio and maintaining stable ROS levels (An et al., 2014; Gao et al., 2016; Li et al., 2015b). Here, an elevation in CmSOS1 and CmSOD was observed in OX plants, suggesting that CmHSF4A might play an intermediate role in the cross-talk between ion homeostasis and oxidative stress.

**Experimental procedures**

**Plant materials and grow conditions**

The chrysanthemum cultivar ‘Jinba’ was obtained from the Chrysanthemum Germplasm Resource Conservation Centre, Nanjing Agricultural University, China. Seedlings of similar size at six to eight leaf stage were planted in pots using a 1 : 3 (w/v) mixture of soil and vermiculite and cultivated in a greenhouse under day and night temperatures of 25/18 °C, respectively, and a 14-h light/10-h dark photoperiod with a relative humidity of 70%.

**Isolation and sequence analysis of CmHSFA4 cDNA**

Full-length CDNA was isolated with the previously reported gene-specific primers CmHSFA4-F/R (Table S1; Xia et al., 2014).
The CmHSFA4 amino acid sequence was aligned with its homologs using the DNAMAN 5.2.2 software and BLAST software online (http://www.ncbi.nlm.gov/blast). A phylogenetic tree was constructed using the neighbour-joining method with MEGA 5.2.2.

Subcellular localization of CmHSFA4

To detect the subcellular localization of CmHSFA4, we generated the p35S::GFP-CmHSFA43 fusion construct. The CmHSFA4 ORF was amplified by PCR using the primer set CmHSFA4-1A-F/R...
harbouring the Xho I and Not I sites. Both the amplified fragment and pENTR™1A were digested with Xho I and Not I; then the corresponding bands were recovered and ligated into pENTR™1A to yield the expression vector pENTR™1A-CmHSFA4. Then, CmHSFA4 was introduced to the vector pMDC43 (Invitrogen) by LR reaction between pMDC43 and pENTR™1A-CmHSFA4. The plasmids including pMDC43-CmHSFA4 and empty pMDC43 vector (negative control) were introduced into onion epidermal cells by a helium-driven particle accelerator (PDS-1000; Bio-Rad, Hercules, California, USA). Onion epidermal cells were incubated for 16 h at 22 °C in the dark before observation of GFP signal via confocal laser scanning microscopy.

Transcriptional activity analysis and DNA-binding assay of CmHSFA4

For transcriptional activity analysis, CmHSFA4 was cloned into the yeast expression vector pGBKT7 by LR reaction using pENTR™1A-CmHSFA4 constructs to generate pGBKT7-CmHSFA4. The fragment CmHSFA4-ΔAHA, which lacked AHA motif, was amplified using the Phusion High Fidelity PCR Kit (New England Biolabs, Ipswich, Massachusetts, USA) with the prime pair CmHSFA4-AHA-F/R (Table S1). Both the PCR products and pGBKT7 vector were digested with BamHI and XhoI, and gel recovered and ligated to yield pGBKT7-CmHSFA4-ΔAHA. The pGBKT7-CmHSFA4 construct, pGBKT7-CmHSFA4-ΔAHA, pCL1 (positive control) and pGBKT7 (negative control) were introduced into Y2HGold yeast cells. Transformants carrying either pGBKT7-CmHSFA4, pGBKT7-CmHSFA4-ΔAHA or pGBKT7 were selected on SD/-Trp medium, whereas pCL1 was selected on SD/-Leu medium.

To examine the DNA-binding ability of CmHSFA4, CmHSFA4 was cloned into the yeast expression vector pGADT7. Y1H assay was performed to examine the DNA-binding ability of CmHSFA4 to HSE using the Matchmaker Gold Yeast One-Hybrid Library Screening System (Clontech, Mountain View, California, USA) as described in the manufacturer's instructions. A HSE consensus sequence element or its mutant form was synthesized with restriction enzyme digestion and cloned into the pAbai vector carrying the AUR1-C gene (Song et al., 2015), kindly provided by Dr. Daolong Dou (Nanjing Agricultural University). Bait yeast cells were then transformed with the pGADT7-CmHSFA4 while the empty vector pGADT7 was used as a negative control.

Expression profile of CmHSFA4 under salinity stress

For salinity treatment, plants at the six to eight leaf stage were subjected to 200 mmol/L NaCl, the 3rd leaf (counted from the
apex) was harvested at 0, 1, 4, 12 and 24 h after salinity treatment for expression profile analysis of CmHSFA4. Three biological replicates of each experiment were conducted.

Generation of CmHSFA4 overexpressing chrysanthemum

The 35S::CmHSFA4 plasmid was transformed into the Agrobacterium tumefaciens EHA105 strain using the freeze–thaw transformation method. The transformation of chrysanthemum was performed as previously described (Li et al., 2015b). After regeneration, DNA was extracted from putative transgenic chrysanthemum plants and wild-type (WT) plants using the Multisource Genomic DNA Miniprep kit (Axygen). The regenerating resistant plants were obtained using PCR with the primer pair Hyg-F/R (Table S1). The RNA of the putative transgenic and resistant plants was extracted using a Quick RNA isolation Kit (Waryong) and reverse-transcribed with the reverse transcription primer Hyg-F/R (Table S1). The primer pair CmEF1a-F/R (Table S1) was used to amplify the reference gene CmEF1a (KF305681). Transcription data with three biological replicates were calculated using the 2^-ΔΔCt method, and the expression level of WT was set as the basal.

DNA-positive lines H4 and H5 with highest expression levels of CmHSFA4 were subjected to DNA gel blotting. The details of DNA gel blotting are included in the Supplemental materials and Figure S2. The CmHSFA4 transcript levels in transgenic plants over 24 h under 200 mM NaCl salinity stress were quantified using procedures mentioned above.

Salinity tolerance of CmHSFA4 overexpressing chrysanthemum

For the salinity tolerance assay, the CmHSFA4 overexpressing plants H4, H5 and wild-type chrysanthemum at six to eight leaf stage were irrigated with 200 mM NaCl for 2 weeks. After treatment, plants were removed from the soil, washed with distilled water, replanted in a fresh mixture of soil and vermiculite (1 : 1, v/v) and left to recover for 2 weeks (Li et al., 2015b). The survival rate of the transgenic and the WT plants was calculated. The experiment included three biological replicates, each replicate with 15 seedlings.

Chlorophyll quantification in CmHSFA4 overexpressing chrysanthemum under salinity

Chlorophyll contents of leaves from entire plant of WT and OX lines H4, H5 plants were determined at day 0 (before salinity treatment) and day 7 after salinity treatment as described by Arnon (1949) with minor modifications. Briefly, approximately 0.1 g (fresh weight) of leaves was incubated in 5 mL ethanol and acetone mixture (1 : 2, v/v) for 48 h in the dark, and then, the absorbance of the supernatant was analysed using a DU 800 UV/Vis spectrophotometer (Beckman Coulter, California, CA), scanning at 665, 649 nm, respectively. The experiment was repeated three times. Each replicate contained five seedlings.

Na^+ and K^+ contents in CmHSFA4 overexpressing chrysanthemum

To estimate Na^+ and K^+ content, plants were subjected to 200 mM/L NaCl treatment for 7 days (An et al., 2014). Roots, stems and leaves were harvested separately on day 7, baked at 80 °C for 3 days, and 0.1 g dry sample was digested in 2 mL 10 mM/L HNO_3 and then metered volume to 10 mL by distilled water. Na^+ and K^+ contents were measured using an Optima 2100DV inductively coupled plasma optical emission spectrometer (Gao et al., 2004). The experiment was repeated three times.

ROS production in CmHSFA4 overexpressing chrysanthemum

Physiological traits of WT and H4, H5 plants were measured at day 0 (before salinity treatment) and day 7 of the salinity test. The quantification of H_2O_2 and O_2^- levels was determined following the previously described method. Briefly, approximately 0.5 g of leaf tissues was homogenized with 5 mL 0.1% (w/v) TCA (trichloroacetic acid) in ice bath. The homogenate was centrifuged at 12 000 × g for 15 min, and 0.5 mL of the supernatant was added to 0.5 mL 10 mM/L potassium phosphate buffer (pH 7.0) and 1 mL 1 mM/L KI. The absorbency of supernatant was read at 390 nm. The content of H_2O_2 was given on a standard curve. Contents of O_2^- were measured by hydroxyamine reaction. Approximately 1.0 g of leaf tissues was homogenized with 250 mM/L phosphate buffer (pH = 8) containing with 10 mM/L PLP (pyridoxal 5-phosphate monohydrate), 1 mM/L Na_3EDTA and 5 mM/L DTT in ice bath. The homogenate was centrifuged at 10 000 × g, 4 °C for 25 min. The absorbency of supernatant was read at 530 nm. The content of O_2^- was given on a standard curve (Velikova et al., 2000; Wang et al., 1990). Diaminobenzidine (DAB) and nitrotriacetic acid blue chlorides (NBT) stainings were used to detect the accumulation of H_2O_2 and O_2^- in the transgenic chrysanthemum plants as previously described (Korásk et al., 2010). After overnight treatment with DAB and NBT separately, the stained leaves were cleared by boiling in 80% ethanol and then destained overnight in absolute ethanol. Representative phenotypes were photographed, and the experiment included three repeats using three different plants for each repeat.

ROS scavenger enzymes activities in CmHSFA4 overexpressing chrysanthemum

The activities of SOD, APX and CAT were assessed as previously described (Aebi, 1983; Fatima et al., 2011; Pan et al., 2006). SOD’s activity of one-unit was defined as the amount of enzyme required to cause a 50% inhibition of NBT. APX’s activity was assayed by the decrease in absorbance at 290 nm as ascorbate oxidase activity. The activity of CAT was determined as the reduction in enzymatic amount in 1 min. Enzymatic activities were expressed as enzyme units per g of protein. Each assay included three replicates of three different plants per line per time point.

Quantification of salinity stress-related genes in CmHSFA4-overexpressing chrysanthemum

To analyse the expression levels of genes responsive to salinity stress, the third leaf from the apex of seedlings was collected. For expression profiles of CmSOS1 and CmHKT2, leaves were sampled at 0 and 7 days after 200 mL/L NaCl exposure. For ROS scavenger genes CmSOD, CmAPX, CmCAT, and CmHSP70 and CmHSP90 analysis, leaves were collected at 0 and 4 h after 200 mL/L NaCl exposure. Each experiment included three biological replicates; samples collected from three individual plants at defined time points were pooled for RNA extraction. CmEF1a was used as the reference gene. The sequences of all relevant primers are listed in Table S1.
Statistical analysis

A one-way analysis of variance, using Tukey’s multiple range test ($P = 0.05$), was employed to identify treatment means that differed statistically. The SPSS v17.0 software (SPSS Inc, Chicago, IL) was used for all statistical analyses.

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Conflict of interest

The authors have no conflicts of interest to declare.

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### Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1** Identification of *CmHSFA4* overexpressing chrysanthemum.

**Figure S2** Diagram of the pMDC43-*CmHSFA4* construct, the structure of the *CmHSFA4* and restriction sites of digestion enzymes.

**Figure S3** DNA gel blotting analysis of genomic DNA isolated from wild type plants using a digoxigenin-labeled *CmHSFA4* probe.

**Figure S4** Osmotic adjustment of WT and *CmHSFA4* overexpressing chrysanthemum subjected to PEG6000 (20%) treatment.

**Table S1** Primer names and sequences used in this study.

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