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ORIGINAL ARTICLE

CaHSP16.4, a small heat shock protein gene in pepper, is involved in heat and drought tolerance

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Abstract

Environmental stress affects growth and development of crops, and reduces yield and quality of crops. To cope with environmental stressors, plants have sophisticated defense mechanisms, including the HSF/HSP pathway. Here, we identify the expression pattern of *CaHSP16.4* in thermo-tolerant and thermo-sensitive pepper (*Capsicum annuum* L.) lines. Under heat stress, R9 thermo-tolerant line had higher *CaHSP16.4* expression level than the B6 thermo-sensitive line. Under drought stress, expression pattern of *CaHSP16.4* was dynamic. Initially, *CaHSP16.4* was downregulated then *CaHSP16.4* significantly increased. Subcellular localization assay showed that *CaHSP16.4* localizes in cytoplasm and nucleus. In the R9 line, silencing of *CaHSP16.4* resulted in a significant increase in malonaldehyde content and a significant reduction in total chlorophyll content, suggesting that silencing of *CaHSP16.4* reduces heat and drought stresses tolerance. Overexpression of *CaHSP16.4* enhances tolerance to heat stress in *Arabidopsis*. Under heat stress, the survival rate of *CaHSP16.4* overexpression lines was significantly higher than wild type. Furthermore, under heat, drought, and combined stress conditions, the *CaHSP16.4*-overexpression lines had lower relative electrolytic leakage and malonaldehyde content, higher total chlorophyll content, and higher activity levels of superoxide dismutase, catalase, ascorbic acid peroxidase, and glutathione peroxidase compared to wild type. Furthermore, the expression levels of the stress response genes in the overexpression lines were higher than the wild type. These results indicate that the overexpression of *CaHSP16.4* enhances the ability of reactive oxygen species scavenging under heat and drought stress.

Keywords *CaHSP16.4* · Pepper · *Arabidopsis* · Heat stress · Drought stress · ROS-scavenging system

Introduction

Plants are exposed to biotic and abiotic stress, such as pathogen infections, pest attacks, extreme temperatures, drought, and salinity (Ahuja et al. 2010; Wang et al. 2003). Plants have a number of defense mechanisms for long-term acclimation to adverse environmental conditions, such as changes in the levels of phytohormone, Ca^{2+} content and reactive oxygen species (ROS) signaling (Cramer et al. 2011). Since the 1950s, global temperature has risen about 0.13 °C every 10 years (IPCC 2007). From 1980 to 2008, the reduction in the yield of maize and wheat were 3.8 and 5.5%, respectively, due to global warming (Lobell et al. 2011). Drought stress can change the abundance of osmotic substances, such as sugar, glycine betaine, amino acid, polyamine, and sugar alcohol, leading to osmotic stress (Zhang and Sonnewald 2017). Heat and drought stress can have opposite effects on physiology

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and morphology of leaves. In the early phase of heat stress, the stomatal opening increases transpiration to reduce the leaf temperature, while drought stress leads to closing of the stomata to reduce water loss. However, combined heat and drought stress leads to elevated leaf temperature by 2~5 °C (Prasch and Sonnewald 2013; Rizhsky et al. 2004). Drought stress has the dominant effect on tomato under combined heat and drought stress (Zhou et al. 2017). In *Arabidopsis*, heat stress leads to elongated thin leaf blades and larger leaf area with reduced of root growth, while drought stress decreases leaf area and increases root growth to increase water uptake (Vile et al. 2012; Wang et al. 2016). Thus, it is extremely important to understand the mechanism of how plants respond to abiotic stress.

The heat shock factor/heat shock protein (HSF/HSP) pathway is one of the major pathways involved in stress response (Jacob et al. 2017). Under heat stress, HSF combined heat shock elements (HSEs), upregulating a range of stress-response genes, a process called heat shock response (HSR) (Giorno et al. 2010; Glazebrook 2001; Mittler et al. 2012). In *Arabidopsis*, HSFA1 is an important regulatory transcription factor for heat stress (Yoshida et al. 2011), and DREB2C positively regulates HSFA3 under heat stress (Chen et al. 2010). In *Solanum lycopersicum*, HSFA2 and HSFA1a jointly regulate HSR (Fragkostefanakis et al. 2016). HSPs serve as molecular chaperones in plants to prevent protein aggregation and resolubilize denatured proteins, maintaining the homeostasis of protein folding to tolerate heat (Basha et al. 2004; Boston et al. 1996; Buchner 1996; Dobson 2003; Haslbeck and Vierling 2015; Hilario et al. 2011; Hilton et al. 2012; Lee et al. 1995; McHaourab et al. 2009; Saibil 2008). Based on molecular weight and sequence homology, plant HSPs are divided into five different families: HSP100s, HSP90s, HSP70s, HSP60s, and HSP20s or small heat shock proteins. Of the five families, HSP20s have molecular weights between 15-42KD (Hartl 1996; Hu et al. 2009; Liberek et al. 2008), and have two characteristics that differ from other HSPs. HSP20s do not use ATP to bind to substrate proteins and have strong ability to bind denatured proteins (Eyles and Gierasch 2010; Haslbeck and Vierling 2015; Tyedmers et al. 2010; Waters 2013).

HSP20s contain a variable N-terminal region that is responsible for binding substrate proteins, and a conserved C-terminal region referred as α-crystallin (ACD) region or HSP20 region, which is responsible for the conformation of heat shock granules and homo-oligomerization. The ACD region consists of two antiparallel sheets made of three to four β sheets. For example, the N-terminal conserved region I (CR I, β2-β3-β4-β5) and C-terminal conserved region II (CRII, β7-β8-β9) are connected by a hydrophobic loop (β6-Loop). In addition to this, HSP20s that function in the endoplasmic reticulum or peroxisome have retention signals for those organellels in the C-terminal extension region, which can be variable in length and sequence (Bondino et al. 2012; Haslbeck and Vierling 2015). In addition, the N-terminal transit/target/signal region is observed in some special cellular compartments and may play a key role in obtaining HSP20s from other cellular compartments (Waters 2013).

Plants contain more HSP20s subfamilies than other HSP families (Kirschner et al. 2000; Vierling 1991; Waters et al. 1996). Based on cellular localization, sequence homology, and function, HSP20s can be divided into different subfamilies. In *Arabidopsis*, HSP20s have 11 subfamilies: six in cytoplasm/nucleus (C I-C VI) and five in different organelles: two in mitochondria (MT I and MT II), one in endoplasmic reticulum (ER), one in chloroplast (CP), and one in peroxisome (PX). However, not all HSP20s belong to these 11 conserved subfamilies (Basha et al. 2006; Bondino et al. 2012; Sarkar et al. 2009; Scharf et al. 2001; Siddique et al. 2008; Sun et al. 2002; Waters et al. 2008). For example, ZmHSP20s include 15 subfamilies in maize (*Zea mays*) (Lopes-Caitar et al. 2013). Under heat stress, C IHSP20s and C IIHSP20s combined together with HSP101 protect protein translation factors in *Arabidopsis* (McLoughlin et al. 2016). *PpHSP16.4* gene plays an important role in heat, salt, and osmotic stress tolerance in *Physcomitrella patens* (Ruibal et al. 2013). MsHSP17.7 is located in cytoplasm, and is involved in heat, salt, drought, and oxidative stress tolerance in *Medicago sativa* (Li et al. 2016b). *AsHSP17* mediates photosynthesis when exposed to abiotic stress and is involved in ABA signaling in *Agrostis stolonifera* (Sun et al. 2016). In *Arabidopsis*, plastid metalloprotease FtsH6 modulates priming phase along with HSP21 under heat stress (Sedaghatmehr et al. 2016). *PtHSP17.8* plays an important role in heat and salt stress tolerances in *Arabidopsis* (Li et al. 2016a). In *Arabidopsis*, HSP21 modulates the development of chloroplast with pTAC5 under heat stress (Zhong et al. 2013). OsHSP18.0-C I and RNA-dependent RNA polymerase (RdRp) modulate tolerance to salt and cadmium stress in rice (Ju et al. 2017). Overexpression of OsHSP18.0-C II resulted in tolerance to heat and salt stress (Kuang et al. 2017). Thus, many members of plant HSP20s are involved in abiotic stress.

Pepper (*Capsicum annuum* L.) is an important crop that is cultivated around the world (Kim et al. 2014). However, environmental stress is a major constraint in pepper production, which can limit pepper growth and development. Abiotic stressors can cause the flower and fruit to drop, which in turn decreases crop yield (Guo et al. 2014, 2015b; Wang et al. 2017). Previously, 35 members of pepper HSP20s were been identified by our laboratory. Among the 35 CaHSP20s, the relative expression level of *CaHSP16.4* was higher under heat stress (Guo et al. 2015a). Here, we further analyzed the subcellular localization and expression pattern of *CaHSP16.4*, and explored the function of *CaHSP16.4* using overexpression (OE) and virus-induced gene silencing (VIGS) in both *Arabidopsis* and pepper. Our results provide further insights into the function of *CaHSP16.4* in plant heat and drought stress response.

Materials and methods

Plant materials and growth conditions

Two pepper lines, thermo-tolerant line "R9" (sweet pepper, introduced from the World-Asia Vegetable Research and Development Center, PP0042-51) and thermo-sensitive line "B6" (hot pepper, selected by the pepper research group, College of Horticulture, Northwest A&F University, Yangling, China) were used in this research (Guo et al. 2014; 2015b; Ma et al. 2013). The pepper lines (R9 and B6) and *Arabidopsis* ecotype Col-0 variety seedlings were grown in a growth chamber having growing conditions of 22/18 °C (day/night), 60% relative humidity, 200 μmol m^{-2} S^{-1} illumination intensity, and 16-h light/8-h dark photoperiod cycle.

RNA extraction and qRT-PCR analysis

Total RNA was extracted from the collected samples using Total RNA kit (Bio Teke, Beijing, China) and cDNA was synthesized using PrimeScript™ kit (Takara, Dalian, China) according to the manufacturer's instructions. Primer pairs for qRT-PCR were designed by NCBI Primer-BLAST (Supplementary Tab. S1). qRT-PCR were performed using the iQ5.0 Bio-Rad iCycler thermocycler (Bio-Rad, Hercules, CA, USA). The SYBR Green Supermix (Takara, Dalian, China) was used in qRT-PCR reaction system according to the manufacturer's instructions. Arabidopsis *Atactin2* gene and pepper ubiquitin binding gene *CaUbi3* (Accession number AY486137) were used as reference genes (Wan et al. 2011). Relative gene expression levels were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Subcellular localization of CaHSP16.4 protein

cDNA for the open reading frame (ORF) of *CaHSP16.4* excluding the termination codon were PCR-amplified using pBI221-CaHSP16.4-F and pBI221-CaHSP16.4-R primers (Supplementary Tab. S1). The resulting *CaHSP16.4* fragment was inserted into the pMD19T vector (Takara, Dalian, China). After digestion with restriction enzymes of Xba I and Kpn I, the *CaHSP16.4* fragment was inserted into the pBI221 vector which containing green fluorescent protein (GFP) to generate the pBI221:CaHSP16.4 construct. Transient expression in the onion epidermal cells was performed by Bio-Rad He/1000 particle delivery system. The empty pBI221 expression vector (without *CaHSP16.4*) was used as control. After cultivated on 1 × MS medium at 28 °C for 24 h, the fluorescence signal of the GFP was observed by A1R laser scanning confocal microscope (Nikon, Tokyo, Japan) as described previously (Guo et al. 2014).

Virus-induced gene silencing (VIGS) of *CaHSP16.4*

A 231-bp fragment of *CaHSP16.4* ORF was PCR amplified using pTRV2-*CaHSP16.4*-F and pTRV2-*CaHSP16.4*-R primers (Supplementary Table S1). The resulting *CaHSP16.4* fragment was inserted into the pMD19T vector (Takara, Dalian, China). After digestion with restriction enzymes of EcoR I and BamH I, the *CaHSP16.4* fragment was inserted into the pTRV2 vector to generate the TRV2:*CaHSP16.4* silencing construct. The TRV2:*CaPDS* (phytoene desaturase gene) was used as positive control. The empty TRV2:00 vector without *CaHSP16.4* was used as negative control. *Agrobacterium tumefaciens* strain GV3101 cells containing TRV2:00, TRV2:*CaPDS*, and TRV2:*CaHSP16.4* were separately injected into the leaves of R9 as described by Wang et al. (2013) and Zhang et al. (2015). When the photobleaching phenotype was observed in pepper seedlings carrying TRV2:*CaPDS*, the silencing efficiency of *CaHSP16.4* gene was assessed by qRT-PCR.

Generation of *CaHSP16.4*-overexpression *Arabidopsis* lines

The full-length ORF of *CaHSP16.4* was amplified using pVBG2307-CaHSP16.4-F and pVBG2307-CaHSP16.4-R primers (Supplementary Tab. S1). The resulting *CaHSP16.4* fragment was inserted into the pMD19T vector (Takara, Dalian, China). After digestion with restriction enzymes of BamH I and Kpn I, the *CaHSP16.4* fragment was inserted into the pVBG2307 overexpression vector to generate the VBG2307:CaHSP16.4 overexpressing construct. Transgenic Arabidopsis plants were generated using the floral dip method by *Agrobacterium tumefaciens* strain GV3101 (Clough and Bent 1998). Transgenic plants were obtained by screening successive generations for kanamycin resistance and PCR verification. Transgenic T3 seeds were used for subsequent experiments.

Experimental treatments and sample collection

We induced heat and drought stress to study stress-induced changes in the expression pattern of *CaHSP16.4*. For heat treatment, R9 and B6 pepper seedlings were incubated at 40 °C, and roots, stems, and leaves were collected at 0-, 0.5-, 1-, 2-, 4-, and 6-h post treatment. We simulated drought conditions using mannitol. Roots of R9 seedlings were soaked in 0.3 M mannitol, and leaves were sampled at 0-, 3-, 6-, 12-, and 24-h post treatment. All samples were immediately frozen in liquid nitrogen and kept at − 80 °C for RNA extraction.

TRV2:CaHSP16.4 and TRV2:00 pepper seedlings were used for abiotic stress treatments. For heat stress, pepper seedlings were exposed to 45 °C for 16 h. For drought stress, pepper seedlings were not watered for 14 days, and then

watered to recover for 3 days. After treatment, pepper leaves were sampled for determination of malonaldehyde (MDA) and total chlorophyll content.

Seeds from *CaHSP16.4* overexpression *Arabidopsis* lines and wild-type Col-0 were germinated on MS plates. For heat stress was induced using a long-term-acquired thermotolerance (LAT) assay, where MS plates with 5-day-old transgenic *Arabidopsis* seedlings were immersed in a water bath at 37 °C for 2 h, then recovered at 22 °C for 2 days, then immersed in a water bath at 46 °C for 2 h followed by recovery at 22 °C for 2 days. The survival rates of the seedlings were measured. In addition, 3-week-old seedlings in pots were exposed to heat stress at 42 °C for 24 h in a controlled temperature growth chamber. During heat stress, we watered *Arabidopsis* seedlings constantly to avoid inducing drought stress. For drought treatment, 3-week-old *Arabidopsis* seedlings were withheld of water for 10 days in a controlled temperature growth chamber. For combined heat and drought stresses, 3-week-old *Arabidopsis* seedlings were withheld of water for 7 days, and then placed in 42 °C for 24 h on the 3rd day. Stress-treated samples and their controls were subjected to analysis of relative electrolytic leakage (REL), MDA, total chlorophyll contents, the activity of superoxide dismutase (SOD), catalase (CAT), ascorbic acid peroxidase (APX), and glutathione peroxidase (GPX), and relative gene expression levels.

Measurement of relative electrolyte leakage (REL), MDA, total chlorophyll content and antioxidant enzymes

REL from the leaf discs of pepper and *Arabidopsis* plants were measured as described by Dionisio-Sese and Tobita (1998). MDA content was measured using thiobarbituric acid reaction according to Buege and Aust (1978). Total chlorophyll content was measured with 80% acetone using the method of Arkus et al. (2005). SOD activity was measured as described by Beauchamp and Fridorich (1971) and Zhou et al. (1997). The reaction system was as follows 0.1 mL Crude extraction enzyme solution + 0.5 mL ddH_2O + 1.5 mL 50 mM phosphate buffer solution (PBS, pH 7.8) + 0.3 mL 130 mM Met + 0.3 mL 0.75 mM NBT + 0.3 mL 0.1 mM EDTA-Na_2 + 0.3 mL 0.02 mM Riboflavin solution. CAT activity was determined using the method of AebiH (1984). The reaction system is as follows 0.1 mL Crude extraction enzyme solution + 0.7 mL 50 mM PBS (pH 7.0) + 0.2 mL 200 mM H_2O_2. APX activity was measured using Nakano and Asada (1981) method. The reaction system is as follows 0.1 mL Crude extraction enzyme solution + 1.7 mL 50 mM PBS (0.1 mM EDTA-Na_2, pH 7.0) + 0.1 mL 5 mM AsA + 0.1 mL 20 mM H_2O_2. GPX activity was measured following methods of Flohé and Günzler (1984). The reaction system is as follows 0.4 mL Crude extraction enzyme solution + 0.4 mL 1 mM GSH + 0.2 mL 1.5 mM H_2O_2 (37 °C) + 4 mL 0.61 mM trichloroacetic acid + 2.5 mL 0.32 M Na_2HPO_4 + 0.5 mL DTNB (0.04% DTNB, 1% trisodium citrate).

Statistical analyses

Data were subjected to analysis of variance (ANOVA). Significance tests for differences between control and stress treatments were performed using student's *t* test at the $p \leq 0.05$ and $p \leq 0.01$ as significance cut-offs. All experiments were performed and analyzed separately with three biological replicates.

Results

Expression of *CaHSP16.4* under heat and drought stresses in pepper

qRT-PCR analysis found that *CaHSP16.4*'s expression pattern under heat stress varies with time (Fig. 1a, b). In the R9 thermo-tolerant pepper line, *CaHSP16.4* expression levels in root, stem, and leaf were highest when treated heat stress for 4, 2, and 4 h, respectively. In the B6 thermo-sensitive pepper line, *CaHSP16.4* expression levels in root, stem, and leaf were highest when treated with heat stress at 0.5, 0.5, and 6 h, respectively. We also treated plants with mannitol to induce drought stress, and found that the expression level of *CaHSP16.4* significantly decreased at 3 h of treatment. However, 6 h of treatment led to significantly increased expression levels of *CaHSP16.4* (Fig. 1c).

Subcellular localization of CaHSP16.4

Subcellular localization of CaHSP16.4 was predicted using WoLF PSORT (https://wolfpsort.hgc.jp/), which predicted that CaHSP16.4 localized mainly to the cytoplasm (Suppl Tab. S2). Using onion epidermal cells, we expressed GFP tagged *CaHSP16.4* under a strong promoter (pBI221:CaHSP16.4), and found that the tagged protein was uniformly distributed in the cytoplasm and nucleus. However, the control CaMV35S::GFP (pBI221) localized throughout the cell (Fig. 2).

Silencing of *CaHSP16.4* decreases tolerance to heat and drought stress

We generated pepper seedlings with a silencing construct against *CaHSP16.4* (TRV2:CaHSP16.4), which reduced *CaHSP16.4* expression by 75% (Suppl Fig. S1a). No obvious difference was observed between TRV2:CaHSP16.4 and control TRV2:00 pepper lines under normal conditions (Suppl Fig. S1b). We then treated plants in heat for 16 h, and

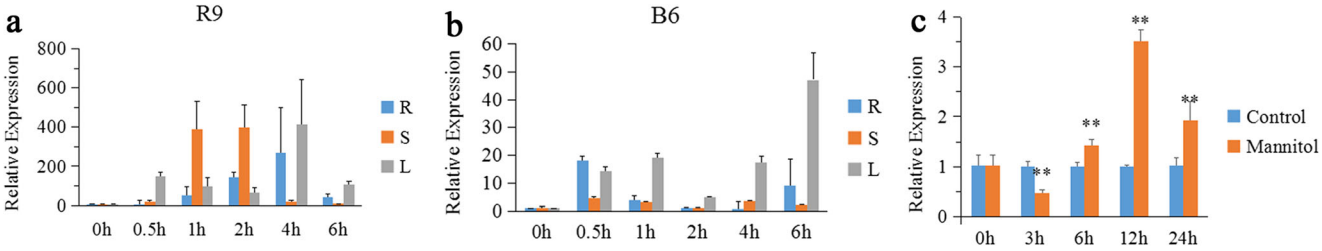

Fig. 1 Relative expression levels of *CaHSP16.4* under heat and drought stresses in pepper. **a**, **b** Relative expression levels of *CaHSP16.4* in heat-treated R9 and B6 pepper lines. **c** Relative expression levels of *CaHSP16.4* in drought-treated R9 pepper line. Bars show the standard deviation of expression levels from three biological replicates. Statistical significance is indicated by a single asterisk ($p < 0.05$) and double asterisks ($p < 0.01$) based on a student's *t* test

CaHSP16.4-silenced pepper showed sunburn-like symptoms while the control TRV2:00 pepper plants were normal (Fig. 3a). In addition, the MDA content in CaHSP16.4-silenced plants was significantly higher than TRV2:00 plants, and the total chlorophyll content in CaHSP16.4-silenced plants was significantly lower than the control (Fig. 3b, c). Next, we treated plants with drought stress by withholding water for 14 days, both CaHSP16.4-silenced and control plant leaves turn yellow and wilted. However, CaHSP16.4-silenced plant leaves showed more severe symptoms. Plants were then allowed to recover for 3 days, and we found that the control plants turned to normal phenotype, while CaHSP16.4-silenced plants did not restore their leaf morphology (Fig. 3d). The variations in the MDA and total chlorophyll contents of the silenced and control plants under drought stress condition showed similar trend as heat stress (Fig. 3e, f).

Overexpression of *CaHSP16.4* enhances tolerance to heat, drought, and combined stress treatments

Arabidopsis transgenic lines overexpressing *CaHSP16.4*, OE1, and OE2 were used to perform abiotic stress treatments (Suppl Fig. S2). No visible difference was observed between CaHSP16.4-OE (overexpression) lines and wild-type (WT) *Arabidopsis* plants under normal growth conditions. We conducted a LAT assay, and found that CaHSP16.4-OE seedlings

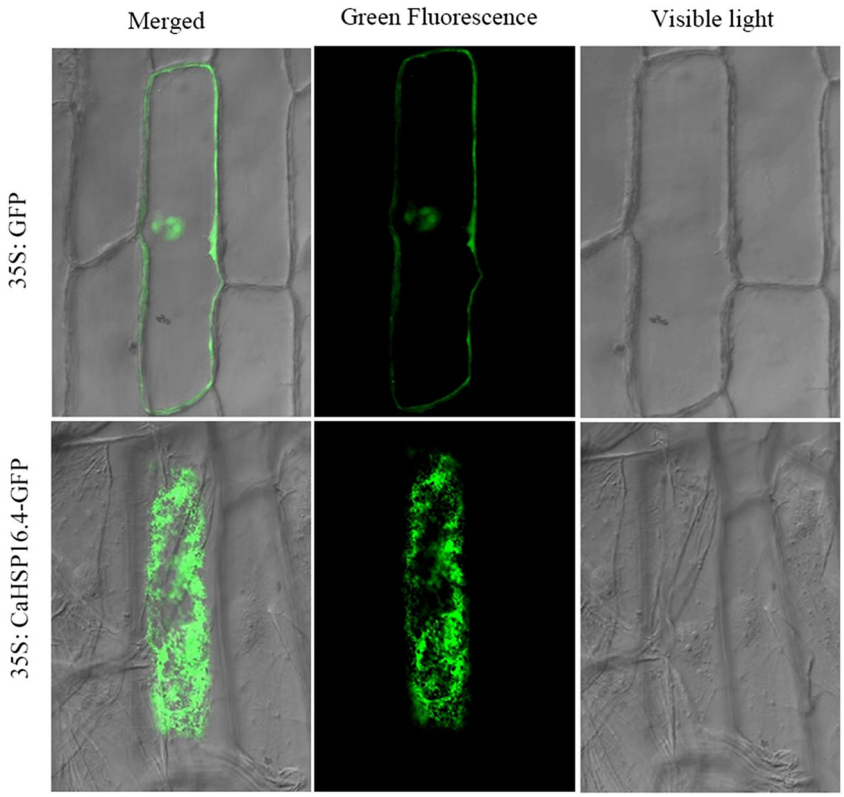

Fig. 2 Subcellular localization of the CaHSP16.4-GFP fusion protein in onion epidermal cells, with CaMV35S::GFP expressing GFP under the cauliflower mosaic virus CaMV35S constitutive promoter. GFP, Bars = 100 μm

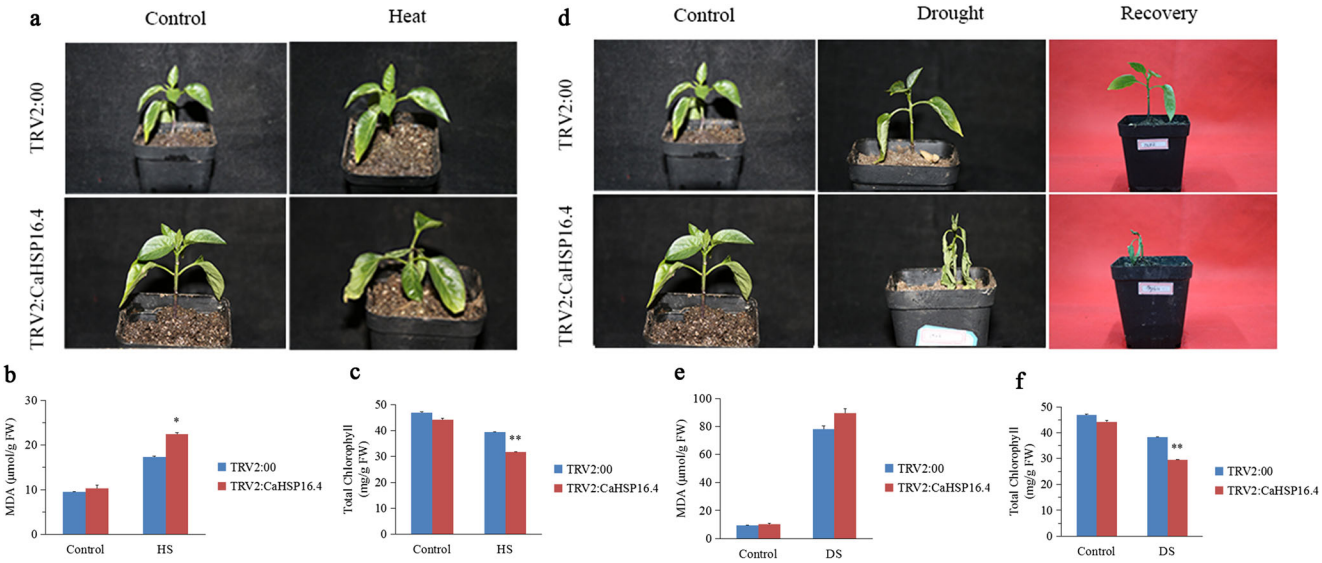

Fig. 3 Silencing of *CaHSP16.4* decreases tolerance to heat and drought stress in pepper. **a**, **d** TRV2:CaHSP16.4 and TRV2:00 pepper seedlings treated with heat stress at 45 °C for 16 h or drought stress with 14 days without water followed by 3 days of recovery. **b**, **c**, **e**, **f** MDA content and total chlorophyll content of TRV2:00 and TRV2:CaHSP16.4 pepper seedlings grown under heat stress, or drought stress. Plants continually grown at 22 °C were used as controls. Bars show the standard deviation of MDA and total chlorophyll contents from three biological replicates. Statistical significance is indicated by a single asterisk ($p < 0.05$) and double asterisks ($p < 0.01$) based on a student's *t* test

showed partly bleached, while WT seedlings totally bleached (Fig. 4a). After heat treatment, the survival rates of CaHSP16.4-OE lines OE1 and OE2 and WT plants were 20, 12, and 2%, respectively (Fig. 4b). After heat treatment at 42 °C for 24 h, severe wilting symptoms were observed in WT plants. Interestingly, we did not observe any changes in morphology in CaHSP16.4-OE seedlings (Fig. 4c). The REL and MDA content of the CaHSP16.4-OE seedlings were significantly lower than WT seedlings (Fig. 4d, e). The total chlorophyll content of the CaHSP16.4-OE seedlings was significantly higher than WT (Fig. 4f). Similar results to total chlorophyll content were observed in SOD, APX, and GPX activities as well (Fig. 4g, i, j). However, no significant change was observed in CAT activity in CaHSP16.4-OE and WT

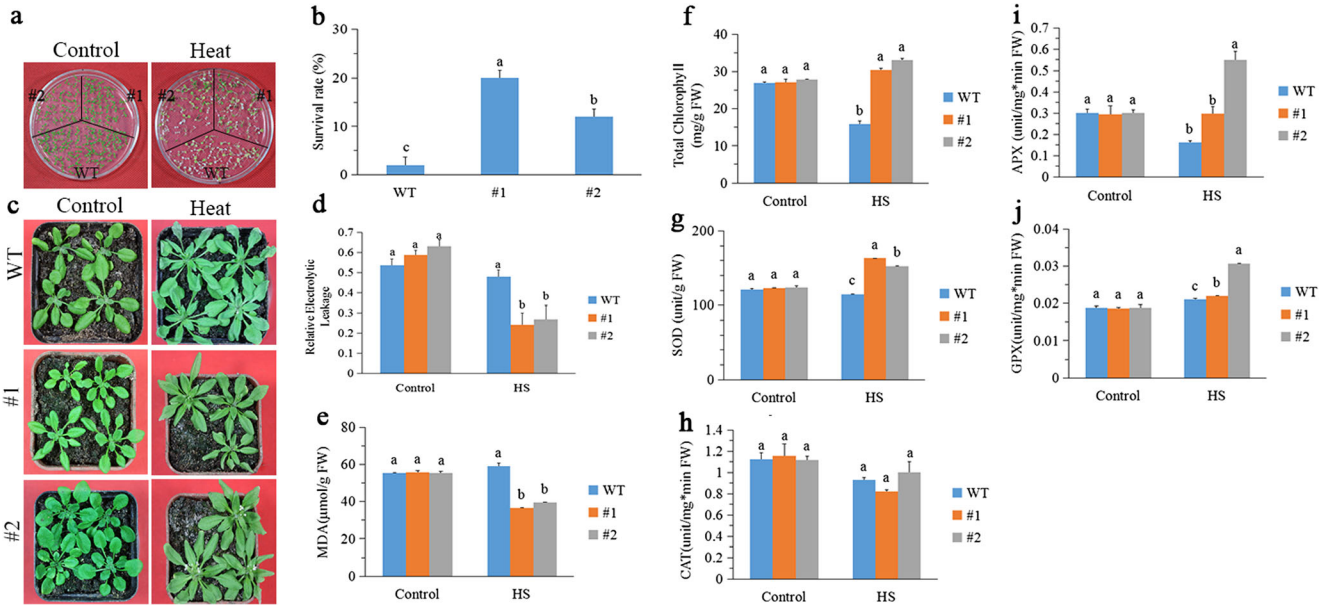

Fig. 4 Overexpression of *CaHSP16.4* enhances tolerance to heat stress. **a**, **b** Phenotype and survival rates of LAT assay on wild type and CaHSP16.4-OE lines. **c**–**j** Phenotype, REL, MDA contents, total chlorophyll content, SOD, CAT, APX, and GPX activity of wild type and CaHSP16.4-OE seedlings treated with heat stress at 42 °C for 24 h. Plants continually grown at 22 °C were used as controls. Bars show the standard deviation of three biological replicates. Different letters denote statistical significance at $p \leq 0.05$

plants (Fig. 4h). The relative expression levels of *AtHSA32*, *AtHSFA7a*, *AtHSFB2a*, *AtHSFB2b*, *AtHSP15.7*, *AtHSP17.6B*, *AtHSP17.6C*, *AtHSP25.3*, *AtHSP70*, *AtHSP90*, *AtHSP101*, *AtAPX2*, *AtSOD*, *AtCAT*, and *AtGPX* were higher in CaHSP16.4-OE plants than in WT (Fig. 5).

We next induced drought stress, and found that WT *Arabidopsis* seedlings showed severe wilting and purpling, while CaHSP16.4-OE *Arabidopsis* seedlings showed no obvious change (Fig. 6). The REL and MDA in CaHSP16.4-OE seedlings are significantly lower than WT seedlings (Fig. 6b, c). The total chlorophyll content in CaHSP16.4-OE seedlings was significantly higher than WT plants (Fig. 6d). Similar results to total chlorophyll content were observed for SOD, CAT, APX, and GPX activities (Fig. 6e–h). The relative expression levels of *AtHSP70*, *AtSOD*, *AtCAT*, *AtAPX1*, *AtGPX*, *AtMYB44*, *AtP5CS*, *AtRD29a*, *AtRAB18*, and *AtNCED3* were higher in CaHSP16.4-OE plants than WT plants (Fig. 7).

Lastly, we treated plants with combined heat and drought stress. WT *Arabidopsis* plants showed severe bleaching and wilting symptoms, while the leaves of CaHSP16.4-OE seedlings turned purple (Fig. 8a). The REL and MDA in CaHSP16.4-OE seedlings were significantly lower than in WT (Fig. 8b, c), and the total chlorophyll content in CaHSP16.4-OE seedlings was significantly higher than in WT (Fig. 8d). Similar results to total chlorophyll content are obtained in SOD, CAT, APX, and GPX activities (Fig. 8e–h). The relative expression levels of *AtHSA32*, *AtHSFA7a*, *AtHSFB2a*, *AtHSFB2b*, *AtHSP15.7*, *AtHSP17.6B*, *AtHSP17.6C*, *AtHSP25.3*, *AtHSP70*, *AtHSP90*, *AtHSP101*, *AtAPX1*, *AtAPX2*, *AtSOD*, *AtCAT*, *AtGPX*, *AtMYB44*, *AtP5CS*, *AtRD29a*, *AtRAB18*, and *AtNCED3* were higher in CaHSP16.4-OE plants compared to WT (Fig. 9).

Discussion

Under normal conditions, expression levels of most HSP20s are moderate while some HSP20s are not expressed. However, under environmental stress conditions, the HSP20s expression increases rapidly. Although HSP20s do not modulate heat stress directly in plants, they can induce the expression of proteins that can influence the plant's response to heat, playing an important role in thermotolerance (Dafny-Yelin et al. 2008; Waters 2013). In different species, HSP20 expression is turned on at different time points after heat stress. For example, in soybean, HSP20s were upregulated within a minute reached to the highest expression level, and then reached to the lowest expression level at 12 h post stress (Kimpel et al. 1990). In contrast, maize HSP20s begin expression 4 h after heat stress, and successively expressed in 20 h

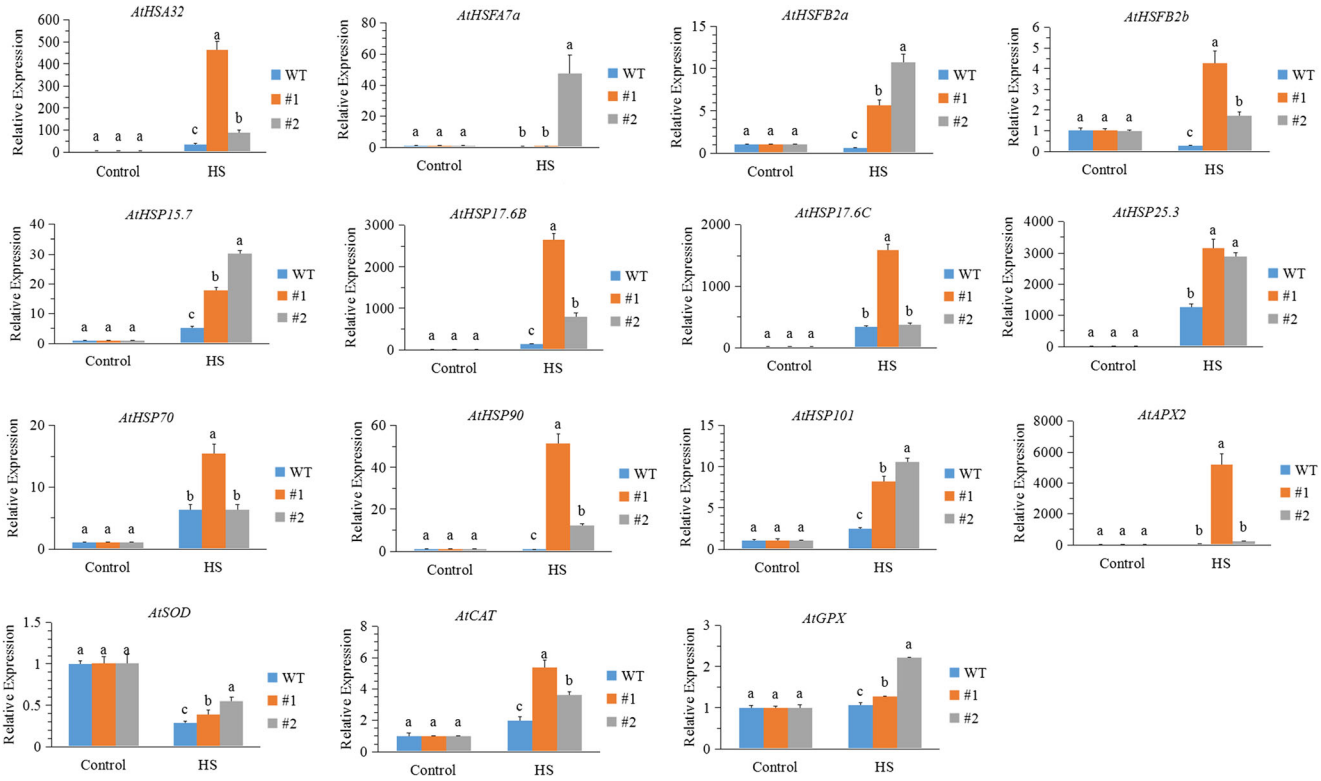

Fig. 5 Relative expression levels of related genes in WT and CaHSP16.4-OE lines under heat stress. Different letters denote statistical significance at $p \leq 0.05$

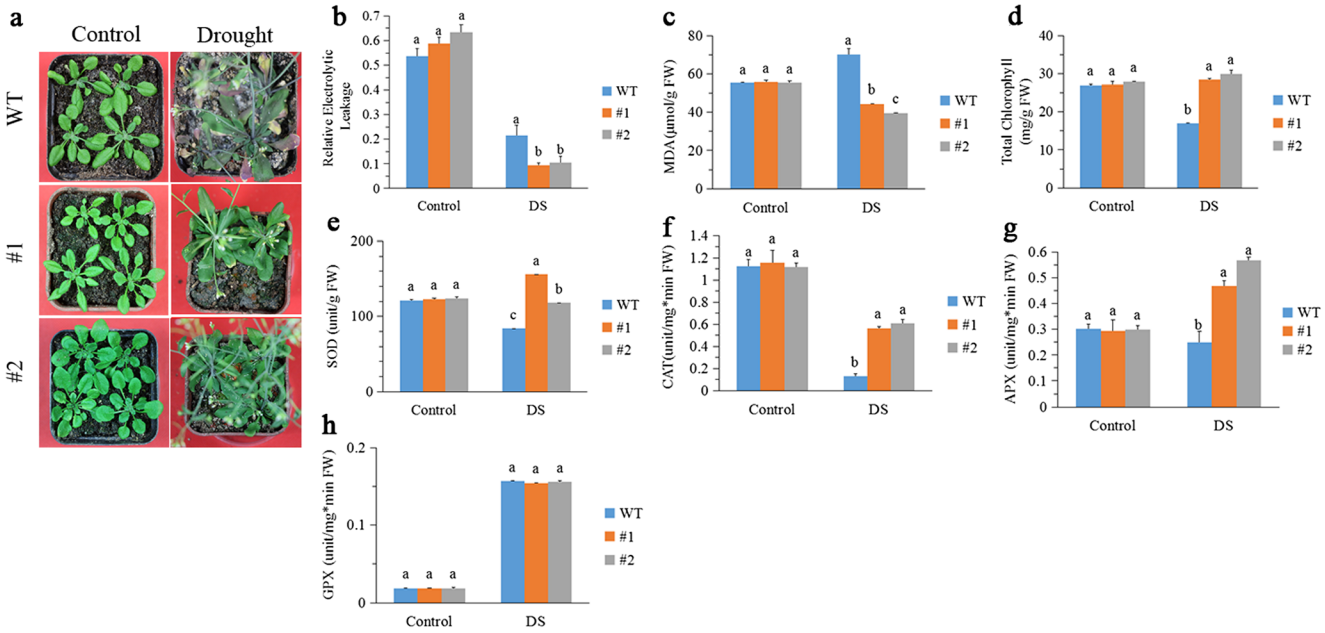

Fig. 6 Overexpression of *CaHSP16.4* enhances tolerance to drought stress. **a**–**h** Phenotype, REL, MDA contents, total chlorophyll content, SOD, CAT, APX, and GPX activity of WT and CaHSP16.4-OE seedlings under drought stress for 10 days. Plants continually grown at normal conditions were used as controls. Bars show the standard deviation of three biological replications. Different letters denote statistical significance at $p \leq 0.05$

after heat stress (Lee et al. 1996). The expression of *AtHSP15.7-P* was successive under heat and anoxia and respond to these stresses (Ma et al. 2006). Here, we found complicated expression pattern of *CaHSP16.4* under heat and drought stress. The expression levels of *CaHSP16.4* was higher in the R9 (thermotolerance line) compared to the B6 (thermosensitive line) under heat stress, suggesting that *CaHSP16.4* is involved in heat shock response.

HSP20s have been found across a wide range of organisms (Waters 2013). *AtHSP21* was localized in the chloroplast (Chen et al. 2017). OsHSP18.0-CII localized in cytoplasm/nucleus (Ju et al. 2017; Kuang et al. 2017). While MT-sHSP23.6 localized in mitochondria (Hüther et al. 2016). The transient expression of *CaHSP16.4* in onion epidermis cells revealed that *CaHSP16.4* is localized in the cytoplasm and nucleus, while prediction tests suggested that *CaHSP16.4* may localized to the cytoplasm. Similarly, in our previous study, *CaHSP16.4* was assigned to cytoplasm/nucleus II (CII) subfamily (Guo et al. 2015a). Thus, we speculate that *CaHSP16.4* may function in the cytoplasm and nucleus.

HSA32 and *HSP101* modulate the acquired heat tolerance in *Arabidopsis* and *Oryza sativa* (Charng et al. 2006b; Wu et al. 2013; Lin et al. 2014). *HSFA2*, *HSFA3*, and *HSFA7a* are main heat shock factors involved in the restoring phase after heat stress, and these three proteins maintain long-term acquired heat tolerance (Charng et al. 2006a; Nishizawa et al. 2006; Schramm et al. 2008). *AtHSFB1* and *AtHSFB2b* suppress the expression of related HSFs under heat stress but play a significant role in the acquired thermotolerance (Ikeda et al. 2011). In this study, the survival rates of *CaHSP16.4* overexpression lines were significantly higher than WT plants after long-term-acquired heat tolerance assay (Fig. 4b). Accordingly, expression levels of heat stress-related genes such as *AtHSA32*, *AtHSFA7a*, *AtHSFB2b*, and *AtHSP101* in the *CaHSP16.4*-overexpressed lines were significantly higher than WT plants (Fig. 5). This indicated that *CaHSP16.4* may be involved in long-term-acquired thermotolerance. *RD29a* and *RAB18* are drought marker genes (Harb et al. 2010; Rasheed et al. 2016). *MYB44* is involved in water management, *NCED3* and *P5CS* are related to biological metabolism, and molecular chaperone *HSP70* is related to drought stress (Rymaszewski et al. 2017). Expression levels of drought stress related genes such as *AtRD29a*, *AtRAB18*, *AtP5CS*, *AtNCED3*, *AtHSP70*, and *AtMYB44* in the *CaHSP16.4*-overexpressed lines were significantly higher than WT plants (Fig. 7). Under heat and drought stress, expression levels of 21 genes related to heat and drought stress were higher in *CaHSP16.4*-overexpressed lines (Fig. 9). Altogether, our study suggests that *CaHSP16.4* is involved in heat and drought stress resistance.

Abiotic stresses can decrease the availability of CO_2, leading to stomatal closure and accumulation of ROS (Zandalinas et al., 2017). ROS can cause damage to cell structures, carbohydrates, proteins, lipids, and nucleic acids that further leading to cell death. However, in spite of their damaging effects, under steady state conditions, ROS plays a major physiological role in intracellular signaling and regulation as secondary

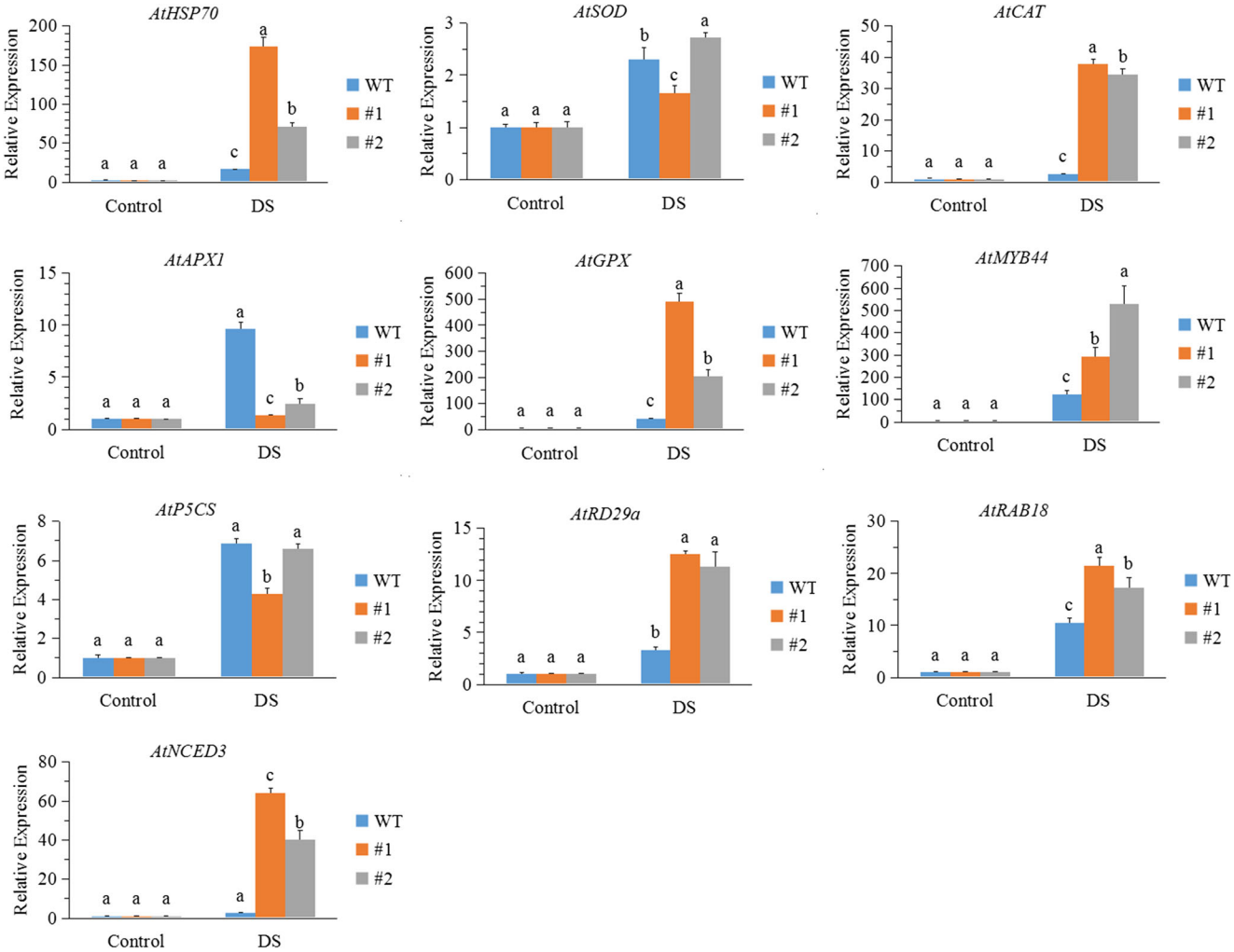

Fig. 7 Relative expression levels of related genes in WT and CaHSP16.4-OE lines under drought stress. Bars show the standard deviation of expression levels from three biological replications. Different letters denote statistical significance at $p \leq 0.05$

messengers (Uzildaya et al., 2011; Volkov et al. 2006). ROS signaling is a common element in heat and drought stress (Pucciariello and Perata 2012; Uzildaya et al., 2011). MDA content of the plant is the physiological index to measure the damage of membrane lipid peroxidation under abiotic stresses (Uzildaya et al., 2011). Here, we found that the silencing of *CaHSP16.4* enhanced MDA content under heat and drought stress, while total chlorophyll contents decreased (Fig. 3). This indicated that silencing of *CaHSP16.4* enhanced the damage of membrane lipid peroxidation in pepper plants under heat and drought stresses. Besides, silencing of *CaHSP16.4* enhanced the decomposition of the chlorophyll and then may decrease the photosynthetic efficiency. Thus, our results suggest that silencing of *CaHSP16.4* decreases tolerance of the pepper plants to heat and drought stress.

To alleviate the damage of ROS accumulation, the levels of ROS-scavenging enzymes and antioxidants, such as SOD, CAT, AsA, and GSH, are increased (Foyer and Noctor 2005; Mittler et al. 2004). Among these ROS-scavenging enzymes, O_2^- is decomposed by SOD to H_2O_2, which is further decomposed by peroxidase in extracellular space and cytosol, and mainly by CAT in peroxisomes (Chaparzadeh et al. 2004; Uzildaya et al., 2011). Overexpression of the *AtHSP17.6* enhances the activity of CAT and modulated abiotic stresses (Li et al. 2017). *MsHSP16.9* positively modulates the ROS system and alleviates detrimental effect of stresses through ABA-dependent or ABA-independent pathways (Yang et al. 2017). SOD, CAT, APX1, and GPX are related to redox reactions (Rymaszewski et al. 2017). In this study, ROS-scavenging enzymatic activity in *CaHSP16.4*-OE lines was significantly higher than WT plants under heat, drought and combined heat and drought stresses (Figs. 4, 6, and 8). Expression levels of *AtSOD*, *AtCAT*, *AtAPX*, and *AtGPX* were similar to the enzymatic activity patterns under heat, drought, and combined stress, suggesting that *CaHSP16.4* is involved in tolerance of heat and drought stress through the ROS-scavenging system.

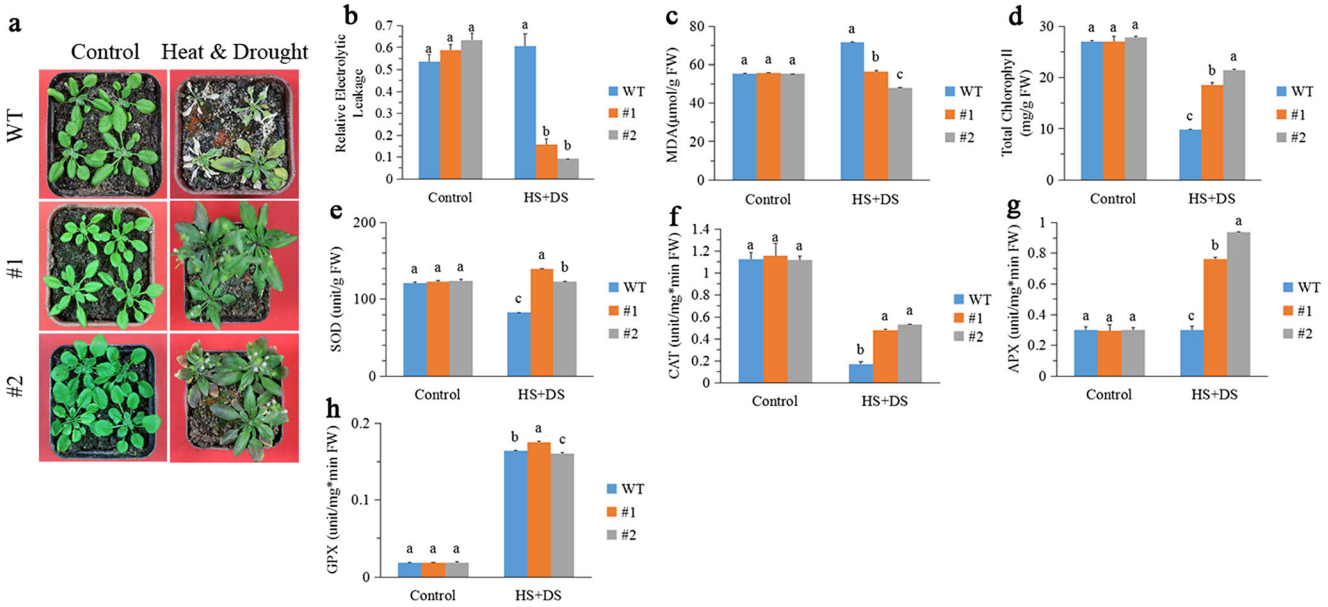

Fig. 8 Overexpression of *CaHSP16.4* enhances tolerance to combined heat and drought stresses. **a**–**h** Phenotype, REL, MDA contents, total chlorophyll content, SOD, CAT, APX, and GPX activity of WT and CaHSP16.4-OE seedlings under combined heat and drought stresses for 7 days. Plants continually grown at normal conditions were used as controls. Bars show the standard deviation of three biological replications. Different letters denote statistical significance at $p \leq 0.05$

Silencing of *CaHSP16.4* reduces the heat and drought stress tolerance, while overexpression of the *CaHSP16.4* enhances the ability of reactive oxygen species scavenging under heat and drought stress. Altogether, *CaHSP16.4* is involved in heat and drought stress tolerance through scavenging of the ROS.

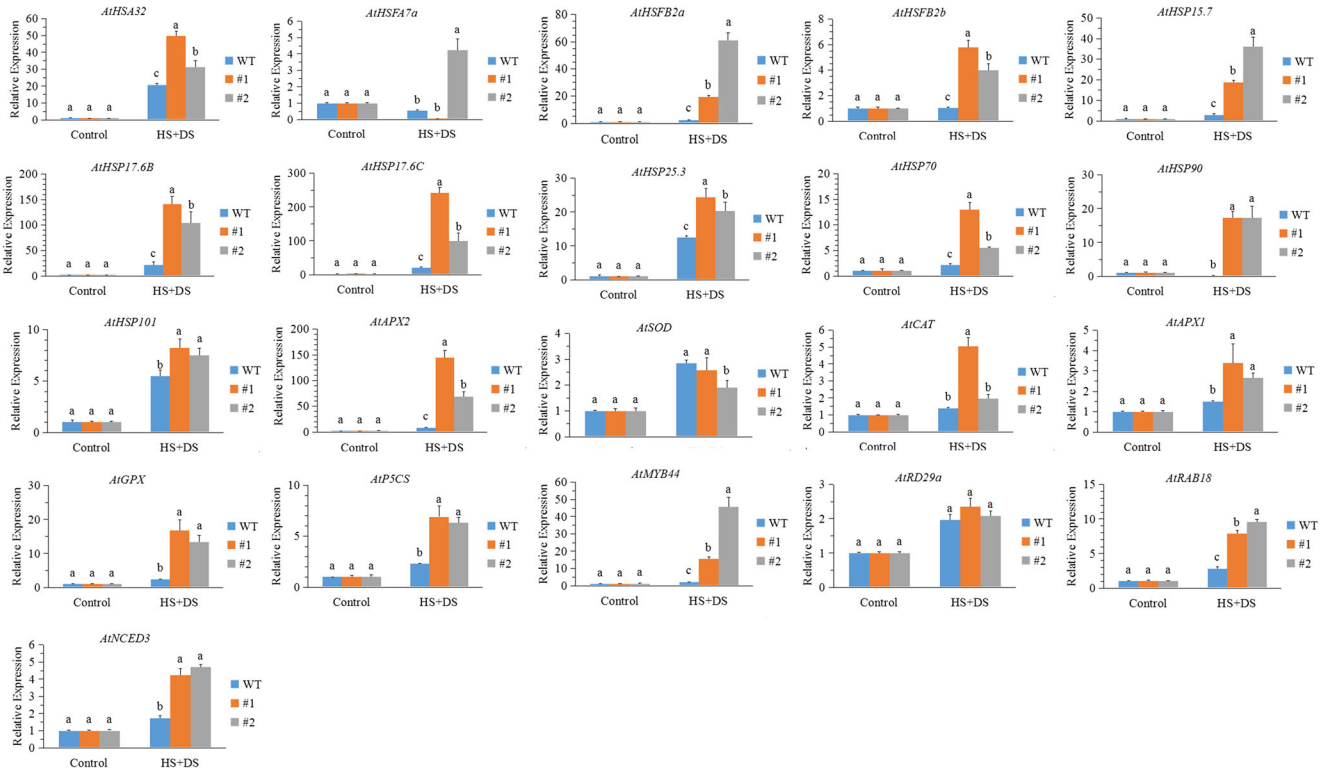

Fig. 9 Relative expression levels of related genes in WT and CaHSP16.4-OE lines under combined heat and drought stresses. Bars show the standard deviation of expression levels from three biological replications. Different letters denote statistical significance at $p \leq 0.05$

Author contributions LH and ZG designed the experiments. LH, GC, AK, AW, QY, and SY performed the research. LH drafted the manuscript. ZG revised the paper. ZG and DL contributed reagents/materials/analysis tools. All authors read and approved the final manuscript.

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Compliance with ethical standards

Competing financial interests The authors declare that they have no competing interests.

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