Chloroplast Small Heat Shock Protein CsHSP24.6 of Tea Plants Positively Regulates Heat, Light, and Salt Stress Tolerance

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Abstract

Small heat shock proteins (sHSPs) function as molecular chaperones, which play crucial roles in plant growth, development, and stress response. However, the function of the sHSP gene in the tea plant (Camellia sinensis L.) has not been extensively investigated. In total, 54 C. Sinensis small heat shock proteins (CssHSPs) in the tea plant genome were screened. Phylogenetic analysis revealed that CssHSPs in the same group have similar conserved domains and motifs; conversely, significant structural differences exist in the different groups. Most CssHSP genes had tissue-specific expression. They also responded to one or more abiotic or biotic stresses, CsHSP24.6 was selected for functional analysis. The results demonstrated that the expression of CsHSP24.6 increased under abiotic stresses such as temperature, light intensity, and NaCl. In addition, under high temperature and high light intensity treatments, CsHSP24.6 and its target gene CspTAC5 interacted to enhance the heat and light resistances of the plant. CsHSP24.6 facilitates resistance to abiotic stresses in tea plants. These results further support that CsHSP24.6 plays an essential role in maintaining plant growth and development under abiotic stress.

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Abstract: Small heat shock proteins (sHSPs) function as molecular chaperones, which play crucial roles in plant growth, development, and stress response. However, the function of the*sHSP* gene in the tea plant (*Camellia sinensis* L.) has not been extensively investigated. In total, 54 *C. Sinensis* small heat shock proteins (CssHSPs) in the tea plant genome were screened. Phylogenetic analysis revealed that CssHSPs in the same group have similar conserved domains and motifs; conversely, significant structural differences exist in the different groups. Most *CssHSP* genes had tissue-specific expression. They also responded to one or more abiotic or biotic stresses,*CsHSP24.6* was selected for functional analysis. The results demonstrated that the expression of *CsHSP24.6* increased under abiotic stresses such as temperature, light intensity, and NaCl. In addition, under high temperature and high light intensity treatments,*CsHSP24.6* and its target gene *CspTAC5* interacted to enhance the heat and light resistances of the plant. *CsHSP24.6*facilitates resistance to abiotic stresses in tea plants. These results further support that *CsHSP24.6* plays an essential role in maintaining plant growth and development under abiotic stress.

Keywords: Tea plant, bioinformatics analysis, abiotic and biotic stresses, *CsHSP24.6.*

1.Introduction

Plants have difficulty avoiding threats due to changes in the environment, such as drought, cold, heat, and salt or heavy metal concentrations[1-3]. When plants are subjected to these stresses, their proteins denature, agglomerate, and lose inherent function, resulting in a substantial decline in crop yield and quality[4]. In general, plants cannot change their positions to escape these stresses; however, they have developed various defense mechanisms to resist them including synthesis of heat shock proteins (HSPs)[5-6]. HSPs are commonly observed in plants responding to heat stress, but studies have shown that HSPs are also involved in the response to other biological and abiotic stresses[7]. As a molecular chaperone, HSPs are involved in protein folding, refolding, assembly, transport, and degradation, facilitating the stabilization of protein and cell membranes under stressful conditions[8-9]. Plant HSPs are divided into five conserved families according to their molecular weights: the HSP100, HSP90, HSP70, HSP60, and small HSP (sHSP) families[10-13]. In particular, HSP20 is a group of ATP-independent HSPs that is a first line of defense for plants at risk of protein aggregation.

HSP20s are key proteins protecting plants from aggregation and enhancing the effectiveness of other HSPs[14]. The monomeric molecular masses of most HSP20s are approximately 15-42 KDa[15-16]. The function of an HSP20 protein is related to its structure, which includes three main functional components: 1) a conserved C-terminal domain called the α-crystallin domain (ACD) or HSP20 domain, which forms a compact axial sandwich structure to help oligomers disintegrate into dimers and bind to nonnatural proteins; 2) a C-terminal extension region, which may be involved in the stabilization and solubilization of oligomeric assemblies; and 3) a variable N-terminal region, which plays a role in transiting, leading, or signaling[17-19]. According to protein homology and intracellular localization, the sHSP group is divided into 12 subfamilies: cytoplasm and nuclei (CIˇ CVII), mitochondria (MI, MII), endoplasmic reticulum (ER), chloroplasts (P), and peroxide (Px)[20]. Different HSP20 proteins have different functions, but most of them are induced by heat, saltand drought stress. In addition, previous studies have shown that HSP20s positively promote plant tolerance to adverse environments. For example, rice*OsHSP26* significantly enhances the tolerance of tall fescue to oxidative and

heat stresses by protecting photosystem II (PSII) and maintaining photosynthesis[21]. In *Arabidopsis thaliana* , *AtHSP21* plays a positive role in the thermotolerance of plants and in extending the thermomemory phase[22]. In addition, overexpression of the *Malus sieversiiMsHSP16.9* gene in *A. thaliana* increased plant tolerance to heat stress by alleviating damage from reactive oxygen species and regulating the expression of stress-related genes[23]. Under adverse conditions, the OsHSP18.2 could promote the germination of rice seeds and cotyledon growth[24]. HSP20s are also involved in plant growth and development. *AtHSP17.4* and*AtHSP17.6* accumulate in maturing seeds and play a protective role in seed development for *A. thaliana* [25-26].

These studies indicate that HSP20s are key for plant resistance to abiotic stress. The sHSP gene families have been investigated for several plant species including *A. thaliana* [27-29], rice[30], tomato[31-32], maize[33], soybean[34], populus[35], and grapes[36]. The functional mechanisms of *sHSPs* in plant stress response have become a common research topic.

The tea plant, *Camellia sinensis* (L.) O. Kuntze, is the source of one of the most widely consumed beverages in the world. The tea plant often suffers biological and abiotic stressors (high temperatures, drought, pests, etc.), affecting its normal development as well as the yield and quality of tea[24]. Therefore, substantial benefits to tea production could be achieved by producing stress-resistant tea plant varieties by using molecular breeding technology. Obtaining these varieties requires studying the mechanisms of tea plant resistance to high temperature and drought and then identifying the related genes. Wang et al. confirmed that*CsHSP17.2* , *CsHSP17.7* , *CsHSP18.1* , and*CsHSP21.8* improve tea tree tolerance to high temperature and drought stresses[37]. However, few other studies have investigated *sHSPs* in tea plants. The genome of the tea plant has been sequenced and published; thus, the identification of genes within the *CssHSP* superfamily is simple and reliable[38].

In this study, 54 *CssHSP* genes were identified, and a comprehensive analysis that included analysis of phylogenetic relationships and conserved motifs was performed. In addition, the responses of all *CssHSP* s to biological stress (methyl jasmonate, [MeJA]) and abiotic stress (cold, drought, salt) were analyzed based on the tea plant genome data. We discovered that only two genes are significantly upregulated for all stresses. The results of this study revealed the molecular characteristics of the *CssHSP* gene superfamily and provide a theoretical basis for future studies of the biological functions of *CssHSP* s under abiotic stresses.

2. Materials and Methods

2.1 Plant Materials, Growth Conditions, and Stress Treatment

Samples of the tea plant cultivar *C. sinensis* (L.) O. Kuntze cv. Shuchazao were collected from the experimental garden of Anhui Agricultural University and the tea plant germplasm resource garden in Guohe Town. The tissue samples used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis were as follows: buds, first leaves, second leaves, third leaves, fourth leaves, mature leaves, old leaves, young stems, and young roots. All samples were immediately frozen in liquid nitrogen and stored at -80 °C.

A. thaliana ecotype Columbia-0 (Col-0) was grown under long-day conditions 16 h of white light and 8 h of darkness at 22 ± 2 °C and a light intensity of 100 mol m^{-2} s^{-1}in the greenhouse at Anhui Agricultural University. Six-week-old*A. thaliana* plants overexpressing *CsHSP24.6* and*CspTAC5* were selected, and wild-type (WT) *A. thaliana* was used as a control. The plants were heat treated once for 6 hours at 45 °C, and were treated continuously at 40,000 lx light for three days, the changes were investigated using a plant chlorophyll fluorometer. To analyze the salt tolerance of *A. thaliana* , transgenic seeds were sowed on Murashige and Skoog (MS) medium. After vernalization at 4 °C for 3 d, the seedlings were grown in the greenhouse for 5 d, after which they were transferred to MS medium containing 100 mM NaCl. After 7 d, photographs were taken to record phenotypes and to measure related physiological indicators. Data processing was done using SPSS. The NaCl treatment method was as described by Johannes Loubser[39].

The expression patterns of *CsHsp24.6* under biological and abiotic stresses were also investigated. Tea branches having the same growth conditions were picked from the tea garden and divided into five groups, after which they were placed in Erlenmeyer flasks containing the same amount of water. After 24 h of

adaptation, the water was replaced with 200 mM NaCl solution for Group 1 and with 25% PEG4000 solution for Group 2. Group 3 was treated by smearing 0.25% MeJA on each leaf. Groups 4 and 5 were treated at 4 °C and 40 °C, respectively. Leaves tissues were collected at seven time points: 0, 6, 12, 24, 36, 42, and 60 h for Group 1; 0, 4, 8, 16, 24, and 36 h for Group 2; at 0, 6, 12, 24, and 38 h for Group 3; 0, 6, 12, 24, 36, 72, and 96 h for Group 4; and 0, 1, 2, 4, 8, 12, and 36 h for Group 5. At each time point, three repeats were performed. Samples were immediately frozen in liquid nitrogen and kept at -80 degC for RNA extraction.

2.2 Database Searches for *CssHSP* Gene Family Members in Tea Plants

Hidden Markov model (HMM) profiles of all sequences containing an HSP domain (PF00011) were used to search the Tea Plant Genome Database (*http://tpia.teaplant.org/index.html*)[38]. The sequences of the *CssHSP* s were analyzed with ExPASy ProtParam (*http://www.expasy.org/tools/protparam.html*) to obtain the number of amino acids, molecular weight, theoretical isoelectric point (pI), and instability index (Table **S1**).

2.3 Phylogenetic Tree Construction and Conserved Motif Analysis

The amino acid sequences of the *sHSP* s of *A. thaliana* and other plants were downloaded from the TAIR (*http://www.arabidopsis.org/*) and NCBI databases; unrooted neighbor-joining phylogenetic trees were constructed using MEGA 7.0 and EvolView (*http://www.evolgenius.info/evolview*). The MEME program (version 4.10.0, http://meme-suite.org/) was used to identify the conserved protein motifs of all *CssHSP* s with the following parameters: number of repetitions was any, maximum number of motifs was 10, and optimum motif widths were from 6 to 200 amino acid residues.

2.4 Expression Analysis and Cloning of *CsHSP24.6* and *CspTAC5*

Total RNA was extracted from young leaves of *C. sinensis* using the RNAiso-Mate kit for plant tissue (Takara, Dalian, China) according to the supplier protocols. The quality and quantity of RNA were verified by 1.2% w/v agarose gel and spectrophotometric analysis. The resulting RNA sample was used as the template for reverse transcription to synthesize the first strand of cDNAs using a PrimeScript RT Reagent Kit (Takara, Dalian, China) by following the manufacturer's protocol.

According to the cDNA sequences of *CsHSP24.6* (TEA033542) and *CspTAC5* (TEA000936), specific primers (Table **S5**) were designed to obtain the open reading frames (ORFs) of *CsHSP24.6* using Phusion High-Fidelity Polymerase (Thermo Scientific, Vilnius, Lithuania). An end-to-end PCR program was performed at 98 degC for 30 s, which was followed by 30 cycles at 98 degC for 10 s and 60 degC for 20 s, 72 degC for 45 s, and finally 10 min at 72 degC. The PCR products were purified from and cloned into a pEASY-Blunt Simple cloning vector following the manufacturer's protocol (TransGen Biotech, Beijing, China) and then transformed into *Escherichia coli* DH5α competent cells for DNA sequencing.

The primers (Table S6) for qRT-PCR were designed using Primer5 software. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is stably expressed in tea tissues[40], was used as an internal standard. The PCR cycling parameters and relative expression level were set and calculated by a published method[41].

2.5 Subcellular Localization

The full-length coding sequences of *CsHSP24.6* and *CspTAC5* lacking its termination codon were separately cloned into the entry vector pDONR207 using the Gateway BP Enzyme mix (Invitrogen) with specific primers. The plasmids newly extracted after sequencing were then introduced into the destination vector named pGWB5 [fused with green fluorescent protein (GFP) at the C-terminal] using the LR Clonase enzyme (Invitrogen). After sequencing, the plasmids pGWB5-*CsHSP24.6* and pGWB5-*CspTAC5* were separately transformed into the *Agrobacterium tumefaciens* strain GV3101 as described above. Selection of a positive colony for cultivation and infiltration of *Nicotiana benthamiana* was performed following a reported protocol[42]. After infection for 48 h, the leaves were examined by using an Olympus FV1000confocal microscope (Japan).

2.6 Pull-Down Experiments

For the pull-down assay, glutathione S-transferase (GST)-*CsHSP24.6* and free GST proteins were coupled to GST Bind Resin (NEB, E8021L). Maltose-binding protein (MBP)-*CspTAC5* and free MBP proteins were incubated with GST-*CsHSP24.6* and free GST coupled to GST Bind Resin in a pull-down buffer (50 mM Tris–HCl and 100 mM NaCl) at 4 °C for 3 h. After incubation, the beads were washed five times with ice-cold pull-down buffer, and the bound proteins were eluted in pull-down buffer containing an additional 20 mM L-glutathione (reduced) and 2.5 mM $CaCl_2$. The eluted proteins were resolved by SDS–PAGE and analyzed by immunoblot[43].

2.7 Bimolecular Fluorescence Complementation

Bimolecular fluorescence complementation (BiFC) assay was performed according to a previous study[44]. Full-length*CsHSP24.6* and *CspTAC5* cDNAs were subcloned into pCambia-CYFP and pCambia-NYFP, respectively, and plasmids were co-transformed into protoplasts (for primers used for fusion constructs; see Table S6 online). Yellow fluorescent protein (YFP) fluorescence was imaged using a confocal laser scanning microscope (Leica SP8).

2.8 Agrobacterium-Mediated Transformation of *A. thaliana*

The ORFs of the *CsHSP24.6* were ligated into the entry vector pDONR207 using the Gateway BP Enzyme mix (Invitrogen, Carlsbad, CA, USA). The primers used for vector construction are listed in Table S1. After sequence confirmation, the entry vectors were introduced into the destination vector pCB2004 using the Gateway LR Clonase enzyme (Invitrogen, Carlsbad, CA, United States). The pCB2004 vectors carrying*CsHSP24.6* were transformed into *A. tumefaciens*strain GV3101 through electroporation. The *A. tumefaciens*were inoculated on solidified LB medium with 50 mg L^{-1}kanamycin and 50 mg L^{-1} rifampicin at 28 degC for approximately 48 h. A positive GV3101 colony harboring the vector pCB2004-*CsHSP24.6* was confirmed through PCR. Then, the *A. tumefaciens* were cultured in liquid LB medium until the OD_{600} of the cell suspension reached 0.6-0.8. The*A. tumefaciens* cells were centrifuged at 5000 x*g* at 4 degC for 10 min and used for genetic transformation of *A. thaliana* by using the floral-dip method as previously described[45]. Seeds harvested from *A. tumefaciens* –infected plants were sterilized and plated on an MS basic medium containing 15 μg mL^{-1} glufosinate ammonium. Herbicide-resistant seedlings were transplanted to soil and grown in a greenhouse. The*A. tumefaciens* -mediated transformation of *CspTAC5* was performed by using an identical method.

2.9 Leaf Area Measurement

Please see the reference method at the following website:

http://www.medtranslation.cn/yixuetuku/20171013/130543.html.

First, a leaf was taken from the leaf base by cutting with a scissors. The length and width of each leaf were measured with a ruler and recorded. A rectangular piece of paper that was larger than the leaf was chosen. The length L , width W , and mass M of the paper were recorded. The paper area was calculated as $A = L \times W$. The leaf was fixed on the paper, and the shape of the leaf was traced using a pencil. The paper was then cut into the shape of the leaf with scissors. The leaf was removed, and the mass of the cut leaf-shaped paper was measured on the electronic balance and recorded as m . The leaf area a was then calculated as$a = A \times (m / M)$ and the shape coefficient was$k = a / (L \times W)$. The shape coefficient of 10 leaves was calculated, and an average value was determined. For subsequent measurements, the length and width of the leaves were measured with a ruler, and the product of these were multiplied by the shape factor to obtain the leaf area. The data and shape factors we measured are shown in the Table **S6** .

3. Results

3.1 Identification and Characterization of the *CssHSP*Gene Family in Tea Plant

A total of 54 *CssHSP* genes were screened from the tea plant genome through an investigation using the

ACD, a conserved carboxyl-terminal domain of approximately 90 amino acids. The results of the analysis of physiological and biochemical properties revealed that the lengths of most *CssHSP* s ranged from 129 (TEA033542) to 288 (TEA003981) amino acids, the molecular weights were between 14.96 kDa (TEA033542) and 32 kDa (TEA003981), and pI ranged from 4.65 (TEA010603) to 10.18 (TEA030325). These data indicate that 18 *CssHSP* proteins were basic with pI >7.0, whereas the remaining 29 proteins were acidic. In addition, the proteins with more than 300 amino acids were TEA028261, TEA024355, TEA019050, TEA029627, TEA031251, TEA006315, and TEA004116. More detailed data, including instability index, aliphatic index, and subcellular localization, were also collected (Table **S1**).

To explore the molecular evolution of and phylogenetic relationships among *sHSP* genes in plants, a phylogenetic tree was constructed from 117 sHSP domain sequences of five different species; the tree included 14 sequences from a monocotyledonous angiosperm (rice) and 103 sequences from dicotyledonous angiosperms (*A. thaliana* , tomato, and tea plant). All *sHSP* genes were categorized into 12 groups (CI–CVII, P, MI–MII, ER, and Px). At least one tea plant sHSP was determined to belong to each group except for Px. Group CI was the largest clade, including 17 (almost 31%) of the *CssHSP* genes identified (Figure **1**). Proteins belonging to the M groups were primarily in groups MI and MII and were closely related to those in P subfamilies, suggesting that they have similar origins and evolutionary history.

Then the protein sequence features of the *CssHSP* families were revealed, that the 10 most conserved motifs were identified and designated motif 1 to motif 10 by using the MEME tool (Figure**2,** Figure **S1**). Members of the same group usually had similar motif compositions, and some motifs were specifically present in one or more groups. For example, most group CI members contained motifs 1, 2, 3, 4, 5, 6, 8, and 9. The locations of the motifs were similar. Motif 9 was unique to group CI. Motifs 7 and 10 were observed only in groups CIII and P. In addition, protein motifs 1, 2, and 3 in the*CssHSPs* were determined to be highly conserved and distributed across almost all members.

3.2 Expression Patterns of *CssHSP* Genes Based on Genome Data

The expression patterns of *CssHSP* genes were presented, eight tea plant tissue genomes that are available online were downloaded (Figure**3A**). The transcripts per million (TPM) values of the*CssHSP* genes in different tissues are listed in Table S2. All*CssHSP* genes had diverse expression in the eight tissues(Figure **S2)** . We found that 10 *CssHSP* genes had higher expression levels [defined as $\log_2(\text{TPM}) > 2$] in these eight tissues. Several *CssHSP* genes exhibited some tissue specificity. For instance, TEA030313 and TEA025531 were only highly expressed in flowers. TEA015431 and TEA003981 were only highly expressed in roots and fruit, respectively. In general, almost all *CssHSP* genes were specifically expressed in one or more tissue (Figure **3A** and Table **S3**), implying that these genes play different roles in the growth and development of the tea plant.

To further demonstrate functions of *CssHSP* genes in responses to abiotic and biotic stresses, the expression values of *CssHSP* s after cold, drought, NaCl, and MeJA treatment were analyzed in the genome data (Table **S4**). Heat maps were created on the basis of expression levels, and upregulated genes are displayed in a Venn diagram (Figure **3**). Under cold, drought, and NaCl treatments,*CssHSP* s with normalized expression values [?]1 was considered to be upregulated genes; in MeJA treatment, *CssHSP* s with normalized expression values [?]3 was considered to be upregulated. Among the 54*CssHSP* genes, 47 genes were upregulated in response to stress, and some were upregulated for more than one stressor. For the abiotic stresses, 40, 8, and 17 *CssHSP* genes response respectively to cold, drought, and salt. Furthermore, 25 genes were observed to be upregulated in the presence of MeJA. For these treatments, the most upregulated genes were TEA033542 and TEA017741 (Figure **3E** and Table **S4**).

3.3 Expression Profiles of *CssHSP* Genes in Response to Stress

The expression pattern of TEA033542 in the tea plant was investigated to understand its response to heat, salt, drought, and MeJA stress and to verify the genomic data. For the basic thermotolerance analysis (Figure**4A**), the expression level of TEA033542 was observed to be strongly upregulated after the 40 degC treatment. Protein expression was relatively high in the leaves at 1 h and then was stable. The TEA033542

expression level was observed to be highest after 24 h MeJA treatment (Figure **4B**). However, in the genomic data, the expression level of TEA033542 was observed to be highest at 48 h. The difference may be due to inconsistent processing conditions. After The expression level of TEA33542 during both the 200 mM NaCl and the 25% PEG400 treatments was highest at 36 h (Figure **4C** and **4D**). Although the expression pattern of TEA033542 was different from that of the genome, analyses indicated that TEA033542 is involved in responses to heat, salt, drought, and MeJA stress.

3.4 Prediction and Identification of CsHSP24.6 Target Genes

Based on the analysis described in 3.3, the protein interactions of *TEA033542* and *TEA017741* were validated by constructing an *A. thaliana* association model using STRING software (version 10.5, *https://string-db.org/*). The amino acid sequence of *TEA033542* was used to retrieve the homologous gene *AtHSP21* . The subnetwork of the hub gene *AtHSP21* is displayed in Figure **5A** , Figure **S3.**displays the subnetwork of the hub gene *AtHSP22.0* , which is similarly homologous with *TEA017741* . In the two interaction networks, the protein–protein relationship scores and the HSP family genes were evaluated, and the correlation between *HSP21* and *pTAC5* had the highest score. On the basis of the gene sequences of *AtpTAC5* , *CspTAC5* was amplified with BLAST to compare cDNA sequences from the NCBI. A full-length ORF of 1200 bp was cloned from young leaves and was observed to encode 399 amino acids. *CspTAC5* contains a peptidoglycan binding-like domain (PG binding) and a DnaJ domain[46] (Figure **S4**). On the basis of the *TEA033542* nucleotide sequence, a full-length ORF consisting of 657 bp was amplified and was named *CsHSP24.6* in accordance with its molecular weight.

To verify the relationship between *CsHSP24.6* and *CspTAC5* , the expression profiles of *CsHSP24.6* and *CspTAC5* in tea plants were characterized by using qRT-PCR analysis. The results revealed that the expression levels of *CsHSP24.6* and *CspTAC5* were higher in mature leaves than in top buds (Figure **5B**). The expression levels of *CsHSP24.6* and *CspTAC5* in mature leaves were approximately 4.28 and 5.17 times higher, respectively, than those in buds (Figure **2B**). GFP fusion is an efficient approach for subcellular protein localization[47]. For subcellular localization of *CsHSP24.6* and *CspTAC5* , a GFP ORF was fused to the C-terminus of an ORF fragment and injected into *N. benthamiana* . Negative GFP controls were also injected into *N. benthamiana* . Confocal microscopy observation was used to determine that the *CsHSP24.6* -GFP fusion protein was localized in the chloroplast (Figure **5C**). These results suggest that there is an interaction between *CsHSP24.6* and *CspTAC5* .

Pull-down and BiFC experiments showed the interaction of *CsHSP24.6* with *CspTAC5* in vitro and in vivo. GST-*CsHSP24.6* could pull down MBP-*CspTAC5* , but free GST protein could not capture *CspTAC5* protein or free MBP protein. Therefore, *CsHSP24.6* and *CspTAC5* interact in vitro (Figure **5E**). A strong fluorescence from YFP was observed when *CsHSP24.6* and *CspTAC5* were co-expressed in *A. thaliana* protoplasts; however, when the combination of *CsHSP24.6* -YFP^N and YFP^C(*CsHSP24.6* -YFP^C and YFP^N) or the combination of *CspTAC5* -YFP^N and YFP^C(*CspTAC5* -YFP^C and YFP^N) was co-transformed into protoplasts, no YFP fluorescence was observed. This result demonstrates that *CsHSP24.6* also interacts with *CspTAC5* in vivo (Figure **5D**).

3.5 *CsHSP24.6* and *CspTAC5* Jointly Resist High Temperature and Light Intensity

Transgenic and WT plants were used to determine whether overexpression of *CsHSP24.6* or *CspTAC5* improved tolerance to adverse temperatures and light intensities. Six-week-old *35S::CsHSP24.6* and *35S::CspTAC5* transgenic plants and WT control plants were analyzed using a plant chlorophyll fluorometer after high-temperature and light treatments.

The fluorescence parameter Fv/Fm represents the maximum quantum yield of PSII, which is related to the potential maximum photosynthetic capacity of the plant. Results for Chl fluorescence imaging and Fv/Fm of the transgenic lines were similar to those of WT under normal growth conditions (Figure **6**). However, upon exposure to high-temperature and high-light stresses, WT exhibited impairment in Chl fluorescence images and significantly lower Fv/Fm compared with the transgenic lines. These results indicate that overexpression of *CsHSP24.6* or *CspTAC5* enhanced the heat and light tolerance of the transgenic plants.

3.6 *CsHSP24.6* Enhances Plant Salt Tolerance

To explore whether *CsHSP24.6* and *CspTAC5* have other common functions, *A. thaliana* seedlings were grown on media supplemented with NaCl. After germination on media containing no NaCl, 5-day-old *A. thaliana* transgenic seedlings of identical sizes were transferred to an MS media supplemented with 100 mM NaCl.

The data from the *CsHSP24.6* -overexpressing plants treated with NaCl are displayed in Figure **7A** . Compared with controls, the main roots and lateral roots of the *35S::CsHSP24.6* transgenic seedlings were substantially longer. The leaves of the *35S::CsHSP24.6* transgenic seedlings were also significantly larger than those of the control plants, although no difference was observed between the transgenic seedlings and the control under normal growth conditions.

No significant difference was observed between *35S::CspTAC5* transgenic plants and control plants (Figure **7B**). Therefore, resistance to NaCl stress is a unique function of *CsHSP24.6* and is not enhanced by *CspTAC5* .

4. Discussion

Small HSPs are known to function as molecular chaperone, protecting plants against abiotic stress in plant growth and evelopment. Earlier, sHSPs were thought to express almost exclusively in vegetative tissues only under heat stress, but recent studies have demonstrated the role of these proteins in diverse stresses like cold, drought, salinity and oxidative stress [29, 30, 33,48-52].The tea plant is an economic woody crop that is cultivated worldwide. However, the shade tolerance and substantial water requirements of tea plants cause them to be more vulnerable to environmental stresses. A decrease in tea yield and quality necessarily affects the tea industry, especially production firms. Therefore, studies have focused on ameliorating tea plant adversity[53-55]. In this study, Small heat shock proteins with different expression were screened out in tea plant genome. What function do they play ? However, the function of sHSPs in tea plants has been inadequately studied. Therefore, a genome-wide analysis of the CssHSP superfamily in the tea plant was performed, and the results provide a strong theoretical basis for future functional studies.

Although 22 CssHSP genes had been discovered in *C. sinensis* based on its genome, no systematic analysis of the *sHSP* gene family has been completed. Because these 22 genes were identified only through BLASTP searches, which used the sHSP protein sequences of *A. thaliana* as queries against two *C. sinensis* genomes, some CssHSPs may have been overlooked. Therefore, additional searches for genes that encode CssHSPs in *C. sinensis* genomes were conducted using HMM profiles. A total of 54 *CssHSP* genes were identified in *C. sinensis* , which is a higher number of *CssHSP* members than identified previously[56].

On the basis of conserved domain and phylogenetic tree analyses, the 54 *CssHSP* genes were divided into 12 distinct subcellular localization groups. No *C. sinensis* sHSPs were found in Px (peroxisome) groups, which was consistent with the results of Chen *et al* .[56]. Because gene expression analysis provides valuable information regarding gene function[57-58], the expression levels of the *CssHSP* genes in different tissues and the levels under abiotic and biological stress (low temperature, NaCl, drought, and MeJA) were investigated. The results revealed that most *CssHSP* genes were expressed in at least one tissue. Some genes were highly expressed in all tissues. Consistent with existing research, sHSPs were found to be induced by stress[33, 59-60]. The results of the genome analysis in this article also reveal that sHSPs can be involved in one or more abiotic and biotic stresses.

According to genome analysis, we cloned *CsHSP24.6* from the tea plant. *CsHSP24.6* belongs to a class of genes responsible for chloroplast localization. The expression of this gene is typical for various stress-inducing characteristics and can quickly respond to stresses. Induced stress substantially increases the expression of all sHSP genes (Figures **3** and **4**). AtHSP21 was revealed to be the only chloroplast-localized sHSP in *A. thaliana.*[27-28, 61-62]. Tea plant sHSP CsHSP24.6 and *A. thaliana* AtHSP21 are both classified in the same branch of the evolutionary tree, have high homology, and have three conserved domains (Figures **1** and **S2**).In this study, *CsHSP24.6* transgenic *A. thaliana* seedlings were treated with NaCl. After treatment, the

transgenic seedlings were observed to have better growth than the controls. However, there was no difference compared with controls for the same treatment with *CspTAC5* transgenic seedlings (Figure **7**). *CsHSP24.6* showed distinctly salt tolerance.

As the photosynthetic organ of plants, chloroplasts are vulnerable to damage when plants are under stress. Many studies have shown that under stress, chloroplast sHSP can maintain the physiological state of the chloroplast and preserve normal function [61, 63-64]. In the gun5 mutant with a yellowing phenotype, the expression of*HSP21* was inhibited. Under heat stress, the mutant had disintegrated thylakoids and lacked stacked basal particles; when the gun5 mutant expressed *AtHSP21* protein, the thylakoid membrane structure was normal and heat tolerance was restored[27]. Therefore, the *A. thaliana* AtHSP21 protein can respond to high temperature stresses by protecting the thylakoid membrane structure in the chloroplast and thereby resisting damage.

It was reported that *AtHSP21* and *pTAC5* are required for chloroplast development under heat stress by maintaining PEP function[28]. Here, the Arabidopsis of overexpressed the*CsHSP24.6* and *CspTAC* 5 genes could maintain a higher Fv/Fm value than controls at high temperatures and under strong light. Electron transfer via PSII was maintained. *CsHSP24.6* interacts with *CspTAC5* to help the plant resist external stress, so the same function was authenticated. *CsHSP24.6* improves the stability of growing development under heat stress (Figure **6**).

Our results indicate that overexpression of *CsHSP24.6* improves*A. thaliana* seeding resistance to high temperature. Meanwhile, it can protect plant from the strong light. In salt stess experiments, the Arabidopsis of overexpressed the *CsHSP24.6* grew better than the control.(Figure **7**) In this context, the *CsHSP24.6* plays an essential role in maintaining plant growth and development under abiotic stress.

Abbreviations

Small heat shock proteins (sHSPs); C. Sinensis small heat shock proteins(CssHSPs); α-crystallin domain (ACD); cytoplasm and nuclei (CI˜ CVII); mitochondria (MI, MII); endoplasmic reticulum (ER); chloroplasts (P); peroxide (Px); photosystem II (PSII); methyl jasmonate (MeJA); quantitative real-time polymerase chain reaction (qRT-PCR); Columbia-0 (Col-0); wild-type (WT); Murashige and Skoog (MS) medium; Hidden Markov model (HMM); theoretical isoelectric point (pI); open reading frames (ORFs); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); green fluorescent protein (GFP); glutathione S-transferase (GST); Maltose-binding protein (MBP); Bimolecular fluorescence complementation (BiFC); Yellow fluorescent protein (YFP); The transcripts per million (TPM); peptidoglycan binding-like domain (PG binding).

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Conflict of Interest

The authors declare no competing interests.

Author contributions

H Y, M J, & X Y performed the experiments and analyzed the data. M. Q. & Y. F. collected experimental materials. G. L. guide data analysis.

Z P. guided experimental drawing.

H Y, M J, & X Y. drafted the manuscript.

H Y, M J, & X Y. designed the experiments.

All authors read and approved the final manuscript.

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Figure 1 . Phylogenetic tree of CssHSPs. Phylogenetic tree of sHSP proteins in tea plant and other plant species were generated by MEGA 7 using neighbor-joining. At: *Arabidopsis thaliana* , Os:*Oryza sativa* , Gm: *Glycine max* , and Sl: *Solanum lycopersicum* ; CI–CVII: cytoplasm I–VII, ER: endoplasmic reticulum, MI–MII: mitochondria I–II, P: plastid, and Px:peroxide.

Figure 2 . Distribution of conserved motifs in CssHSP. Putative motifs are represented by a number in a colored box.CI-CVII: cytoplasm CI-CVII; ER: endoplasmic reticulum, MI: mitochondria I; P: plastid, and Px: peroxide

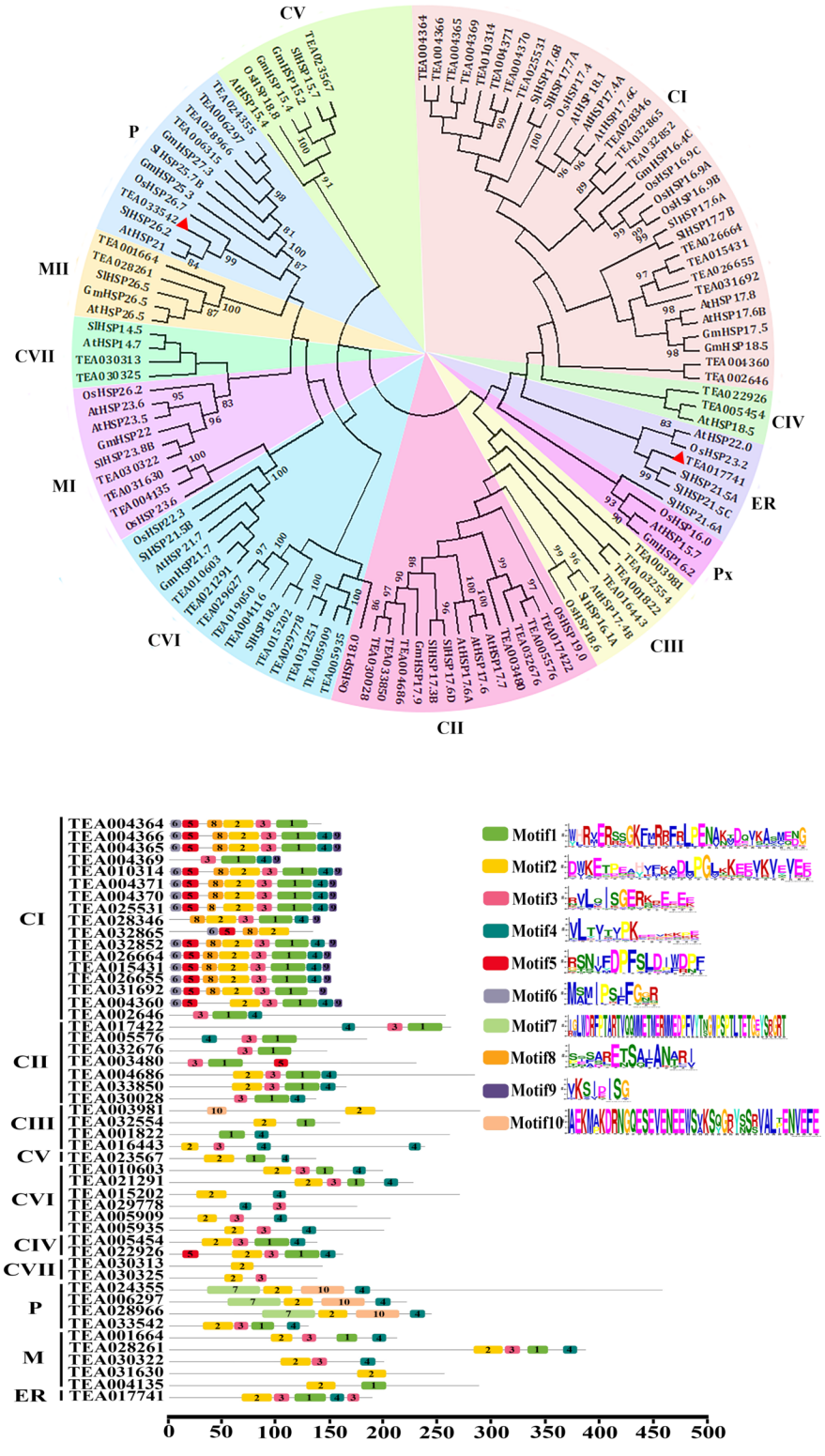
Figure 3 . Expression profiles of *CssHSP* genes. (A)Expression profiles of *CssHSP* genes in different tissues. (A)Cold treatment included CK, CA1 and de-acclimated (CA3);(B) MeJA treatment; (C)200mM NaCl treatment for 0, 24, 48, and 72 h; (D) Drought treatment: 25% polyethylene glycol (PEG) treatment for 0, 24, 48 and 72 h. (E) Venn diagram of *CssHSP* genes expressed in four treatments. The heatmap was generated by Heml software using the *CssHSP*genes' expression data, and normalized $\log_2$ transformed values were used with hierarchical clustering. The TPM values of the*CssHSP* genes in different tissues are listed in Table S2 and normalized $\log_2$(TPM treatment/TPM_control) transformed values were used with hierarchical clustering(A, B, C, E). Blue represents low expression, and red represents high expression. The venn diagram was finished by omicshare website.

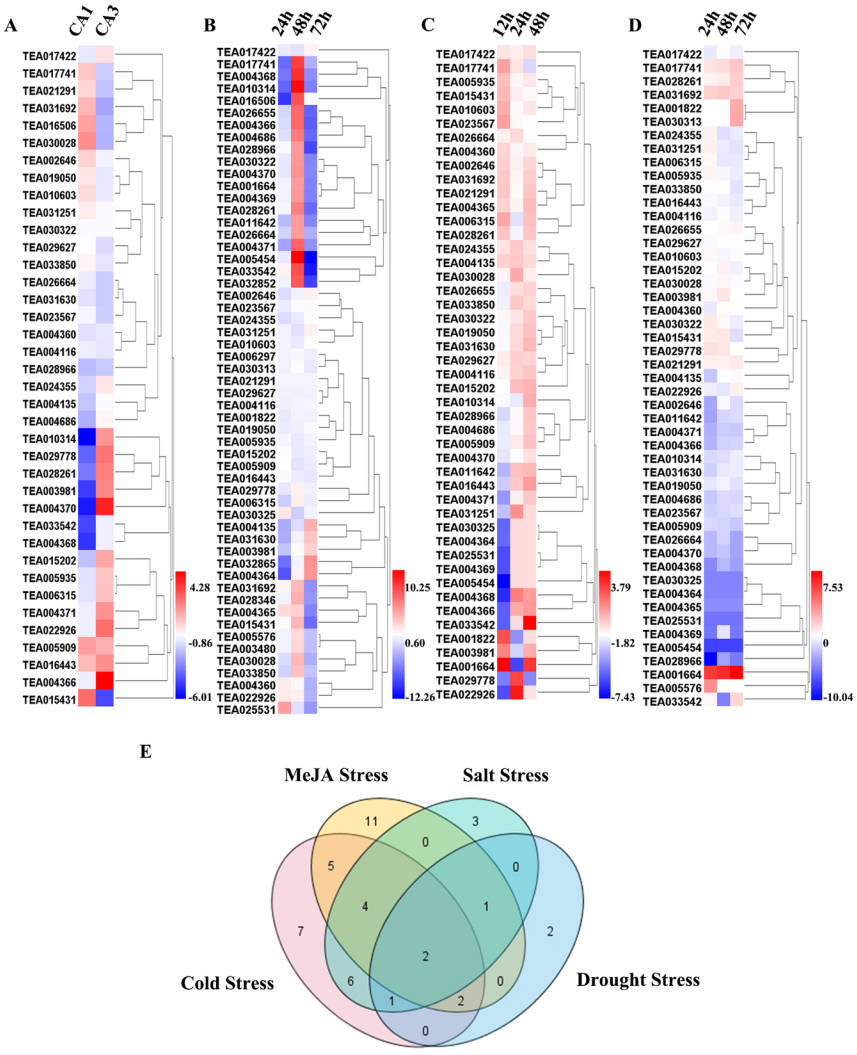
Figure 4 Expression pattern of TEA033542 under heat, salt, drought and MeJA stress in tea plant leaves. (A) Expression levels of TEA033542 following heat stress in different time points: 40 °C treatment for 0 h, 1 h, 2 h, 4 h, 8 h, 12 h. (B) Expression levels of TEA033542 following 0.25% MeJA treatments in different time points: 0 h, 12 h, 24 h, 48 h, 72 h.(C) Expression levels of TEA033542 following salt treatment in different time points: 200 mM NaCl treatment for 0 h, 6 h, 12 h, 24 h, 36 h, 48 h, 60h. (D) Expression levels of TEA033542 following drought treatment in different time points: 25% PEG4000 treatment for 0 h, 4 h, 8 h, 12 h, 24 h, 36 h. Data are means with standard deviations of three biological replicates. Different letters denote statistical significance at p[?]0.05.

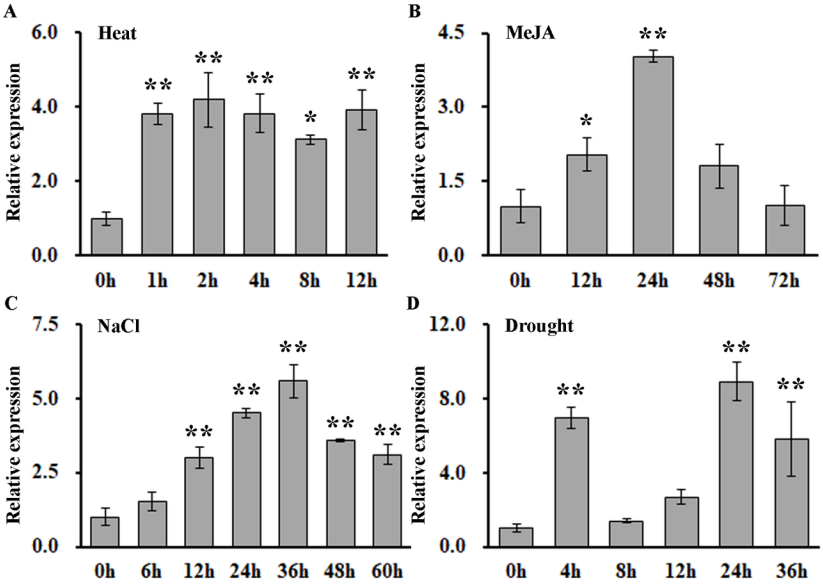
Figure 5 . Interaction between *CsHSP24.6* and*CspTAC5* in tea plants. (A) Putative interaction network of*TEA033542* in tea plants. Homologous proteins in tea plant and Arabidopsis are shown in red and black, respectively. (B)qRT-PCR data show expression profiles of *CsHSP24.6* and *CspTAC5* in buds, leaves (different stages), young stems, and roots. (C)Images obtained using confocal microscopy indicated chloroplast localization of the*CsHSP24.6* -GFP protein and *CspTAC5* -GFP protein in leaf epidermal cells of Nicotiana benthamiana. GFP alone were used as controls. Bars = 30 μm. (D) In vivo interaction between *CsHSP24.6*and *CspTAC5* examined by BiFC. The YFP confocal microscopy images show that Arabidopsis protoplasts express instantaneously combination encoding fusion proteins. Each image represents at least three independent experiments. Bars = 5 μm. (E) In vitro interaction between*CsHSP24.6* and *CspTAC5* analyzed by pull down. GST-*CsHSP24.6* and free GST proteins coupled to GST binding resin were incubated with MBP-*CspTAC5* and free MBP proteins. Bound proteins were separated by SDS-PAGE and immunoblotted with MBP or GST antibodies. The results were repeated twice.

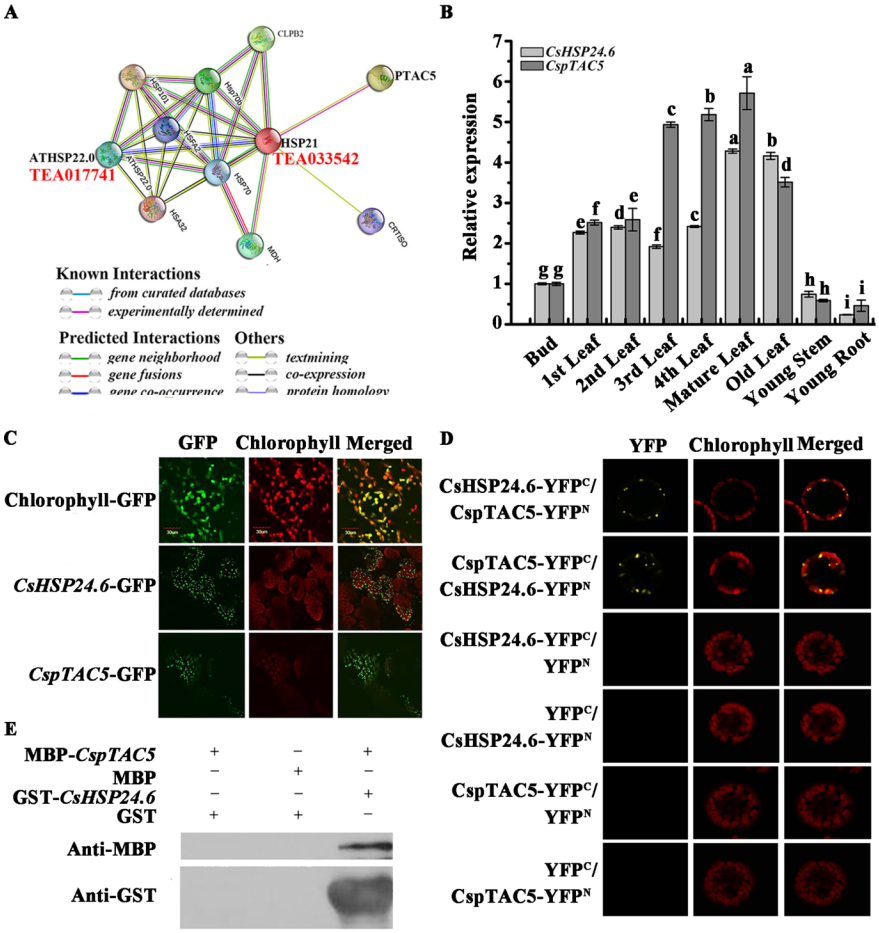
Figure 6 Overexpression of *CsHSP24.6* and *CspTAC5*in transgenic arabidopsis confers enhanced heat and hight tolerance. (A and B) Chl fluorescence imaging (A) and Fv/Fm (B) of transgenic lines and WT before and after the heat treatment. 6-week-old Arabidopsis plantlet in 45 °C for 6 h. Control,22 ;Heat,45 . (C and D) Chl fluorescence imaging (C) and Fv/Fm (D) of transgenic lines and WT before and after the light treatment. 6-week-old Arabidopsis plantlet in high light (40000 lx) for 3 days. Control,8000 lx;Light,40000 lx. All date is mean of 3 biological Replicates, Bar is +-SD; Statistical significant was analyzed by SPSS software, n=3, ** P <0.01

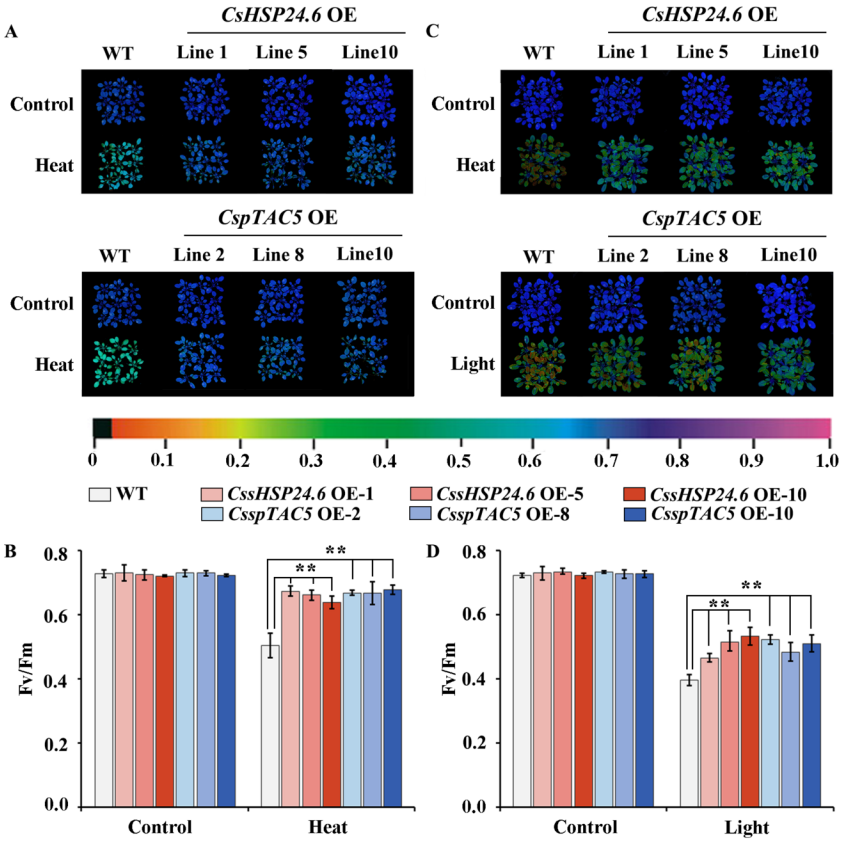
Figure 7 Growth and biochemical analysis of *CsHSP24.6*and *CspTAC5* overexpressing plants after 100 mM NaCl for 7 days.(A) Growth status of control and *CsHSP24.6* transgenic Arabidopsis plants after 100 mM NaCl for 7 days and normal condition on the left. Corresponding to the right are the physiological indicators of the control and *CsHSP24.6* transgenic plants such as main root, lateral root, and total leaf area. (B) Growth status of control and*CspTAC5* transgenic Arabidopsis plants after 100 mM NaCl for 7 days and normal condition on the left. Corresponding to the right are the physiological indicators of the control and *CspTAC5*transgenic plants such as main root, lateral root, and total leaf area. All date is mean of 50 biological Replicates, Bar is +-SD; Statistical significant was analyzed by SPSS software, n=50, * P <0.05,** P <0.01.

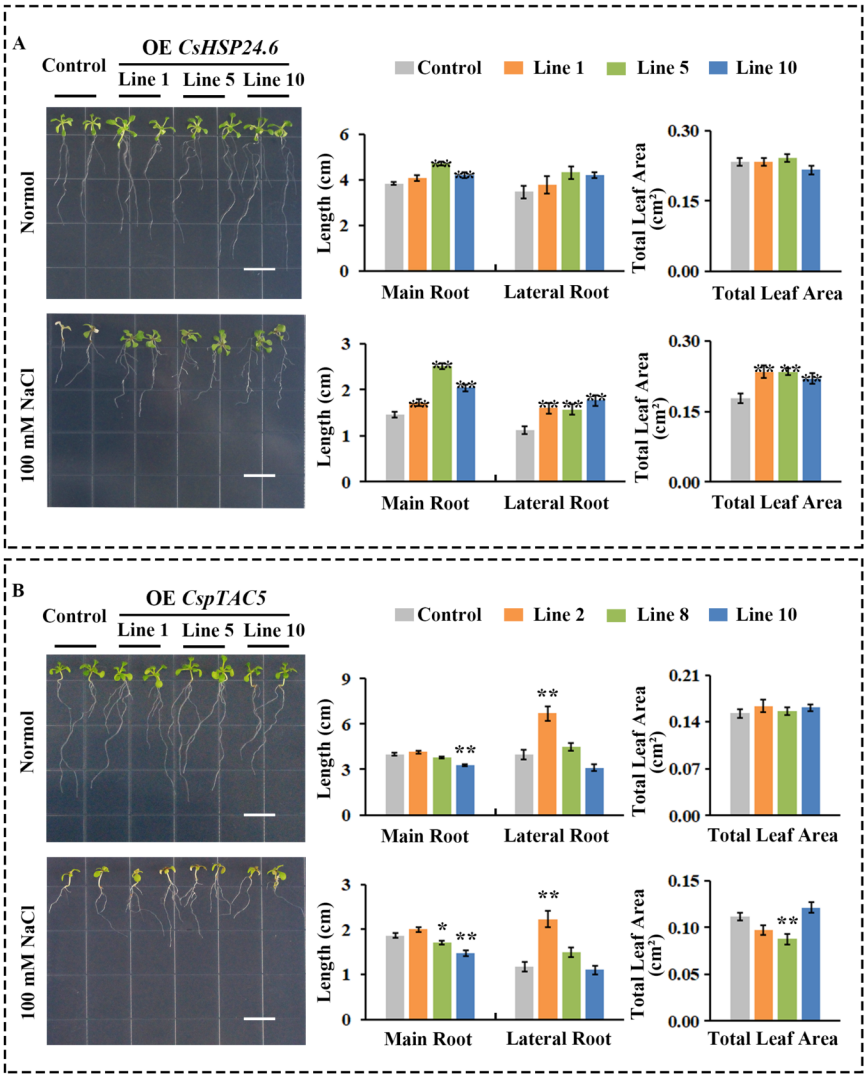












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