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Chrysanthemum CmHSP90.5 as a Tool to Regulate Heat and Salt Stress Tolerance

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Abstract: Heat shock proteins (HSPs) play important roles in various stress conditions. In this study, CmHSP90.5, whose expression is induced by heat and salt, was cloned from a chrysanthemum (Chrysanthemum morifolium) ‘Jinba’ and expressed in Arabidopsis. We found that CmHSP90.5 localized in the chloroplast. The heterologous expression of CmHSP90.5 weakened the heat tolerance of Arabidopsis and reduced the activity of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), indicating that CmHSP90.5-mediated heat stress sensitivity may be partially due to the regulation of active oxygen cleavage. The levels of expression of AtHSP101, AtHSP15.7, and AtHSP17.6C in CmHSP90.5-overexpressing plants decreased compared with those in wild-type (WT) plants under heat stress, indicating that these HSPs and CmHSP90.5 coregulate a plant’s heat stress tolerance. In addition, the salt stress tolerance of the CmHSP90.5-overexpressing Arabidopsis decreased compared with that of WT plants; CmHSP90.5-overexpressing plants showed increased Na⁺ levels and decreased K⁺ and proline levels compared with those of WT plants. Interestingly, the expression of stress-related genes, such as the Na⁺/H⁺ antiporter encoding gene SOS1, high-affinity K⁺ transporter encoding gene HKT1;1, and proline synthesis gene AtP5CS1, decreased in CmHSP90.5-overexpressing plants under salt stress compared with those expressions in WT plants. Our findings lay a foundation for understanding the roles of CmHSP90.5 in response to abiotic stresses in chrysanthemum.

Keywords: heat stress; salinity; CmHSP90.5; chrysanthemum; transgenic Arabidopsis

1. Introduction

Plants, as sessile organisms, are constantly subjected to heat and salinity challenges. Both heat and salinity stress are the main factors hampering plant growth, development, and yield; these stresses may even cause plant death [1–4]. Heat stress may affect protein stability, membrane permeability, cytoskeleton structure, and enzyme activity in plants, leading to major physiological dysfunction and metabolic disorders and impeding plant growth and development [5]. Moreover, it has been reported that yields decrease with temperature increases [6]. The adverse effects of salinity on plants result from a reduction in water availability in the soil as well as from sodium and chlorine ion toxicity [4]. Plants frequently phase both heat and salinity stresses during hot summers, especially under protected cultivation.

Heat stress proteins (HSPs) are molecular chaperone proteins that have been known for a long time [7]. Based on their molecular weight, HSPs are divided into HSP110, HSP90,
HSP70/80, HSP60, and small molecular HSP (sHSP) [8]. Among these HSP families, HSP90 members play an important role in the response to both biotic and abiotic stresses [9]. As typical molecular chaperone proteins, HSP90s contain three domains, including an N-terminal conserved ATP-binding domain, a middle domain, and a C-terminal dimerization domain which usually has the pentapeptide "MEEVD" [10]. In Arabidopsis, for example, seven HSP90 genes have been identified [11]. Among them, AtHSP90.1/.2/.3/.4 contain the pentapeptide “MEEVD” at their C-terminal end; this pentapeptide is necessary for protein cytoplasmic localization. AtHSP90.1/.2/.3/.4 are functionally redundant. In contrast, AtHSP90.5/.6/.7 are predicted to be localized in the chloroplast, mitochondria, and endoplasmic reticulum, respectively, based on their N-terminal signal peptides [12].

All three HSP90 protein domains play a crucial role on binding to other proteins, such as auxiliary proteins, regulatory factors, and substrate proteins [13].

Under normal conditions, HSP90s account for 1–2% of the total protein and bind other proteins to maintain their “non-active state” in an ATPase-dependent manner [14,15]. During the onset of stress, HSP90s depolymerize; released interaction proteins, i.e., transcription factors, especially heat stress factors (HSFs), kinases, and chaperones, are now free to activate or repress a series of signal transduction and regulatory processes in response to the triggering stress [16].

Released HSF monomers trimerize and bind to heat shock response elements (HSEs) to regulate downstream gene expression, including that of HSP90s [17,18]. HSP genes are induced not only by heat stress but also by salt, drought, and oxidative stresses [19–22]. An inhibitor of HSP90 may upregulate heat-related genes, improving Arabidopsis’s heat tolerance [23]. The overexpression of AtHSP90.2 in Arabidopsis enhances its tolerance to oxidative stress by suppressing HSFA2 transcription [24]. ROF1, a member of the FK506-binding protein family, interacts with AtHSP90.1 in the cytoplasm; a ternary complex with HSFA2 is then formed and transferred to the nucleus to regulate the expression of sHSPs in response to heat stress [25,26]. Recent studies have shown that HSP90 interacts with its auxiliary protein SGT1 to stabilize the auxin receptor transport inhibitor response 1 (TIR1) auxiliary protein to mediate the growth of Arabidopsis seedlings at high temperatures [27,28].

Overexpressing HSP90s in rice and soybean enhanced stress tolerance compared with that of the wild types (WTs) [29,30]. Furthermore, it has been reported that organelle localization of HSP90 is intimately related to the response of plants to stress. Transgenic seeds and seedlings of Arabidopsis overexpressing AtHSP90.5 and AtHSP90.7, which are localized in chloroplasts and the endoplasmic reticulum, respectively, were more sensitive to NaCl and drought stress than were plants which overexpressed AtHSP90.2, a cytoplasmic protein [19].

The chrysanthemum is an important ornamental plant worldwide. High temperatures hamper growth and flower bud differentiation and cause poor flowering and fading of flower color [31]. In addition, salinization of soil decreases the yield and quality of chrysanthemums [32]. In hot summers, heat stress is always accompanied by salt stress in chrysanthemum-producing areas. However, only a few genes related to heat tolerance, such as CmCPL1, and genes related to salt tolerance, such as CcSOS1 [33], DgWRKY4 [34], CmHSFA4 [32], CiCBF1 [35], and CmWRKY17 [36], have been functionally characterized in chrysanthemum. Moreover, genes involved in the response to both heat and salinity stresses remain largely unexplored, limiting the innovation and breeding progress of germplasm with tolerances to above two stresses. In our previous study, it was found that CmHSP90.5 was differentially expressed when chrysanthemum plants were under heat or salt stress; however, whether CmHSP90.5 confers tolerance to both salt and heat stresses remains unknown. In our study, we found that the overexpression of CmHSP90.5 reduced the tolerance of Arabidopsis to both heat and salt both by regulating the activity of several antioxidant enzymes and the expression of other HSPs and by modifying the Na+/K+ ion homeostasis and osmotic proline cell content. These results suggest that CmHSP90.5, as a potentially valuable breeding gene, might play an important role in both heat and salt tolerance. This laid the foundation for revealing the function of CmHSP90.5 in the regulation of heat and salt stress tolerance mechanisms in the chrysanthemum.
2. Materials and Methods

2.1. Plant Materials and Growth Condition

*Chrysanthemum* ‘Jinba’ plants were obtained from the *Chrysanthemum* Germplasm Resource Conservation Center, Nanjing Agricultural University, China. The plants were potted in a 1:1 (v/v) mixture of soil and vermiculite and grown in a greenhouse. *Arabidopsis* ecotype *Columbia* (Col-0) plants were grown in a 3:1 (v/v) mixture of vermiculite and soil in a growth chamber with a 16 h light period at 22 °C, followed by an 8 h dark period at 18 °C, with a relative humidity of 70%.

2.2. Cloning and Sequence Analyses of CmHSP90.5

Total RNA was extracted with Trizol reagent (TaKaRa, Tokyo, Japan) from *Chrysanthemum* ‘Jinba’ leaves, and 1 µg samples were used to obtain cDNA using a reverse transcription kit (TaKaRa). *CmEF1α* (KF305681) was amplified with primers *CmEF1α*-F/R (Table 1) and used as the internal reference gene. HSP90.5 gene sequences annotated in the *Chrysanthemum* transcriptome database were used to design amplification primers *CmHSP90.5*-F/R. The full-length *CmHSP90.5* cDNA sequence was obtained using Pfu DNA polymerase (TaKaRa Ex Taq®).

### Table 1. Primer names and sequences used in this study.

| Primer Name          | Sequence (5’-3’)                      |
|----------------------|---------------------------------------|
| 35S                  | GACGCAACATCCCACATCC                   |
| Actin2-F             | GGTAAATGTTGCTCAGTGGTG                |
| Actin2-R             | AACGACCTTAATCTTCATGTCG               |
| qAtHSP15.7-F         | TGCCGGAGAGTGTGAAATGTTG               |
| qAtHSP15.7-R         | CGATTTTGAAGTGTGCTCTTTAGG             |
| qAtHSP17.6c-F        | AAGAATGACAGTGGCAGCTGTTG              |
| qAtHSP17.6c-R        | GGCATTGATTTCTCTCCTATCTTACC           |
| qAtHSP101-F          | TGCAATTAGTTGCTGCTTGT                |
| qAtHSP101-R          | CCAACGGCACATAGATTGG                  |
| qCmHSP90.5-F         | TTATGAAATGACAGTGGCAGCTGCTTT          |
| qCmHSP90.5-R         | TCGCTCTCATTCTCTGATGCTCT              |
| qAtSOS1-F            | TTATGAAATGACAGTGGCAGCTGCTTT          |
| qAtSOS1-R            | CCCTCATAAAGACATCCCCAGTA              |
| qAtHKT1;1-F          | TCGATGCAATGAGAAGCTGGTG               |
| qAtHKT1;1-R          | CAGGACCATCGTGAT                 |
| qAtP5CS1-F           | TACAGCCCGAGAGCCCAT                 |
| qAtP5CS1-R           | T TTCGACTTCAACGGCCACTAGA           |
| CmEF1α-F             | TGTAACATTGTGCTCTCTCGTCCTTG          |
| CmEF1α-R             | TCGCCCTCAAACCCAGAACTCAA            |
| CmHSP90.5-F          | ATGGCTCCAGTCTCTCTCCAGA             |
| CmHSP90.5-R          | TCAAGTACTCCATGGTGGTCTTT            |
| pORE_R4-CmHSP90.5-F  | CAGGATCCACATGGTGGCCTCTT          |
| pORE_R4-CmHSP90.5-R  | CGGAATCATTCTGAGAAGCTGCTCCTG       |
| pORE_R4-CmHSP90.5−70-F| CGGAGATCCAAGCTGCTCACTGTCTCTG      |
| pORE_R4-CmHSP90.5−70-R| CGGAATCATTCTGAGAAGCTGCTCCTG       |

The predicted amino acid sequence for CmHSP90.5 was generated using DNAMAN 6.0 [37], and a phylogenetic tree was constructed using the neighbor-joining method employing MEGA 5.0 software [38] with 1000 bootstrap replicates. The conserved motifs on the full-length HSP90 family proteins were analyzed using the online MEME tool (Multiple Expectation Maximization for Motif Elicitation, http://memesuite.org/tools/meme, accessed on 15 July 2021) [39]. The maximum motif search value was set at 6. The protein transit peptide was analyzed using ChloroP (http://www.cbs.dtu.dk/services/ChloroP/, accessed on 15 July 2021).
2.3. Subcellular Localization of CmHSP90.5

To detect the subcellular localization of CmHSP90.5, HSP90.5<sub>1-70</sub> CDS were amplified and inserted into the pORE-R4-GFP vector under the control of the CaMV 35S promoter to produce a 35S:HSP90.5<sub>1-70</sub>:GFP plasmid as previously reported [40]. The plasmids pORE-R4-CmHSP90.51-70-GFP and pORE-R4-GFP (empty vector: negative control) were infiltrated into Nicotiana benthamiana abaxial leaf cells. Transformed N. benthamiana leaves were incubated for 16 h at 22 °C in the dark before observing the GFP signal via confocal laser scanning microscopy.

2.4. Expression Profile of CmHSP90.5

For the transcription profile analysis of CmHSP90.5, tissue samples were obtained from the root, stem, third leaves from the apex, and apical buds of Chrysanthemum 'Jinba' at the vegetative phase as well as from the root, stem, third leaves from the apex, tubular flowers, and ray flowers at the reproductive stage. Chrysanthemum seedlings at the six- to eight-leaf stage were selected for stress treatment. For the heat treatment, Chrysanthemum seedlings were exposed to 40 °C [41]. For the salt treatment, 200 mM NaCl was applied to the seedlings as previously reported [19]. All the seedlings were cultivated under a 16 h light/8 h dark photoperiod with a relative humidity of 70% and 120 µmol·m<sup>−2</sup>·s<sup>−1</sup> light intensity. The samples were collected at 0, 0.5, 1, 2, 4, and 6 h after beginning the heat treatment and at 0, 1, 4, 12, and 24 h of salt treatment. The total RNA was extracted with Trizol reagent (TaKaRa, Tokyo, Japan) and the cDNA was obtained using a reverse transcription kit (TaKaRa). Three independent biological replicates were used, and the qRT-PCR data were calculated using the 2−ΔΔCt method [42].

2.5. Generation of CmHSP90.5-Overexpressing Arabidopsis

For genetic transformation, we generated the 35S::CmHSP90.5:GFP fusion construct following the methods of Zhang et al. [43]. The CmHSP90.5 open reading frame was amplified by PCR using the primer set CmHSP90.5-R4-F/R (Table 1) harboring BamHI and EcoRI restriction sites. The amplicons digested with BamHI and EcoRI were directly cloned into the pORE-R4 cassette containing the CaMV 35S promoter, to generate the plasmid pORE-R4-CmHSP90.5-GFP. Arabidopsis plants were transformed with R4-CmHSP90.5 through the floral dip method [44]. Transformants were screened by germination of seeds on 1/2 MS medium containing 25 µg·mL<sup>−1</sup> kanamycin. DNA was extracted from putative transgenic lines and WT Arabidopsis plants using the Multisource Genomic DNA Miniprep kit (Axygen, Hangzhou, China). The regenerating resistant plants were obtained using PCR with the primer pair 35S-F and qCmHSP90.5-R (Table 1). The expression level of CmHSP90.5 in T3 generations was detected by RT-PCR using the primer pair qCmHSP90.5-F/R (Table 1).

2.6. Heat Tolerance Analysis of CmHSP90.5-Overexpressing Arabidopsis Plants

Col-0 and CmHSP90.5-overexpressing Arabidopsis seedlings were planted in a 3:1 (v/v) mixture of vermiculite and soil under a 16 h light/8 h dark photoperiod at 22 °C with a relative humidity of 70%. Next, 3-week-old seedlings were exposed to 42 °C for 24 h in an incubator (Sanyo, MIR-154, Shanghai, China) [45]. Each experiment included three replicates, and each replicate included 40 individual plants. All samples were frozen in liquid nitrogen and stored at −80 °C.

2.7. Salt Treatment on Transgenic Arabidopsis Plants

Transgenic and WT 3-week-old Arabidopsis plants were irrigated with 200 mM NaCl for two weeks. A group comprised of 20 plants was used to calculate the plant survival rate after a 2-week salinity treatment. The physiological indexes and the expression of stress-related genes were also determined using a 20-plant group under a 2-week salinity treatment. The experiment was repeated three times.
2.8. Determination of the Physiological Indexes of Plants under Stress Conditions

Cytoplasmic membrane permeability was measured by relative electrolyte leakage [46]. The chlorophyll content was determined by the 80% acetone extraction method [47]. The enzyme activity index was determined using a total superoxide dismutase SOD assay kit (WST-1 method), a catalase CAT assay kit (visible light method), and an ascorbate peroxidase APX test kit (ultraviolet colorimetric method) [48]. Both the methods and reagents for the determination of osmotic indicators were provided by the Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China). Each experiment included three biological replicates. For weight determination, water on the surface of plants was wiped, and then the fresh weight was determined.

For measuring Na\(^+\) and K\(^+\) content, transgenic and WT Arabidopsis plants were harvested. The plants were then dried at 80 °C for 3 days, and 0.1 g dry samples were digested in 2 mL HNO\(_3\). Finally, the sample volume was adjusted to 10 mL with distilled water. The Na\(^+\) and K\(^+\) contents were measured using an Optima 2100DV inductively coupled plasma optical emission spectrometer, as previously described [49].

2.9. Expression Profiles of Stress-Related Genes in CmHSP90.5-Overexpressing Arabidopsis Plants

To analyze the expression of stress-responsive genes, cDNA was synthesized using RNA from the leaves of WT and transgenic Arabidopsis subjected to heat or salt stress as a template. The stress-related genes of AtHSP15.7 [50], AtHSP17.6c [51], AtHSP101 [52], AtSOS1, AtHKT1;1, and AtP5CS1 [53] were monitored. AtActin2 was used as the reference gene. The sequences of all relevant primers are listed in Table 1.

2.10. Statistical Analysis

SPSS 26.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. The Tukey’s range test \((p = 0.05)\) was used to analyze the results after the one-way analysis of variance was performed.

3. Results

3.1. Identification and Characterization of CmHSP90.5

The CmHSP90.5 encoding the heat shock protein HSP90.5 from Chrysanthemum was cloned. CmHSP90.5 has an open reading frame of 2376 base pairs, encoding a 791-amino acid protein (Figure 1). In order to elucidate the evolutionary relationship of CmHSP90.5, HSPs proteins from four species, including seven from Arabidopsis thaliana, four from rice and tomato, and nine from soybean, were selected for further study. Phylogenetic analysis showed that CmHSP90.5 is evolutionarily closely related to the soybean chloroplast protein GmHSP90.5 (Figure 2A). The conserved motifs in CmHSP90.5 and other HSP90.5s were analyzed using the online MEME tool; this analysis showed that HSP90.5s had six highly conserved motifs (Figure 2B). It is worth noting that the motif 3 in the N-terminal end corresponds to an ATPase-domain (red box in Figure 1), which is an ATP/ADP binding site related to ATPase activity. Other motifs were closely related to the structure of HSP90s. Interestingly, analyzed HSP90.5s did not harbor the “MEEVD” pentapeptide in their C-terminal domains, suggesting that CmHSP90.5 could not bind proteins containing a tetratricopeptide repeat (TPR) domain. This interaction is generally important for the growth and development of plants.

Figure 2 shows the alignment of the deduced polypeptide sequences of CmHSP90.5 with those of other plant HSP90.5s.
their C-terminal domains, suggesting that CmHSP90.5 could not bind proteins containing a tetratricopeptide repeat (TPR) domain. This interaction is generally important for the growth and development of plants.

**Figure 1.** Amino acid alignment among HSP90.5 proteins. Red box includes the conserved ATPase-domain in HSP90.5 proteins.

**Figure 2.** Characterization of the CmHSP90.5 polypeptide sequence. (A) A phylogenetic analysis of the CmHSP90.5 amino acid sequence. Phylogenetic tree was constructed using the Neighbor-Joining method with 1000 bootstrap replicates. AtHSP90s of *Arabidopsis thaliana* were obtained from TAIR (the *Arabidopsis* Information Resource, https://www.arabidopsis.org/, accessed on 15 July 2021). Other sequences of HSP90 homologs were obtained from GenBank database, and their accession numbers were as follows: *Arabidopsis thaliana* AtHSP90.1: AT5G52640, AtHSP90.2: AT5G56030, AtHSP90.3: AT5G56010, AtHSP90.4: AT5G56000, AtHSP90.5: AT2G04030, AtHSP90.6: AT3G07770, AtHSP90.7: AT4G24190; GmHSP90-A1: NP_001304616; GmHSP90.6: XP_003545075.4; GmHSP90.5: XP_003516650.1; GmHSP90.5.1: NP_001344318.1; GmHSP90.6.1: XP_003519663; GmHSP90: XP_003545075; GmHSP90.2: NP_001236599; GmHSP90.1: NP_001236612; GmHSP90.A2: XP_006599549; SlHSP80: NP_001234439.1; SlHSP90.6: XP_004243554.1; SlHSP90.5: XP_004239010.1; SlHSP90: NP_001308492; SlHSP90.1: NP_001234436; OsHSP74.8: XP_015612552.1; OsHSP50.2: EEE70117.1; rHSP90: BA9A90487.1; OsHSP80.2: XP_015611111.1. (B) Conserved motifs analysis of HSP90.5 proteins using MEME tools. Conserved motifs are showed in colored boxes.
3.2. Expression Profiles of CmHSP90.5 in Different Tissues of Chrysanthemum and in Response to Heat and Salt Treatment

The relative expression pattern of CmHSP90.5 showed that its highest expression was in the leaf and its lowest level was in the root at both vegetative (Figure 3A) and reproductive growth stages (Figure 3B). The expression level of CmHSP90.5 was induced rapidly by heat stress, and it was 11.75-fold higher than that of the control at 1 h after the beginning of the heat treatment; the expression levels of CmHSP90.5 were maintained at a higher level compared with those of the control at 2–6 h after the beginning of the heat treatment (Figure 3C). In addition, the 200 mM NaCl treatment induced the expression of CmHSP90.5 rapidly; its expression was 2.98-fold higher than that of the control at 4 h and remained at a higher level after 24 h (Figure 3D). These results suggest that CmHSP90.5 might be involved in the response to both heat and salt stresses.

![Figure 3](image_url)

**Figure 3.** Expression profiling of CmHSP90.5 in Chrysanthemum ‘Jinba’. (A) Transcript abundances of CmHSP90.5 in different tissues at vegetative growth stages. (B) Transcript abundances of CmHSP90.5 in different tissues at reproductive growth stages. CmHSP90.5 expression was induced by heat stress (C) and NaCl treatment (D). Error bars represent ± SE. ** Statistically significant differences were compared with Col-0 plants under different conditions. Value of *p* < 0.01 was considered to be statistically significant.

3.3. Subcellular Localization of CmHSP90.5

CmHSP90.5 contains a predicted 70-aa chloroplast transit peptide at its N-terminal region, suggesting that it might localize in chloroplasts. To verify the predicted chloroplast localization of CmHSP90.5, we constructed a 35:CmHSP90.570:GFP vector carrying 70-aa from the N-terminal end of CmHSP90.5 fused to GFP. The construct was infiltrated into *N. benthamiana* leaves. A strong overlap between the fluorescence pattern of the CmHSP90.5:GFP fusion protein and chloroplast autofluorescence was observed, suggesting a chloroplast localization for CmHSP90.5. In contrast, the GFP fluorescence and chloroplast autofluorescence did not overlap in plants transformed with an empty vector (Figure 4).

![Figure 4](image_url)

**Figure 4.** Subcellular localization of CmHSP90.5 in leaves of *N. benthamiana* (A) 35S-CmHSP90.5:GFP vector carrying 70-aa from the N-terminal end of CmHSP90.5 fused to GFP. (B) GFP fluorescence and chloroplast autofluorescence in leaves of *N. benthamiana* transformed with an empty vector.

3.4. Overexpression of CmHSP90.5 Reduced Heat Tolerance in Transgenic Arabidopsis Plants

To test whether CmHSP90.5 played a role in regulating heat tolerance, CmHSP90.5 was introduced into Col-0 via floral dip transformation. Two transgenic lines (#1 and #5) were confirmed by PCR using Kan-F/R primers (Figure 5A), and CmHSP90.5 transcription was detected in the two lines via semi-quantitative PCR analysis (Figure 5B).
Figure 4. Subcellular localization of CmHSP90.5. 35: CmHSP90.5-GFP, GFP-fused protein was transiently expressed in epidermal cells of *Nicotiana benthamiana*, and GFP-fluorescent signals and chlorophyll (autofluorescence) were monitored by confocal microscopy. Bars: 20 µm, GFP: green fluorescent protein.

Figure 5. Validation of transgenic plants. (A) PCR analysis of genomic DNA extracted from kanamycin-resistant lines. (B) Transcription levels of *CmHSP90.5* in Col-0 and transgenic lines determined by RT-PCR. *AtActin2* was used as the reference gene.

The heat tolerance of the T3 generation of lines #1 and #5 was then assayed. After heat treatment at 42 °C for 24 h, severe wilting symptoms were observed in the two transgenic lines compared with the control (Figure 6A). In response to the heat treatment, the relative electrolytic leakage content in the transgenic lines was 4–5-fold higher than that in WT plants (Figure 6B), while the total chlorophyll content in the transgenic lines was significantly lower than that of the WT (Figure 6C). The activities of several antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX), in transgenic lines were lower (1.5-fold, 1.8-fold, and 1.4-fold, respectively) than those in the WT under heat treatment (Figure 6D–F). Importantly, there were no significant differences in antioxidant enzyme contents between the transgenic lines and WT plants under normal conditions.

In order to better understand the molecular mechanism of decreased thermal stress tolerance in *CmHSP90.5* transgenic *Arabidopsis*, the expression patterns of *ATHSP101*, *ATHSP15.7*, and *ATHSP17.6C* under thermal stress were studied. After heat treatment at 42 °C for 0.5 h, the relative expression levels of *ATHSP101*, *ATHSP15.7*, and *ATHSP17.6C* were lower in the transgenic lines than in the WT. However, the differences between the expression levels of these genes diminished with the duration of the heat treatment (Figure 7). *CmHSP90.5* might hamper the regulation of *sHSPs* expression, causing a decrease in heat tolerance.
Figure 6. Phenotypic analysis and physiological index determination of overexpression CmHSP90.5 in Arabidopsis under heat stress. (A) Phenotype of normal plants and plants under heat stress. (B) The relative electrolytic leakage in transgenic lines and Col-0 plants under normal and heat stress. (C) Chlorophyll of transgenic lines and Col-0 plants under normal and heat stress. (D–F) The activities of SOD, POD, and CAT in transgenic lines and Col-0 plants under normal and heat stress. Statistically significant differences were compared with Col-0 plants under different conditions. Error bars represent ± SE. * Value of \( p < 0.05 \) was considered to be statistically significant.

Figure 7. The expression level of AtHSPs genes in CmHSP90.5 transgenic Arabidopsis and Col-0 plants after heat treatment. The expression level of AtHSP15.7 (A), AtHSP101 (B), AtHSP17.6 (C) was measured after heat treatment. The Arabidopsis AtActin2 gene was used as the reference gene. Error bars represent ± SE. ** Statistically significant differences were compared with Col-0 plants under different conditions. Value of \( p < 0.01 \) was considered to be statistically significant.
3.5. Overexpression of CmHSP90.5 Reduced Salt Tolerance in Transgenic Arabidopsis Plants

To assess whether CmHSP90.5 decreased salt tolerance in Arabidopsis, CmHSP90.5-overexpressing transgenic lines (#1 and #5) were treated with 200 mM NaCl. Under non-stressed conditions, there were no significant differences in physiological indicators, including fresh and dry weight and ion and proline contents, between WT and transgenic lines either in roots or leaves. However, after salt stress treatments, the transgenic lines showed worse performance than the WT, especially transgenic line #5 (Figure 8A). Both fresh and dry weights of transgenic plants were significantly lower than those of WT plants (Figure 8B,C).

![Figure 8](image)

**Figure 8.** Overexpression CmHSP90.5 in Arabidopsis decreases survival rate under 200 mM NaCl treatment. (A) Phenotype of plants under salt stress and after 2-week recovery. (B,C) Fresh and dry weight measured after 2-week recovery. Error bars represent ± SE. * Statistically significant differences were compared with Col-0 plants under different conditions. Value of p < 0.05 was considered to be statistically significant.

To explore the role of CmHSP90.5 in salt stress response, the relative expression levels of stress-related genes were measured. Under salt treatment, the expression of AtSOS1, AtHKT1;1, and AtP5CS1 was lower in transgenic lines than in WT plants (Figure 9A–C). Consistent with the changes observed in gene expression, the Na⁺ content of the transgenic lines was higher than that of WT plants, whereas the content of K⁺ in transgenic lines was lower than that of WT plants (Figure 10A,B) both in leaves and roots. CmHSP90.5 transgenic lines had a lower proline content than the WT lines (Figure 10C). These results suggest that CmHSP90.5 plays a negative regulatory effect on osmotic regulation under salt stress.

![Figure 9](image)

**Figure 9.** Relative expression levels of stress-related genes in transgenic and WT plants under salt stress. (A–C) Graphs showing the expression levels of AtSOS1, AtHKT1;1, and AtP5CS1, respectively. Error bars represent ± SE. * Statistically significant differences were compared with Col-0 plants under different conditions. Value of p < 0.05 was considered to be statistically significant.

![Figure 10](image)

**Figure 10.** Changes in ion contents in transgenic and WT plants under salt stress. (A) Na⁺ content. (B) K⁺ content. (C) Proline content. Error bars represent ± SE. * Statistically significant differences were compared with Col-0 plants under different conditions. Value of p < 0.05 was considered to be statistically significant.
was considered to be statistically significant. (Figure 1B). It is well-known that molecular chaperones, including HSPs, are necessary for plant responses to various stresses. In our study, we identified an HSP90 family gene, CmHSP90.5, from Chrysanthemum which shared high sequence similarity with GmHSP90.5. 

**Figure 9.** The expression level of salinity-related genes in CmHSP90.5 transgenic Arabidopsis and Col-0 plants after salt treatment. The expression level of AtSOS1 (A), AtHKT1:1 (B), AtP5CS1 (C) was measured after salt treatment. The Arabidopsis AtActin2 gene was used as the reference gene. Error bars represent ± SE. * Statistically significant differences were compared with Col-0 plants under different conditions. Value of $p < 0.05$ was considered to be statistically significant.

**Figure 10.** Na⁺, K⁺, and proline contents in Col-0 and CmHSP90.5-overexpressed plants under 200 mM NaCl. (A, B) Na⁺ and K⁺ contents in roots and leaves of the plant. (C) Proline content in CmHSP90.5 transgenic Arabidopsis and Col-0 plants. Error bars represent ± SE. ** Statistically significant differences were compared with Col-0 plants under different conditions. Value of $p < 0.01$ was considered to be statistically significant.

4. Discussion

4.1. CmHSP90.5 Is a Heat Shock Protein Family Member and Is Chloroplast-Localized

Heat stress proteins play an important role as molecular chaperones involved in plant responses to various stresses. In our study, we identified an HSP90 family gene, CmHSP90.5, from Chrysanthemum which shared high sequence similarity with GmHSP90.5 (Figure 1B). It is well-known that molecular chaperones, including HSPs, are necessary for the correct folding and final confirmation of many proteins in vivo [53]. The ATP-binding domain at the N-terminal region plays an important role in the function of HSPs. The
conformational transition of HSP90s between the open and closed states relies on ATP binding and hydrolysis [54]. Mutations in the ATP-binding domain may cause the abolition of HSP90 functions both in vivo and in vitro [55]. Our report showed that CmHSP90.5 has a conserved ATPase domain at motif 3 (Figure 1A), similar to that of other HSP90.5s, indicating that it could function as a molecular chaperone. The other five motifs position CmHSP90.5 as a member of the HSP90 family of proteins (Figure 1C). Interestingly, those HSP90.5s that lack the five-peptide domain “MEEVD” in their C-terminal end (this domain is related to cytoplasmic localization of the protein and vital for interacting with proteins with a TPR domain) might be located in an organelle (Figure 1A) [53]. HSP90s, constituting a multimer family, have been found to localize in the cytoplasm and different organelles in plants. Using Arabidopsis as an example, the signal pentapeptide “MEEVD” at the C-terminal end is important for the subcellular localization of HSPs; all AtHSP90.1/.2/.3/.4 present this domain and are located in the cytoplasm. In contrast, AtHSP90.5 is guided into chloroplasts by a polypeptide consisting of 60 amino acids at its N-terminal region, while AtHSP90.6 is located in mitochondria due to the presence of a 48-amino acid polypeptide at its N-terminal end. The endoplasmic reticulum-located AtHSP90.7 proteins have 18-30-amino acid signal sequences at their N-terminal ends and a “KDEL” endoplasmic reticulum resident sequence in their C-terminal regions [56]. In our study, the Chrysanthemum ‘Jinba’ CmHSP90.5 contained a 70-amino acid chloroplast transit peptide at its N-terminal end, implying that it might be located in the chloroplast. Our subcellular localization assay showed that the CmHSP90.51−70:GFP fluorescence and chloroplast autofluorescence overlapped (Figure 3). Therefore, CmHSP90.5 is located in the chloroplast due to the presence of its N-terminal signal peptide. Meanwhile, the expression level of CmHSP90.5 was highest in the leaf at both vegetative (Figure 3A) and reproductive growth stages (Figure 3B), consistent with its subcellular location in chloroplast of Chrysanthemum. CmHSP90.5 might function in regulating the response of the leaves in Chrysanthemum upon salt and heat stresses.

4.2. CmHSP90.5 Confers Sensitivity to Heat Stress by Regulating Antioxidants and Heat Shock Proteins

Both high temperature and salinity seriously damage organisms. Plants may adapt or comply with extreme environments in different ways [57], including physical, anatomical, and physiological responses, such as changes in leaf orientation and stomatal conductance to overcome high temperature and shifts in developmental stages, retention of root hydraulic conductance, or accumulation of protective substances under saline as well as high temperature stresses. Moreover, at the onset of a stressful condition, plants may adjust their hormone balance, including changes in abscisic acid, ethylene, and jasmonic acid, and produce secondary metabolites. In addition, plants can regulate gene expression and synthesize stress-related biomolecules and proteins when facing challenging situations [58]. HSPs, as marker symbols related with stresses, are quickly synthesized.

In our study, CmHSP90.5 was induced both by heat and salt stresses, indicating that it might be involved in the response of the Chrysanthemum to both conditions. To explore the function of CmHSP90.5 in the plant’s response to heat stress, we generated CmHSP90.5-overexpressing Arabidopsis and found that CmHSP90.5 decreased the tolerance of Arabidopsis to heat. Reactive oxygen species (ROS), as signal molecules, are not only involved in the regulation of different pathways when under biotic or abiotic stress but also function as toxic byproducts of stress metabolism, causing plant damage or even plant death [59]. Under heat stress, plants accumulate a large amount of ROS (e.g., O$_2^·$, H$_2$O$_2$, OH·, and ¹O$_2$), resulting in the oxidative damage of cell membranes, affecting the membrane permeability and leading to intracellular electrolyte extravasation and increased osmotic potential [60]. Here, the overexpression of CmHSP90.5 led to increased cell membrane permeability (Figure 5C), implying that transgenic line cells were more damaged at high temperatures than WT cells were. Electrolyte leakage, as a symbol of stress responses in plant cells, is widely used to measure the degree of plant stress tolerance [60].
Therefore, the higher the electrolyte leakage is, the lesser the tolerance to heat stress that was observed in plants overexpressing CmHSP90.5. At the same time, ROS accumulation is the main reason for chlorophyll content decreases in plants [61]. In this study, the total chlorophyll content in transgenic lines was significantly lower than in WT plants under heat stress, indicating that the overexpression of CmHSP90.5 accelerated chlorophyll decomposition (Figure 5D). Under stresses, antioxidant enzymes, such as SOD, CAT, and APX, are conducive to maintaining the balance between the production and clearance of ROS in plant cells, preventing membrane lipid peroxidation. Therefore, the protective enzyme activity of plants under heat stress is often regarded as an important adversity indicator [62]. In our study, the activities of SOD, CAT, and APX increased under heat stress in both transgenic and WT plants; however, such activities were significantly lower overall in transgenic plants than in WT plants (Figure 5E–G), suggesting that CmHSP90.5 hampers heat stress tolerance via the regulation of the ROS-scavenging system.

It has been reported that several HSP family members, such as HSP101 and sHSPs, are involved in responses to various stresses. Overexpression of Arabidopsis AtHSP101 in rice significantly enhanced the heat tolerance of plants [63]. sHSPs such as AtHSP15.7 and AtHSP17.6C play a key role in assisting plant cells in avoiding heat stress damage [41]. In this study, the relative expression levels of AtHSP101, AtHSP15.7, and AtHSP17.6C were lower in transgenic lines than in WT plants under heat stress (Figure 6), suggesting that the CmHSP90.5-hampered resistance of Arabidopsis to heat tolerance might restrain the expression of these heat-related HSPs.

4.3. CmHSP90.5 Confers Salt Stress Sensitivity by Regulating Ion Homeostasis and Osmosis

Soil salinization is one of the main abiotic stresses faced by plants during growth and development. Salt stress can cause ion toxicity and osmotic stress, leading to nutrient deficiency and oxidative damage, and may severely inhibit plant growth, yield, and even survival [64]. The overexpression of AtHSP90.5 in Arabidopsis weakens the tolerance to salt stress [19]. Similarly, in our study, we found that CmHSP90.5 increased plant salt sensitivity, suggesting that the role of HSP90.5 in salt response might be conserved between Arabidopsis and Chrysanthemum.

The Na\(^+\) and K\(^+\) homeostasis in plant cells is an important indicator of salt tolerance in plants [65]. In this study, ion homeostasis changed upon CmHSP90.5 overexpression; the Na\(^+\) content was higher and the K\(^+\) content was lower in transgenic lines than in WT plants (Figure 9A,B). The salt overly sensitive (SOS) signaling pathway, a well-known regulatory pathway, is required in plants to respond to several abiotic stresses. As an important member of the SOS pathway, the Na\(^+\) transporter AtSOS1 functions in Na\(^+\) tolerance under salinity stress in plants. In this study, the expression level of SOS1 decreased in transgenic lines under salt stress compared with that of WT plants (Figure 8A). In addition, HKTs play a vital role in salt tolerance, and the expression level of AtHKT1;1 was lower in transgenic lines compared with that of the WT under salt stress. Therefore, Na\(^+\) accumulation and K\(^+\) diminishment in CmHSP90.5-overexpressing plants might be due to an altered regulation of the expression of ion transporter genes such as AtSOS1 and AtHKT1;1 under salt stress.

In Arabidopsis, an excessive number of HSP90s in organelles may slow down the secretion and targeting of ion transporters in the plasma or vacuolar membranes to either exclude or absorb excessive cytoplasmic Na\(^+\) or K\(^+\) ions. Therefore, overexpression of AtHSP90.7 may cause changes in toxic ion accumulation, resulting in reduced plant resistance [66]. In addition, the interaction between HSP90 and its substrate is based on dynamic changes in relative binding affinity; thus, an increased availability of HSP90 may delay the maturation or transport of the client protein [12]. It has been reported that excessive HSP90s in the endoplasmic reticulum may slow down the secretion and targeting of ion transporters in the plasma or vacuole membranes, leading to excessive cytoplasmic Na\(^+\) excretion rates [65]. However, the determination of the mechanism underlying the regulation of the expression of a number of stress tolerance-related genes by the chloroplast-localized protein CmHSP90.5 remains to be illustrated in the future.
Plant cells undergo osmotic regulation by accumulating osmotic substances under salt stress. As a highly water-soluble amino acid, proline is an osmotic regulation substance that plays an important role in preventing cell dehydration and osmotic stress. The accumulation of proline caused by stress is positively correlated to the extent of injury [64,67]. Salt stress causes an accumulation of large amounts of proline in plants. Here, we showed that the expression level of AtP5CS1 in the CmHSP90.5 transgenic lines was significantly lower than that of the WT; consequently, the proline content in the CmHSP90.5 transgenic lines decreased. Therefore, the decrease in salt tolerance observed in CmHSP90.5-overexpressing plants might be partially due to a diminishment in osmotic tolerance.

In summary, CmHSP90.5, a HSP90 family gene, was induced by heat and salt stress. CmHSP90.5, which is chloroplast-localized, contributed to the heat and salt sensitivity of the CmHSP90.5-overexpressing Arabidopsis. Overexpression of CmHSP90.5 might affect its interaction proteins’ function, which could regulate the activity of antioxidant enzymes, ion homeostasis, small HSP proteins, and osmotic substances, consequently regulating the tolerance of heat and salt stress in Chrysanthemum (Figure 11).

![Figure 11. Working model of CmHSP90.5 in response to heat and salt stresses. Light green oval: chloroplast, yellow circle: nucleus, pink oval: chloroplast-located CmHSP90.5.](image)

However, the characteristics of CmHSP90.5 and the CmHSP90.5-mediated signal pathways in response to environmental stress in chrysanthemum remain to be illustrated. Actual experimentation in Chrysanthemum itself needs to be performed to make any lasting conclusions about the heat or salt tolerance qualities of CmHSP90.5. All in all, these results provide a new insight into the roles of the HSP90 machinery response to adversity stress and laid a foundation for better understanding of the molecular mechanisms of the chrysanthemum in response to environmental stress.

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