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Ectopic over-expression of HaFT-1, a 14-3-3 Protein from Haloxylon ammodendron, leads to enhanced acquired thermotolerance of transgenic Arabidopsis

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Abstract

Haloxylon ammodendron, an important shrub for desert afforestation, can adapt to harsh ecological environments such as drought, saline and extreme high temperature. Therefore, the study of *H. ammodendron* stress adaptation mechanism is of great practical significance for ecological improvement in desert areas. Previously, a heat stress (HS) responsive *H. ammodendron* 14-3-3 protein gene *HaFT-1* with HS memory characteristics found in transcriptome was cloned and its transgenic *Arabidopsis* lines were obtained. In this study, its role in HS tolerance was further investigated. qRT-PCR analysis showed that HS priming enhanced the expression of *HaFT-1* under the second HS and subsequent recovery treatment, indicating expression of *HaFT-1* had transcriptional memory to HS. Subcellular localization showed that YFP-HaFT-1 fusion protein located in the cytoplasm of protoplasts. The survival rates of *HaFT-1* overexpression seedlings were higher than that of WT after priming-and-triggering and non-primed control treatments. Cell death staining showed that cell death in *HaFT-1* overexpression lines was more significantly inhibited than that in WT seedlings when treated with HS. Transcriptome analysis showed that energy generation, protein metabolism, signal transduction, biosynthesis, proline metabolism, autophagy, chlorophyll metabolism, and peroxisome were related to resistance of *HaFT-1* transgenic plants to the second HS. Growth physiology analysis showed that priming-and-triggering treatment in *HaFT-1* overexpression *Arabidopsis* promoted regulation on proline content and strengthened reactive oxygen species (ROS) scavenging activity. These results demonstrated that overexpression of *HaFT-1* strengthened not only HS priming but also tolerance to the second HS of transgenic *Arabidopsis*, suggesting HaFT-1 as a positive regulator in acquired thermotolerance.

Introduction

Plants can be primed by recurrent stresses to obtain stress adaption, which is related to "stress memory" (Vriet et al. 2015, Avramova 2015, Hilker et al 2015, Lämke et al. 2017; Avramova 2018). Stress memory represents an intrinsic response to repeated stress events (Avramova 2015). It is involved in modifications at different levels, including morphological, physiological, transcriptional, translational, and epigenetic levels (Kinoshita and Seki 2014; Baulcombe and Dean 2014; Sun et al. 2018; Srikant and Drost 2021). Heat stress (HS) is one of the major abiotic stresses in crop growth and productivity. Studies have shown that HS induces stress response in higher plants, leading to photosynthesis inhibition, chlorophyll degradation, protein denaturation and aggregation, loss of plasma membrane integrity, and accumulation of reactive oxygen species (ROS), thus disturbing cell metabolism and homeostasis (Dobrá et al 2015; Bäurle 2016; Lämke and Bäurle 2017; Ohama et al 2017). In recent years, studies on *Arabidopsis*, rice and other plants have found that the response of plants to HS is not limited to immediate responses to HS or heat shock response (HSR) (Grinevich et al. 2019). In nature HS is often recurring, many plants have evolved the ability to “memorize” the previous HS exposure (HS memory), by maintaining some stress-related changes, thereby preparing them for a better response to future HS stress. HS memory can last for several days under normal growth temperature, which is also called "acquired heat tolerance" (Bäurle

2016; Ohama et al 2017). HS memory has a unique adaptive significance of plants growing in the environment of long-term high temperature or frequent high temperature stress (Liu et al. 2019a).

14-3-3 proteins are members of a highly conserved dimer protein family. A typical 14-3-3 protein contains three regions: the N-terminal and C-terminal regions with high variability, and the conserved core region. The N-terminal region is the key region for protein dimer formation, and the C-terminal region is directly involved in protein-protein interaction (Gökirmak et al 2015, Chen et al. 2018). As a molecular chaperone, 14-3-3 protein mainly exists in the form of homodimer or heterodimer in plants. It binds to the target protein in a sequence specific and phosphorylation dependent manner, and deeply participates in the protein-protein interaction of various signaling pathways. The distribution of 14-3-3 proteins in cells is diverse, mainly in the cytoplasm, but also in nucleus, Golgi apparatus, mitochondria, chloroplast and plasma membrane (Stevers et al. 2018; Obsilova et al 2020). 14-3-3 protein genes have been found in soybean, tobacco, tomato, *Arabidopsis*, rice and barley. In plants, 14-3-3 proteins have been shown to regulate many biological processes, such as metabolism, light and hormone signaling, apoptosis, cell cycle control and protein transport. More and more reports have described the role of 14-3-3 proteins in plant stress response by interacting with key proteins in biotic and abiotic stresses (Gökirmak et al. 2015; Lyu et al. 2021; Mikhaylova et al. 2021). Studies on *Arabidopsis* 14-3-3 protein GRF9 showed that its function was related to the synthesis of isopentenyl diphosphate, the common precursor of cytokinin (CTK) and abscisic acid (ABA) biosynthesis. Under long-term HS, the expression of GRF9 was promoted in *Arabidopsis* leaves and roots. In *Arabidopsis*, the expression of GRF9 was still up-regulated within 2–6 hours after HS, indicating that the function of GRF9 may be related to HS adaptation (Dobrá et al. 2015).

Haloxylon ammodendron is a perennial shrub belonging to *Haloxylon* of *Chenopodiaceae*. It grows in gravel desert, clay desert, sandy or saline alkali land. It plays an important role in ecological restoration and maintaining the stability of ecological structure. It is an effective tool for vegetation reconstruction in arid desert (Yu et al. 2012; Wang et al. 2018). Adult *H. ammodendron* plants have strong stress tolerance to heat, drought, cold and salinity stresses, while the survival rates of *H. ammodendron* seedlings are very low under these abiotic stresses, indicating that its seedlings may acquire stress memory through priming in the growth process to adapt to environmental stresses (Wang et al. 2021). However, there are few studies on the role of memory related genes in *H. ammodendron* seedlings under HS. Previous transcriptome analysis of *H. ammodendron* indicated that its 14-3-3 gene *HaFT-1* (NCBI ACCESSION: KU565486) might be involved in the regulation of HS memory (unpublished data). *HaFT-1* gene has been cloned and its transgenic *Arabidopsis* lines have been obtained. Expression of *HaFT-1* was found to be upregulated by salt and dehydration stresses in *H. ammodendron* seedlings (Li et al. 2017; Liu 2019b). In this study, we further analyzed the function and mechanism of *HaFT-1* gene in thermotolerance from aspects of its expression, subcellular localization, and phenotype of *HaFT-1* overexpression *Arabidopsis* under priming-and-triggering treatment. It will expand our knowledge on the role of *H. ammodendron* 14-3-3 family in stress tolerance, and will lay a foundation for studying the molecular mechanism of 14-3-3 protein in plant adaptability to HS.

Materials And Methods

Plant Materials and Growth Conditions

The seeds of *H. ammodendron* were collected from Turpan Desert Botanical Garden in Xinjiang. The surface-sterilized seeds sown in Murashige-Skoog (MS) solid medium plates at 25℃±2℃ were grown under a diurnal cycle of 16 h light and 8 h dark with light intensity of 1500–2000 LX. One month old *H. ammodendron* seedlings were treated with HS priming-and-triggering procedure as described previously (Wang et al. 2020). In brief, samples grown at 26℃ (CK') were primed at 37℃ for 1 d (S1'), followed by 3 d recovery at 26℃ (R1'), then triggering was performed at 45℃ for 4 h (S2'), followed by 10 d recovery at 26℃ (R2'); while samples directly subjected to triggering at 45℃ for 4 h (FS2'), followed by 10 d recovery at 26℃ (FR2') as the non-primed control. At the end of each time point (CK', S1', R1', S2', R2 ', FS2', and FR2'), samples were frozen in liquid nitrogen and stored in a refrigerator at -80℃. The three biological replicates were taken from each sample.

Wild-type (WT) *Arabidopsis* (Col-0) and *HaFT-1* transgenic *Arabidopsis* (Col-0 background) heterozygous T2 lines were obtained from previous research (Liu 2019b). Screening of T3 *HaFT-1* transgenic homozygous lines were carried out as previously described (Wang et al. 2017). 2-week-old *Arabidopsis* seedlings were subjected to HS priming-and-triggering procedure as described in the reference (Charng et al. 2007). In brief, samples grown at 22℃ (CK) were primed at 37℃ for 90 min (S1), followed by 2 d recovery at 22℃ (R1), then triggering was performed at 47℃ for 1 h (S2), followed by 2 d recovery at 22℃ (R2); while samples directly subjected to triggering at 47℃ for 1 h (FS2) followed by 2 d recovery at 22℃ (FR2) as the non-primed control. At the end of each time point (CK, S1, R1, S2, R2, FS2, and FR2), samples were frozen in liquid nitrogen and stored in a refrigerator at -80℃. The three biological replicates were taken from each sample.

RNA Extraction, Reverse Transcription and PCR

Total RNA from each sample was extracted with Trizol reagent (Takara). Quality of total RNA was determined by using agarose gel electrophoresis and nano drop nd-1000 full wavelength spectrophotometer. About 2 μg of total RNA was reverse transcribed into cDNA using cDNA first strand synthesis kit (*TransScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix) (TaKaRa) in a 20 μL reaction. Reverse transcription PCR (RT-PCR) was conducted by 35 cycles, with *18S rRNA* as internal controls for *H. ammodendron*. Quantitive real-time PCR (qRT-PCR) was performed with an ABI 7500 fast real-time PCR system (Applied Biosystems). qRT-PCR reaction was performed using TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) (TaKaRa) according to the manufacture instructions. *18S rRNA* gene was used as the internal reference gene (Wang et al. 2018), primers used are listed in Supplementary Table 1. The relative expression levels were calculated using the relative $2^{-\triangle\triangle CT}$method (Livak and Schmittgen 2001) in three independent biological samples with three technical replicates.

Vector Construction and Subcellular Localization

Full length *HaFT-1* coding region was amplified by using TRANS high fidelity DNA polymerase (Transgene) with specific primers (Supplementary Table S1). The sequence-confirmed PCR product was ligated into the gateway pDONR221 vector and transferred to the 35S promoter driven plant overexpression vector pEarleygate104 according to the manufacturer's instructions (Invitrogen). The construct was introduced into *Arabidopsis* protoplasts for subcellular localization with procedure as described in the reference (Yoo et al. 2007). YFP signal was observed with a ZEISS LSM510 confocal laser scanning microscope.

Cell Death Staining

Cell death staining was performed according to the references (Zhu et al. 2015;Wang et al. 2021). For Hoechst 33342 staining, roots were soaked in assay buffer containing Hoechst 33342 for 30 min at 4℃ using the Apoptosis and Necrosis Assay Kit (Beyotime Institute of Biotechnology). After washing twice with ddH2O, samples were observed with a confocal laser scanning microscope (ZEISS LSM510) with an excitation filter of 350 nm. For trypan blue staining, leaves were soaked in PBS buffer containing trypan blue (0.4%) for 3–5 min at room temperature. After washing with PBS buffer and fixing with methanol for 30 min, decolorize samples with 75% ethanol. Samples were observed with an optical microscope (ECLIPSE Ts2R-FL, Nikon). All images were processed with ZEN 2009 Light Edition and Adobe Photoshop. The percentage of cells death compared with the total cell number was estimated from three different samples, each containing at least 30 cells. One-way analysis of variance (ANOVA) was performed with SPSS13.0.

DAB Staining

Peroxide levels were measured by 3,3'-diaminobenzidine (DAB) staining (Orozco-Cardenas and Ryan 1999, Kawai-Yamada et al. 2004). Seedlings were immersed in freshly prepared 0.1% (w/v) DAB-HCl (pH 3.8) in potassium phosphate buffer (pH 7.8) containing 10 mM NaN_3. After vacuum infiltration, samples were incubated in darkness at 22℃ for 10 h for DAB staining. For peroxide determination, seedlings were placed in acetic acid: glycerol: ethanol (1: 1: 3, by vol.) solution at 95℃ for 10 min and then stored in 95% ethanol until photographed.

Measurment of Chlorophyll and Proline Contents and ROS Scavenging Activity

WT and *HaFT-1* transgenic *Arabidopsis* chlorophyll content were monitored as described previously (Li and He 2021). Proline content, SOD and POD activity were measured with proline test kit, plant POD test kit and plant SOD test kit (all from Nanjing Jiancheng Biology) following the kit instructions. Three replicates of individual leaves were used for each sample.

Transcriptome Analysis

2-week-old *HaFT-1* transgenic and WT *Arabidopsis* seedlings taken at CK, S2, and FS2 time points were used for transcriptome analysis. A total of 2 mg RNA per sample was used as the input material for the RNA sample preparations. Sequencing libraries were generated using a NEBNext UltraTM RNA Library

Prep Kit for Illumina (NEB, United States) following a manufacturer's recommendations. After the library was constructed, Qubit2.0 fluorometer was used for preliminary quantification. The library was diluted to 1.5 ng/ul, and then Agilent 2100 Bioanalyzer was used to detect the insert size of the library. After passing the library inspection, different libraries were pooled according to the requirements of effective concentration and target offline data volume, and then Illumina sequencing was carried out. All downstream analyses were performed with high quality clean data. About 3 Gb of clean data for each sample were used to perform the transcriptional analysis. Statistically significant differentially expressed genes (DEGs) were discovered using the DESeq2 R package, and DEG was defined by the adjusted P-value (FDR) < 0.05 and Fold Change ≥ 2.

Results

Expression of *HaFT-1* during heat stress priming-and-triggering treatment

Previous transcriptome analysis showed that *HaFT-1* expression is significantly promoted by triggering at 45℃ after priming at 37℃ in *H. ammodendron* seedlings (unpublished data). To further verify whether *HaFT-1* has HS primed expression pattern at transcriptional level, qRT-PCR was used to detect expression of *HaFT-1* during HS priming-and-triggering treatment (CK', S1', R1', S2', R2') and non-primed control treatment (CK', FS2 ', FR2') (Fig. 1a). As shown in Fig. 1b, with priming, the relative expression level of *HaFT-1* was significantly induced at triggering (S2') and subsequent recovery stages (R2'); while in non-primed *H. ammodendron* seedlings, the relative expression level was much lower at triggering (FS2') and subsequent recovery stages (FR2') compared to that in primed plants. These results indicated that HS priming enhanced the expression of *HaFT-1* during the second HS and subsequent recovery phase, thus suggesting that *HaFT-1* has the transcriptional memory to HS in *H. ammodendron*.

Subcellular localization of HaFT-1

CNLS Mapper (http://nls-mapper.iab.keio.ac.jp) prediction did not find any nuclear localization signal in HaFT-1 protein. In order to further determine the subcellular localization of HaFT-1, recombinant vector pEarleygate104-HaFT-1 expressing YFP-HaFT-1 fusion protein was transformed into *Arabidopsis* protoplasts for microscopical observation. The results showed the YFP-HaFT-1 fluorescent signals in the cytoplasm of *Arabidopsis* protoplasts (Fig. 2), indicating subcellular localization of HaFT-1 in the cytoplasm.

HaFT-1 overexpression enhanced thermotolerance of transgenic *Arabidopsis* seedlings

T3 generation homozygous lines of *HaFT-1* overexpression *Arabidopsis* were obtained from previous research. In this study, P1 and P8, which showed the highest expression level of *HaFT-1* among all T3 homozygous lines, were selected using semi-quantitative PCR screening (Supplementary Fig. 1). To assess whether overexpression of *HaFT-1* led to enhances HS tolerance of transgenic *Arabidopsis*, we

designed experiments to qualitatively evaluate growth morphology and cell death characteristics of *HaFT-1* transgenic *Arabidopsis* during HS priming-and-triggering treatment (CK, S1, R1, S2, R2) and non-primed control treatment (CK, FS2, FR2) (Fig. 3a). The growth of P1, P8 lines, and WT *Arabidopsis* was inhibited at S2, FS2, R2, and FR2 stages, and the inhibition of WT *Arabidopsis* growth was more obvious than that of P1 and P8 lines (Fig. 3b). Furthermore, the survival rates of P1, P8 lines, and WT *Arabidopsis* at the R2 stage were higher than those at the FR2 stage. Moreover, the survival rates of P1and P8 lines were higher than those of WT *Arabidopsis* at the R2 and FR2 stages (Fig. 3c). In addition, qRT-PCR showed that at the S1 stage, the expression levels of *HsfA2* (AT2G26150), *HSP21* (AT4G27670), and *HSP101* (AT1G74310) in P1 and P8 were much higher than those in WT *Arabidopsis*, while at other stages, their expression levels did not significantly differ compared to the S1 stage (Fig. 3d). These results showed that *HaFT-1* overexpression not only promotes HS priming and resistance to second HS of transgenic *Arabidopsis*, but also improves thermotolerance of transgenic *Arabidopsis* without HS priming.

HaFT-1 overexpression inhibited cell death of transgenic Arabidopsis seedlings after heat stress with or without HS priming

To investigate the mechanism of promoted survival rates of *HaFT-1* transgenic *Arabidopsis* after HS, cell death of *Arabidopsis* overexpressing *HaFT-1* and WT *Arabidopsis* was analyzed by cell staining. Trypan blue staining was used to observe cell death in leaves. With TB staining, the dead cells were colored in the dark blue, while living cells were not stained. At S2 and FS2 stages, the numbers of stained cells in the same blade area in WT *Arabidopsis* leaves were higher than that of P1 and P8 leaves (Fig. 4a). The cell death rates of P1 and P8 leaves were significantly lower than that of WT *Arabidopsis* at the S2 and FS2 stages. Furthermore, the leaves' cell death rates of P1, P8, and WT *Arabidopsis* at the S2 stage were significantly lower than those at the FS2 stage (Fig. 4b). Next, roots of *HaFT-1* overexpression lines and WT *Arabidopsis* were stained with Hoechst33342 (a fluorescent dye that can bind to nuclear DNA, where the dead cells are deeply stained due to chromatin concentration) to show the vitality and morphology of the cells (Fig. 4c). Hoechst33342 staining showed that the number of dead cells in the main root meristem zone of P1 and P8 at the S2 and FS2 stages were significantly lower than that of WT *Arabidopsis*. Moreover, similar to leaves, the roots cell death rates of P1, P8, and WT *Arabidopsis* at the S2 stage were significantly lower than those at the FS2 stage (Fig. 4d). To sum up, the results of TB and Hoechst33342 staining further proved that *HaFT-1* overexpression reduces the cell mortality of transgenic *Arabidopsis* seedlings after HS and after HS priming.

Transcriptome analysis of *HaFT-1* overexpression lines under HS priming-and-triggering treatment

To further investigate the mechanism of enhanced survival rate of *HaFT-1* transgenic *Arabidopsis* after second HS on the transcriptional level, RNA-seq was used to monitor differential expression of genes after priming-and-triggering treatment. Compared to untreated control (CK), 3427 and 2701 genes, were up- and downregulated in P1 plants at the S2 stage, while in WT plants, 3255 and 3094 genes were up- and downregulated at the S2 stage, respectively (Fig. 5). At the FS2 stage, compared to CK, 4882 and

5033 genes were up- and downregulated in P1 plants, respectively, while in WT plants, 4585 genes were upregulated and 4934 genes downregulated (Fig. 5). Among the upregulated genes, 1134 and 1236 genes were specifically expressed in P1 plants at S2 and FS2 stages, respectively (Fig. 5); among all downregulated genes, 930 and 1372 genes were specifically expressed in P1 plants at S2 and FS2 stages, respectively (Fig. 5).

KEGG enrichment was then applied for genes upregulated and downregulated in P1 plants at S2 (P1S2-UP and P1S2-DOWN) and FS2 (P1FS2-UP and P1FS2-DOWN) stages, respectively. According to the value of -log10(padj), the top 20 KEGG enrichment pathways in P1S2-UP, P1S2-DOWN, P1FS2-UP, and P1FS2-DOWN are shown in Fig. 6. In P1S2-UP and P1FS2-UP groups, up-regulated genes (1134 genes and 1236 genes, respectively; Fig. 5) specifically expressed in P1 plants were mainly included in protein and energy metabolism pathways, such as proteasome, oxidative phosphorylation, aminoacyl-tRNA biosynthesis and fatty acid degradation pathways (Fig. 6). Compared to the P1FS2-UP group, pathways including pyruvate metabolism and citrate cycle (TCA) were specifically enriched in the P1S2-UP group (Fig. 6). Moreover, down-regulated genes in P1S2-DOWN and P1FS2-DOWN groups (930 genes and 1372 genes, respectively; Fig. 5) expressed in P1 plants were included in pathways related to signal transduction and biosynthesis (Fig. 6). Compared to down-regulated pathways enriched at the FS2 stage, pathways including plant hormone signal transduction, spliceosome and mRNA surveillance pathways were specifically enriched in P1 plants at the S2 stage (Fig. 6).

Pathways related to cell death inhibition and stress resistance were also found in KEGG enrichment. Among the top 20 KEGG enrichment pathways in each group, autophagy was enriched in P1S2-DOWN and P1FS2-UP, and arginine and proline metabolism was enriched in P1S2-DOWN and P1FS2-DOWN, and peroxisome was enriched in P1FS2-DOWN (Fig. 6). In addition to the top 20 KEGG enrichment pathways, there were more KEGG enrichment pathways related to cell death inhibition and stress resistance. Autophagy was enriched in P1S2-UP (No.23), arginine and proline metabolism was enriched in P1S2-UP (No.26) and P1FS2-UP (No.35), peroxisome was enriched in P1S2-UP (No.44), P1S2-DOWN (No.58) and P1FS2-DOWN (No.84), porphyrin and chlorophyll metabolism was enriched in P1S2-UP (No.53), P1S2-DOWN (No.40), P1FS2-UP (No.89), and P1FS2-DOWN (No.44) (Supplementary Table 2 to 5). Therefore, among all KEGG enrichment pathways, proline metabolism, autophagy, chlorophyll metabolism and peroxisome pathways were enriched in P1S2-UP, P1S2-DOWN and P1FS2-DOWN, while these three pathways, other than autophagy were enriched in P1FS2-UP.

To sum up, these results showed that energy generation and protein metabolism were promoted, while signal transduction and biosynthesis were depressed after the second HS in *HaFT-1* transgenic plants. In addition, the regulation of proline metabolism, autophagy, chlorophyll metabolism and reactive oxygen species (ROS) scavenging was also related to the resistance of *HaFT-1* transgenic plants to the second HS.

Effect of *HaFT-1* overexpression on physiological changes of seedlings under heat stress priming-and-triggering

treatment

To analyze the effect of *HaFT-1* overexpression on proline metabolism, chlorophyll metabolism and ROS scavenging at the physiological level, enzyme activities of SOD and POD, proline and chlorophyll contents in P1, P8 and WT were measured during HS priming-and-triggering treatment.

DAB staining showed that P1 and P8 had no obvious staining at CK or S2 stage and slight staining at the FS2 stage, but WT was stained deeper than P1 and P8 after both S2 and FS2 treatment (Fig. 7a). At S1 and R1 stages, enzyme activities of SOD and POD in P1 or P8 were higher than those in WT (Fig. 7b, c), suggesting that *HaFT-1* overexpression promoted the accumulation of SOD and POD during HS priming. At the S2 stage, there was no significant difference in POD activity between P1, P8, and WT, but SOD activity was higher than that of WT (Fig. 7b, c). Chlorophyll content of P1 and P8 seedlings were significantly lower than that of WT at S1 and R1 stages. Contrary to that, chlorophyll contents of P1 and P8 seedlings were significantly higher than that of WT at the S2 stage ((Fig. 7d).

The expression of key genes in proline biosynthesis was further explored (Fig. 8). qRT-PCR analysis showed that at the S1 stage, the expression level of *P5CS2* in P1 and P8 was much higher than it in WT *Arabidopsis*. At the S2 stage, the expression level of the *P5CS2* (AT3G55610) gene in P1 and P8 was slightly up-regulated compared to its expression level in WT *Arabidopsis.* At the FS2 stage, the expression levels of *P5CS2* in P1, P8, and WT were significantly different from those at the S2 stage (Fig. 8). These results showed that compared with WT, the expression of *P5CS* might be important to enhance tolerance to the second HS in *HaFT-1* overexpression *Arabidopsis*, and more expression of *P5CS* at the S1 stage was likely to lead to more accumulation of proline at the R1 stage in *HaFT-1* overexpression *Arabidopsis*. At the R1 stage, proline contents in P1 or P8 were higher than that in WT. However, at the S2 stage, proline contents in P1 or P8 were lower than that in WT. By contrast, at the FS2 stage, proline contents of P1 and P8 were significantly higher than that of WT, and the proline contents of P1, P8 and WT at FS2 stage was significantly higher than these at S2 stage (Fig. 8).

In summary, our data suggested that *HaFT-1* overexpression enhances ROS scavenging ability and proline biosynthesis at the HS priming stage, probably contributing to maintaining the stability of chlorophyll and reducing cell death under second HS.

Discussion

HS usually causes assimilation branches of *H. ammodendron* seedlings to turn yellow and stop growing, resulting in the death of seedlings that are not deeply rooted when exposed to HS-induced drought stress (Yu et al. 2012). Therefore, acquiring the adaptability to HS through heat priming is of great significance for the survival of *H. ammodendron* seedlings and the natural regeneration of the *H. ammodendron* population.

14-3-3 proteins, a family of conserved molecules that target several protein clients through their ability to recognize well-defined phosphorylated motifs and regulate activities of a wide array of target proteins via

protein-protein interactions, are involved in many biologically important processes, including cell cycle regulation, metabolism control, apoptosis, protein trafficking, stress response, and control of gene transcription (Yang et al. 2006; Chen et al. 2013; Mikhaylova et al. 2021). At present, the function and regulation mechanism of *H. ammodendron* 14-3-3 gene *HaFT-1* are ambiguous. The study on *H. ammodendron* 14-3-3 gene *HaFT-9* and heat shock factor gene *HaHsfA5* showed that their expression has transcriptional memory to HS in *H. ammodendron* (Liu 2019b; Wang et al. 2020). In this study, we further discovered that the expression patterns of *HaFT-1* were similar to that of *HaFT-9* and *HaHsfA5* during HS priming-and-triggering treatment (Fig. 1), indicating that *HaFT-1* can regulate acquired thermotolerance. Unlike *HaFT-9* or *HaHsfA5*, *HaFT-1* is obviously up-regulated during the second HS and subsequent normal temperature recovery stage but repressed at the HS priming stage and unresponsive during the whole control treatment. However, it should be noted that stress memory regulating factors, including DNA methylation, chromatin remodeling, microRNA and histone modification, can affect and modulate *HaFT-1* expression, which needs to be further explored. Transient expression of HaFT-1::YFP fusion protein in *Arabidopsis* protoplasts showed HaFT-1 localized in the cytoplasm (Fig. 2) indicating that as a molecular chaperon, HaFT-1 may interact with its target proteins, many of which act as acquired thermotolerance regulators in the cytoplasm. However, to date, there has been no report about target proteins of HaFT-1 or other 14-3-3 proteins in *H. ammodendron*.

Growth observation and cell death staining showed that *HaFT-1* overexpression enhances HS priming and thermotolerance after priming-and-triggering treatment and non-primed control treatment in transgenic *Arabidopsis* (Fig. 3 and Fig. 4). These results confirmed the important role of HaFT-1 in thermotolerance. Furthermore, the phenotype difference between *HaFT-1* transgenic lines and the WT *Arabidopsis* after the second HS was more obvious than that after control treatment, indicating that there might be additional regulation on HaFT-1 protein and its interaction with target proteins after HS priming. These results further suggest that the major role of HaFT-1 is to maintain the stability of acquired thermotolerance regulators. qRT-PCR further showed that at the HS priming stage, genes including *HsfA2*, *HSP21*, and *HSP101* in P1 and P8 were highly expressed compared to those in WT *Arabidopsis*, implying that HaFT-1 may interact with certain transcriptional regulators, which promote the expression of heat shock factors and heat shock proteins at HS priming stage.

In this study, the transcriptomic analysis revealed more hints for understanding the function of HaFT-1. Venn diagrams showed genes that are up- and down expressed in *HaFT-1* transgenic plants at the S2 stage (Fig. 5). Furthermore, KEGG enrichment analysis showed that energy generation and protein metabolism pathways were promoted among those genes, while signal transduction and biosynthesis pathways were repressed after second HS in *HaFT-1* transgenic plants (Fig. 6). HaFT-1 might bind to its target proteins to facilitate the expression of genes in pathways specifically enriched in transgenic plants after second HS. This data suggested that upregulated genes in energy generation and protein metabolism pathways were more likely to promote HS-resistant activities after the second HS, while downregulated genes in signal transduction and biosynthesis may inhibit some metabolic activities from saving energy for promoting HS tolerance. Bioinformatics prediction further suggested that HaFT-1 may

interact with HSFs, NACs, and other transcription factor family members (Liu 2019). Therefore, target proteins of HaFT-1 need to be further explored.

KEGG enrichment showed that genes specifically expressed in *HaFT-1* overexpression lines and related to cell death inhibition and stress resistance belonged to proline metabolism, autophagy, chlorophyll metabolism, and ROS scavenging pathways. There is a close relationship between stress-related genes expression, ROS scavenging, and proline synthesis (Altuntaş et al. 2020; Medina et al. 2021; Li et al. 2022; Wang et al. 2022). A study on desiccation-tolerant resurrection plant *Craterostigma plantagineum* showed that after being primed by drought, expression of four representative stress-related genes and ROS pathway-related genes gradually increased, accompanied by increased SOD activity, proline content, and sucrose content, and a decrease in H_2O_2 content and electrolyte leakage (EL) (Liu et al. 2019c). Research on wheat and tall fescue confirmed that drought-priming leads to HS cross adaptation with higher leaf water potential, chlorophyll content, and photochemical efficiency. The improved protection results in higher grain yield and increased tolerance compared to the non-primed plants (Liu et al. 2017; Zhang et al. 2019). These examples suggest a positive role of HS priming in elevating chlorophyll content.

In this study, DAB staining showed higher staining in WT compared to P1 and P8 after both S2 and FS2 treatment (Fig. 7), indicating the accumulation of ROS in P1 and P8 plants was lower than that in WT at S2 and FS2 stages. These results suggested that the ROS scavenging ability of *HaFT-1* transgenic plants was stronger than that of WT at S1 and R1 stages, resulting in less peroxide accumulation in *HaFT-1* transgenic plants compared to that of WT at the S2 stage. Furthermore, DAB staining of WT and *HaFT-1* transgenic plants at the FS2 stage was significantly higher than that at the S2 stage, which further showed that HS priming might reduce the accumulation of peroxide at the S2 stage. Meanwhile, expression of *P5CS2* increased at the HS priming stage, accompanied by increased SOD, POD, and proline contents at S1, R1, and S2 stage, and chlorophyll content at the S2 stage in *HaFT-1* transgenic *Arabidopsis* (Fig. 7, 8). Therefore, enhanced peroxide scavenging ability and proline biosynthesis during HS priming might contributed to maintaining chlorophyll stability and reduce cell death at the second HS stage in *HaFT-1* transgenic *Arabidopsis*, leading to a gain of HS tolerance and HS memory.

Taken together, this study characterized *HaFT-1* for its role in thermotolerance. *HaFT-1* has the transcriptional memory to HS in *H. ammodendron*. Overexpression of *HaFT-1* enhances HS priming and thermotolerance of transgenic *Arabidopsis*. Priming-and-triggering treatment in *Arabidopsis* overexpressing *HaFT-1* promoted energy generation and protein metabolism, depressed signal transduction and biosynthesis, and strengthened physiological regulation of proline content and peroxide scavenging activity. This study showed that HaFT-1 is involved in the regulation of acquired thermotolerance rather than basic thermotolerance and its upregulated expression depends on HS priming in *H. ammodendron.*

Declarations

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Competing Interests

The authors declare that they have no competing interests. The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

BW conceived the study, designed the experiments, and wrote the paper. RP designed and performed all the experiments, and wrote the first draft of the manuscript, which was further elaborated by XD and BW. XD and HZ participated in designing the experiments. WR performed some physiological assays and gene expression assays. All authors read and approved the manuscript.

Data Availability

The data underlying this article are available in the GenBank Nucleotide Database at https://www.ncbi.nlm.nih.gov/bioproject and can be accessed with accession No. PRJNA849331.

References

1. Altuntaş C, Demiralay M, Muslu AS, Terzi R (2020) Proline-stimulated signaling primarily targets the chlorophyll degradation pathway and photosynthesis associated processes to cope with short-term water deficit in maize. Photosynth Res 144(1):35–48. https://doi.org/10.1007/s11120-020-00727-w
2. Avramova Z (2015) Transcriptional 'memory' of a stress: transient chromatin and memory (epigenetic) marks at stress-response genes. Plant J 83:149–159. https://doi.org/10.1111/tpj.12832
3. Avramova Z (2018) Defense-related priming and responses to recurring drought: two manifestations of plant transcriptional memory mediated by the ABA and JA signaling pathways. Plant Cell Environ 42(3):983–997. https://doi.org/10.1111/pce.13458
4. Baulcombe DC, Dean C (2014) Epigenetic regulation in plant responses to the environment. CSH Perspect Biol 6(9):a019471. https://doi.org/10.1101/cshperspect.a019471
5. Bäurle I (2016) Plant Heat Adaptation: priming in response to heat stress. F1000Res 5: F1000. https://doi.org/10.12688/f1000research.7526.1. Faculty Rev-694
6. Charng Y, Liu H, Liu N, Chi W, Wang C, Chang S, Wang T (2007) A heat-inducible transcription factor, HsfA2, is required for extension of acquired thermotolerance in *Arabidopsis*. Plant Physiol 143:251–262. https://doi.org/10.1104/pp.106.091322
7. Chen Q, Guo CL, Wang P, Chen X, Wu K, Li K, Yu Y, Chen L (2013) Up-regulation and interaction of the plasma membrane H(+)-ATPase and the 14-3-3 protein are involved in the regulation of citrate

exudation from the broad bean (*Vicia faba* L.) under Al stress. Plant Physiol Bioch 70:504–511. https://doi.org/10.1016/j.plaphy.2013.06.015

8. Chen Q, Zhuo W, Li J, Lu L, Li L (2018) Cloning, bioinformatics and expression pattern analysis of *Nt14-3-3*. Tob Sci Technol 50(8):1–9. https://doi.org/10.16135/j.issn1002-0861.2017.0138
9. Dobrá J, Černý M, Štorchová H, Dobrev P, Skalák J, Jedelský PL, Lukšanová H, Gaudinová A, Pešek B, Malbeck J, Vanek T, Brzobohatý B, Vanková R (2015) The impact of heat stress targeting on the hormonal and transcriptomic response in *Arabidopsis*. Plant Sci 231:52–61. https://doi.org/10.1016/j.plantsci.2014.11.005
10. Gökirmak T, Denison FC, Laughner BJ, Paul AL, Ferl RJ (2015) Phosphomimetic mutation of a conserved serine residue in *Arabidopsis thaliana 14-3-3ω* suggests a regulatory role of phosphorylation in dimerization and target interactions. Plant Physiol Bioch 97:296–303. https://doi.org/10.1016/j.plaphy.2015.10.022
11. Grinevich DO, Desai JS, Stroup KP, Duan J, Slabaugh E, Doherty CJ (2019) Novel transcriptional responses to heat revealed by turning up the heat at night. Plant Mol Biol 101:1–19. https://doi.org/10.1007/s11103-019-00873-3
12. Hilker M, Schwachtje J, Baier M, Balazadeh S, Bäurle I, Geiselhardt S, Hincha D, Kunze R, Mueller-Roeber B, Rillig MC, Rolff J, Romeis T, Schmülling T, Steppuhn A, van Dongen J, Whitcomb SJ, Wurst S, Zuther E, Kopka J (2015) Priming and memory of stress responses in organisms lacking a nervous system. Bio Rev 91(4):1118–1133. https://doi.org/10.1111/brv.12215
13. Kawai-Yamada M, Ohori Y, Uchimiya H (2004) Dissection of *Arabidopsis* Bax Inhibitor-1 Suppressing Bax-, Hydrogen Peroxid-, and Salicylic Acid-Induced Cell Death. Plant Cell 16(1):21–32. https://doi.org/10.1105/tpc.014613
14. Kinoshita T, Seki M (2014) Epigenetic memory for stress response and adaptation in plants. Plant Cell Physiol 55:1859–1863. https://doi.org/10.1093/pcp/pcu125
15. Lämke J, Bäurle I (2017) Epigenetic and chromatin-based mechanisms in environmental stress adaptation and stress memory in plants. Genome Biol 18(1):124. https://doi.org/10.1186/s13059-017-1263-6
16. Li L, He G (2021) Experimental Guidance of Plant Physiology. Higher Education Press, Beijing
17. Li P, Lin P, Zhao Z, Li Z, Liu Y, Huang C, Huang G, Xu L, Deng Z, Zhang Y, Zhao X (2022) Gene Co-Expression Analysis Reveals Transcriptome Divergence between Wild and Cultivated Sugarcane under Drought Stress. Int J Mol Sci 23(1):569. https://doi.org/10.3390/ijms23010569
18. Li Y, Zhang H, Jiang Y, Ma H (2017) Cloning and expression analysis of HaFT-1 and HaFT-2 genes in *Haloxylon ammodendron*. Acta Agric Boreali-occidentalis Sinica 26(3):455–462. https://doi.org/10.7606/j.issn.1004-1389.2017.03.018
19. Liu J, Feng L, Gu X, Deng X, Qiu Q, Li Q, Zhang Y, Wang M, Deng Y, Wang E, He Y, Bäurle I, Li J, Cao X, He Z (2019a) An H3K27me3 demethylase-HSFA2 regulatory loop orchestrates transgenerational thermomemory in *Arabidopsis*. Cell Res 29(5):379–390. https://doi.org/10.1038/s41422-019-0145-8

20. Liu S, Li X, Larsen DH, Zhu X, Song F, Liu F (2017) Drought priming at vegetative growth stage enhances nitrogen-use efficiency under post- anthesis drought and heat stress in wheat. J Agron Crop Sci 203:29–40. https://doi.org/10.1111/jac.12190
21. Liu S (2019b) Analysis of stress-resistance function of 14-3-3 genes *HaFT-1* and *HaFT-9* in *Haloxylon ammodendron*. Dissertation. Xinjiang Agricultural University
22. Liu X, Challabathula D, Quan W, Bartels D (2019c) Transcriptional and metabolic changes in the desiccation tolerant plant *Craterostigma plantagineum* during recurrent exposures to dehydration. Planta 249:1017–1035. https://doi.org/10.1007/s00425-018-3058-8
23. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402–408. https://doi.org/10.1006/meth.2001.1262
24. Lyu S, Chen G, Pan D, Chen J, She W (2021) Molecular analysis of *14-3-3* genes in *Citrus sinensis* and their responses to different stresses. Intl J Mol Sci 22(2):568. https://doi.org/10.3390/ijms22020568
25. Medina E, Kim SH, Yun M, Choi W (2021) Recapitulation of the function and role of ROS generated in response to heat stress in plants. Plants 10(2):371. https://doi.org/10.3390/plants10020371
26. Mikhaylova YV, Puzanskiy RK, Shishova MF (2021) Evolution of 14-3-3 Proteins in Angiosperm Plants: Recurring Gene Duplication and Loss. Plants 10(12):2724. https://doi.org/10.3390/plants10122724
27. Obsilova V, Obsil T (2020) The 14-3-3 proteins as important allosteric regulators of protein kinases. Int J Mol Sci 21(22):8824. https://doi.org/10.3390/ijms21228824
28. Ohama N, Sato H, Shinozaki K, Yamaguchi-Shinozaki K (2017) Transcriptional regulatory network of plant heat stress response. Trends Plant Sci 22(1):53–65. https://doi.org/10.1016/j.tplants.2016.08.015
29. Orozco-Cardenas M, Ryan CA (1999) Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. PNAS 96(11):6553–6557. https://doi.org/10.1073/pnas.96.11.6553
30. Srikant T, Drost H (2021) How stress facilitates phenotypic innovation through epigenetic diversity. Front Plant Sci 11:606800. https://doi.org/10.3389/fpls.2020.606800
31. Stevers LM, Sijbesma E, Botta M, MacKintosh C, Obsil T, Landrieu I, Cau Y, Wilson AJW, Karawajczyk A, Eickhoff J (2018) Modulators of 14-3-3 protein-protein interactions. J Med Chem 61(9):3755–3778. https://doi.org/10.1021/acs.jmedchem.7b00574
32. Sun R, Lin C, Zhang X, Duan L, Qi X, Gong Y, Deng X (2018) Acclimation-induced metabolic reprogramming contributes to rapid desiccation tolerance acquisition in *Boea hygrometrica*. Environ Exp Bot 148:70–84. https://doi.org/10.1016/j.envexpbot.2018.01.008
33. Vriet C, Hennig L, Laloi C (2015) Stress-induced chromatin changes in plants: of memories, metabolites and crop improvement. Cell Mol Life Sci 72(7):1261–1273. https://doi.org/10.1007/s00018-014-1792-z

34. Wang B, Du H, Zhang Z, Xu W, Deng X (2017) BhbZIP60 from Resurrection Plant *Boea hygrometrica* Is an mRNA Splicing-Activated Endoplasmic Reticulum Stress Regulator Involved in Drought Tolerance. Front Plant Sci 8:245. https://doi.org/10.3389/fpls.2017.00245
35. Wang B, Du H, Yao Z, Ren C, Ma L, Wang J, Zhang H, Ma H (2018) Validation of reference genes for accurate normalization of gene expression with quantitative real-time PCR in *Haloxylon ammodendron* under different abiotic stresses. Physiol Mol Biol Pla 24:455–463. https://doi.org/10.1007/s12298-018-0520-9
36. Wang B, Man L, Zhang H (2020) Identification of Heat Shock Factor Family Gene HaHsfA5 from *Haloxylon ammodendron* and Analysis of Its Expression in Heat Stress Memory. Mol Plant Breed 19(15):5157–5163. https://doi.org/10.13271/j.mpb.018.000858
37. Wang B, Xue Y, Pan R, Ren W, Zhang H (2021) Analysis of cell death characteristics of *Haloxylon ammodendron* seedlings during the establishment of Adaptability to heat stress. Mol Plant Breeding 19(15):5157–5163. https://doi.org/10.13271/j.mpb.019.005157
38. Wang C, Zhou Y, Yang X, Zhang B, Xu F, Wang Y, Song C, Yi M, Ma N, Zhou X, He J (2022) The Heat Stress Transcription Factor LlHsfA4 Enhanced Basic Thermotolerance through Regulating ROS Metabolism in Lilies (*Lilium Longiflorum*). Int J Mol Sci 23(1):572. https://doi.org/10.3390/ijms23010572
39. Yang X, Lee W, Sobott F, Papagrigoriou E, Robinson CV, Grossmann JG, Sundström M, Doyle D, Elkins JM (2006) Structural basis for protein-protein interactions in the14-3-3 protein family. PNAS 103(46):17237–17242. https://doi.org/10.1073/pnas.0605779103
40. Yoo SD, Cho YH, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: A versatile cell system for transient gene expression analysis. Nat Protoc 2:1565–1572. https://doi.org/10.1038/nprot.2007.199
41. Yu T, Ren C, Zhang J, He X, Ma L, Chen Q, Qu Y, Shi S, Zhang H, Ma H (2012) Effect of high desert surface layer temperature stress on *Haloxylon ammodendron* (C.A. Mey.) Bunge. Flora 207(8):572–580. https://doi.org/10.1016/j.flora.2012.06.008
42. Zhang X, Xu Y, Huang B (2019) Lipidomic reprogramming associated with drought stress priming-enhanced heat tolerance in tall fescue (*Festuca arundinacea*). Plant Cell Environ 42:947–958. https://doi.org/10.1111/pce.13405
43. Zhu Y, Wang B, Phillips J, Zhang ZN, Du H, Xu T, Huang L, Zhang X, Xu G, Li W, Wang Z, Wang L, Liu Y, Deng X (2015) Global transcriptome analysis reveals acclimation-primed processes involved in the acquisition of desiccation tolerance in *Boea hygrometrica*. Plant Cell Physiol 56:1429–1441. https://doi.org/10.1093/pcp/pcv059

Figures

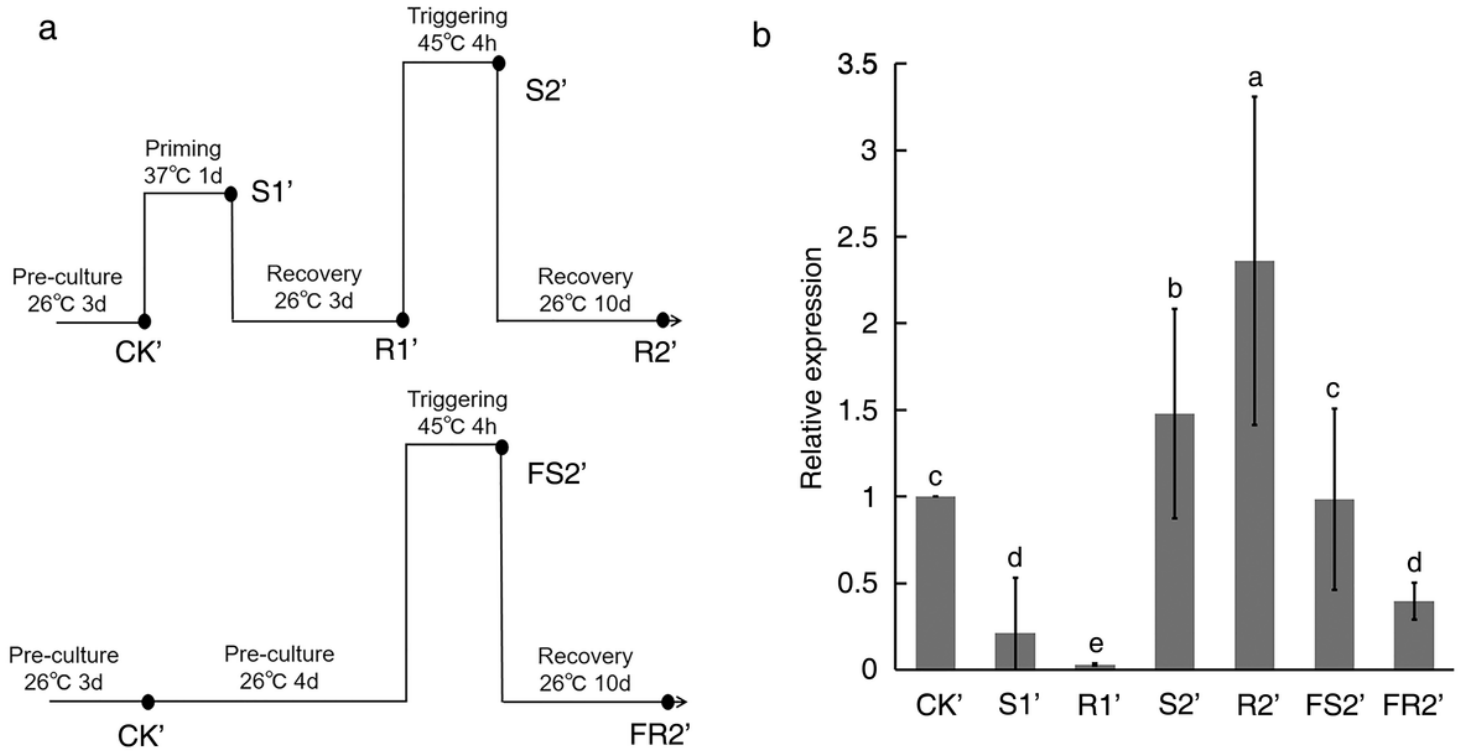


Figure 1

The transcriptional memory of *HaFT-1* under HS priming-and-triggering treatment

(a) Schematic representation of the experimental procedure; dots indicate the time of sampling. Samples were taken immediately at each sampling time point (CK', S1', R1', S2', R2', FS2', FR2'). (b) Relative expression of *HaFT-1* at each sampling time point. Statistical significance was determined with Student's t-test ($P<0.05$), and is labeled with lower case letters.

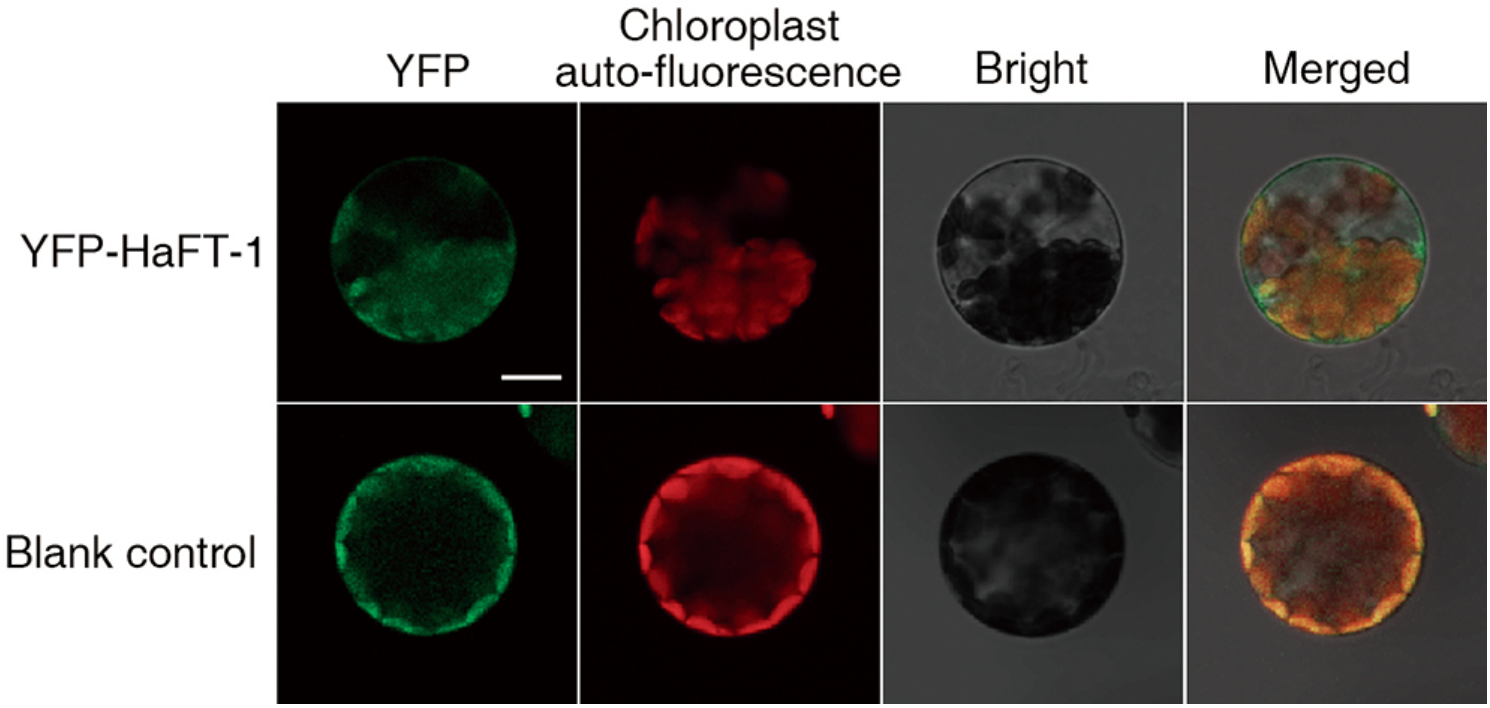


Figure 2

Subcellular localization of HaFT-1

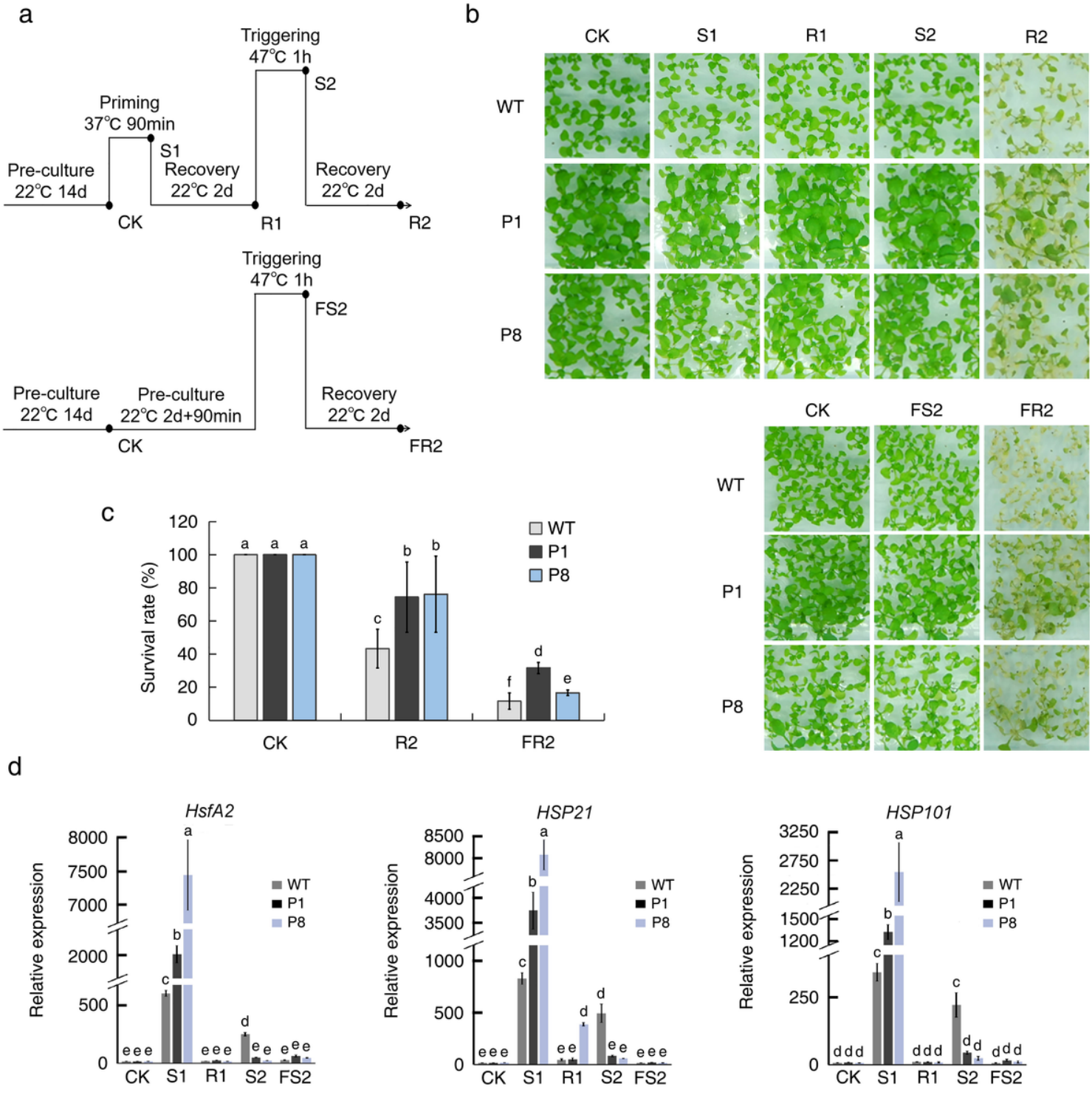


Figure 3

Effect of *HaFT-1* overexpression on growth of seedlings under heat stress priming-and-triggering treatment

(a) Schematic representation of the experimental set-up; dots indicate the time of sampling. Samples were taken immediately after the end of each sampling time point (CK, S1, R1, S2, R2, FS2, and FR2). Growth phenotype (b), survival rate (c) and expression of partial genes (d) in P1, P8, and WT *Arabidopsis*.

Statistical significance was determined with Student's t-test (P<0.05), and is labeled with lower case letters.

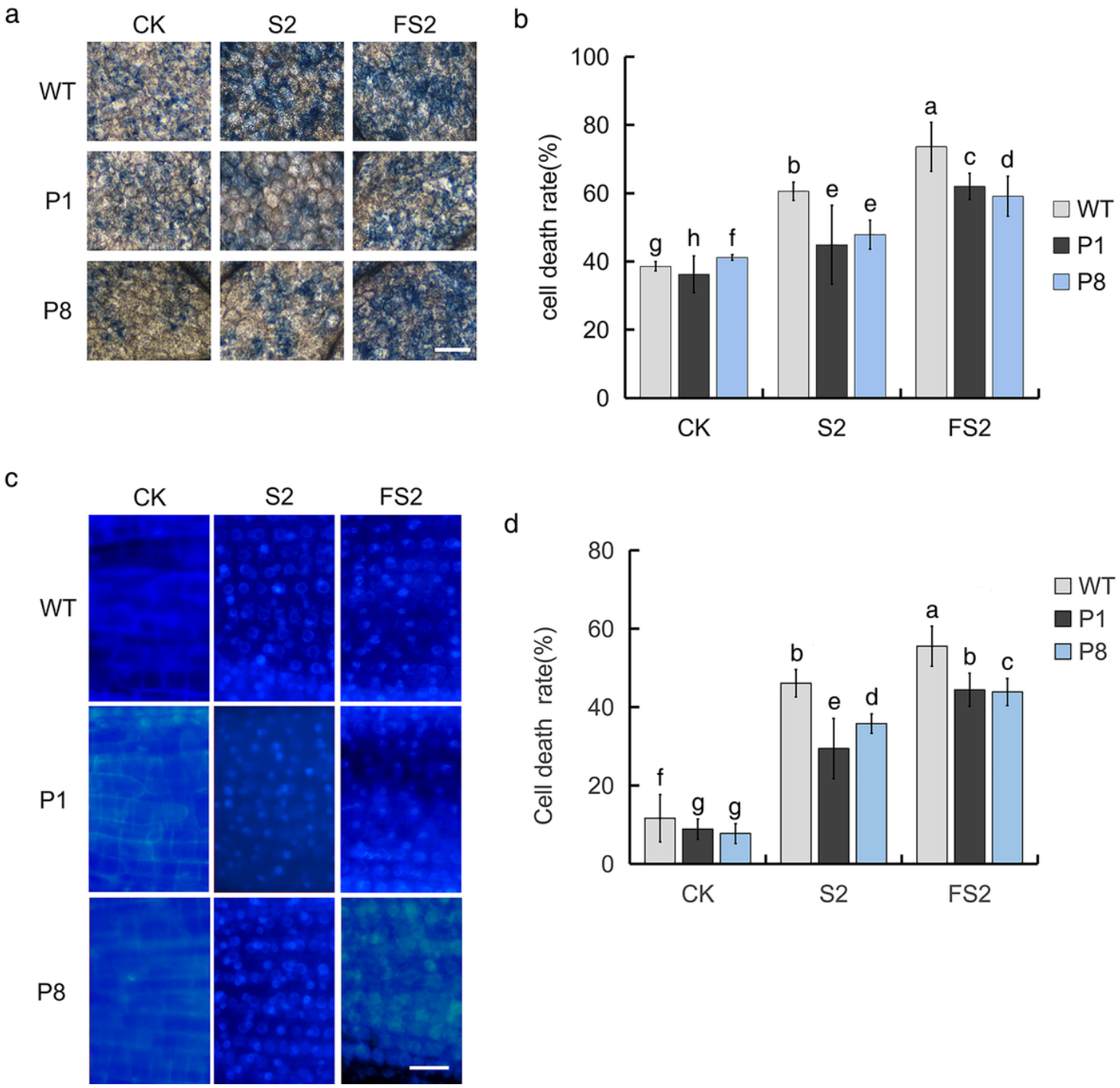


Figure 4

Effect of *HaFT-1* overexpression on cell death of seedlings under heat stress priming-and-triggering treatment

(a) Trypan blue staining (bar=50 μm). (b) Cell death rates in leaves. (c) Hoechst33342 staining (bar=50 μm). (d) Cell death rates in roots. Statistical significance was determined with Student's t-test (P<0.05),

and is labeled with lower case letters.

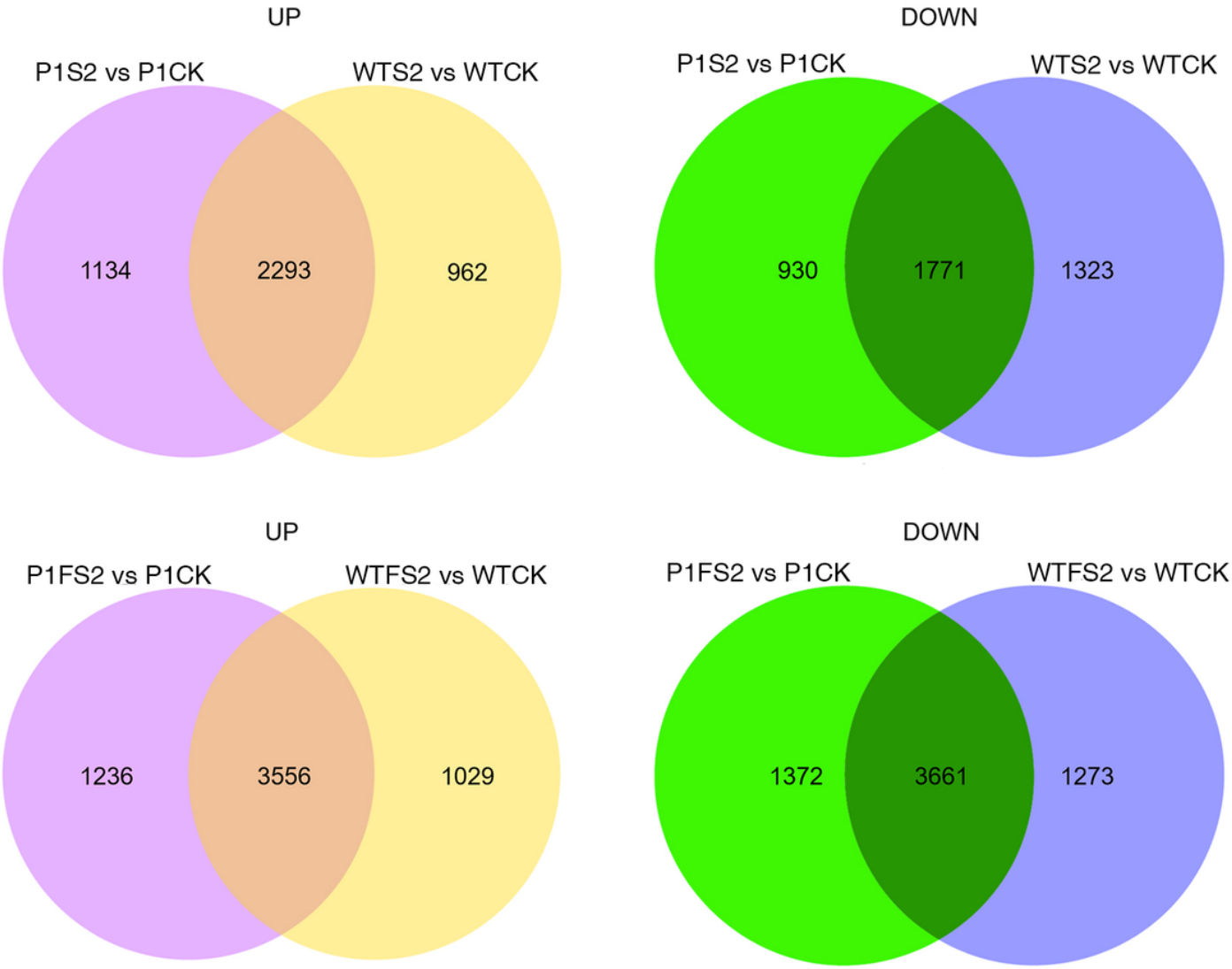


Figure 5

Venn diagram summarizing the genes overlapping between P1 plants and WT *Arabidopsis* at S2 and FS2 stages.

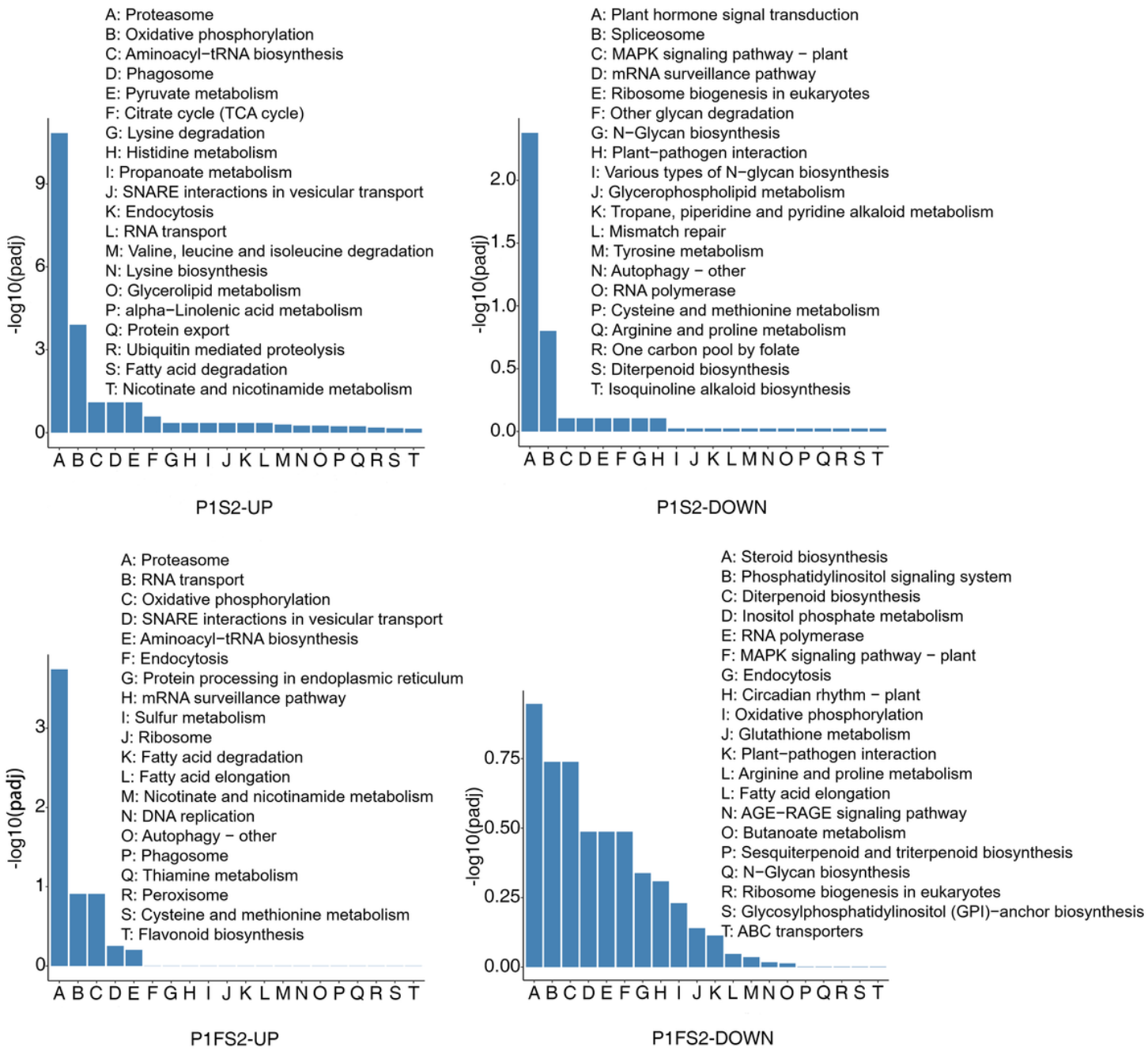


Figure 6

KEGG-enriched pathways among genes specifically induced or repressed in P1 plants at S2 and FS2 stages

Figure 7

ROS scavenging activity and chlorophyll content of P1, P8, and WT *Arabidopsis* under HS priming-and-triggering treatment

(a) DAB staining. (b) Enzyme activity of POD. (c) Enzyme activity of SOD. (d) Chlorophyll content. Statistical significance was determined with Student's t-test($P<0.05$), and is labeled with lower case letters.

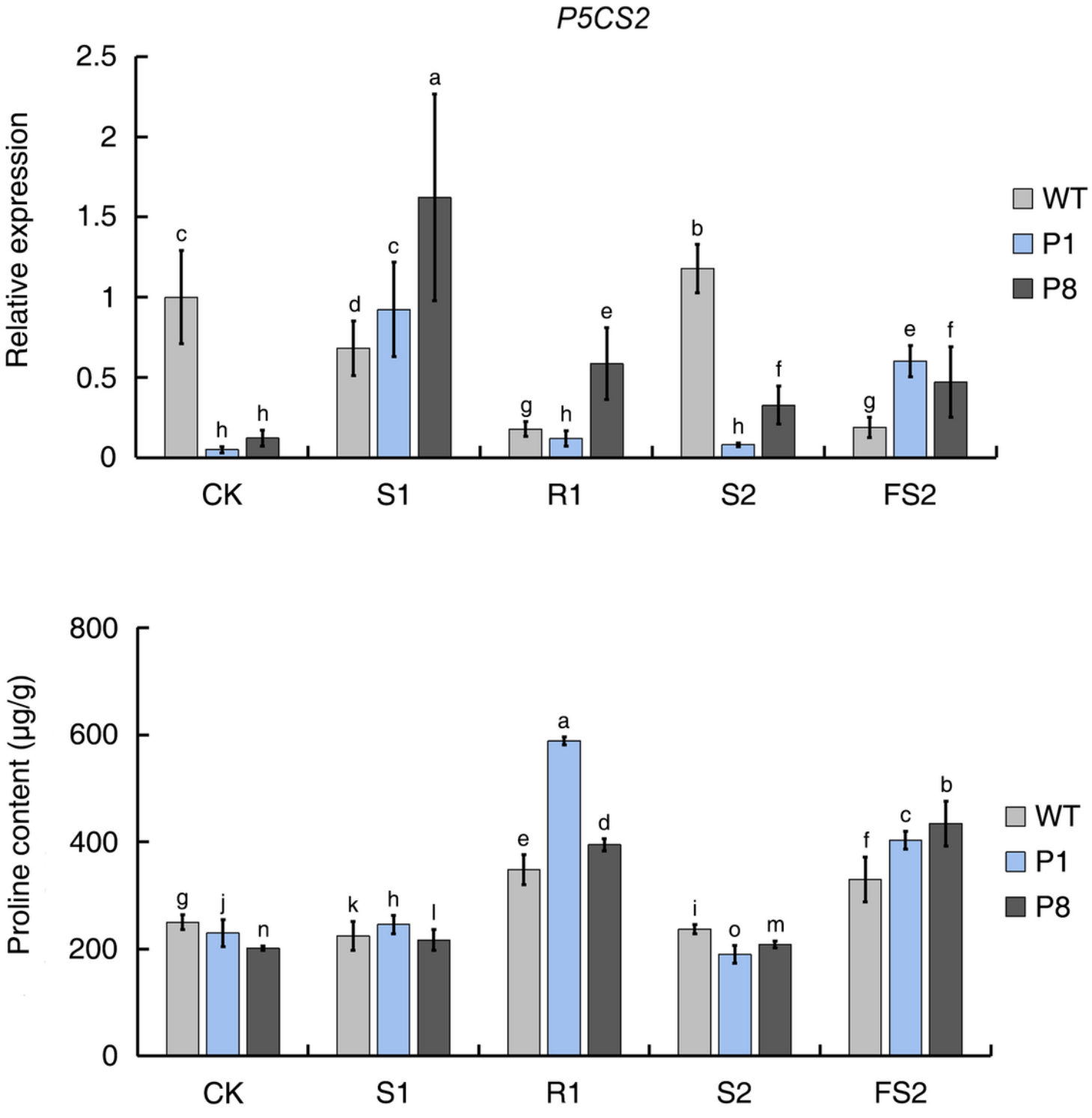


Figure 8

Expression of *P5CS2* and proline content of P1, P8 and WT *Arabidopsis* during HS priming-and-triggering treatment

Supplementary Files

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