Overexpression of Seagrass Nucleotide Exchange Factor Gene ZjFes1 Enhances Heat Tolerance in Transgenic Arabidopsis

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ABSTRACT
Nucleotide exchange factors (NEFs) play an important role in plant abiotic stress response, but their characteristics and functions in seagrass have not been studied. Zostera japonica (Z. japonica) is one of the most widely distributed seagrass species in China and are distributed in subtropical and temperate coastal areas. Z. japonica is intertidal seagrass, which often undergoes heat stress during summer when the tide ebbs. Overexpression of ZjFes1 in Arabidopsis results in an increase in heat tolerance. We found that ZjFes1 associates with ZjHsp70 in vivo by yeast two-hybrid and bimolecular fluorescence complementarity (BiFC). Overexpression of ZjFes1 leads to selective reduction of Hsp70 transcription and an increase in Hsp101. In conclusion, our results suggest that ZjFes1 may be an active regulator of heat tolerance.

Introduction
High temperature is one of the worst environmental factors in plant growth, which limits plant development and crop productivity, and further leads to huge economic losses. Temperature is the key factor to control the growth of seagrass. It can affect the physiological and biochemical processes of seagrass. The change of water temperature beyond the optimum temperature range for growth will have a negative impact on the biochemical process of Zostera marina. The photosynthetic rate and respiratory rate of seagrass increased with the increase of temperature in a certain range, but when the temperature was higher than a certain threshold, the respiratory rate was higher than the photosynthetic rate, resulting in a negative net photosynthetic rate. In order to resist high temperature, plants have evolved many complex mechanisms. All organisms respond to heat stress by inducing heat-shock proteins (Hsps). Hsp70s are the most abundant type of Hsps and play important roles in preventing misfolding and aggregation of newly synthesized proteins. Hsp70s have a highly conserved ATPase domain and a substrate-binding domain (SBD). ATP binds to ATPase domain and induces conformational changes of SBD, which opens the substrate-binding pocket and temporarily binds to hydrophobic region in the peptide. Under the stimulus of substrate binding and synergistic chaperone J domain, Hsp70 hydrolyzed ATP to ADP, triggered the conversion of SBD to closed conformation, and captured the substrate. NEFs promote the release of ADP, and SBD returns to open conformation and releases substrates. Under physiological conditions, the nucleotide exchange step is a speed-limiting step, so it is very important for the functional cycle of Hsp70s. Many proteins and co-partners participate in the Hsp70 ATPase cycle. Proteins containing J domain, such as DnaJ in E. coli, Ydj1p in yeast, Hsp40 in animal cells and J domain proteins in Arabidopsis thaliana, are ubiquitous, which can stimulate ATP hydrolysis. For different chaperone systems, nucleotide exchange reaction is accelerated by different NEFs. The NEFs are represented by GrpE in E. coli, Fes1p and Ssb1p in yeast, and Bag-1 and HspBP-1 in animals.

Hsp70 binding protein-1 (HspBP-1) was initially identified as binding to human Hsp70, and was subsequently found to be a functional NEF of cytosolic Hsp70s. Thereafter, it was found to inhibit the carboxyl end of the Hsp70 interacting protein (CHIP), which is a ubiquitin ligase that directs client proteins to proteasomes. Therefore, HspBP-1 was considered to be a regulator of the fate of substrate proteins. Hspbp-1 homologues have also been found in other eukaryotes. For example, the yeast Fes1p, a cytoplasmic protein, is an orthologue of HspBP-1 and serves as an NEF.

In Arabidopsis thaliana, AtFes1A not only plays an important role in heat resistance, but also plays a major role in heat response signal transduction pathway. Therefore, nucleotide exchange factors play an important role in the molecular mechanism of plant resistance to abiotic stress and improve plant tolerance to abiotic stress.

J proteins are a kind of molecular chaperone with J domain, also called Hsp40s, which interact with Hsp70s. J protein localized in chloroplast maintained the stability of photosystem II under chilling stress. Tomato chloroplast localized J protein protects Rubisco activity under heat stress. The synergistic effect of Sis1 with Hsp70 and E3 ligase Ubr1 promotes the degradation of misfolded cytoplasmic proteins. Sis1 plays a key role in transferring substrates to Hsp104. The interaction between Hsp101 and proteasome promotes the removal of ubiquitinated protein aggregates. Hsp101 is essential for the formation of acquired thermotolerance in plants and other organisms. The interaction between Hsp101 and Hsa32 (heat-stress-associated 32 kD protein) in Arabidopsis prolongs thermal
acclimation memory by post-transcriptional regulation. During the recovery period, Hsp101 promoted the translation of Hsa32, and Hsa32 delayed the degradation of Hsp101. Under heat stress, if mitochondrial inhibitors such as sodium azide and dinitrophenol (DNP) were present, the expression of Hsp101 was inhibited and thermotolerance decreased in cultured Arabidopsis cells.21

Zostera japonica belongs to the Zosteraceae family. It is a unique species of seagrass in Asia. It mainly distributes in Japan, Korea, and China. Seagrass meadows are considered as one of the most productive ecosystems,22 which provides ecosystem services including: important habitat for marine organisms, food source,23 and they play an important role in the global circulation of C, N and P. Seagrasses provide key ecological services, including organic carbon production and export, nutrient cycling, sediment stabilization, enhanced biodiversity, and trophic transfers to adjacent habitats in tropical and temperate regions.24,25 Z. japonica is one of the most widely distributed seagrass species in China and are distributed in subtropical and temperate coastal areas. At present, there is no study on the molecular mechanism of heat tolerance of Z. japonica. In our previous study, we isolated and identified a heat-resistant NEF gene ZjFes1 from Z. japonica (unpublished data). In this study, we cloned the nucleotide exchange factor ZjFes1 from Z. japonica and overexpressed it in Arabidopsis, and found that ZjFes1 increased the tolerance of transgenic plants to heat stress.

Materials and methods

Isolation of ZjFes1 and construction of overexpression vector

Total RNA from Z. japonica leaves was isolated using an RNAPrep Pure Plant Kit (DP441, Tiangen, Beijing, China) according to the manufacturer’s instructions and reversely transcribed. The first-strand cDNA was used as a template for amplification with the primers ZjFes1-pGWB17-F and ZjFes1-pGWB17-R (Table 1). PCR involved use of 2× TransStart® FastPfu PCR SuperMix (-dye) (Trans, China) and was as follows: preheating at 95°C for 5 min, then 40 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 20 s and extension at 72°C for 1 min, then a final extension at 72°C for 5 min. The product was cloned into TOPO® vector (ThermoFisher, USA) using the pENTR™ Directional TOPO® Cloning Kits (K2400-20, Invitrogen, USA) according to the manufacturer’s instructions and sequenced. The 35S::ZjFes1 construct was generated by cloning the fragment under the control of a CaMV35S promoter in the pGWB17 binary vector using Gateway® BP Clonase™ II Enzyme Mix (11789–020, Invitrogen, USA) according to the manufacturer’s instructions.

Generation of Arabidopsis transgenic lines

The 35S: ZjFes1 recombinant plasmid was introduced into Agrobacterium tumefaciens C58 by freeze-thaw method and transformed into Arabidopsis thaliana (ecotype Columbia) by floral dip method.26 The seeds of transformed plants were seeded on agar plate with hygromycin (25 µg/ml), then cultured in 16 h of light and 8 h of darkness for 14 days. Resistant seedlings were transplanted into the soil and continued to grow. The transformed plants were identified by RT-PCR with the primers ZjFes1-F, ZjFes1-R, Actin-RT-F, Actin-RT-R (Table 1). RT-PCR involved use of 2× EasyTaq® PCR SuperMix (Tran, China) and was as follows: preheating at 94°C for 2 min, then 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s, then a final extension at 72°C for 5 min. The seeds were harvested separately. Homozygous progenies of T3 were screened by hygromycin resistance for further experiments.

Heat treatment of Arabidopsis

Three 35S: ZjFes1 transgenic lines and wild-type plants were planted in a square dish (10 cm wide) with 1/2MS medium in parallel, stratified in 4°C for 3 days, and then cultured in an incubator for 21°C. We measured the acquired thermotolerance using a stepwise temperature-increasing program (STI). In the STI program, 5-day-old seedlings were acclimated for 2 h at 38°C, and immediately subjected to a lethal heat treatment of 45°C for varying periods. The plants were then transferred to normal growth conditions for recovery.

Yeast two-hybrid test

Yeast two-hybrid analysis was carried out according to the user manual of Matchmaker Gold yeast two-hybrid system. The pGBK T7 and pGADT7 vectors (Clontech) were digested by BamHI and EcoRI and purified by column. The coding region of ZjFes1 and ZjHsp70 was amplified from plasmids by PCR, and sequenced. The first-strand cDNA was used as a template for PCR amplification. The primers are listed in Table 1. Yeast two-hybrid test

Table 1. Primer in this study.

| Name | Sequence |
|------|----------|
| ZjFes1-pGWB17-F | CACTATGGCGAAGGACGTTGATAATCC |
| ZjFes1-pGWB17-R | CTTGCAACGCTGCAACGGCCTACAAGT |
| ZjFes1-F | ATGCTCACAAGCCGCTACAAGT |
| ZjFes1-R | GCGATCTGGACGCTGCTGAGGAC |
| Actin-RT-F | GAGATCAAGCTGCAAGCAGG |
| Actin-RT-R | CTGTTTGATATTAGTGGTTT |
| ZjFes1-pSYNE-35S-F | TCTAGATTCGACGACGCTAAATG |
| ZjFes1-pSYNE-35S-R | TCTAGATTCGACGACGCTAAATG |
| ZjHsp70-pSYCE-35S-F | TCTAGATTCGACGACGCTAAATG |
| ZjHsp70-pSYCE-35S-R | TCTAGATTCGACGACGCTAAATG |
| Hsp101-F | TTTCCTCCACCA CCTATCCT |
| Hsp101-R | TTTCCTCCACCA CCTATCCT |
| Hsp70-F | CTTGTTTGATATTAGTGGTTT |
| Hsp70-R | TCTAGATTCGACGACGCTAAATG |
| shSHP Class I-F | TCAAGCAGTCGACGCTCAAGC |
| shSHP Class I-R | TCAAGCAGTCGACGCTCAAGC |
| Hsf21-F | GCCCTGCAAATGAATACTG |
| Hsf21-R | GCCCTGCAAATGAATACTG |
| Hsp60-F | ACCATGTCAAAAGCAGGCTGTCACCA |
| Hsp60-R | ACCATGTCAAAAGCAGGCTGTCACCA |
respectively. The junctions contained 24 base sequences homologous to ZiFes1 or ZjHsp70 and 15 base sequences homologous to the linearized end of pGBK7 or pGADT7 vectors, respectively. ZiFes1 and pGBK7 were mixed, ZjHsp70 and pGADT7 were mixed, and in-Fusion enzyme was used for fusion cloning. The yeast transformation system 2 (provided in the kit) was used to transform 100 ng expression vector pGBK7-ZiFes1 or pGADT7-ZjHsp70 into the yeast strain Y2H Gold or Y187 (Clontech) respectively. 100 μl of 1/10 dilution was applied to a 90 mm plate containing selective mediums without tryptophan (SM-W) or leucine (SM-L). The plate was incubated at 30°C until the colony appeared. 2–3 mm clones were selected for small-scale mating operation. Two clones were added to the same 1.5 ml centrifugal tube containing 500 μl 2 X YPDA. The centrifugal tube was incubated with shaking at 200 rpm overnight at 30°C for 24 hr. 100 μl of 1/10 dilution was spread onto a 90 mm plate containing selective mediums without tryptophan and leucine (DDO). The plate was incubated at 30°C until the colony appeared. The transformers were transferred to selective medium without leucine, tryptophan, histidine and adenine, but added with X-a-Gal and aureobasidin (QDO/X/A). The interaction between p53 and T proteins and Lam and T proteins were used as positive and negative controls, respectively. When the detected gene was co-transformed with pGADT7 or pGBK7 empty vector, autoactivation was analyzed by a growth experiment. The results are based on three independent biological repeats.

BiFC

The BiFC analysis was based on the previously described method and slightly modified.27 ZiFes1 or ZjHsp70 were fused into N-terminal of YFP or C-terminal of mVenus respectively and transformed into Agrobacterium strain C58. Overnight cultures of Agrobacterium were collected by centrifugation and were suspended to 0.6 OD600 in a suspension buffer (10 mM MES, 10 mM MgCl2, 150 μM acetyleneugenol), mixed with C58 expressing p1300-p19 and incubated at room temperature for 2 h. Agrobacterium suspension was injected into 3-week-old Nicotiana Benthamiana leaves with a 1 ml syringe (without metal needles). After injection, plants were cultured under 16 h light and 8 h darkness for 3 days. By fluorescence microscope (OLYMPUS, BX51), leaves from the infiltrated tobacco plants were illuminated with blue light (460–495 nm) to make GFP green (510–550 nm).

qRT-PCR analysis of heat-responsive gene expression in 35S:ZjFes1 transgenic Arabidopsis thaliana

Ten-day-old 35S:ZjFes1 seedlings and wild-type plants grown on 1/2MS medium were incubated for 2 hours at 38°C or 45°C respectively, and harvested. Total RNA was extracted and reversely transcribed. Primer pairs for qRT-PCR are described.14 Primer sequences are listed in Table 1. CFX96 Real-Time PCR Detection System (Bio-Rad) was used for qRT-PCR amplification. The reaction system was 20μl, containing 100 ng cDNA, 0.4 μM of each primer, and 1× TB Green Premix Ex Taq II (Tli RNaseH Plus) (RR420Q, Takara, Japan). The conditions for the qRT-PCR amplification were as follows: incubation at 95°C for 30 sec, 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Each reaction was performed in triplicate, including three non-template controls. Statistical analysis of gene relative expression level was calculated by the 2^(-ΔΔCT) method. Actin2 was used as a reference gene. The relative mRNA level of wild type (0 h) without heat treatment was normalized to 1.

Western blot

Plant protein was extracted from seedlings with Plant protein extraction kit (CW0885M, CWBIO, China) according to the manufacturer’s instructions. We added SDS-PAGE Sample Loading Buffer (P0015, Beyotime, China) to the collected protein samples according to the manufacturer’s instructions. We used PageRuler Prestained Protein Ladder (26616, Thermo Scientific, USA) to observe the electrophoretic effect and membrane transfer effect, as well as to judge the protein molecular weight. We used BeyoGel™ SDS-PAGE Precast Gel (P0053A, Beyotime, China) according to the manufacturer’s instructions. During electrophoresis, SDS-PAGE Electrophoresis Buffer (P0014B, Beyotime, China) was used. Low voltage constant voltage electrophoresis was used in the upper layer, while high voltage constant voltage electrophoresis was used in the lower layer. When the bromophenol blue reached the bottom of the gel, the electrophoresis was stopped. We used nitrocellulose membranes. The Filter Paper for Blotting Transfer (FFP51, Beyotime, China) was used for membrane transfer. The Western Transfer Buffer (P0021B, Beyotime, China) was used for membrane transfer. We set the film transfer current to 300mA and the film transfer time to 60 min. We put the film transfer tank in the ice bath for film transfer. After the membrane transfer, we immediately put the protein-membrane into TBS-T (T9142, Takara, Japan), rinsed for 1 min to remove the membrane transfer solution. We added Western BLOT Blocking Buffer (T7131A, Takara, Japan), shake it slowly on the shaker, and blocked it at room temperature for 10 min. Primary antibodies were anti-Hsp101/ClpB heat-shock protein N-terminal polyclonal antibody (AS07 253; Agrisera, http://www.agrisera.com), HSC70 (plant) monoclonal antibody (5B7) (ADI-SPA-817; Enzo, http://www.enzolifesciences.com) and Actin polyclonal antibody (AS13 2640; Agrisera, http://www.agrisera.com). We added TBS-T, and washed it slowly on the shaker for 5 min. We washed three times in total. Secondary antibodies were ProteinFind® Goat Anti-Rabbit IgG (H + L) HRP Conjugate (HS101-01, Tran, China) and ProteinFind® Goat Anti-Mouse IgG (H + L), HRP Conjugate (HS101-01, Tran, China). We added TBS-T, and washed it slowly on the shaker for 5 min. We washed three times in total. We used TaKaRa DAB Substrate (MK210, Takara, Japan).

Results

Overexpression of ZjFes1 enhances thermotolerance of transgenic Arabidopsis thaliana

In order to study the function of ZjFes1 (GenBank accession no. MK124711) in plants, we constructed transgenic Arabidopsis lines carrying 35S:ZjFes1 construct (Figure 1a). Seven independent transgenic lines were identified by hygromycin resistance.
T3 homozygous progenies of transgenic lines (OE1, OE2, and OE3) were selected for further study. The transcript level of ZjFes1 was detected by RT-PCR (Figure 1b). Under normal growth conditions, there was no phenotypic difference between transgenic Arabidopsis and wild type. Transgenic Arabidopsis and wild type were grown in 0.5× MS medium containing 1% sucrose for 10 days to evaluate the thermotolerance of transgenic Arabidopsis. In order to study the thermotolerance of transgenic Arabidopsis, STI program was used to measure the thermotolerance.

In STI treatment (Figure 1c), 5-day-old seedlings were acclimated at 38°C for 2 h, and were immediately treated at 45°C lethal temperature for different periods of time. Transgenic Arabidopsis are more heat-resistant than wild type (Figure 1c).

ZjFes1 binds ZjHsp70 in vivo

Protein–protein interactions (PPIs) play important roles in various biological processes. ZjFes1 is a homologous gene of Arabidopsis AtFes1A, which binds Hsp70 in vivo and in vitro, without NEF activity in vitro. In our previous studies, we found that ZjFes1 was located in the cytoplasm and ZjHsp70 in the cytoplasm and nucleus, and their expression was induced by heat (unpublished data). We, therefore, tested the association between ZjFes1 and ZjHsp70 (GenBank accession no. MK359368) by yeast two-hybrid assay. The result showed that ZjFes1 interacted with ZjHsp70 in vivo (Figure 2a).

In the study of biochemistry, genetics and imaging methods of PPIs, visualization of PPIs in living cells is the key to understand its cellular function. BiFC analysis is one of the imaging tools for direct observation of PPIs in living cells. In order to determine the in planta site of interaction between ZjFes1 and ZjHsp70, BiFC experiments were carried out in tobacco epidermal cells. Strong fluorescence signals were observed in the cytoplasm, while no fluorescence signals were observed in the negative control (Figure 2b).

Regulation of heat response by ZjFes1

The expression of heat shock transcription factors (Hsfs) and Hsp increased significantly under heat stress, which was closely related to the acquisition of thermotolerance. In order to find out the possible reasons for overexpression of ZjFes1 enhances thermotolerance of transgenic Arabidopsis thaliana, we used qRT-PCR to quantitatively analyze the transcription levels of heat tolerance-related Hsfs and Hsps at different temperatures (Figure 3). HsfA2 is a heat-inducible transcription factor, which is required for extension of acquired thermotolerance in Arabidopsis. Hsp70 affects the regulation of Hsfs, and the acquisition of thermotolerance in transgenic Arabidopsis thaliana. Hsp101 is one of the key Hsps essential for acquired thermotolerance in Arabidopsis thaliana and other plants, which is a member of the Hsp100/ClpB chaperones in the AAA+ family. All the genes tested were expressed at low levels at normal temperature (21°C). When plants were incubated at 38°C for 2 h, the transcription level of Hsp70 in transgenic Arabidopsis was significantly lower than that of wild type, while there was no significant difference in other genes. qRT-PCR data analysis
(Livak method) was shown in Data S1. These results suggest that ZjFes1 overexpression specificity led to a strong down-regulation of Hsp70 transcription. Therefore, we concluded that ZjFes1 mediates the regulation of heat response.

Figure 2. ZjFes1 interacted with ZjHsp70 in vivo. (a) Yeast two-hybrid analysis of the interaction between ZjFes1 and ZjHsp70. The interaction between p53 and T proteins and Lam and T proteins were used as positive and negative controls, respectively. DDO indicates selective mediums without tryptophan and leucine. QDO/X/A indicates selective medium without leucine, tryptophan, histidine and adenine, but added with X-α-Gal and aureobasidin. (b) BiFC analysis showed that ZjFes1 interacted with ZjHsp70. N. benthamiana was co-transformed with ZjFes1-nYFP and ZjHsp70-VC155.

Figure 3. Differential transcription of heat-induced genes in the wild type (WT) and transgenic lines (OE1 and OE2). 10-day-old seedlings were incubated at a specified temperature for 2 h and then used to extract RNA. Transcription levels of thermotolerance-related genes HsfA2 (AT2G26150), HsfB1 (AT4G36900), Hsp60 (AT2G28000), Hsp70 (AT3G09440), Hsp101 (AT1G74310), sHsp class I (AT5G46230) and sHSP class II (AT5G12030). Quantitative analysis was performed by qRT-PCR. Actin2 was used as the reference gene.
Role of cytoplasmic Hsp70 in heat response\textsuperscript{31,32} implies that the down-regulation of Hsp70 transcription in transgenic Arabidopsis should result in the reduction of Hsp70 protein, which in turn promotes the expression of other Hsps.\textsuperscript{33} In fact, there was no difference in the expression level of other heat tolerance-related Hsfs and Hsps between wild type and transgenic Arabidopsis. Therefore, we measured the cytoplasmic Hsp70 level (Figure 4). After heat stress, the level of Hsp70 protein in transgenic Arabidopsis did not differ from that in wild type, which was inconsistent with the significant downregulation of its transcripts (Figure 4). We measured the level of Hsp101 because it was associated with acquired thermotolerance.\textsuperscript{34} Figure 4 shows that Hsp101 in transgenic Arabidopsis is higher than that of wild type, while there is no significant difference in transcription level. Therefore, the increase of Hsp101 in transgenic Arabidopsis after heat stress is a new discovery.

**Discussion**

In this study, we cloned the nucleotide exchange factor ZjFes1 from Z. japonica and overexpressed it in Arabidopsis, and found that ZjFes1 increased the tolerance of transgenic plants to heat stress. OE1, OE2, and OE3 exhibited different thermotolerance pattern, and this may be due to the different expression level of ZjFes1.

When exposed to different temperatures, the accumulation of heat shock proteins is different, and the level of heat shock proteins is positively correlated with the acquired thermotolerance. The role of Hsp101 in thermotolerance has been extensively studied. During the recovery period of an intermittent temperature-increasing program (ITI), heat shock proteins such as Hsp101 accumulated continuously.\textsuperscript{35} The higher the levels of Hsp, the greater the thermotolerance. In Arabidopsis thaliana, Hsp101, Hsp70, small Hsps, and substrates form complex after severe heat stress,\textsuperscript{36} so Hsp101 should be cleared with its partners. On the contrary, Hsp101 mRNA has the internal ribosome entry site (IRES) structure that promotes translation, which makes Hsp101 protein accumulate continuously.\textsuperscript{36} In our work, the HSP101 protein level in transgenic Arabidopsis was higher than that of wild type (Figure 4). Therefore, we suspect that the increase of thermotolerance of transgenic Arabidopsis is related to the increase of Hsp101.

In our work, overexpression of ZjFes1 specifically inhibited the transcription of Hsp70 (Figure 3), but there was no difference in Hsp70 protein level between wild type and transgenic Arabidopsis (Figure 4). We speculate that this is the result of selective slowdown of Hsp70 degradation, rather than the acceleration of Hsp70 translation, because translation regulation usually affects the synthesis of many proteins at the same time. The degradation of Hsp70 is regulated by the Hsp70 binding protein CHIP. Hsp70 and its substrates can form complex with CHIP, and the substrates bound by Hsp70 are ubiquitinated preferentially.\textsuperscript{35} When ubiquitinated Hsp70 complexes bind proteasomes, misfolded substrates are degraded and subsequently bound Hsp70 is proteolyzed.\textsuperscript{37} Several factors are involved in the degradation of Hsp70 substrate complex. For example, HspBP-1 inhibits the degradation of immature cystic fibrosis transmembrane-conductance regulator (CFTR) induced by CHIP, thus stimulating the maturation of CFTR. HspBP-1 is considered to be a regulatory factor that can lead chaperone-substrate complexes to the refolding pathway.\textsuperscript{15} AtFes1A prevents ubiquitination of Hsp70 substrate complex in Arabidopsis thaliana.\textsuperscript{14} There was no difference in protein level (Figure 4), while down-regulation of transcription level of Hsp70 (Figure 3), strongly suggesting that overexpression of ZjFes1 inhibited degradation of Hsp70. The results showed that the nucleotide exchange factor could inhibit Hsp70 degradation in both Arabidopsis thaliana and seagrass. In our study, the characteristics of ZjFes1 indicate the complexity of the regulatory mechanism affecting plant Hsp70 activity.

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**Author Contributions**

SC designed the study and performed the laboratory experiments. GQ designed the fieldwork. SC wrote the main part of the manuscript. All authors reviewed the manuscript and added details to it.

**Data Availability**

All data generated or analyzed during this study are included in this published article. The nucleotide and deduced amino acid sequence data of ZjFes1 and ZjHsp70 was registered in the GenBank (No. MK124711, MK359368). We have requested that our data are to be held confidential until December 2, 2019 and February 4, 2020, respectively. They will not be released to the public database until this date, or until the data or accession numbers appear in print, whichever is first.

![Figure 4](image-url). The effect of high temperature on the accumulation of Hsp101 and Hsp70 proteins in wild type and transgenic lines. The 10-day-old seedlings grown on 1/2MS medium were treated at a specified temperature for 2 h for protein extraction. Immunoreactive bands of Actin in a duplicate SDS-PAGE gel were used to confirm equal protein loading.
Disclosure of interest
The authors report no conflict of interest.

Ethical approval
This article does not contain any studies with human participants or animals performed by any of the authors.

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