Characterization of a wheat HSP70 gene and its expression in response to stripe rust infection and abiotic stresses

Ying-Hui Duan · Jun Guo · Ke Ding · Shu-Juan Wang ·
Hong Zhang · Xi-Wei Dai · Yue-Ying Chen ·
Francine Govers · Li-Li Huang · Zhen-Sheng Kang

Abstract Members of the family of 70-kD heat shock proteins (HSP70 s) play various stress-protective roles in plants. In this study, a wheat HSP70 gene was isolated from a suppression subtractive hybridization (SSH) cDNA library of wheat leaves infected by *Puccinia striiformis* f. sp. *tritici*. The gene, that was designated as *TaHSC70,* was predicted to encode a protein of 690 amino acids, with a molecular mass of 73.54 KDa and a pI of 5.01. Further analysis revealed the presence of a conserved signature that is characteristic for HSP70s and phylogenetic analysis demonstrated that TaHSC70 is a homolog of chloroplast HSP70s. *TaHSC70* mRNA was present in leaves of both green and etiolated wheat seedlings and in stems and roots. The transcript level in roots was approximately threefold less than in leaves but light–dark treatment did not charge *TaHSC70* expression. Following heat shock of wheat seedlings at 40°C, *TaHSC70* expression increased in leaves of etiolated seedlings but remained stable at the same level in green seedlings. In addition, *TaHSC70* was differentially expressed during an incompatible and compatible interaction with wheat-stripe rust, and there was a transient increase in expression upon treatment with methyl jasmonate (MeJA) treatment. Salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) treatments had no influence on *TaHSC70* expression. These results suggest that TaHSC70 plays a role in stress-related responses, and in defense responses elicited by infection with stripe rust fungus and does so via a JA-dependent signal transduction pathway.

Keywords Wheat · HSP70 · Stripe rust · Expression profile · Abiotic stress

Ying-Hui Duan and Jun Guo contributed equally to this work.

Y.-H. Duan · J. Guo · K. Ding · S.-J. Wang · H. Zhang ·
X.-W. Dai · Y.-Y. Chen · L.-L. Huang · Z.-S. Kang (✉)
College of Plant Protection and Shaanxi Key Laboratory of
Molecular Biology for Agriculture, Northwest A & F University,
Yangling 712100, Shaanxi, People's Republic of China
e-mail: kangzs@nwsuaf.edu.cn

F. Govers
Laboratory of Phytopathology, Plant Sciences Group,
Wageningen University, Binnenhaven 5, 6709 PD Wageningen,
The Netherlands

Introduction

Heat shock proteins (HSPs) are a class of ubiquitous and highly conserved proteins which show increased expression in response to an elevated temperature or other forms of environmental stress [1]. They are proposed to be fundamental and essential for cell survival [2]. HSPs can be produced at particular stages of the cell cycle or during development in the absence of stress, or constitutively present in both normal and stressed cells [3]. The 70-kDa heat shock proteins (HSP70) are encoded by one of the major HSP multigene family, and are ubiquitous in both eukaryotes and prokaryotes. In eukaryotes, the members of HSP70s are located in major subcellular compartments, including the cytoplasm, the lumen of the endoplasmic reticulum, the matrix of mitochondria and in chloroplasts of plants. The various subcellular localizations implies both functional distinction and phylogenetic divergence [3, 4]. HSP70s belong to one of the most conserved protein families [5]. The characteristic features of a HSP70 protein are a conserved amino (N)-terminal ATPase domain of

approximately 44-kDa [6] and a carboxyl (C)-terminal peptide binding domain of approximately 25-kDa which is further subdivided into a *b*-sandwich subdomain of 15 kDa and a C-terminal *a*-helical subdomain [7]. HSP70s function as molecular chaperones in the folding and refolding of nonnative proteins to prevent irreversible aggregation [8–10], and play roles in protein transport and assembly processes [11, 12]. They are also involved in interaction with signal transduction proteins [13], and this is not necessarily related to function as a chaperone [14].

HSP70s are fundamental in developmental processes and function in environmental stress, including heat, cold, heavy metal, water deficit, oxidative stress, wounding and so on [15–19]. In addition, HSP70 also are important in pathological processes. In animals, HSP70s exert activities in processes such as oncogenesis, neurodegenerative and autoimmune diseases and viral infections [20–22]. In plants, it was reported that HSP70 and HSP90 are essential components in the INF1-mediated hypersensitive response in *Nicotiana benthamiana*, and that they function upstream or independent of the MAPK cascade [23]. Moreover, various reports describe that HSP70 expression in plants is induced upon infection with pathogens [23–26], but more detailed analysis of HSP70 expression profiles during plant-pathogen interactions are scarce.

In a previous study, we constructed a suppression subtractive hybridization (SSH) cDNA library of wheat leaves infected by *Puccinia striiformis* f. sp. *tritici* to identify genes that are differentially expressed and involved in defense responses of wheat [27]. In the wheat SSH cDNA library, one of the expressed sequence tags (ESTs) that we sequenced was highly homologous to the rice HSP70 gene. In this study, we obtained a full-length cDNA of wheat HSP70 gene using rapid amplification of cDNA ends (RACE) and denominated the gene *TaHSC70*. The characteristics of the *TaHSC70* cDNA sequence and deduced amino acid sequences were analyzed by a series of bioinformatic tools. Moreover, we analysed the expression patterns of *TaHSC70* in wheat plants exposed to a variety of abiotic stress treatments and upon inoculation with the stripe rust fungus.

Materials and methods

Plant material, pathogen inoculation and abiotic stress treatment

Wheat (*Triticum aestivum* L.) cultivar Suwon11 and stripe rust (*Puccinia striifromis* f.sp. *tritici*) strains CYR23 and CYR31 were used throughout this study. Suwon11 displays HR upon infection with strain CYR23, but is susceptible to CYR31. Suwon 11 is presumed to contain the stripe rust

resistant gene *YrSu* [28, 29]. Wheat plants were grown and maintained following the description of Kang and Li [30]. Freshly collected stripe rust urediospores were applied with a paintbrush to the surface of the primary leaves of 8-day old wheat seedlings. After inoculation, the seedlings were kept in a high humid chamber at 100% humidity for 24 h to ensure the maximal rate of infection. Subsequently, the seedlings were transferred to a growth chamber at 15°C with a regular day–night cycle. A control inoculation was made with sterile water. Wheat leaves were sampled at 0, 12, 18, 24, 36, 48, 72, 96 and 120 h post-inoculation (hpi), quickly frozen in liquid nitrogen and stored at −80°C prior to extraction of total RNA.

For light–dark treatment, 8-day old wheat seedlings were transferred to the dark for 24 h, after which they were returned to the light for 24 h. For heat-shock treatment, 8-day old wheat seedlings and etiolated seedlings grown in the dark chamber, were incubated at 40°C for 2 h. For chemical treatment, leaves of wheat seedlings were sprayed with 100 μM SA, 100 μM ET, 100 μM MeJA and 100 μM ABA according to Zhang's method [31]. Distilled water containing 0.025% (w/v) Tween-20 was used as a mock treatment. The samples were harvested at the time points as indicated in each experiment. For tissue specificity analysis, leaves, stems and roots of normal wheat seedlings and leaves of etiolated seedlings were sampled.

Total RNA extraction and reverse transcription

Total RNA was extracted using RNeasy Plant Mini kit (Qiagen) according to the manufacturer's protocol. DNaseI treatment was applied to remove genomic DNA. First-strand cDNA was synthesized using SMART™ reverse transcription Kit (Clontech) for RACE (rapid amplification of cDNA ends) or M-MLV reverse transcriptase (Promega) with pd(N)6 random primer (Takara) in the presence of recombinant RNasin ribonuclease inhibitor (Promega) for gene expression analysis according to the manufacturer's instruction.

Cloning of the 5′ and 3′ ends of cDNA

The 5′ and 3′ ends of wheat HSP70 cDNA were obtained with a forward primer (5′-GACTCGCAGAGAACAGCAA CAAAGGATG-3′) and reverse primer (5′- GTCCCCAG ATGTGGAAAGCACCTCAAAT-3′), respectively, using the SMART™ RACE cDNA Amplification Kit (Clontech). Primers for 3′ ends of cDNA were designed from the EST LWSRC242 sequence (annotated as putative HSP70, GenBank accession no. EV253937) and primers for 5′ ends of cDNA were designed from the cDNA sequence obtained by 3′ RACE. PCRs were performed in a PTC-200 thermo-

cycler (MJ Research, Watertown, USA) with the following Program: 5 cycles of 95°C for 30 s, 72°C for 3 min; 5 cycles of 95°C for 30 s, 70°C for 30 s, 72°C for 3 min; 25 cycles of 95°C for 30 s, 68°C for 30 s, 72°C for 3 min; followed by 72°C for 10 min. PCR products were gel purified and cloned into pGEM-T Easy Vector. Ten positive clones were sequenced using an ABI PRISM 3130XL Genetic analyzer (Applied Biosystems). To verify the full-length cDNA after completing 5′- and 3′-RACE, a RT-PCR was performed with a pair of primers (a forward primer: 5′-TTCACTAGGGTTAGGGTTTACG-3′ and reverse primer: 5′-ACTCAGGGTCTATGTATCACTCAC-3′) that were designed based on the ends of 5′ and 3′ cDNA sequences.

Sequence analysis

cDNA sequence data were analyzed using DNASTAR software (http://www.dnastar.com), BLAST (http://www.ncbi.nlm.nih.gov/blast/), and ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/ gorf.html). Protein sequences were analyzed using Compute pI/MW tool (http://www.expasy.org/tools/pi_tool.html) for computation of the theoretical iso-electric point and protein molecular weight, InterPro-Scan (http://www.ebi.ac.uk/InterProScan/) and PROSITE Scan (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prosite.html) for prediction of the conserved domains and motifs, TargetIP (http://www.cbs.dtu.dk/services/SignalP/) for prediction of the signal peptide, TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) for transmembrane analysis, PSORT (http://psort.nibb.ac.jp/form.html) for prediction of subcellular localization, ClustalX 1.83 program for the sequence alignment and MEGA 3.1 software for constructing a phylogenetic tree.

Real time-PCR analysis

Real time-PCR was performed on a light cycler ABI Prism 7500 SDS (Applied Biosystems). Specific primers for *TaHSC70* gene and the housekeeping gene of wheat 18S ribosomal RNA (18S rRNA) were designed using the Primer Express Software (Applied Biosysterms) or primer premier 5.0. Real time-PCR reaction was performed with 25 μl 2× SYBR *Premix Ex Taq*™ (Takara), 1.0 μl ROX Reference Dye II, 2.0 μl 20 × first-strands cDNA, 0.2 μM of each primer in a total reaction of 50 μl. The reactions were subjected to 95°C for 1 min, 40 cycles at 95°C for 10 s, 58°C for 20 s, and 72°C for 40 s. Each reaction was done in triplicate and three non-template controls were included. Specificity of the amplicon was verified at the end of the PCR run using ABI Prism Dissociation Curve

Analysis software. Real time-PCR data were analysed according to Pfaffl's model (ΔΔCt method of relative gene quantification) [32] with 18S rRNA gene for normalization. Two parameters, relative quantity of RNA at least two-fold higher or lower than mock and *P*-value ≤0.005, were used to assess the significance of difference between time points. The primers designed for Real time-PCR were: wheat 18 s rRNA forward primer: 5′-TTTGACTCAACACGGGGA AA-3′, reverse primer: 5′-CAGACAAATCGCTCC- AC-CAA-3′; *TaHSC70* gene forward primer: 5′-TCAATGAC TCGCAG AGAACAG-3′, reverse primer: 5′-TGGAAAG CA CCTCAAAT ACAC -3′.

Results

Isolation and sequence analysis of *TaHSC70* cDNA

Total RNA samples were isolated from wheat leaves at 24, 48 and 72 hpi with CYR23 and used to synthesize first-strand cDNA. Two cDNA fragments of 844 bp and 1906 bp were obtained by 5′-RACE and 3′-RACE, respectively, and sequenced. DNASTAR software (www.dnastar.com) was used to combine the two fragments into an 2534 bp consensus sequence including an open reading frame (ORF) of 2073 nucleotides, a 5′-untranslated region (UTR) of 37 nucleotides and a 3′-UTR of 424 nucleotides plus a poly(A) tail (Supplementary Fig. 1). Furthermore, RT-PCR with gene specific primers corresponding to the 5′-UTR and 3′-UTR confirmed the combined sequence. BLAST analysis showed that the 2534 bp sequence shared significant identity (87%) to the rice full-length HSP70 gene (GenBank accession no. NM_001073018). Hence, we named the encoding gene *TaHSC70* (GenBank accession no. GQ280382).

The ORF of *TaHSC70* encodes a polypeptide of 690 amino acids. The protein sequence was calculated to have a molecular mass of 73.54 KDa and a pI of 5.01. The protein conserved domain search using Interproscan indicated that TaHSC70 protein contained a N-terminal ATPase domain and a C-terminal peptide binding region, but no apparent signal peptide sequences. In addition, PSORT algorithm allowed the prediction of a chloroplast localization for TaHSC70.

Alignment of TaHSC70 with full-length chloroplast HSP70s from other plant species (*Oryza sativa*, *Ipomoea nil*, *Spinacia oleracea*, *Arabidopsis thaliana*) revealed a strong amino acid sequence conservation (81.3 to 91.2% identity). Sequence alignment also revealed that TaHSC70 differed from other plant chloroplast HSP70s in the N and C termini (Supplementary Fig. 2). However, the C-terminal sequence (underlined residues in Supplementary Fig. 2) of TaHSC70 and other chloroplast HSP70s was highly

conserved. The conserved sequence was reported as a predictive localization motif for organelle HSP70s [33]. To illustrate this finding, 20 HSP70 sequences from plants, human, yeast, and prokaryotes were subjected to multiple alignment and a neighbor-joining phylogenetic tree was constructed (Supplementary Fig. 3). The results indicated that HSP70s can be divided into four major groups, which reflect the cellular compartment in which the proteins are located. The chloroplast HSP70s, including TaHSC70, appeared to form a distinct monophyletic group with the DnaKs of *Synechocystis* sp. PCC6803.

TaHSC70 expression in wheat tissues and its responses to heat shock and light–dark treatments

Because *TaHSC70* is localized within chloroplasts, it was of interest to determine its expression pattern in photosynthetic and nonphotosynthetic tissues, and by regulation of light. Figure 1a showed that *TaHSC70* was present in the tested wheat tissues, including green and etiolated leaf, stem and root of seedling. However, it was noticed that the transcripts level was about threefold less in the root than in the green leaves. For light–dark treatment, the transcripts of *TaHSC70* in wheat leaf did not change remarkably by a 24 h dark–light cycle (Supplementary Fig. 4).

Expression profiles of *TaHSC70* during heat-shock treatment were determined in leaves of wheat seedlings that were exposed to 40°C for 2 h. In etiolated wheat leaves transcript levels of *TaHSC70* increased more than 2.8-fold upon heat shock for 1 h, but in green leaves the transcript level did not change (Fig. 1b).

Expression of TaHSC70 upon infection with stripe rust fungus

Real time-PCR showed that in an incompatible interaction *TaHSC70* expression increased between 24 to 48 hpi in (Fig. 2). It then slightly decreased (72 hpi) but then increased again at 96 hpi and stayed at a high level until 120 hpi. The highest level was reached at 96 hpi, at which timepoint the transcript level was about threefold over the control. In a compatible interaction, the highest accumulation of *TaHSC70* transcripts was observed at 18 hpi, about twofold over the control, but thereafter (from 24 hpi to 120 hpi), the transcript level steadily decreased. When comparing the expression profiles of *TaHSC70* between incompatible and compatible interactions, at each time point after inoculation, it is obvious that the transcriptional profiles are different: a transient expression early after infection in the compatible interaction and a continuous higher level of expression in the incompatible interaction.
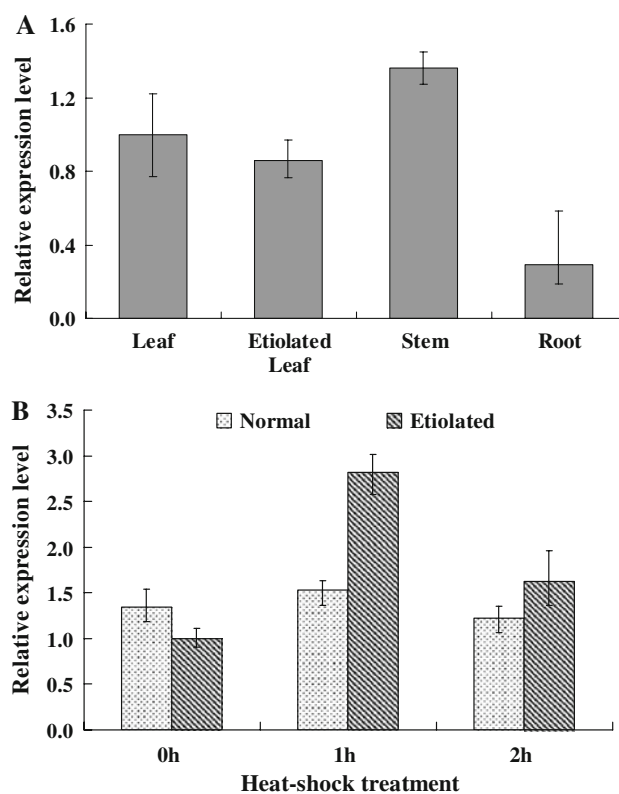

Fig. 1 Expression patterns of *TaHSC70* in wheat tissues and by heat-shock treatment. **a** Expression profiles of *TaHSC70* in wheat tissues. **b** Expression profiles of *TaHSC70* in wheat leaves by heat-shock treatment. The transcriptional patterns displayed are typical of three sets of independent experiments. *Bars* indicate standard deviation of the mean
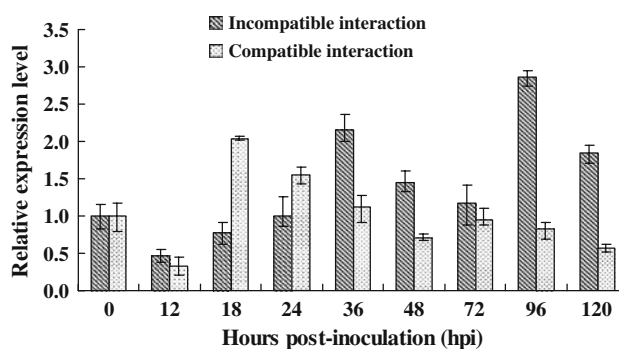

Fig. 2 Expression profiles of *TaHSC70* gene in wheat leaves infected by stripe rust fungus at different time points. Incompatible interaction-wheat cultivar Suwon11 was inoculated with CYR23, Compatible interaction-wheat cultivar Suwon11 was inoculated with CYR31. The transcriptional patterns displayed are typical of three sets of independent experiments. *Bars* indicate standard deviation of the mean

Expression pattern of *TaHSC70* to chemical stimuli

When treated with exogenous plant hormones, *TaHSC70* was induced by MeJA, but did not respond significantly to

SA, ET and ABA (Fig. 3). After treatment with MeJA, the accumulation of *TaHSC70* transcripts elevated transiently to a peak within 2 h and then decreased to a normal level.

Discussion

As diverse characteristics of HSP70s had been described in various plants, a few wheat HSP70s had been studied in some aspects, including physical location [34], expression profiles analysis [35] and heat shock signal transduction [36], and so on. In this study, we isolated a putative HSP70 gene *TaHSC70* from wheat leaves infected by stripe rust. TaHSC70 protein contains a highly conserved N-terminal ATPase domain and a C-terminal substrate binding region, suggesting that TaHSC70 might function as a molecular chaperone. Sequence alignment of TaHSC70 and HSP70s from other plants revealed strong sequence conservation except variation in the N and C-termini, where the information for subcellular localization and for intramolecular and intermolecular interactions resides [4, 5]. It was noticed that a highly conserved and diagnostic motif in the C-terminus of the chloroplast HSP70s existed in TaHSC70. In addition, the phylogenetic analysis in the present study showed that the chloroplast HSP70s were clustered as a distinct monophyletic group with the DnaKs of *Synechocystis* sp. PCC6803. This result was also in agreement with previous phylogenic analyses of HSP70 in yeast, plants, and other organisms [4, 5, 33, 37], and suggested that TaHSC70 was a homolog of chloroplast HSP70s with prokaryotic origin.

The chloroplast HSP70s are known to show tissue specific expression and to be regulated by environmental factors, such as light and heat-shock [38, 39]. Here, we determined the expression patterns of *TaHSC70*. *TaHSC70* mRNA was present in all tested wheat tissues, but approximately threefold less in roots than in leaves. It is
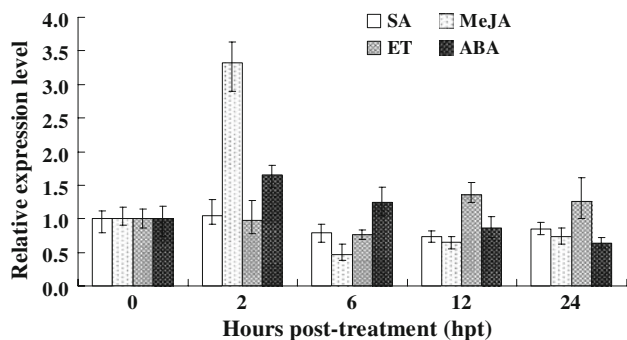

Fig. 3 Expression profiles of *TaHSC70* in wheat leaves treated with four plant hormones, salicylic acid (SA), ethylene (ET), methyl jasmonate (MeJA) and abscisic acid (ABA). *Bars* indicate standard deviation of the mean

possible that, similar to the spinach chloroplast HSP70 Chsp70, TaHSC70 is not strictly localized to the chloroplast but may occur in various plastid types [38]. Lower expression levels could reflect the lower number of plastids in root. In addition, *TaHSC70* was expressed at a high level in etiolated wheat leaf and was not found to be induced by light–dark treatment. This may imply that TaHSC70 is an important factor in chloroplasts, but not involved in photosynthesis. Upon the heat-shock treatment, transcription of *TaHSC70* was increased in etiolated wheat leaves, but stayed at the same stable level in green leaves. Since chloroplasts are not fully developed in etiolated leaves and could be sensitive to heat, TaHSC70 might play a particular thermoprotective role thereby maintaining the physiological process intact despite the environmental stress. However, unlike other inducible HSP70s, TaHSC70 cannot be apparently induced by cold (4°C), drought, salinity treatments (data not shown).

In plants, the relation between HSP70s and disease defence response has been implicated. However, the function of HSP70s during pathogen infection remains uncertain. In this study, expression profiles of *TaHSC70* in wheat cultivar Suwon 11 infected by two races of the stripe rust fungus (avirulent and virulent) were analyzed. *TaHSC70* was up-regulated during the incompatible interaction. In addition, the *TaHSC70* expression patterns differed between the incompatible and compatible interactions. Previously, histopathological and histochemical analysis revealed that hypersensitive cell death of host cells around infection sites occurs in the incompatible interaction, while no similar responses appeared in the compatible interaction [40]. During hypersensitive response (HR), a mass of proteins becomes denatured following shrinkage of the protoplast and collapse of the cytoplasm. According to current knowledge, HSP70s are recruited for folding and refolding of nonnative proteins to prevent irreversible aggregation of stress-denatured proteins [10]. Upregulation of *TaHSC70* during the incompatible interaction might function in ensuring that the cells surrounding the necrotic lesion survive. In addition, at the pre-penetration stage during appressorium formation of both stripe rust races (CYR23 and CYR31), reactive oxygen species (ROS) are induced in the guard cells. Prior to or during HR, sustained accumulation of ROS occurred at the early infection stage from 12 to 24 hpi in the incompatible interaction, but could not be detected in the compatible interaction [40]. ROS accumulation can generate oxidative stress in the chloroplasts, the major site of ROS production. Scarpeci discovered a rapid increase of chloroplast HSP70 by methyl viologen (MV), a superoxide anion propagator in the light [41]. Therefore, we speculate that the accumulation of *TaHSC70* in the compatible (at 18 hpi) and incompatible interaction (after 24 hpi) is related to a role for *TaHSC70* in

protecting for oxidative stress. However, the accumulation of *TaHSC70* was earlier and fewer in the compatible interaction than in the incompatible interaction. It was implied that *TaHSC70* might be regulated by a pathogen-induced signaling pathway. To date, a variety of signaling molecules, such as SA, ethylene (ET), jasmonic acid (JA) and ABA, have been proposed to be involved in plant defences [42, 43]. In mammals, heat-induced HSP70s can be increased through prior treatment with moderate doses of SA [44, 45]. In wheat, expression of *TaHSC70* was induced by MeJA treatments in the early stage (2 h after being treated), but this was not found after treatments with SA, ET and ABA. JA plays a crucial role in plant development and defence responses to wounding, insect pests and plant pathogens [46–48]. Therefore, *TaHSC70* was presumed to be involved in basal defense through JA signaling pathway. However, because of the limited insight in signaling pathways that play a role in wheat-stripe rust interactions, it is unclear how *TaHSC70* is regulated by JA. In another respect, the transcriptional regulation of genes enconding HSP is known to due to the activation of the correlative transcription factors, which bind to the HSP promoter elements [19, 49]. Thereby mining these transcription factors and DNA elements could help to further investigate the biological functions of *TaHSC70* in response to the environmental stress.

In summary, *TaHSC70* was isolated from wheat seedlings infected by stripe rust. Its expression patterns were analysed in different wheat tissues and upon light and heat-shock treatment. In addition, *TaHSC70* is expressed differentially during incompatible and compatible interactions with wheat-stripe rust, and was upregulated by MeJA treatment. It is speculated that *TaHSC70* plays roles in stress-related responses, and possibly in pathogen infection.

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