Stress granule-associated TaMBF1c confers thermotolerance through regulating specific mRNA translation in wheat (Triticum aestivum)

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Introduction

High temperature adversely affects plant growth and severely causes crop yield loss worldwide, especially for chimonophilous wheat, which prefers an optimal daytime growing temperature during reproductive development of 15°C (Bita & Gerats, 2013; Akter & Rafiqul Islam, 2017; Ni et al., 2018). Model predictions indicate that global wheat production will fall by 6% per 1°C increase above optimum temperature (Asseng et al., 2015), which will significantly impact food security. Because of their sessile nature, plants have evolved sophisticated defense mechanisms to survive and acclimatize themselves to hostile conditions (Kotak et al., 2007; Mittler et al., 2012; Ohama et al., 2017). Therefore, understanding the molecular responses of plants to heat stress would be helpful in improving yield potential in crop breeding programs.

The transcriptome profile changes extensively in response to heat stress in wheat, and thousands of responsive genes enriched in diverse biological functions have been identified (Liu et al., 2015). Of these genes, transcription factors (TFs) can bind to specific cis-acting elements of target genes and contribute to gene expression alteration when plants are subjected to heat stress. Similarly, transcriptional coactivators also play an important role in controlling variations in downstream gene transcript abundance by communicating with TFs and/or other regulatory components and the core transcription machinery. MULTIPROTEIN BRIDGING FACTOR 1 (MBF1) is a typical transcriptional coactivator that mediates transcriptional activation by physically bridging TFs, such as c-Jun, GCN4, FTZ-F1, Ad4BP and ATF1, with TATA-box binding protein (TBP) to participate in the regulation of diverse developmental processes in various organisms (Takemaru et al., 1997, 1998; Kabe et al., 1999; Brendel et al., 2002; Busk et al., 2003; Liu et al., 2003). Interestingly, MBF1 has also been identified as a critical regulator for stress responses in plants, including heat stress. In Arabidopsis (Arabidopsis thaliana), knockout of AtMBF1c significantly reduces heat tolerance, whereas

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overexpression confers enhanced thermotolerance in terms of seedling survival rate at 45°C for 2 h (Suzuki et al., 2005, 2008). Genetic analyses of AtMBF1c demonstrated that AtMBF1c probably functions upstream of the salicylic acid and ethylene-related pathways, but is not required for the expression of HEAT SHOCK TRANSCRIPTION FACTOR A2 (HSFA2), some heat shock proteins (HSPs) and ASCORBATE PEROXIDASE 1 during heat stress (Suzuki et al., 2005, 2008). In addition, transcriptome comparisons suggested that AtMBF1c can regulate expression levels of 36 genes, including dehydrogenase-responsive element-binding protein 2A (DREB2A), HSFB2A and HSFB2B in response to heat stress (Suzuki et al., 2011). Furthermore, the expression of two heat shock proteins, HSA32 and HSP70T-2, were also activated by AtMBF1c and contribute to heat tolerance (Kim et al., 2015). Interestingly, it was suggested that MBF1 could regulate the mRNA translation process. For example, an MBF1 mutation leads to changes in ribosomal frameshifting rate and consequently influences translation fidelity in yeast (Culbertson et al., 1982; Costanzo et al., 1986; Hendrick et al., 2001). MBF1 has also been identified as a polyadenylated mRNA-binding protein in yeast as well as in human (Baltz et al., 2012; Klass et al., 2013; Kwon et al., 2013). In addition, an affinity purification assay demonstrated that archaebal MBF1 protein from Sulfolobus solfataricus can bind to the 30S ribosomal subunit during translation, suggesting its physiological function linked to translation (Blombach et al., 2014). Although a set of studies have tried to understand how MBF1c mediates heat tolerance, whether it is directly involved in regulating translation in response to heat stress is still unknown, especially in plants.

Stress granules (SGs) are a conserved cytoplasmic aggregate induced by various environmental stresses, containing untranslated mRNAs, translation initiation factors, RNA binding proteins and the 40S ribosomal subunit (Kedersha et al., 2005; Anderson & Kedersha, 2008; Prother & Parker, 2016). This complex often acts as a triage center to help stabilize specific mRNAs, which facilitates the storage and/or transfer of mRNA to other RNA nucleoproteins during adaptation to environmental stresses (Kedersha & Anderson, 2002; Decker & Parker, 2012). Our previous analysis identified a TaMBF1c in wheat, and its overexpression improved heat tolerance in rice (Qin et al., 2015). However, its underlying molecular mechanism remains ambiguous. In this study, we demonstrate that TaMBF1c is colocalized with SGs, and show that TaMBF1c contributes to heat tolerance at least partially by regulating mRNA translation efficiency (TE) of a subset of genes in wheat, which are enriched in the ‘sequence-specific DNA binding’ category, consistent with its biological function as a transcriptional coactivator.

Materials and Methods

Plant materials and growth conditions

Spring wheat cultivar ‘CB037’ (Triticum aestivum L.) was used for gene cloning, expression analyses and genetic transformation. Twenty-one diploid progenitor species (nine AA, six SS (possibly modified BB) and six DD), nine tetraploid wheat species (AABB) and 100 hexaploid wheat cultivars (AABBDD) were used for nucleotide sequence divergence analysis (Supporting Information Table S1). Chinese Spring (CS) nulli-tetrasomic (NT) lines (N7A1T7B, N7A1T7D, N7B1T7A, N7B1T7D, N7DT1T7A, N7DT1T7B) were used for chromosomal localizations. The wheat materials were grown hydroponically in 1 : 1 Hoagland solution in a glasshouse at 22°C : 18°C, day : night, 16 h : 8 h, light : dark, 60% relative humidity and light intensity of 3000 lx (Master GreenPower CG T 400W E40; Philips). In addition, Arabidopsis ecotype Columbia-0 used for genetic transformation was grown in 1 : 2 Murashige and Skoog (MS) medium agar plates or soil, and Nicotiana benthamiana used for transient transfection was grown in soil in the same glasshouse as described above.

At least three independent experiments were performed for each assay. All primer and probe sequences used in this study are listed in Table S2.

Gene cloning and sequence analysis

Genomic DNA was isolated from 10-d-old wheat seedling leaves using CTAB (Coolaber, Beijing, China). Based on the potential sequences of three TaMBF1c homologous genes obtained from the International Wheat Genome Sequencing Consortium (IWGSC; http://www.wheatgenome.org/), gene-specific primers were designed for PCR amplification. PCR assays were performed using Tks Gflex DNA Polymerase (Takara, Dalian, China), and PCR products were subcloned and sequenced. Sequence alignment and similarity comparisons were performed by DNAMAN and CLUSTALX, and a neighbour-joining tree was constructed by MEGA6.

cDNA synthesis and expression analysis

Total RNA was extracted from leaves of 10-d-old CB037, TaMBF1c-overexpression and TaMBF1c-RNAi seedlings heat stressed at 38°C for 0, 1 and/or 6 h using TRIzol reagent (Invitrogen). Genomic DNA removal and cDNA synthesis were done using the HiScript Q RT SuperMix (Vazyme, Nanjing, China). Gene-specific primers for real-time quantitative PCR (RT-qPCR) were designed on the basis of the cDNA sequence polymorphisms of three TaMBF1c homologous genes. RT-qPCR was conducted using SYBR Green Realtime PCR Master Mix (Takara) on a CFX96 real-time PCR machine (Bio-Rad Laboratories). The average values of 2–ΔΔct were used to calculate the relative expression of genes (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_040980.pdf). Triplicate measurements were made for each cDNA sample, and the gene expression values were normalized to the wheat β-actin gene (TraesCS5B01G124100).

Transient expression and transactivation assays

Various TaMBF1c-7B promoter-driven β-glucuronidase (GUS) reporters (P1:GUS, P2:GUS and P3:GUS) were constructed by recombining the PCR-amplified DNA fragments (200, 525 and 1500 bp) upstream of GUS in the pCAMBIA1300 vector using Exnase (Vazyme). The GUS reporters and an internal control (35S:RFP) were cointroduced into the N. benthamiana leaf
epidermal cells via Agrobacterium tumefaciens strain GV3101 (Liu et al., 2010), and the empty pCAMBIA1300 vector (p0:GUS) was used as a negative control. Plants were kept at 22 °C for 3 d before being induced by heat stress (38 °C for 1 h). Infiltrated leaves before and after heat treatment were harvested and used for GUS expression analysis. MAS:TaHsfA constructs for transactivation assays were generated by cloning the coding sequences of 35 TaHsfA genes (Table S3) from CB037 into the p-super1300 vector through recombination ligation, respectively. A mixture of an effector (MAS:TaHsfA), a reporter (P3:GUS) and an internal control (35S:RFP) was cotransfected into N. benthamiana as described above, and the empty p-super1300 vector was used as a negative control. Three days after infiltration, the infected leaves were harvested and used for GUS expression analysis. GUS activity in all samples was normalized against the red fluorescent protein (RFP) activity.

Electrophoretic mobility shift assay

The full-length coding sequence of TaHSFA6e was cloned into the pGEX6P-1 vector fused with glutathione S-transferase (GST). Expression of recombiant proteins in Transetta (DE3) Escherichia coli (TransGen, Beijing, China) was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in Luria Bertani (LB) buffer overnight at 16 °C. The cells were subsequently harvested, washed and resuspended in 30 ml of phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich) and half a tablet of protease inhibitor cocktail (Roche), and cells were sonicated for 1 h and centrifuged at 13 000 g for 45 min. The supernatant was filtered through a 0.22 μm membrane into a 50 ml tube. The supernatant was mixed with 1 ml of GST MAG Agarose Beads (Novagen, Madison, WI, USA) and shaken overnight at 4 °C. The GST beads were washed with 5 ml of PBS, four times, and the fusion proteins were eluted from the beads by incubation at 4 °C for more than 4 h with 50 mM Tris–HCl (pH 8.0) supplemented with 10 mM reduced glutathione. Protein concentrations were determined using a Nano Drop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The biotin-probe was 5’ end-labeled with biotin. The double-stranded oligonucleotides used in the assays were annealed by cooling from 100 °C to room temperature in annealing buffer. The DNA-binding reactions were performed in 20 μl with 1× binding buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol (DTT); pH 7.5), 10% glycerol, 0.5 mM EDTA, 7.5 mM MgCl2, 14 mM 2-mercaptoethanol, 0.05% (v/v) NP-40 and 50 ng μl−1 poly(dI-dC)). Competition analysis was used to test the specificity of the TaHSFA6e to the binding motif. A 5-, 20- and 200-fold molar excess of an unlabelled DNA fragment was added to the binding reaction, 5 min before the probe was labeled. After incubation at room temperature for 30 min, samples were loaded onto a 6% native polyacrylamide gel. Electrophoretic transfer to a nylon membrane and detection of the biotin-labeled DNA was performed using a LightShift Chemiluminescent EMSA Kit according to the manufacturer’s instructions (Thermo Scientific).

Thermotolerance test

For the thermotolerance assay in yeast, the coding sequences of TaMBF1c-7B, MBF1 domain and HTH domain were recombined into yeast expression vector pYES2 (driven by the GAL1 promoter), respectively. The recombinant plasmids and the empty pYES2 control plasmid were then transformed into Saccharomyces cerevisiae strain INVSc1 using the lithium acetate method according to the manufacturer’s instructions (Invitrogen). A single positive clone of the transgenic yeast lines was shaken cultivated in synthetic dropout (SD) liquid medium lacking uracil (SD/-Ura) at 30 °C for 12 h. Next, the cultures were resuspended and diluted to an OD600 of 0.4 using inducible nitrogen base liquid medium containing 2% galactose and 1% raffinose but lacking uracil (IN/-Ura). After induction culture at 30 °C for 20 h, the yeast cell densities were redetected and unified, and exposed to 48 °C for 1 h. Then, 10 μl of 10-fold serial dilutions was dotted on SD/-Ura plates and incubated at 30 °C for 2 d.

For the thermotolerance assay in Arabidopsis, the coding sequences of TaMBF1c-7B fused in-frame to a C-terminal MYC epitope tag, MBF1 domain and HTH domain were recombined into p-super1300 vector (driven by the MAS promoter), respectively. The constructs were then transformed into Columbia-0 using a floral-dip method by Agrobacterium tumefaciens strain GV3101 (Clough & Bent, 1998). Positive transgenic plants were screened on MS agar medium containing Basta (glufosinate ammonium) and identified by RT-qPCR. Seven-day-old seedlings of T3 homozygous transformants and wild-type (WT) lines grown on MS medium under normal conditions were incubated at 45 °C for 2 h, and survival rates were calculated after 3 d of recovery.

For the thermotolerance assay in wheat, the overexpression, RNAi suppression and CRISPR/Cas9-based gene editing constructs of TaMBF1c were generated using vectors pWMB122, pWMB006 and pBUE411, as previously described (Chen et al., 2016; Liu et al., 2020). The resulting constructs were introduced into wheat cultivar ‘CB037’ via A. tumefaciens-mediated transformation using strain EHA105 (Ishida et al., 2015). Positive transgenic plants were screened and identified through PCR and RT-qPCR. Stable T3 homozygous transgenic lines were used for phenotypic and molecular analyses. Seven-day-old overexpression (OE) and CB037 (WT) lines grown in Hoagland solution under normal conditions were treated at 42 °C (16 h photoperiod) for 5 d, and then scored for fresh weight. Two-day-old RNAi (Ri), knockout (KO) and CB037 lines grown in Hoagland solution under normal conditions were treated at 38 °C (16 h light cycle) for 10 d, and then scored for plant height and fresh weight.

Confocal microscopic analysis and stress granule quantification

The TaMBF1c-7B-GFP and AtRBP47-RFP fusion gene expression cassettes constructed by PCR were inserted into p-super1300 vector under control of the MAS promoter, respectively. Both constructs were then introduced into A. tumefaciens strain GV3101. Coinfiltration of N. benthamiana was performed...
Yeast two-hybrid screening and interaction assays

A wheat cdNA library was prepared in the prey pGADT7 vector using RNA isolated from 10-d-old CB037 seedlings treated at 38°C for 1 h according to the Yeast Protocols Handbook (Clontech, Palo Alto, CA, USA). The full-length TaMBF1c coding sequence (CDS) was cloned into the bait vector pGBKKT7. Yeast two-hybrid screening interaction assays were performed by transforming pairs of pGBKKT7- and pGADT7-based plasmids harboring genes of interest into the yeast strain AH109 according to the user manual supplied with the Matchmaker GAL4 Two-Hybrid System (Clontech). To verify the protein–protein interactions, we expressed the full-length TaG3BP and S20 proteins as a translational fusion to the yeast GAL4 activation domain (AD), and fused the TaMBF1c-7B to the GAL4 binding domain (BD). The transformants were grown on SD/-Trp-Leu-His-Ade medium at 30°C for 3 d.

Immunoprecipitation and MS analysis

Total proteins from 7-day-old MAS:MYC and MAS:TaMBF1c-7B-MYC transgenic plants treated at 38°C for 1 h were extracted with extraction buffer (50 mM Tris (pH 7.5), 1 mM EDTA (pH 8.0), 150 mM NaCl, 20% glycerol, 2% Triton X-100, 1× Complete protease inhibitor cocktail (Roche), 1 mM PMSF, 50 µM MG132 (Sigma-Aldrich) and 5 mM DTT). Protein extracts were subsequently immunoprecipitated using MYC-Trap_A beads (Chromotek, Planegg, Germany) according to the manufacturer’s instructions. Immunoprecipitated proteins were digested with trypsin and identified using LC-MS/MS analysis as previously described (Wang et al., 2016). Peptide spectra were searched by MaxQuant software against the UniProt database and TAIR10 database.

Antibody preparation and immunoblot analysis

A specific rabbit anti-TaMBF1c polyclonal antibody was custom-made by AppTec (Shanghai, China) using purified TaMBF1c protein expressed in prokaryotic system. Protein immunoblot analyses were conducted as described previously (Yang et al., 2016). In brief, total proteins from wheat were extracted with lysis buffer (200 mM Tris (pH 6.8), 40% glycerol, 8% SDS and 20% β-mercaptoethanol) at 100°C for 10 min. Protein extracts were then separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), and electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore). Protein detection was carried out using anti-MYC antibody (Calibo, San Francisco, CA, USA) or anti-TaMBF1c as primary antibody, and anti-rabbit IgG-HRP or antimouse IgG-HRP (Sigma-Aldrich) as secondary antibody according to the manufacturer’s instructions. The signal was developed by chemiluminescence using ECL Prime (GE Healthcare, Beijing, China) and shown via X-ray film.

Polysome profiling assays

Ten-day-old KO-1 and CB037 seedlings were treated at 42°C for 3 h before 2 h of recovery, and then ground to a fine powder in liquid nitrogen. Then, 1.5 g of seedlings was lysed in 5 ml ice-cold extraction buffer containing 200 mM Tris–HCl (pH 9.0), 200 mM KCl, 35 mM MgCl2, 2 mM EGTA, 1% Triton X-100, 1% Tween 20, 2% polyoxyethylene, 2.5 mg heparin, 5 mM DTT, 100 µg ml–1 chloramphenicol and 100 µg ml–1 cycloheximide. After 20 min in an ice-bath, cell debris was removed by centrifugation at 20 800 g for 15 min at 4°C. The supernatant was layered on top of a 1.7 M sucrose cushion, and ultracentrifuged at 218 300 g for 3 h at 4°C. The supernatant was removed and the pellet was resuspended in resuspension buffer containing 20 mM Tris–HCl (pH 8.0), 200 mM NaCl, 5 mM MgCl2, 1 mM DTT, 100 µg ml–1 chloramphenicol and 100 µg ml–1 cycloheximide. Five thousand A260 units polyosomes were layered over a 20–60% (w/v) sucrose density gradient poured over with Gradient Master (BioComp, Fredericton, NB, Canada), and the preparation was then ultracentrifuged at 159 300 g (SW55Ti rotor; Beckman) for 2.5 h at 4°C. After speed reduction without brake, Gradient Profiler (BioComp) was used to separate different sucrose components, and measure their optical density at 254 nm. Nine to 14 fractions were mixed for polysome-bound RNA extracted using TRIzol reagent. For ribosome-protein isolation, 3–8 fractions were precipitated respectively by twice the volume of ethanol for 8 h at 4°C, and centrifuged at 20 800 g for 1 h at 4°C. The precipitate was then treated with 4× protein loading buffer and boiled for 10 min.

RNA sequencing and data analysis

The samples of total RNA and polysome-bound RNA were used to prepare 150 bp paired-end RNA-sequencing (RNA-seq) libraries according to the manufacturer’s protocol of the Illumina Standard mRNA-seq library preparation kit and sequenced on a Novaseq 6000 platform. The raw reads were processed with Fastp (v.0.19.4; Chen et al., 2018) with parameters ‘-3 -5 -W 6 -l 30 -c’. Finally, c. 6 Gb high-quality clean reads were generated from each library. The high-quality reads were then mapped to the Chinese Spring wheat reference genome (IWGSC RefSeq v1.0) using STAR (v.2.5.3a; Dobin et al., 2013) with parameters ‘-alignEndsType EndToEnd -outFilterMultimapNmax 1 -outFilterMismatchNmax 3 -twopassMode Basic’. The R package deseq2 (v.1.24.0; Love et al., 2014) was used for differential expression analysis. Differentially expressed genes between conditions were identified according to fold change values > 2 and false discovery rate (FDR)-adjusted P<0.05. Gene Ontology (GO) analysis was conducted using the R package clusterProfiler.
Construction of coexpression networks

All differentially expressed genes at transcriptional and translational levels were used to construct the coexpression networks by using the WGCNA R package (v.1.69; Zhang & Horvath, 2005). The raw reads count was normalized using the regularized logarithm (rlog) from DESEQ2. The first power to exceed a scale-free topology fit index of 0.8 was considered to be the most appropriate threshold, which is 12, and all the other important parameters in the blockwiseModules() function include ‘corType = pearson, TOMType = unsigned, mergeCutHeight = 0.30’. The top 2% genes with the highest module eigengene-based connectivity value in each module were identified as hub genes for further analysis and network visualization. Networks were visualized using CYTOSCAPE (v.3.7.0; Shannon et al., 2003); to show this more intuitively, all homeologs were merged into the same node.

Translational efficiency calculation

Only genes with > 10 normalized reads in at least one library were considered to be expressed genes. TE was calculated as the ratio of polysomal RNA to total RNA. TEKO/TEWT was calculated and then log2-transformed to compare TE variations, and genes with a z-score value > 2 or < -2 were considered as up- or downregulated genes in TE, respectively.

Results

Heat stress responsive TaMBF1c is evolutionarily conserved during polyploidization and artificial selection history

Our previous transcriptome profiling found that TaMBF1c (GenBank accession number: GQ370008) was rapidly upregulated when subjected to high temperature in wheat seedlings (Qin et al., 2008), and its overexpression resulted in enhanced heat tolerance in rice (Qin et al., 2015). These lines of evidence indicate that TaMBF1c plays an important role in regulating heat tolerance in plants, but little is known about the underlying mechanism. Since common wheat is a hexaploid species with most of the genes present as triplicate homeologs, we first identified its homeologous genes on chromosomes 7A, 7B and 7D, respectively, by a BLAST search against the wheat reference genome sequence (IWGSC RefSeq v.1.1). Those locations were further confirmed by amplifying homeolog-specific fragments from the genomic DNA of the Chinese Spring nullisomic–tetrasomic lines (Fig. 1a), which lack one pair of chromosomes but have extra homeologous chromosomes in compensation. The amplicons disappeared when the corresponding chromosome pair was substituted. Therefore, these three TaMBF1c loci were considered to be homeologs and were designated as TaMBF1c-7A, TaMBF1c-7B (the one used for rice transformation in our previous study, Qin et al., 2015) and TaMBF1c-7D, respectively. The three homeologs were all composed by only one exon and shared 98.1% protein sequence similarity between each other (Fig. S1a).

Next, we examined the expression patterns of TaMBF1c homeologs by RT-qPCR and the results showed that their transcript
amounts were all sharply increased in response to heat stress with similar patterns (Fig. 1b). To gain further insight into the nucleotide sequence divergence of TaMBF1c homeologs during the evolution history of wheat and artificial selection process, we isolated its counterparts in 21 diploid, nine tetraploid and 100 hexaploid wheat species using homeolog-specific primers (Tables S1, S2). The sequences were highly conserved between diploid/tetraploid progenitors and hexaploid wheat species, and 99.58–100, 97.88–100 and 100% sequence identity was revealed in subgenome A, B and D homeologous groups, respectively, among these wheat species.

Class A HSFs regulate heat stress-induced TaMBF1c-7B expression

To elucidate the underlying mechanism responsible for the transcriptional regulation of TaMBF1c under heat stress conditions, we first analyzed the promoter sequences of the three homeologs. Although their promoter sequences showed obvious divergence, they all contained heat shock elements (HSEs) (Fig. S1b), which have been validated to be bound by heat shock transcription factors (HSFs) to activate target gene expression in response to heat stress (Xue et al., 2014). Our previous study reported that TaMBF1c-7B overexpression led to increased heat tolerance in rice (Qin et al., 2015), so we selected the TaMBF1c-7B homeolog as the target gene for further analysis. Three HSEs at positions –166, –204 and –530 bp were identified in the TaMBF1c-7B promoter region (Figs 1c, S1b). We next examined whether the number of HSEs in the TaMBF1c-7B promoter is related to its expression level. To this end, we constructed four GUS reporter fusion vectors containing no HSEs (p0:GUS, 0 bp), one HSE (p1:GUS, 200 bp), two HSEs (p2:GUS, 525 bp) and three HSEs (p3:GUS, 1500 bp), respectively (Fig. S1b). The results showed that deletion of HSE affects GUS expression in response to heat stress, and that GUS activity was proportional to the number of HSEs (Fig. 1b).

Since several class A HSF members bind to HSEs in the AtMBF1c promoter to upregulate its expression in response to heat stress in Arabidopsis (Tsuda & Yamazaki, 2004; Ogawa et al., 2007; Yoshida et al., 2011; Bechtold et al., 2013), we further examined which class A HSF members in wheat could trigger elevation of TaMBF1c transcript abundance using transactivation assays. Of 33 wheat class A HSFs, six could highly induce the TaMBF1c transcript abundance compared with the control (Fig. S2a–c; Table S3). An electrophoretic mobility shift assay (EMSA) was performed to examine whether TaHSF6a6c can bind to the HSE motif in vitro. The recombinant GST-TaHSF6a6c protein and GST alone were incubated with the labeled oligonucleotide probes. In the EMSA, the probe was shifted when incubated with the GST-TaHSF6a6c-enriched extract, but it was not shifted with a GST control protein. Moreover, the binding capacity of the probe was effectively outcompeted by a molar excess of unlabeled probe but not mutated competitor (Fig. S2d). Collectively, these findings indicated that HSEs are required for HSFs to regulate a proper expression pattern of TaMBF1c under heat stress conditions.

TaMBF1c contributes to heat tolerance in wheat

To further determine the biological significance of TaMBF1c in the heat response, wheat transgenic lines with ubiquitin promoter-driven overexpression of TaMBF1c-7B and RNAi-induced downregulation of three TaMBF1c homeologs (hereafter TaMBF1c) were generated. Expression levels of TaMBF1c-7B were detected in three independent overexpression lines (OE-1, OE-2 and OE-3) under normal conditions, whereas the expression abundance of TaMBF1c was analyzed in knockdown lines (Ri-1, Ri-2 and Ri-3) under heat-stressed conditions. There was a clear increase of TaMBF1c-7B and reduction of TaMBF1c mRNA abundance in OE lines and knockdown lines, respectively, compared to the wild-type (CB037; hereafter WT) (Fig. S3). Under normal growth conditions, no significant phenotypic variation was observed between TaMBF1c transgenic lines and the WT. However, under heat stress conditions, the overexpression and knockdown lines were more resistant and sensitive to heat stress than the WT at the seedling stage, respectively. Specifically, the fresh weight of the three TaMBF1c overexpression transgenic lines was significantly higher than that of the WT (0.161, 0.164 and 0.159 g vs. 0.128 g, on average) under heat stress conditions (42°C, 5 d) (Fig. 2a). By contrast, the three TaMBF1c knockdown transgenic lines exhibited significantly lower fresh weight than that of the WT (0.153, 0.152 and 0.149 g vs. 0.181 g, on average) under heat stress conditions (38°C, 10 d) at the seedling stage. In addition, the two knockdown lines (Ri-2 and Ri-3) showed reduced plant height compared with the WT (10.83 and 10.74 cm vs. 12.4 cm, on average) under heat stress conditions at the seedling stage (Fig. 2b).

Next, we generated knockout mutants (KO-1, KO-2 and KO-3) of the three homeologs of TaMBF1c simultaneously in the wheat cultivar CB037 background via CRISPR/Cas9-based gene editing (Fig. S4), which also exhibited significantly decreased heat tolerance compared to the WT (Fig. 2c). Specifically, the three TaMBF1c knockout lines exhibited significantly lower plant height and fresh weight than that of the WT (8.23, 8.47 and 8.57 cm vs. 11.13 cm, and 0.113, 0.117 and 0.121 g vs. 0.157 g, on average) under heat stress conditions at the seedling stage (Fig. 2c). Notably, knockout of only one homeolog of TaMBF1c did not significantly change the heat response in wheat (data not shown), suggesting their functional redundancy in response to heat stress. Collectively, these results indicated that TaMBF1c is required for heat tolerance in wheat.

Functional validation of N- and C-terminal domain of TaMBF1c-7B protein in response to thermotolerance in both yeast and Arabidopsis

The TaMBF1c protein was predicted to contain an MBF1 domain and a helix–turn–helix (HTH) domain at the N- and C-terminus of the deduced amino acid sequence, respectively. To examine their potential function, we overexpressed the full-length (TaMBF1c-pYES2), MBF1 domain (80 amino acids; MBF1-pYES2) and HTH domain (76 amino acids; HTH-pYES2) of TaMBF1c-7B protein in an INVSc1 yeast strain,
respectively (Fig. 3a). Under normal conditions, all yeast strains exhibited similar growth status. However, after heat stress, the TaMBF1c-pYES2 and MBF1-pYES2 transgenic yeast cells showed improved viability compared to the WT strain, whereas the HTH-pYES2 transgenic yeast cells exhibited suppressed viability, indicating the HTH domain has a dominant-negative effect on heat tolerance when constitutively expressed in yeast (Fig. 3b). These observations were further confirmed in transgenic Arabidopsis lines. Under normal growth conditions, no obvious phenotypic variation was detected between transgenic lines and the WT. However, under heat stress conditions, the TaMBF1c-7B and MBF1 domain-overexpressed lines (MAS: TaMBF1c-7B and MAS:MBF1) were more resistant to heat stress than the WT in terms of seedlings survival rate (50 and 33% vs 27%). Yet, MAS:HTH transgenic Arabidopsis seedlings exhibited significantly reduced heat tolerance (18%) (Fig. 3c). Together, these results suggest that TaMBF1c is evolutionarily conserved in regulating heat tolerance, and the HTH domain has a dominant-negative effect on heat tolerance when constitutively expressed in yeast and Arabidopsis.

TaMBF1c is a component of stress granule under heat stress conditions

To explore the underlying molecular mechanisms of wheat TaMBF1c function in the heat response, we investigated the subcellular localization of TaMBF1c-7B using transient expression assays of N. benthamiana leaf epidermal cells with MAS: TaMBF1c-7B-GFP constructs. Under normal conditions, the TaMBF1c-7B protein was randomly distributed in nuclear and cytoplasm, whereas heat stress treatment induced an association of the GFP fluorescent signal with prominent cytoplasmic foci (Fig. 4a). To investigate the identity of these foci, MAS:TaMBF1c-7B-GFP and MAS:AtRBP47-RFP (RNA Binding
Protein 47, SG marker) (Weber et al., 2008) were transiently cotransfected into N. benthamiana leaf epidermal cells. Approximately 80% TaMBF1c-7B proteins were colocalized with SGs after heat stress (Fig. 4a), indicating an association of the TaMBF1c-7B protein with SGs in response to heat stress.

To further confirm our observations, we performed a yeast two-hybrid screen using the full length of TaMBF1c-7B as a bait against a cDNA library of heat-treated wheat seedlings, and found 35 potential interaction proteins in response to heat stress (Table S4). Of these, RNA-binding Ras-GAP SH3 binding protein TaG3BP (TraesCS7B02G179500) and 40S ribosomal subunit protein S20 (TraesCS2A02G485400) were selected for further analysis. Yeast cells cotransformed with GAL4-AD-TaG3BP and GAL4-BD-TaMBF1c-7B protein fusions showed normal growth on selective medium (SD/-Trp-Leu-His-Ade medium) (Fig. 4b), but not with GAL4-AD-S20 and GAL4-BD-TaMBF1c-7B. Interestingly, it has been reported that G3BP binds to 40S ribosomal subunits, which is necessary for SG formation in mammals and Arabidopsis (Kedersha et al., 2016; Krapp et al., 2017). Thus, we next examined whether TaMBF1c-7B can interact with S20 indirectly. To this end, we performed a sucrose density gradient centrifugation assay and isolated 40S, 60S and 80S monosomes as well as polysomes from WT and KO-1 plants under normal and heat-stressed conditions, respectively. Western blot analysis showed that TaMBF1c protein signal was detected only in the 40S ribosomal subunit fraction in the WT after heat stress using anti-TaMBF1c polyclonal antibody (Fig. S5). These results indicated that TaMBF1c can probably coprecipitate with 40S ribosomal subunit 20S of wheat in response to heat stress. In addition, immunoprecipitation of an MYC-tagged TaMBF1c-7B (MAS:TaMBF1c-7B-MYC) assay was conducted in Arabidopsis followed by LC-MS/MS analysis. In total, 48 coprecipitated proteins were identified (Table S5), and noticeably 23 were annotated as components of SGs, including ribosomal subunit compositions (RPSs and RPLs), translation factors (eEF4A-1/2/3, eEF1B and eEFTu), and RNA binding proteins (GRPs). Together, these data suggested that the TaMBF1c protein is a component of SGs in response to heat stress in wheat.

TaMBF1c influences heat response via regulating specific gene translational efficiency

Our observations above suggested that knockdown and knockout of TaMBF1c contributed to reduced heat tolerance, at least, partially by regulating gene translation process via SGs. To test this hypothesis, we exploited polysome profiling technology to

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**Fig. 3** Thermotolerance assays of different domains in TaMBF1c protein in yeast (Saccharomyces cerevisiae) and Arabidopsis (Arabidopsis thaliana). (a) TaMBF1c contains the MBF1 domain and HTH domain. (b) Thermotolerance assay in yeast. Growth of S. cerevisiae strains expressing TaMBF1c-7B, MBF1 domain or HTH domain protein are assessed in response to heat stress by spotting on SD/-Ura media. CK: 30°C; HS: 48°C for 60 min. (c) Thermotolerance assay in Arabidopsis. Seven-day-old seedlings were incubated at 45°C for 2 h. Survival rates were calculated 3 d after recovery. Sixty plants per line were used for each experiment; values are means ± SD from three biological replicates. Single and double asterisks represent statistically significant differences at \( P < 0.05 \) and \( P < 0.01 \), respectively, determined by Student's t-test between transgenic and wild-type plants.
compare TE of WT and TaMBF1c knockout plants (KO-1) under normal growth conditions (22°C; CK), heat stress conditions (42°C for 3 h; HS) and recovery conditions (22°C for 2 h after heat stress; RE). Comparative analysis of polysome profiles demonstrated that heat stress leads to a reduction of the polysome fraction in both WT and KO-1 mutant (Fig. S5). We then isolated polysome-bound, translationally active mRNAs (P-mRNA) of WT and KO-1 by density gradient centrifugation, and performed RNA-seq to identify translating mRNA changes caused by the TaMBF1c mutation. Simultaneously, we performed transcriptome (T-mRNA) profile analysis using the same materials.

We first compared total RNA and polysomal RNA populations globally using principal component analysis, and found that total RNA and polysomal RNA deviated from each other in both the mutant and WT, and heat stress intensified the discrepancy of transcriptome and translatome between the WT and mutant (Fig. 5a). Finally, we identified 1831, 3424 and 1027 differentially expressed genes in transcriptome profiles between the WT and mutant at normal conditions, heat stress conditions and recovery conditions, respectively. For the translatome, 757, 3866 and 2929 genes exhibited expression variation between each other (Fig. S6; Tables S6, S7). GO enrichment analysis was performed to distinguish functional distribution between the WT and mutant. Compared with the WT, downregulated genes in the KO mutant were significantly enriched in ‘sequence-specific DNA binding (GO: 0043565)’ terms at both the transcriptional and translational level in heat-stressed and recovery conditions (Fig. S7). This observation was consistent with the biological function of the transcriptional coactivator of TaMBF1c. Subsequently, we analysed the candidate genes affected by TaMBF1c in terms of TE in response to heat stress, and identified 520 and 389 genes exhibiting altered TE between the WT and mutant at heat stress conditions and recovery conditions (Tables S8, S9).

TaMBF1c influenced the heat response at both transcriptome and translatome levels in wheat. Thus, we subsequently combined these data and clustered the gene expression patterns into five modules using the WGCNA package (Fig. 5b; Table S10). Interestingly, these five groups were enriched in different GO terms. Module blue was enriched in binding-related category terms, whereas Module brown was enriched in RNA processing-related terms (Fig. 5c). More importantly, Module turquoise containing TaMBF1c showed significant enrichment in ‘response to heat (GO: 0009408)’, ‘unfolded protein binding (GO: 0051082)’, ‘heat shock protein binding (GO: 0031072)’ and ‘endoplasmic reticulum lumen (GO: 0005788)’ related categories. By comparison, the proportion of genes with downregulated TE in TaMBF1c knockout plants under heat treatment and recovery conditions (56% and 66%, respectively) was higher in the turquoise module than in other modules (Fig. S8). To further understand this module, we performed gene expression network analysis, and found that TaMBF1c was closely associated with HSPs, including HSP17.7, DnaJ, HSP17.6, HSP17.4, HSP70 and HSP23.5, among which five showed downregulated TE in response to heat stress between the TaMBF-1c knockout mutant and WT (Fig. S9; Table S10). All these lines of evidence confirmed TaMBF1c as an important regulator in response to heat stress in wheat, and it functions at least partially via selectively altering TE of heat responsive genes under stress conditions.

Discussion

Global warming is a major threat to agriculture and food security. Chimonophilous wheat is one of the most important staple food crops and is sensitive to heat stress, which has caused and will continue to cause severe yield reduction and quality loss worldwide (Akter Islam & Rafiqul, 2017; Yang et al., 2017). Deciphering the molecular mechanisms of the heat response in wheat would be helpful to improve yield potential in breeding programs.

We previously reported that overexpression of wheat TaMBF1c-7B altered gene transcription of heat shock protein and trehalose phosphate synthase-related genes in rice and contributed to heat tolerance (Qin et al., 2015). However, the underlying molecular mechanism of the TaMBF1c-mediated thermostolerance is still unknown in wheat. As a highly conserved cofactor, MBFs are expected to mediate the interaction between TFs and the basal transcriptional machinery to control gene expression. In the present study, we performed transcriptome
profiling of TaMBF1c knockout mutants together with the WT under normal conditions, heat stress conditions and recovery stage, and found that a proportion of heat responsive genes were downregulated at the transcriptional level in the TaMBF1c mutant compared with WT, and interestingly these genes were significantly enriched in the ‘sequence-specific DNA binding’ GO category, consistent with its predicted biological function as a transcriptional coactivator. Consistently, AtMBF1c is also involved in the regulation of gene transcription in Arabidopsis, and the atmbH1c mutation leads to misregulation of 36 genes compared with the WT in response to heat stress (Suzuki et al., 2011). Specifically, AtMBF1c accumulates rapidly in response to heat stress and influences the transcription abundance of TREHALOSE PHOSPHATE SYNTHASE 5, HSA23 and HSP70T-2 to regulate thermotolerance in Arabidopsis (Suzuki et al., 2008; Kim et al., 2015). Collectively, MBF1c contributes to heat tolerance in plants, at least partially by regulating the transcription abundance of a subset of heat responsive genes.

In addition to the potential role in regulating gene transcription, TaMBF1c is also probably involved in the regulation of gene translation, because we observed that TaMBF1c colocalized with SGs, which is a complex usually caused by environmental stress. They are assumed to protect mRNA against degradation in response to stress and rapidly release them for retranslation at the recovery stage (Anderson & Kedersha, 2008; Protter & Parker, 2016; Merret et al., 2017). Furthermore, TaMBF1c can interact with SG component TaG3BP and is only detected in the 40S ribosomal subunit fraction in response to heat stress, and thus we

Fig. 5 Translational and transcriptional mRNA sequencing data analysis in wheat (Triticum aestivum). (a) The PCA analysis was based on gene expression patterns of 12 samples. The x-axis and y-axis indicate two principal components (PC), PC1 and PC2, respectively. The mutant and wild-type are color-coded and total RNAs (T-mRNA) and polysome-bound RNAs (P-mRNA) are labeled with different shapes. Three biological duplicates were performed for each sample. (b) The expression pattern of hub genes of wgcna modules. The x-axis represents different samples; the y-axis indicates log2-transformed TPM value. The modules were smoothed using the LOESS method. P, polysome-bound mRNA; T, total mRNA; CK, normal conditions; HS, heat stress conditions; RE, recovery stage; WT, wild-type; KO, TaMBF1c knockout mutant. (c) GO enrichment analysis of hub genes from each wgcna module. The top 10 significantly enriched GO terms were selected for plotting; A–E, module blue, brown, green, turquoise and yellow, respectively.
hypothesized that TaMBF1c probably participates in specific mRNA TE in response to heat stress but not at a genome-wide level, since only a subset of heat responsive genes showed translational variation in wheat, including HSPs and sequence-specific DNA binding genes. HSPs are widely recognized as molecular chaperones assisting in protein conformational folding in response to heat stress, and their rapid translation after heat stress would aid plants to rescue misfolding proteins and to recover from harmful conditions (Wang et al., 2004; Al-Whaibi, 2011; Lang et al., 2021). TFs are essential for the regulation of gene expression under stress conditions, because many of the primary response genes encode TFs, which modulate secondary response gene expression (Winkles, 1997). Not surprisingly, in this study, ‘sequence-specific DNA binding’ category-related genes showed downregulated translational efficiency in TaMBF1c knockout mutant compared with the WT under heat stress conditions. Although previous studies have not demonstrated that MBF1c participates in controlling gene translation in response to heat stress in plants, several reports suggest that MBF1c might be associated with this process. For example, a coimmunoprecipitation assay showed that MBF1 can interact with the 30S ribosomal subunit during translation in Sulfolobus solfataricus (Blombach et al., 2014). Moreover, MBF1 has also been identified as a polyadenylated mRNA-binding protein in yeast as well as in human (Baltz et al., 2012; Klass et al., 2013; Kwon et al., 2013), and the MBF1 mutation results in ribosomal frameshifting rate variation and consequently influences translation fidelity in yeast (Culbertson et al., 1982; Costanzo et al., 1986; Hendrick et al., 2001). Together, these observations support that TaMBF1c plays a nonnegligible role in regulating the heat response at the translational level in wheat. However, according to the present data, we can only conclude that TaMBF1c contributes to heat tolerance at both the transcriptional and translational level in wheat, but we cannot differentiate which process plays the major role. Probably due to its indispensable role, we found that TaMBF1c is highly conserved in wheat species. Common wheat is a typical allohexaploid species originating from two independent hybridization events involving three diploid species, Triticum urartu (AA), Aegilops speltoides (BB) and Aegilops tauschii (DD) (Gill & Friebe, 2002). Theoretically, each gene has three homeologs at similar positions in A, B and D subgenomes, respectively, including TaMBF1c. During the evolutionary history of wheat, many homeologs were subjected to sequence diversification, but TaMBF1c is highly conserved and only a few variations were detected in the CDS region among three homeologs, which shared 98.1% sequence identity between each other in hexaploid wheat, and all TaMBF1c homeologs were induced in response to heat stress according to our analysis. Moreover, TaMBF1c homeologs also shared high sequence similarity in diverse hexaploid wheat varieties as well as in diploid and tetraploid progenitor species. To identify the potential role of TaMBF1c in heat tolerance during wheat polyploidization, we examined the heat response of a subset of diploid and tetraploid progenitors as well as hexaploid wheat, but we did not find any rules of heat tolerance from an evolutionary perspective. These lines of evidence suggest that TaMBF1c itself cannot fully explain the heat response variations during the wheat polyploidization event, probably because heat tolerance is a quantitative trait controlled by multiple genes with minor effects. In addition, different ecological environments would also promote adaptation variation. Yet, we believe that wheat progenitors possibly contain many superior alleles conferring heat tolerance, which merits further study.

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Author contributions

MX, HP, ZN and QS conceived the project. XT, ZQ, YZ, FW, AZ and HP collected the plant materials. XT, ZQ, JW, DQ, LZ and TL performed the research. ZQ, KY, ZH and YY analyzed the data. MX, YZ, IDS and HP wrote and revised the manuscript.

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Data availability

The data that support the findings of this study are openly available at the NCBI Bioproject under accession no. PRJNA741399.

References


**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

**Fig. S1** Sequence alignment of TaMBF1c homeologs.

**Fig. S2** Transactivation and EMSA analysis of class A HSFs on the TaMBF1c promoter.

**Fig. S3** Expression detection of TaMBF1c in wheat transgenic lines.

**Fig. S4** Strategy used for generating the TaMBF1c knockout mutant.

**Fig. S5** Western blot analysis of TaMBF1c protein in 40S, 60S and 80S monosome fractions.

**Fig. S6** Overview of differentially expressed genes between knockout mutant and wild-type at the transcriptional and translation level.

**Fig. S7** GO enrichment analysis of downregulated genes in the TaMBF1c knock-out mutant.

**Fig. S8** Proportion of genes with changed translation efficiency in WGCNA modules.

**Fig. S9** Gene expression network of the turquoise module.

**Table S1** Information for wheat accessions used in this study.

**Table S2** Primers and probes used in this study.

**Table S3** Gene IDs of 33 class A Hsf genes in wheat.

**Table S4** Candidates of TaMBF1c-interacting protein from Y2H screening.

**Table S5** Interaction proteins with TaMBF1c in Arabidopsis.

**Table S6** RNA-sequencing data under different conditions.

**Table S7** List of differentially expressed genes in RNA-sequencing data.

**Table S8** Gene translation efficiency matrix under various conditions.

**Table S9** List of genes with varied translation efficiency between the WT and KO under various conditions.

**Table S10** Genes used to construct the network and their corresponding coexpression modules.

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