Check for updates

New Phytologist

Research

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

Xuejun Tian[1]*, Zhen Qin[1]*, Yue Zhao[1]*, Jingjing Wen[1], Tianyu Lan[1], Liyuan Zhang[1], Fei Wang[1], Dandan Qin[1], Kuohai Yu[1], Aiju Zhao[2], Zhaorong Hu[1], Yingyin Yao[1], Zhongfu Ni[1], Qixin Sun[1], Ive De Smet[3,4], Huiru Peng[1] and Mingming Xin[1]

[1]State Key Laboratory for Agrobiotechnology, Key Laboratory of Crop Heterosis Utilization (MOE), Beijing Key Laboratory of Crop Genetic Improvement, China Agricultural University, Beijing 100193, China; [2]Hebei Academy of Agriculture and Forest Sciences, Shijiazhuang 050035, China; [3]Department of Plant Biotechnology and Bioinformatics, Ghent University, Ghent B-9052, Belgium; [4]VIB Center for Plant Systems Biology, Ghent B-9052, Belgium

Authors for correspondence:
Mingming Xin
Email: mingmingxin@cau.edu.cn

Huiru Peng
Email: penghuiru@cau.edu.cn

Received: *27 July 2021*
Accepted: *7 November 2021*

New Phytologist (2022) **233:** 1719–1731
doi: 10.1111/nph.17865

Key words: polysome profiling, stress granule, *TaMBF1c*, thermotolerance, wheat.

Summary

- Heat stress is a major limiting factor for global wheat production and causes dramatic yield loss worldwide. The *TaMBF1c* gene is upregulated in response to heat stress in wheat. Understanding the molecular mechanisms associated with heat stress responses will pave the way to improve wheat thermotolerance.
- Through CRISPR/Cas9-based gene editing, polysome profiling coupled with RNA-sequencing analysis, and protein–protein interactions, we show that TaMBF1c conferred heat response via regulating a specific gene translation in wheat.
- The results showed that *TaMBF1c* is evolutionarily conserved in diploid, tetraploid and hexaploid wheat species, and its knockdown and knockout lines show increased heat sensitivity. TaMBF1c is colocalized with the stress granule complex and interacts with TaG3BP. TaMBF1c affects the translation efficiency of a subset of heat responsive genes, which are significantly enriched in the 'sequence-specific DNA binding' term. Moreover, gene expression network analysis demonstrated that TaMBF1c is closely associated with the translation of heat shock proteins.
- Our findings reveal a contribution of TaMBF1c in regulating the heat stress response via the translation process, and provide a new target for improving heat tolerance in wheat breeding programs.

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

*These authors contributed equally to this work.

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation
This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

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 *dehydration-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 archaeal 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 mRNA, translation initiation factors, RNA binding proteins and the 40S ribosomal subunit (Kedersha *et al.*, 2005; Anderson & Kedersha, 2008; Protter & 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 (N7AT7B, N7AT7D, N7BT7A, N7BT7D, N7DT7A, N7DT7B) were used for chromosomal locations. The wheat materials were grown hydroponically in 1 : 10 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* homeologous 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* homeologous 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 $2^{-\Delta ct}$ 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

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation

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 33 *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 recombinant 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 Na_2HPO_4, 2 mM KH_2PO_4) 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 $MgCl_2$, 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 OD_{600} 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 *A. 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

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation

as described above. Transfected plants were cultured at 22°C for 3 d before being induced by heat stress (38°C for 30 min). Confocal imaging and colocalization analyses of infiltrated leaves were performed using a Zeiss 510 META confocal laser scanning microscope with excitation wavelength 488 nm and emission wavelengths 505–530 nm for gren fluorescent protein (GFP) and excitation wavelength 543 nm and emission wavelengths 560–615 nm for RFP. Statistical analysis was performed using ImageJ.

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-7B* coding sequence (CDS) was cloned into the bait vector pGBKT7. Yeast two-hybrid screening interaction assays were performed by transforming pairs of pGBKT7- 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 (Cali-Bio, 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 $MgCl_2$, 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 $MgCl_2$, 1 mM DTT, 100 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} cycloheximide. Five thousand A260 units polysomes 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 v.1.0) 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

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation

(v.3.12.0; Yu *et al.*, 2012) with the GO annotations of the IWGSC RefSeq v.1.1 transcriptome, which was download from the Ensembl Plant Biomart database (http://plants.ensembl.org/).

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. TE_{KO}/TE_{WT} was calculated and then $\log_2$-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

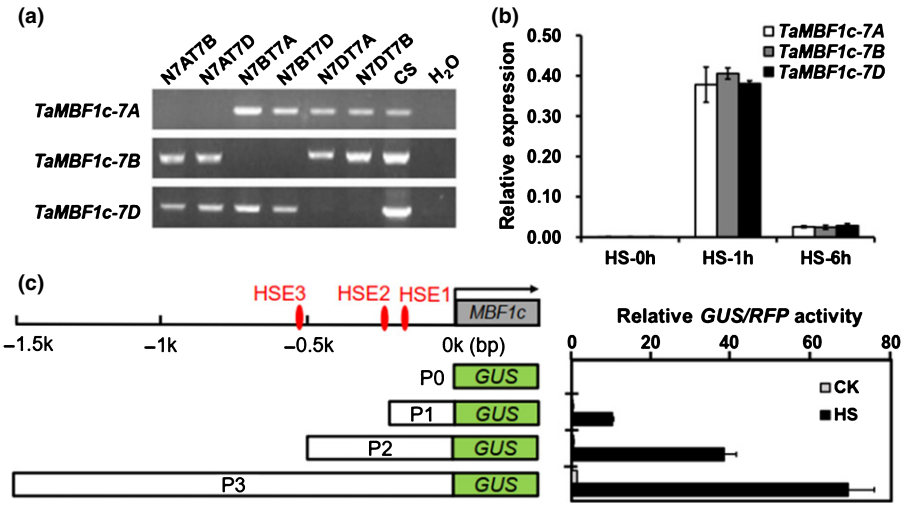

Fig. 1 Chromosomal location, expression pattern and promoter activity analysis of TaMBF1c. (a) Chromosomal locations of three homeologous *TaMBF1c* genes. The nomenclature represents different constitution of A, B or D genomes; for example, N7AT7B represents nullisomic 7A-tetrasomic7B; CS: Chinese Spring. (b) RT-qPCR analysis of expression patterns of wheat *TaMBF1c* genes in response to heat stress. Total RNA was isolated from leaves of wheat seedlings. The wheat *β-actin* gene was used as an internal reference. Error bars indicate the standard deviation (SD). (c) Transcriptional activation analysis of the *TaMBF1c-7B* promoter in response to heat treatment in *Nicotiana benthamiana* upon transient expression of *pTaMBF1c-7B:GUS*. Red dots indicate predicted HSE motifs located in the promoter region of *TaMBF1c-7B*. The empty pCAMBIA1300 vector (P0) was used as a control, and 200, 525 or 1500 bp upstream from the start codon of *TaMBF1c-7B* were designed as P1, P2 or P3, respectively, which were fused with the *GUS* reporter gene. Infiltrated leaves before and after heat treatment (38°C for 1 h) were harvested and used for *GUS* expression analyses. The levels of *GUS* activity were normalized by *RFP*. Data are means ± SD of three biological replicates.

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation

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 TaHSFA6e can bind to the HSE motif *in vitro*. The recombinant GST-TaHSFA6e protein and GST alone were incubated with the labeled oligonucleotide probes. In the EMSA, the probe was shifted when incubated with the GST-TaHSFA6e-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) at the seedling stage under heat stress conditions (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,

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation

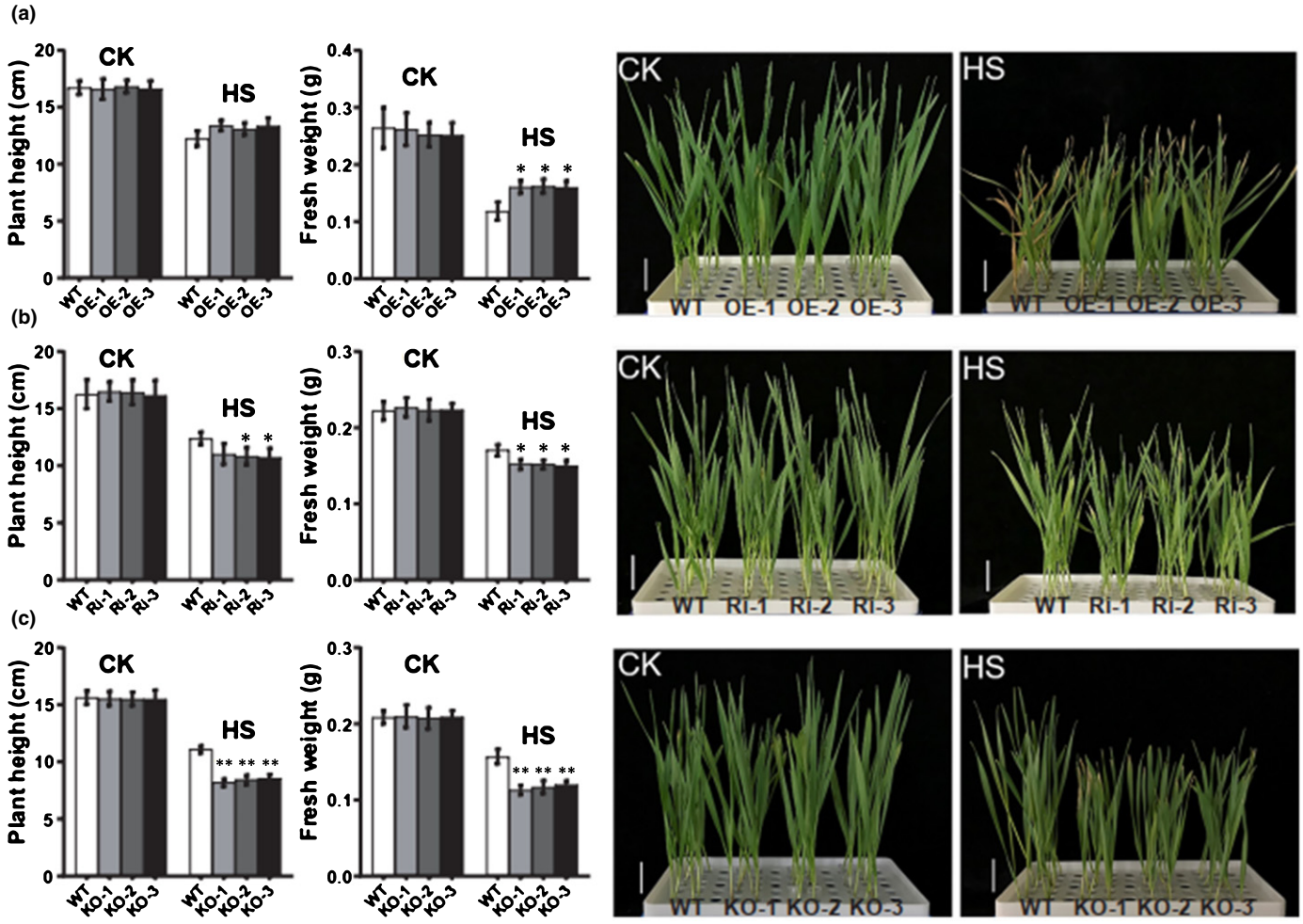

Fig. 2 Thermotolerance assays of *TaMBF1c* overexpression (OE), knockdown (Ri) and knockout (KO) lines in wheat. (a) Phenotypic and statistical analysis of plant height and fresh weight in response to heat stress in wheat *TaMBF1c* OE lines and WT. Bar, =2 cm. Each bar shows mean ± SD ($n \geq 7$). Single asterisk represents statistically significant differences at $P < 0.05$ determined by Student's *t*-test. (b) Phenotypic and statistical analysis of plant height and fresh weight in response to heat stress in wheat *TaMBF1c* knockdown lines and WT. Bar, =2 cm. Each bar shows mean ± SD ($n \geq 7$). Single asterisk represents statistically significant differences at $P < 0.05$ determined by Student's *t*-test. (c) Phenotypic and statistical analysis of plant height and fresh weight in response to heat stress in wheat *TaMBF1c* knockout lines and WT. Bar, =2 cm. Each bar shows mean ± SD ($n \geq 7$). Double asterisks represent statistically significant differences at $P < 0.01$ determined by Student's *t*-test. CK, normal conditions; HS, heat stress conditions.

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

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation

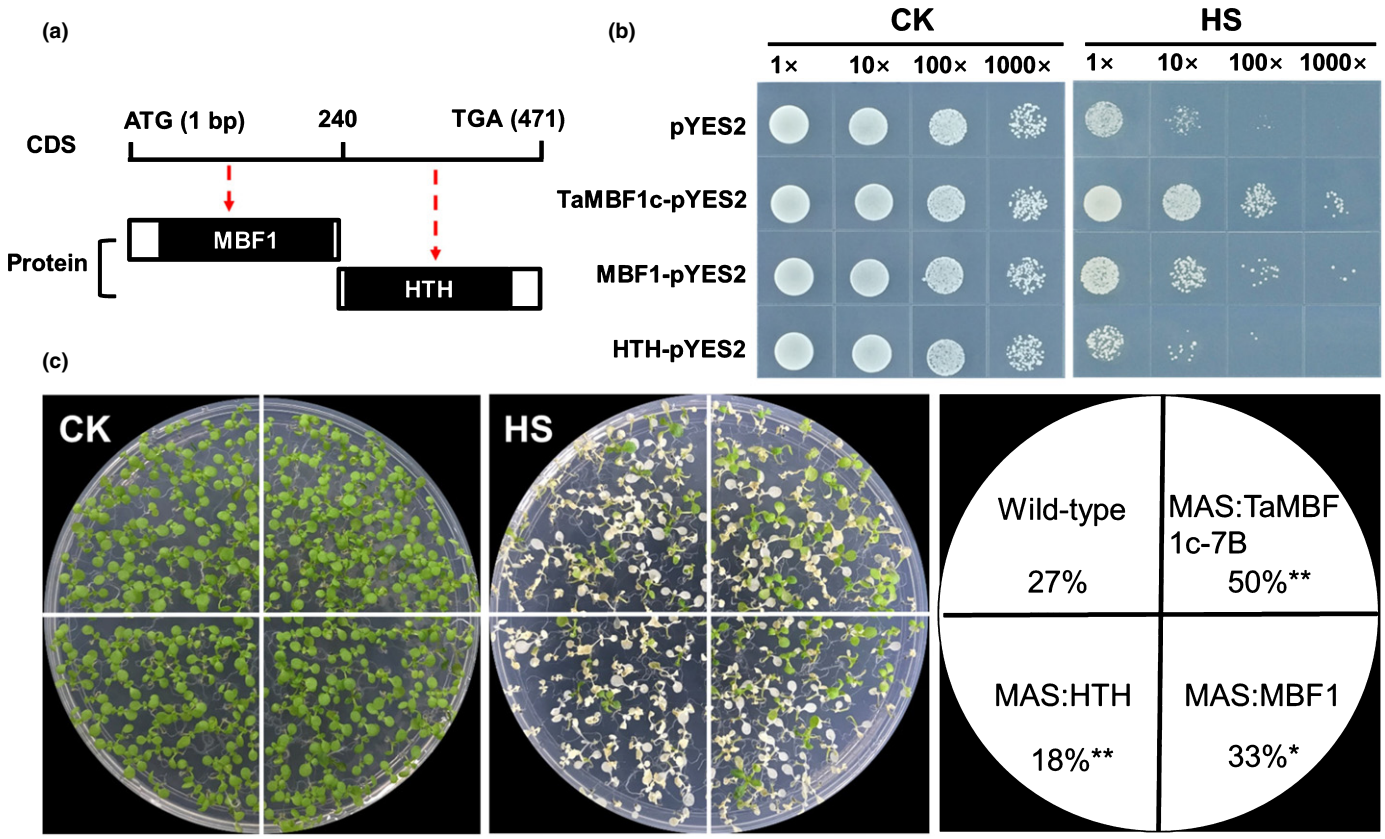

Fig. 3 Thermotolerance assays of different domains in TaMBF1c protein in yeast (*Saccharomyces cerevisiae*) and Arabidopsis (*Arabidopsis thaliana*). (a) TaMBF1c contains the MBF1 domain and HTH domain. (b) Thermotolerance assay in yeast. Growth of *S. cerevisiae* strains expressing TaMBF1c-7B, MBF1 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.

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 (eiF4A-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

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation

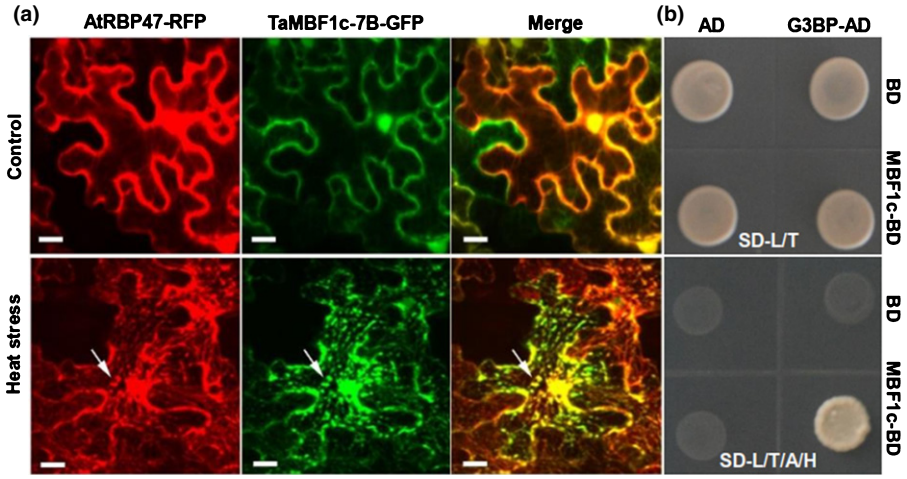

Fig. 4 Subcellular localization and protein interaction analyses of TaMBF1c. (a) Subcellular localization of TaMBF1c was analyzed in tobacco (*Nicotiana benthamiana*) epidermal cells under normal growth conditions and heat stress conditions (38°C for 30 min), respectively. RFP-tagged AtRBP47 served as a stress granule marker. Bar, 10 μm. White arrows denote colocalization. (b) Interaction analysis between TaMBF1c and G3BP in yeast (*Saccharomyces cerevisiae*). Yeast cells cotransformed with BD (pGBKT7) and AD (pGADT7), BD and G3BP-AD, as well as MBF1c-BD and AD vectors were set as controls. SD-L/T: SD-Leu$^-$/Trp$^-$ culture medium; SD-L/T/A/H: SD-Leu$^-$/Trp$^-$/Ade$^-$/His$^-$ culture medium.

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 thermotolerance 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

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation

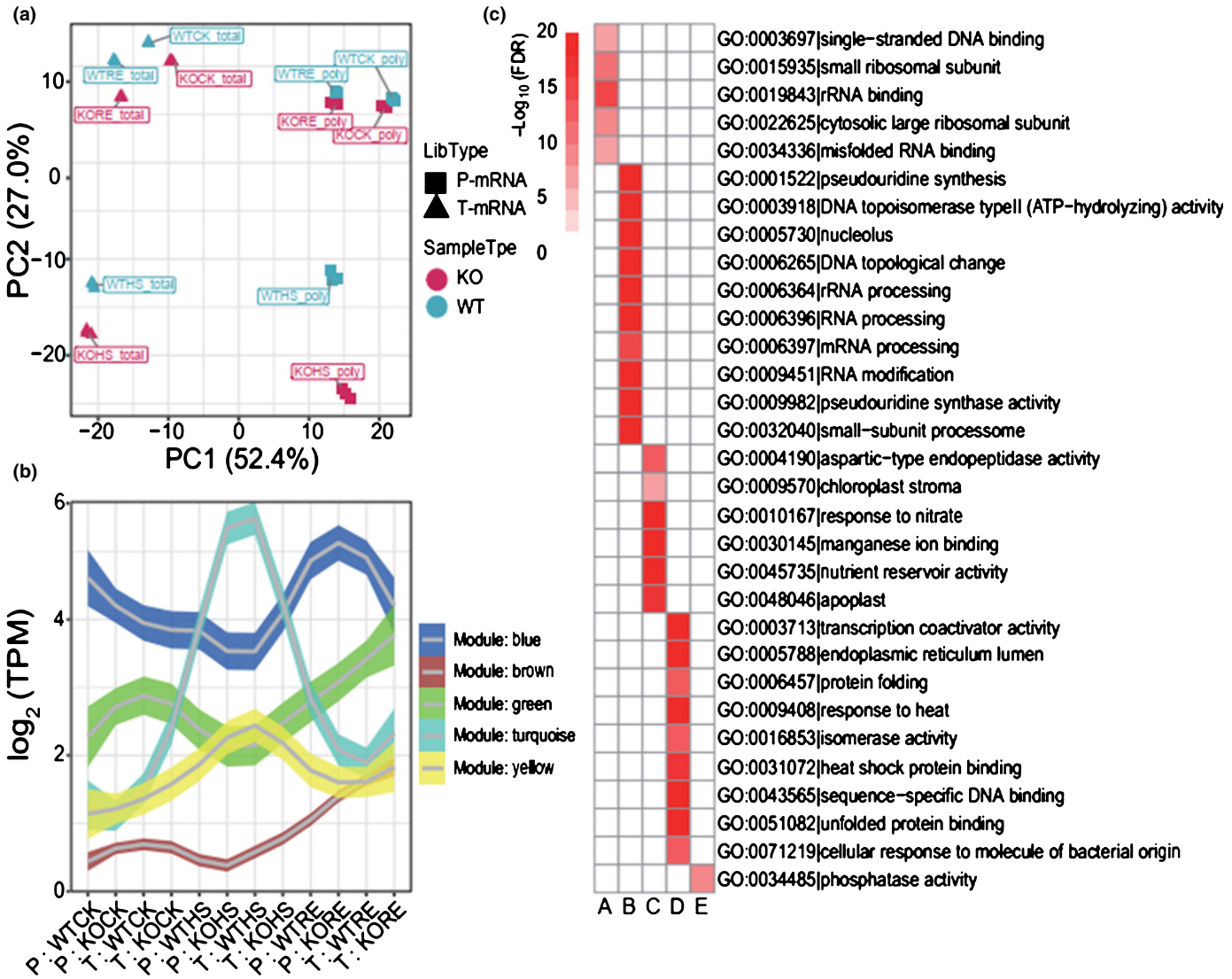

Fig. 5 Translational and transcriptional mRNA sequencing data analysis in wheat (*Triticum aestivum*). (a) The PCA analysis was based on gene expression patterns of 12 samples. The *x*-axis and *y*-axis indicate two principal components (PC), PC1 and PC2, respectively. The mutant and wild-type are color-coded 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 $\log_2$-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.

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 *atmbf1c* 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, *TaMBF1*c 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

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation

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.

Acknowledgements

This work was supported by the National Natural Science of China (31971942, 31571747 and 32130078).

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.

ORCID

Ive De Smet https://orcid.org/0000-0003-4607-8893
Zhaorong Hu https://orcid.org/0000-0002-1815-648X
Tianyu Lan https://orcid.org/0000-0002-7915-7363
Zhongfu Ni https://orcid.org/0000-0003-4524-7720
Huiru Peng https://orcid.org/0000-0002-8329-9428
Zhen Qin https://orcid.org/0000-0001-7185-3965
Qixin Sun https://orcid.org/0000-0002-3819-6892
Xuejun Tian https://orcid.org/0000-0002-0299-5115
Mingming Xin https://orcid.org/0000-0003-4010-4165
Yingyin Yao https://orcid.org/0000-0003-4122-4118

Data availability

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

References

Akter N, Rafiqul Islam IM. 2017. Heat stress effects and management in wheat. A review. *Agronomy for Sustainable Development* **37**. doi: 10.1007/s13593-017-0443-9.

Al-Whaibi MH. 2011. Plant heat-shock proteins: a mini review. *Journal of King Saud University – Science* **23**: 139–150.

Anderson P, Kedersha N. 2008. Stress granules: the Tao of RNA triage. *Trends in Biochemical Sciences* **33**: 141–150.

Asseng S, Ewert F, Martre P, Rötter RP, Lobell DB, Cammarano D, Kimball BA, Ottman MJ, Wall GW, White JW *et al.* 2015. Rising temperatures reduce global wheat production. *Nature Climate Change* **5**: 143–147.

Baltz A, Munschauer M, Schwanhäusser B, Vasile A, Murakawa Y, Schueler M, Youngs N, Penfold-Brown D, Drew K, Milek M *et al.* 2012. The mRNA-bound proteome and its global occupancy profile on protein-coding transcripts. *Molecular Cell* **46**: 674–690.

Bechtold U, Albihlal WS, Lawson T, Fryer MJ, Sparrow PAC, Richard F, Persad R, Bowden L, Hickman R, Martin C *et al.* 2013. Arabidopsis HEAT SHOCK TRANSCRIPTION FACTORA1b overexpression enhances water productivity, resistance to drought, and infection. *Journal of Experimental Botany* **64**: 3467–3481.

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation

Bita C, Gerats T. 2013. Plant tolerance to high temperature in a changing environment: scientific fundamentals and production of heat stress-tolerant crops. *Frontiers in Plant Science* **4**: 273.

Blombach F, Launay H, Snijders AL, Zorraquino V, Wu H, de Koning B, Brouns SJ, Ettema TG, Camilloni C, Cavalli A *et al.* 2014. Archaeal MBF1 binds to 30S and 70S ribosomes via its helix–turn–helix domain. *Biochemical Journal* **462**: 373–384.

Brendel C, Gelman L, Auwerx J. 2002. Multiprotein bridging factor-1 (MBF-1) is a cofactor for nuclear receptors that regulate lipid metabolism. *Molecular Endocrinology* **16**: 1367–1377.

Busk PK, Wulf-Andersen L, Strøm CC, Enevoldsen M, Thirstrup K, Haunsø S, Sheikh SP. 2003. Multiprotein bridging factor 1 cooperates with c-Jun and is necessary for cardiac hypertrophy *in vitro*. *Experimental Cell Research* **286**: 102–114.

Chen S, Zhou Y, Chen Y, Gu J. 2018. FASTP: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**: i884–i890.

Chen T, Xiao J, Xu J, Wan W, Qin BI, Cao A, Chen W, Xing L, Du C, Gao X *et al.* 2016. Two members of TaRLK family confer powdery mildew resistance in common wheat. *BMC Plant Biology* **16**: 27.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**: 735–743.

Costanzo MC, Mueller PP, Strick CA, Fox TD. 1986. Primary structure of wild-type and mutant alleles of the PET494 gene of *Saccharomyces cerevisiae*. *Molecular and General Genetics* **202**: 294–301.

Culbertson MR, Gaber RF, Cummins CM. 1982. Frameshift suppression in *Saccharomyces cerevisiae*. V. Isolation and genetic properties of nongroup-specific suppressors. *Genetics* **102**: 361–378.

Decker CJ, Parker R. 2012. P-bodies and stress granules: possible roles in the control of translation and mRNA degradation. *Cold Spring Harbor Perspectives in Biology* **4**: a012286.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. 2013. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**: 15–21.

Gill BS, Friebe B. 2002. Cytogenetics, phylogeny and evolution of cultivated wheats. In: Curtis BC, Rajaram S, Gomez-Macperson H, eds. *Bread wheat improvement and production*. Rome, Italy: FAO, 71–88.

Hendrick JL, Wilson PG, Edelman II, Sandbaken MG, Ursic D, Culbertson MR. 2001. Yeast frameshift suppressor mutations in the genes coding for transcription factor Mbf1p and ribosomal protein S3: evidence for autoregulation of S3 synthesis. *Genetics* **157**: 1141–1158.

Ishida Y, Tsunashima M, Hiei Y, Komari T. 2015. Wheat (*Triticum aestivum* L.) transformation using immature embryos. *Methods in Molecular Biology* **1223**: 189–198.

Kabe Y, Goto M, Shima D, Imai T, Wada T, Morohashi K, Shirakawa M, Hirose S, Handa H. 1999. The role of human *MBF1* as a transcriptional coactivator. *Journal of Biological Chemistry* **274**: 34196–34202.

Kedersha N, Anderson P. 2002. Stress granules: sites of mRNA triage that regulate mRNA stability and translatability. *Biochemical Society Transactions* **30**: 963–969.

Kedersha N, Panas MD, Achorn CA, Lyons S, Tisdale S, Hickman T, Thomas M, Lieberman J, McInerney GM, Ivanov P *et al.* 2016. G3BP–Caprin1–USP10 complexes mediate stress granule condensation and associate with 40S subunits. *Journal of Cell Biology* **212**: 845–860.

Kedersha N, Stoecklin G, Ayodele M, Yacono P, Lykke-Andersen J, Fritzler MJ, Scheuner D, Kaufman RJ, Golan DE, Anderson P. 2005. Stress granules and processing bodies are dynamically linked sites of mRNP remodeling. *Journal of Cell Biology* **169**: 871–884.

Kim G-D, Cho Y-H, Yoo S-D. 2015. Regulatory functions of evolutionarily conserved AN1/A20-like Zinc finger family proteins in Arabidopsis stress responses under high temperature. *Biochemical and Biophysical Research Communications* **457**: 213–220.

Klass DM, Scheibe M, Butter F, Hogan GJ, Mann M, Brown PO. 2013. Quantitative proteomic analysis reveals concurrent RNA–protein interactions and identifies new RNA-binding proteins in *Saccharomyces cerevisiae*. *Genome Research* **23**: 1028–1038.

Kotak S, Larkindale J, Lee U, von Koskull-Döring P, Vierling E, Scharf K-D. 2007. Complexity of the heat stress response in plants. *Current Opinion in Plant Biology* **10**: 310–316.

Krapp S, Greiner E, Amin B, Sonnewald U, Krenz B. 2017. The stress granule component G3BP is a novel interaction partner for the nuclear shuttle proteins of the nanovirus pea necrotic yellow dwarf virus and geminivirus abutilon mosaic virus. *Virus Research* **227**: 6–14.

Kwon SC, Yi H, Eichelbaum K, Föhr S, Fischer B, You KT, Castello A, Krijgsveld J, Hentze MW, Kim VN. 2013. The RNA-binding protein repertoire of embryonic stem cells. *Nature Structural & Molecular Biology* **20**: 1122–1130.

Lang BJ, Guerrero ME, Prince TL, Okusha Y, Bonorino C, Calderwood SK. 2021. The functions and regulation of heat shock proteins: key orchestrators of proteostasis and the heat shock response. *Archives of Toxicology* **95**: 1943–1970.

Liu H, Li H, Hao C, Wang K, Wang Y, Qin L, An D, Li T, Zhang X. 2020. TaDA1, a conserved negative regulator of kernel size, has an additive effect with TaGW2 in common wheat (*Triticum aestivum* L.). *Plant Biotechnology Journal* **18**: 1330–1342.

Liu L, Zhang Y, Tang S, Zhao Q, Zhang Z, Zhang H, Dong L, Guo H, Xie Q. 2010. An efficient system to detect protein ubiquitination by agroinfiltration in *Nicotiana benthamiana*. *The Plant Journal* **61**: 893–903.

Liu Q-X, Jindra M, Ueda H, Hiromi Y, Hirose S. 2003. *Drosophila* MBF1 is a co-activator for Tracheae defective and contributes to the formation of tracheal and nervous systems. *Development* **130**: 719–728.

Liu Z, Xin M, Qin J, Peng H, Ni Z, Yao Y, Sun Q. 2015. Temporal transcriptome profiling reveals expression partitioning of homeologous genes contributing to heat and drought acclimation in wheat (*Triticum aestivum* L.). *BMC Plant Biology* **15**: 152.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**: 550.

Merret R, Carpentier M-C, Favory J-J, Picart C, Descombin J, Bousquet-Antonelli C, Tillard P, Lejay L, Deragon J-M, Charng Y. 2017. Heat shock protein HSP101 affects the release of ribosomal protein mRNAs for recovery after heat shock1. *Plant Physiology* **174**: 1216–1225.

Mittler R, Finka A, Goloubinoff P. 2012. How do plants feel the heat? *Trends in Biochemical Sciences* **37**: 118–125.

Ni Z, Li H, Zhao Y, Peng H, Hu Z, Xin M, Sun Q. 2018. Genetic improvement of heat tolerance in wheat: recent progress in understanding the underlying molecular mechanisms. *Crop Journal* **6**: 32–41.

Ogawa D, Yamaguchi K, Nishiuchi T. 2007. High-level overexpression of the Arabidopsis *HsfA2* gene confers not only increased themotolerance but also salt/osmotic stress tolerance and enhanced callus growth. *Journal of Experimental Botany* **58**: 3373–3383.

Ohama N, Sato H, Shinozaki K, Yamaguchi-Shinozaki K. 2017. Transcriptional regulatory network of plant heat stress response. *Trends in Plant Science* **22**: 53–65.

Protter DSW, Parker R. 2016. Principles and properties of stress granules. *Trends in Cell Biology* **26**: 668–679.

Qin D, Wang F, Geng X, Zhang L, Yao Y, Ni Z, Peng H, Sun Q. 2015. Overexpression of heat stress-responsive *TaMBF1c*, a wheat (*Triticum aestivum* L.) multiprotein bridging factor, confers heat tolerance in both yeast and rice. *Plant Molecular Biology* **87**: 31–45.

Qin D, Wu H, Peng H, Yao Y, Ni Z, Li Z, Zhou C, Sun Q. 2008. Heat stress-responsive transcriptome analysis in heat susceptible and tolerant wheat (*Triticum aestivum* L.) by using wheat genome array. *BMC Genomics* **9**: 432.

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Research* **13**: 2498–2504.

Suzuki N, Bajad S, Shuman J, Shulaev V, Mittler R. 2008. The transcriptional co-activator MBF1c is a key regulator of thermotolerance in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **283**: 9269–9275.

Suzuki N, Rizhsky L, Liang H, Shuman J, Shulaev V, Mittler R. 2005. Enhanced tolerance to environmental stress in transgenic plants expressing the transcriptional coactivator multiprotein bridging factor 1c. *Plant Physiology* **139**: 1313–1322.

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation

Suzuki N, Sejima H, Tam R, Schlauch K, Mittler R. 2011. Identification of the MBF1 heat-response regulon of *Arabidopsis thaliana*. *The Plant Journal* **66**: 844–851.

Takemaru K, Harashima S, Ueda H, Hirose S. 1998. Yeast coactivator MBF1 mediates GCN4-dependent transcriptional activation. *Molecular and Cellular Biology* **18**: 4971–4976.

Takemaru K, Li F-Q, Ueda H, Hirose S. 1997. Multiprotein bridging factor 1 (*MBF1*) is an evolutionarily conserved transcriptional coactivator that connects a regulatory factor and TATA element-binding protein. *Proceedings of the National Academy of Sciences, USA* **94**: 7251–7256.

Tsuda K, Yamazaki K. 2004. Structure and expression analysis of three subtypes of Arabidopsis *MBF1* genes. *Biochimica et Biophysica Acta (BBA) – Gene Structure and Expression* **1680**: 1–10.

Wang W, Vinocur B, Shoseyov O, Altman A. 2004. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. *Trends in Plant Science* **9**: 244–252.

Wang Z, Li N, Jiang S, Gonzalez N, Huang X, Wang Y, Inzé D, Li Y. 2016. SCF(SAP) controls organ size by targeting PPD proteins for degradation in *Arabidopsis thaliana*. *Nature Communications* **7**: doi: 10.1038/ncomms11192.

Weber C, Nover L, Fauth M. 2008. Plant stress granules and mRNA processing bodies are distinct from heat stress granules. *The Plant Journal* **56**: 517–530.

Winkles JA. 1997. Serum- and polypeptide growth factor-inducible gene expression in mouse fibroblasts. *Progress in Nucleic Acid Research and Molecular Biology* **58**: 41–78.

Xue G-P, Sadat S, Drenth J, McIntyre CL. 2014. The heat shock factor family from *Triticum aestivum* in response to heat and other major abiotic stresses and their role in regulation of heat shock protein genes. *Journal of Experimental Botany* **65**: 539–557.

Yang H, Liu X, Xin M, Du J, Hu Z, Peng H, Rossi V, Sun Q, Ni Z, Yao Y. 2016. Genome-wide mapping of targets of maize histone deacetylase *HDA101* reveals its function and regulatory mechanism during seed development. *Plant Cell* **28**: 629–645.

Yang X, Tian Z, Sun L, Chen B, Tubiello FN, Xu Y. 2017. The impacts of increased heat stress events on wheat yield under climate change in China. *Climatic Change* **140**: 605–620.

Yoshida T, Ohama N, Nakajima J, Kidokoro S, Mizoi J, Nakashima K, Maruyama K, Kim J-M, Seki M, Todaka D *et al.* 2011. Arabidopsis *HsfA1* transcription factors function as the main positive regulators in heat shock-responsive gene expression. *Molecular Genetics and Genomics* **286**: 321–332.

Yu G, Wang L-G, Han Y, He Q-Y. 2012. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS: A Journal of Integrative Biology* **16**: 284–287.

Zhang B, Horvath S. 2005. A general framework for weighted gene co-expression network analysis. *Statistical Applications in Genetics and Molecular Biology* **4**: Article17.

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.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation