Virus Genes (2019) 55:209–217
https://doi.org/10.1007/s11262-018-1626-4

Triticum aestivum heat shock protein 23.6 interacts with the coat protein of wheat yellow mosaic virus

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Received: 13 August 2018 / Accepted: 12 December 2018 / Published online: 18 December 2018

Abstract
The role of heat shock proteins (HSPs) in viral replication has been described in numerous publications. Wheat yellow mosaic virus (WYMV) belongs to the genus *Bymovirus* (family *Potyviridae*), which causes yellow mosaic and dwarf symptoms in wheat (*Triticum aestivum*). In this study, the *T. aestivum* heat shock protein 23.6 (TaHSP23.6), which belongs to the small heat shock protein family, was shown to interact with the WYMV coat protein (CP) in a yeast two-hybrid screen. The co-localization and interaction between TaHSP23.6 and WYMV CP were additionally verified in *Nicotiana benthamiana* by co-localization assays and bimolecular fluorescence complementation (BiFC). Not only the transcription of *TaHSP23.6* but also that of other HSP family members (*TaHSP70, TaHSP90,* and *TaHSP101*) was up-regulated in WYMV-infected leaves, as shown by semi-quantitative PCR assays. Interestingly, the expression levels of the *T. aestivum* heat stress transcription factor A2 (*TaHSFA2*) members were varied in response to WYMV infection. Thus, our results provide insights into the interaction between TaHSP23.6 and WYMV infection.

Keywords Wheat yellow mosaic virus · *Triticum aestivum* heat shock protein 23.6 · Coat protein · Interaction · Chaperone

Introduction

Wheat yellow mosaic virus (WYMV) is one of the economically important pathogens of wheat (*Triticum aestivum*) causing a yellow mosaic disease, which was first reported in Japan and has resulted in serious yield reductions in East Asia [1, 2]. WYMV belongs to the genus *Bymovirus* (family *Potyviridae*) with bipartite plus-sense RNA genomes, and is transmitted by an obligate soil-inhabiting fungal-like organism, *Polymyxa graminis* (order *Plasmodiophorales*) [3]. RNA1 of WYMV encodes a large polyprotein that is cleaved into eight mature proteins: P3, 7K, cylindrical inclusion protein (CI), 14K, genome-linked viral protein (VPg), nuclear inclusion protein a-proteinase (NIa-Pro), nuclear inclusion protein b (NIb), and coat protein (CP). RNA2 encodes a polyprotein that gives rise to two proteins of P1 and P2 [3–5]. As a multifunctional viral protein of *Potyviridae* family members, apart from accurate assembly of viral particles, the CP is also involved in the inhibition of viral RNA translation, RNA replication, host-specific long-distance transport, and virus transmission by vector [6–9]. The WYMV encodes CP associated with the nucleocytoplasmic shuttling of VPg possibly by assisting VPg to attain a proper conformation during WYMV infection [10]. In addition, the WYMV CP is predicted to be involved in cell-to-cell movement based on the functions of well-characterized homologues of viruses in the genus *Potyvirus* [11]. However, detailed functional analysis of WYMV CP is still not well defined.

The small heat shock proteins (sHSPs) are found in all kingdoms of life that be proposed as the first line of defense with subunit molecular masses of 12–43 kDa, which have a multitude of crucial functions in normal and pathological conditions [12]. Overall, 163 TaHSP20 genes have been

Edited by Karel Petrzik.

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identified and classified in wheat with functional divergences, a much higher number of transcribed genes than in the wheat genome [13, 14]. In addition to the molecular chaperone-like activity in preventing aggregation of proteins/peptides, sHSPs are also involved in several plant–virus infections. The transcript levels of three *Solanum lycopersicum HSP20* genes (*SlHSP11.9, SlHSP17.6D,* and *SlHSP27.1*) were up-regulated, and another three genes (*SlHSP15.2, SlHSP17.7B,* and *SlHSP15.7*) were down-regulated by tomato spotted wilt virus (TSWV) in tomato [15]. The rice (*Oryza sativa*) OsHSP20 and its homologue (NbHSP20) were highly induced during rice stripe virus (RSV) infection and interacted with the viral RNA-dependent RNA polymerase (RdRp) and Pc4 of RSV [16, 17]. The subcellular localization and distribution pattern of HSP20 were altered in RSV-infect plants [16]. In addition to sHSPs, many other HSP family members, such as HSP100, HSP90, and HSP70, were also found to play multiple roles in replication, viral assembly, movement, and other steps of the viral life cycle [15, 18–21]. The rice HSP70 was also found to be involved in RSV replication by interacting with RdRp as well as OsHSP20 [22]. HSP70, HSP90, and its co-chaperones were involved in the establishment of tomato yellow leaf curl virus (TYLCV) infection [23]. TYLCV CP recruits HSP70 to promote CP translocation into the nuclei of infected cells [23]. Some functional link between these plant–virus interactions is unknown. The considerable evidence supports the important function of plant HSPs during virus infection, while a full understanding of the importance of wheat sHSPs and relevant chaperones during virus infection is lacking.

In eukaryotes, the expression of HSP genes is governed by heat shock transcription factors (HSFs) [24]. HSF binding to heat shock elements (HSEs) leads to transcriptional regulation of HSPs. Plants have a large HSF family involved in the activation of complex heat stress response networks. There are 56 TaHSF members identified in wheat, and they are classified into A, B, and C classes [25]. The heat-induced expression profiles of many HSP genes are paralleled by those of A2, which are the most strongly induced proteins during heat stress [25]. Recent studies demonstrated that capturing of HSFs by viral proteins could suppress the transcriptional activation of heat stress response genes [26]. All six TYLCV-encode proteins could interact with tomato HSFA2 in vitro [27]. Moreover, the CP developed complexes with HSFA2 in the nuclei [27]. The transcriptional activation of *HSFA2* and *HSFB1* and the downstream genes *HSP17* and *Apx1/2* was suppressed in TYLCV-infected tomatoes [23].

To gain insight into the potentially roles of CP during WYMV infection, we obtained a key member of the sHSP family in wheat, TaHSP23.6 (Accession Number: AF104108), and carried out functional analysis by checking the interaction between TaHSP23.6 and WYMV CP in vitro and vivo. Meanwhile, the possible interaction mechanism was discussed by identifying differential expression levels of *TaHSP23.6* and related chaperones in response to WYMV infection, as well as the TaHSFA2 transcription factors. Finally, these data demonstrate the association of TaHSP23.6 with WYMV infection.

Materials and methods

Plant materials and plasmids

WYMV-infected wheat plants (*T. aestivum*, cultivar Linmai4) were derived from a field in Linyi, Shandong Province of China, and prepared as described previously [28]. *Nicotiana benthamiana* plants were grown in a glasshouse at 22–24 °C with a photoperiod of 16-h light/8-h dark. The pCV-nYFP/pCV-cYFP and pCV-GFP/RFP vectors were described previously [22].

Yeast two-hybrid assay

Yeast two-hybrid assays were performed by using a GAL4-based system as described in the protocol provided by the manufacturer (Clontech). The full length of *TaHSP23.6* (Accession Number: AF104108) and WYMV *CP* were cloned and fused to the Gal DNA-binding domain (BD, vector: pGBKT7) or Gal4 activation domain (AD, vector: pGADT7), using the primers listed in Table 1. Bait and prey plasmids were co-transformed into the yeast strain Y2HGold cells using standard PEG/LiCl transformation protocols. The transformation mixture was grown on selection plates SD/-Trp-Leu-Ade-His solid medium with 20 mg/L of X-α-gal for 3–4 days at 30 °C. The co-transformed pGBKT7-53 and pGADT7-T plasmids were used as the positive control, and the co-transformed pGBKT7-lam and pGADT7-T plasmids were used as the negative control. The cells were diluted to four concentrations: 1, 1/10, 1/100, 1/1000.

Subcellular localization and bimolecular fluorescence complementation (BiFC) assays

The plant binary vectors used in the subcellular localization and BiFC experiments have been described previously [22]. The full-length coding regions of *TaHSP23.6* and WYMV *CP* were cloned from WYMV-infected wheat and fused into the pCV-GFP/RFP vectors, respectively, forming pCV-TaHSP23.6-RFP and pCV-WYMV CP-GFP. The primer sequences are listed in Table 1. *Escherichia coli* strain DH5α was used for transformation. For subcellular localization assay, pCV-TaHSP23.6-RFP or pCV-WYMV CP-GFP was introduced into *Agrobacterium tumefaciens*

Table 1 Primer sequences for yeast two-hybrid, subcellular localization, BiFC, and semi-qPCR assays

Primer	Sequence(5′ to 3′)	Accession number
TaHSP23.6 for YTH	*CATATG*ATGGCTTCCGCCGTCGATT and *GGATCC*CTACTC GACCTTGACGTGG	AF104108
TaHSP23.6 for localization	*GGATCC*ATGGCTTCCGCCGTCGATT and *GGTACC*CTCGAC CTTGACGTGGAAG	AF104108
TaHSP23.6 for BiFC	*GGTACC*ATGGCTTCCGCCGTCGATT and *GAGCTC*CTACTC GACCTTGACGTGG	AF104108
WYMV CP for YTH	*CATATG*GCAGCTGACACACAAACAGAC and *GAATTC*TTA GGTTAGTTCTGGGTGTCC	NP_697040
WYMV CP for localization	*GGTACC*ATGGCAGCTGACACACAAACAGAC and *GTCGAC* GTTAGTTCTGGGTGTCCATC	NP_697040
WYMV CP for BiFC	*GGTACC*GCAGCTGACACACAAACAGAC and *GAGCTC*TTA GGTTAGTTCTGGGTGTCC	NP_697040
TaHSP23.6 for semi-q PCR	ATGGCTTCCGCCGTCGATTGCAAG and CGTCAGCAGAGG GCACCTCGA	AF104108
TaHSP70 for semi-qPCR	GGAGAGGGCAACGAGAAGGT and CTTGTTCTTCTGCCC CGTCG	AF005993, AK360096
TaHSP90 for semi-qPCR	CAGCCTCAAGGACTACGTCAC and CTCCTCCATGATGCC GTTCTC	GQ240772, GQ240773, GQ240774
TaHSP101 for semi-qPCR	GCAAGGTCATCCTCTTCATC and CATGATGTACCTCGCCGA GA	AJ970534, AJ970535, AJ970536, KT355890
WYMV CP for semi-qPCR	GCAGCTGACACACAAACAGAC and TTAGGTTAGTTCTGG GTGTCC	NP_697040
Actin for semi-qPCR	CCTTCGTTTGGACCTTGCTGGCCG and AGGGCCACGTAA GCGAGCTTCTCC	AB181991

The enzyme cutting sites are marked with italics

YTH yeast two-hybrid assays, *semi-qPCR* semi-quantitative PCR assay

strain EHA105, respectively, and agroinfiltrated at an OD_{600} of 0.6–0.8 into fully expanded leaves of 3-week-old *N. benthamiana*. Empty pCV-GFP/RFP vectors were used as control. For co-localization assay, EHA105 strains co-expressing pCV-TaHSP23.6-RFP and pCV-WYMV CP-GFP were adjusted to an OD_{600} of 0.8:0.8 before leaf infiltration. BiFC assays were performed as described in the co-localization assay. Binary plasmids carrying full-length coding regions of *TaHSP23.6* or WYMV *CP* either fused to the N-terminal (pCV-nYFP) or C-terminal (pCV-cYFP) regions of yellow fluorescent protein (YFP), generating recombinant plasmids pCV-nYFP-TaHSP23.6 and pCV-cYFP-WYMV CP. The combinations pCV-nYFP-TaHSP23.6/ pCV-cYFP and pCV-nYFP/ pCV-cYFP-WYMV CP were used as the negative controls. Fluorescence was visualized between 2 and 4 days post-infiltration (dpi) by confocal microscopy (Leica). GFP and RFP was excited at 488 nm and the emitted light captured between 500 and 550 nm, and YFP was excited at 514 nm and the emitted light captured between 530 and 600 nm [20].

RNA isolation and semi-quantitative PCR analysis

Total RNA was extracted from healthy and WYMV-infected wheat leaves using RNAiso Plus (TaKaRa) and treated with RNase-free DNase I (TaKaRa). For cDNA synthesis, 1 μg of total RNA of each sample was reverse transcribed using RevertAid H Minus Reverse transcriptase according to the manufacturer's protocol (Fermentas). Transcription analyses were performed by semi-quantitative polymerase chain reaction (semi-qPCR) using gene-specific primers. Primers were designed according to the conserved domains of *TaHSPs* members in the NCBI database (Table 1). Accession numbers of the *TaHSPs* genes are provided in Table 1. Primers designed for *TaHSFs* were described previously [25, 26]. The *T. aestivum Actin* gene (Accession Number: AB181991.1) was used as the internal reference gene for analysis. To confirm the systemic infection of WYMV, viral RNAs in a new leaf were amplified by reverse transcriptase-PCR (RT-PCR) with primers designed from the WYMV *CP* sequence. The amplicons were separated in 2% agarose

gels, and the gene specificity was confirmed by directly sequencing all RT-PCR products. The grayscale values of DNA bands were analyzed by ImageJ and Date processing system (DPS). The band of the first sample was used as the standard, and then, the ratios of the grayscale value between the other bands and a standard strip were calculated.

Western blotting

Total proteins of plant samples were extracted with lysis buffer (100 mM Tris-HCl, pH 8.8, 60% SDS, 2% β-mercaptoethanol). Proteins were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto 0.45 μm nitrocellulose membranes (Amersham) by wet electroblotting. Host proteins were detected using primary anti-HSP70 (1:3000) and anti-HSP90 (1:3000) antibodies (Agrisera) and a secondary polyclonal alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (1:10,000) (Sigma) secondary antibody. Antibody of WYMV CP was produced as described previously [10]. β-Actin dyed by Ponceau S was used as a loading control. The antigen–antibody complexes were visualized using nitrotetrazolium blue chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) buffer (Sigma) under standard conditions.

Results

TaHSP23.6 interacted with WYMV CP in vitro

CP is a multifunctional protein in the process of virus infection. We investigated whether some host protein in wheat were involved in these biological processes interacted with WYMV. The GAL4-based yeast two-hybrid screens between WYMV CP and HSP family members in wheat (*T. aestivum*) were performed. A small HSP was identified and sequencing result confirmed it was a nuclear-encoded mitochondrion-localized sHSP with an ORF of 651 bp coding for 216 amino acids in wheat (TaHSP23.6, Accession Number: AF104108). The full length of TaHSP23.6 and WYMV CP was cloned and fused to the Gal DNA-binding domain (BD) and Gal4 activation domain (AD), respectively. The recombinants (BK-TaHSP23.6 and AD-WYMV CP) were co-transformed into Y2H Gold cells. Yeast cells co-expressing pGBKT7-53 and pGADT7-T were used as positive controls. Transformants containing TaHSP23.6 and CP appeared blue on selective Leu-/Trp-/His-/Ade-SD medium with X-α-gal, as well as the positive control (Fig. 1a). The results showed that TaHSP23.6 interacted with WYMV CP in vitro.

The subcellular location of TaHSP23.6 and WYMV CP in *N. benthamiana* was confirmed by confocal microscopy.

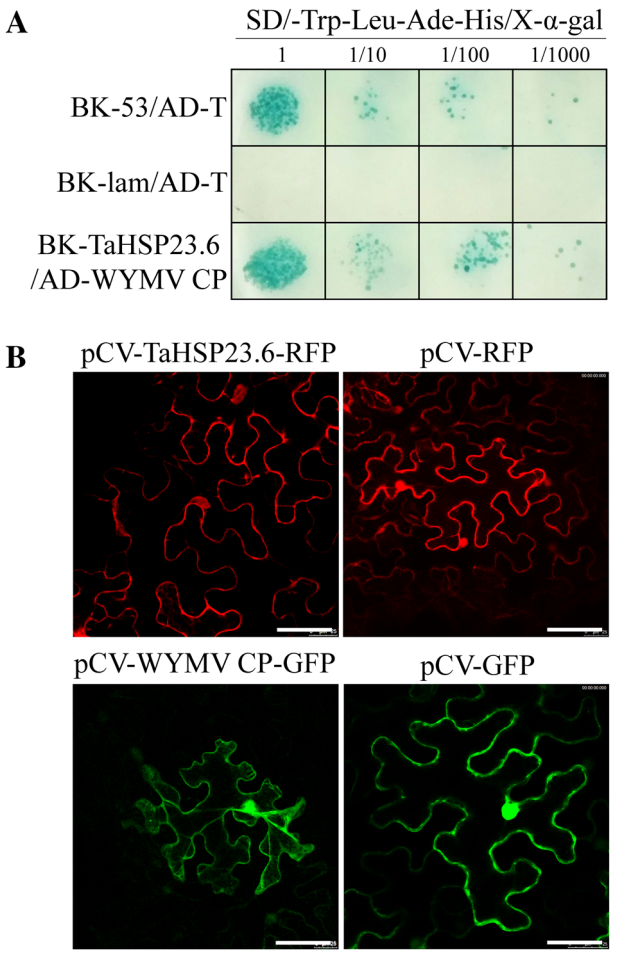

Fig. 1 TaHSP23.6 interacts with WYMV CP in yeast two-hybrid analysis and subcellular localization of TaHSP23.6 and WYMV CP. **a** The interaction of TaHSP23.6 with WYMV CP was determined by yeast two-hybrid assays. Co-transformed yeast cells harboring BD-TaHSP23.6 and AD-WYMV CP were plated on selection plates SD/-Trp-Leu-Ade-His/X-α-gal solid medium. Y2H Gold strains carrying AD-T/BK-53 or AD-T/BK-lam were used as the positive control or negative control. The cells were diluted to four concentrations: 1, 1/10, 1/100, 1/1000. For each experiment, yeast strains were maintained at 30 °C for 3–4 days until blue colony formed on media. **b** Localization of TaHSP23.6 and WYMV CP protein within *N. benthamiana* leaf epidermal cells is agroinfiltrated with pCV-TaHSP23.6-RFP or pCV-WYMV CP-GFP by RFP or GFP assay. Empty pCV-GFP/RFP vectors were used as controls. Fluorescence photographs were taken at 48 hpi. Scale bar, 50 μm

RFP-fused TaHSP23.6 or GFP-fused CP was expressed alone in *N. benthamiana* leaves and the subsequent fluorescence was examined under confocal microscopy. The RFP fluorescence and GFP fluorescence were both detected at the cytoplasm and the nucleus in cells expressing RFP-fused TaHSP23.6 or GFP-fused CP, respectively (Fig. 1b), indicating that TaHSP23.6 and WYMV CP were all distributed in both the cytoplasm and the nucleus.

TaHSP23.6 interacted with WYMV CP in *N. benthamiana*

The co-localization of TaHSP23.6 with WYMV CP was investigated. RFP-fused TaHSP23.6 and GFP-fused CP were co-expressed in *N. benthamiana* epidermal cells using *Agrobacterium*. At 3 dpi, RFP-fused TaHSP23.6 co-localized with GFP-fused CP in the cytoplasm and nucleus (Fig. 2a), with no significant difference as they expressed alone in the cell. Our results indicated that WYMV CP and TaHSP23.6 functions in the same cellular space, further proving the possible interaction between the two proteins in vivo. To confirm this hypothesis, BiFC analysis was performed to detect the interaction between the WYMV CP and TaHSP23.6 (Fig. 2b). Fusion proteins of full-length CP or TaHSP23.6 with either the n-YFP or c-YFP, respectively, were expressed in pairwise combinations using agro-infiltration of *N. benthamiana* leaves. As controls, single BiFC constructs of TaHSP23.6 or CP were infiltrated. Strong fluorescent signals were present at the cell periphery and many aggregations were formed during of co-expression TaHSP23.6 and WYMV CP (Fig. 2b). The results further corroborated the observation that the TaHSP23.6 interacts with WYMV CP in vivo.

TaHSPs abundance changed in WYMV-infected wheat

Host HSPs have been reported to be induced by virus infection and participate in multiple steps of the viral cycle of different viruses [12, 15, 19–22, 29–31]. The expression level of *TaHSP23.6* was detected by semi-qPCR to determine whether WYMV could induce the expression of *HSP* genes in wheat. Total RNA was extracted from healthy and WYMV-infected wheats. The specificity of cDNA amplification of *HSP* gene was confirmed by sequencing the RT-PCR products. The results showed that *TaHSP23.6* was markedly increased in virus-infected wheat that was hard

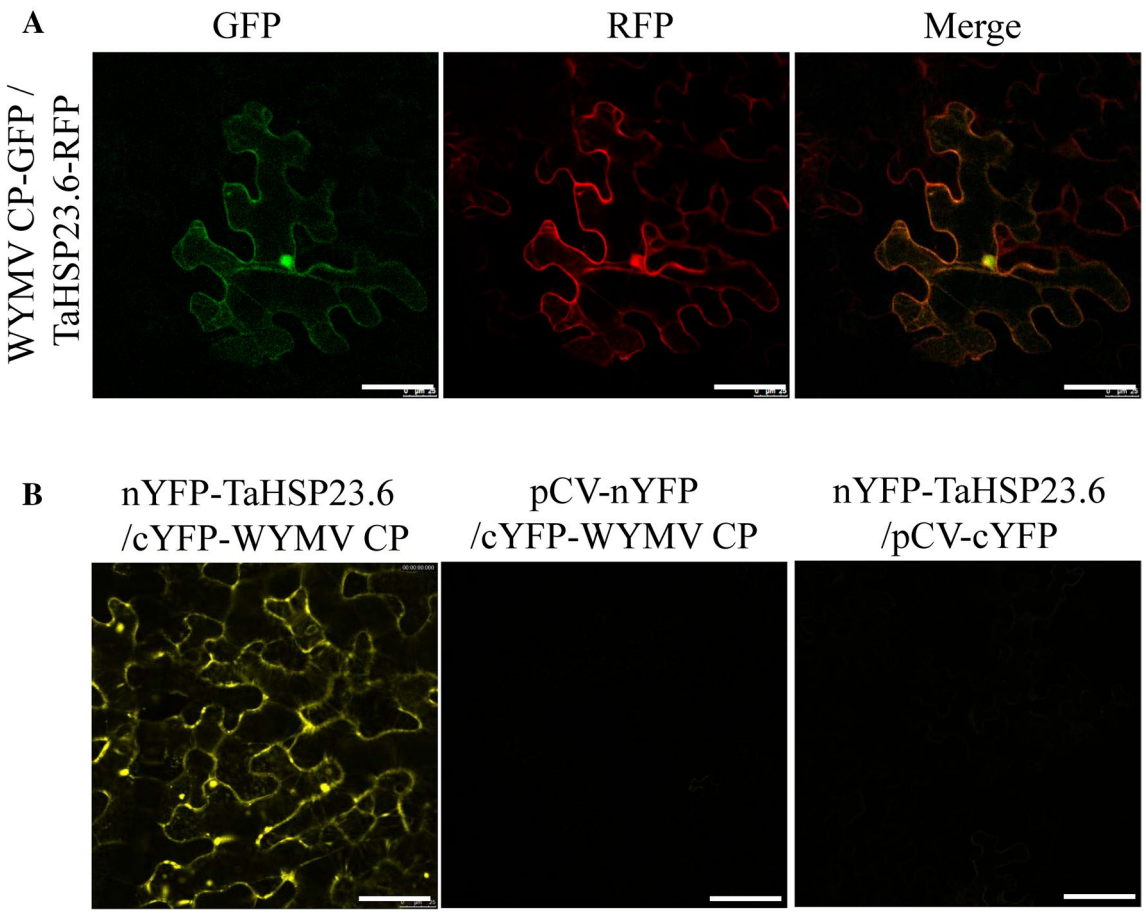

Fig. 2 The interaction between TaHSP23.6 and WYMV CP was confirmed in *N. benthamiana* by co-localization analyses and bimolecular fluorescence complementation (BiFC) assays. **a** Confocal micrographs of *N. benthamiana* co-expressing pCV-TaHSP23.6-RFP and pCV-WYMV CP-GFP at 48 hpi. The red fluorescence coincided with the green fluorescence in cells co-expressing TaHSP23.6-RFP and WYMV CP-GFP. Scale bar, 50 µm. **b** BiFC assay of TaHSP23.6 and WYMV CP interactions in *N. benthamiana*. Non-fused empty vectors (pCV-cYFP and pCV-nYFP) were used as negative controls. Fluorescence photographs were taken at 3 dpi. Scale bar: 50 µm

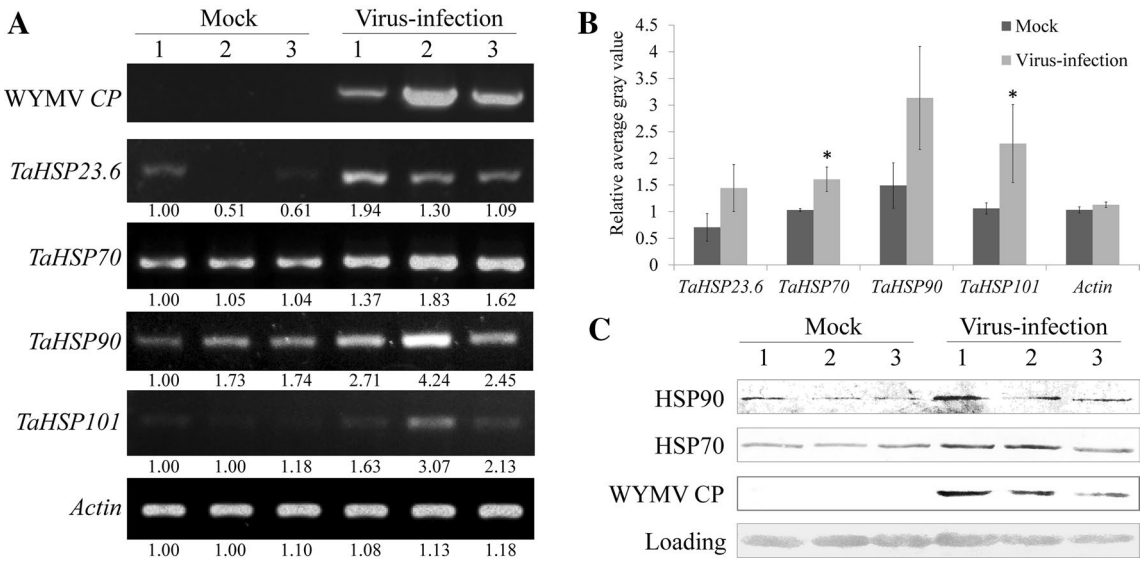

Fig. 3 Relative levels of *TaHSP23.6, TaHSP70, TaHSP90*, and *TaH101* in wild-type plants and virus-infected wheat. **a** The expression levels of *TaHSP* genes detected by semi-quantitative polymerase chain reaction (semi-qPCR) assay in a 2% agarose gel. Total RNAs were isolated from heathy wheat leaves (mock) or leaves infected by WYMV (virus infection). The systemic infection of WYMV was confirmed by detecting the expression level of WYMV *CP* gene. The *Actin* gene (bottom) was used as an internal control. The gray values were indicated below the bands analyzed by ImageJ. **b** Relative average gray values of *TaHSP* genes in mock and virus-infected plants. Date processing system (DPS) was used to analyze the gray value of DNA bands. Results were presented as means ± standard deviation from three replicates. Bars represent the standard errors of the means. A two-sample unequal variance directional *t* test was used to test the significance of the difference (*$P < 0.05$). **c** Protein expression levels of TaHSP70 and TaHSP90 were detected by Western blot. The systemic infection of WYMV was confirmed by detecting the accumulation of CP protein. β-actin dyed by Ponceau S was used as a loading control. All experiments were repeated at least three times

to be detected in healthy plants (Fig. 3a, b). As a molecular chaperone, the sHSPs interacted with denaturing proteins and presented them to the ATP-dependent HSP70 and HSP100 chaperones [32]. To investigate possible interactions of other major chaperones during plant–WYMV interactions in *T. aestivum*, we also designed universal primers based on the conserved sequences and examined the gene expression levels of the *TaHSP70, TaHSP90,* and *TaHSP101* family members. Semi-qPCR results showed that the transcripts of the *TaHSP70s* (AF005993 and AK360096), *TaHSP90s* (GQ240772, GQ240773, and GQ240774), and *TaHSP101s* (AJ970534, AJ970535, AJ970536, and KT355890) exhibited a high level in WYMV-infected plants compared to in healthy plants (Fig. 3a, b). The protein accumulation levels of TaHSP70 and TaHSP90 were also increased in response to WYMV infection, as shown by Western blot assay (Fig. 3c). The detection signal of TaHSP23.6 was too weak to distinguish the difference in protein accumulation between samples for lower molecular weight by Western blot analysis and the result did not show. Collectively, these results indicated that TaHSP23.6 as well as other major TaHSPs members is involved in WYMV infection.

Differential expression of *TaHSFA2* members in response to WYMV infection

To elucidate the regulatory mechanism of TaHSPs responded on WYMV infection, the transcript levels of TaHSFA2 and TaHSFA6 subclass were analyzed, as these proteins play important roles during HSP expression regulation in *T. aestivum* [25]. Six *TaHSFA2* members (*A2a, A2b, A2c, A2d, A2e*, and *A2f*) were tested by semi-qPCR. Gray scale analysis showed that most *TaHSFA2* members were induced at various rates by WYMV, in which the *TaHSFA2a* and *TaHSFA2e* were significantly increased while the *TaHSFA2d* was decreased in viral-infected plants (Fig. 4). The levels of *TaHSFA6* members were also detected but showed no significant differences (results not shown).

Discussion

sHSPs are an evolutionarily conserved class of ATP-independent chaperones that are proposed to be a first line of defense and protect cells against proteotoxic stress [12]. Recent studies have demonstrated induction of some plant sHSPs in response to viral infection [15, 16]. Based on

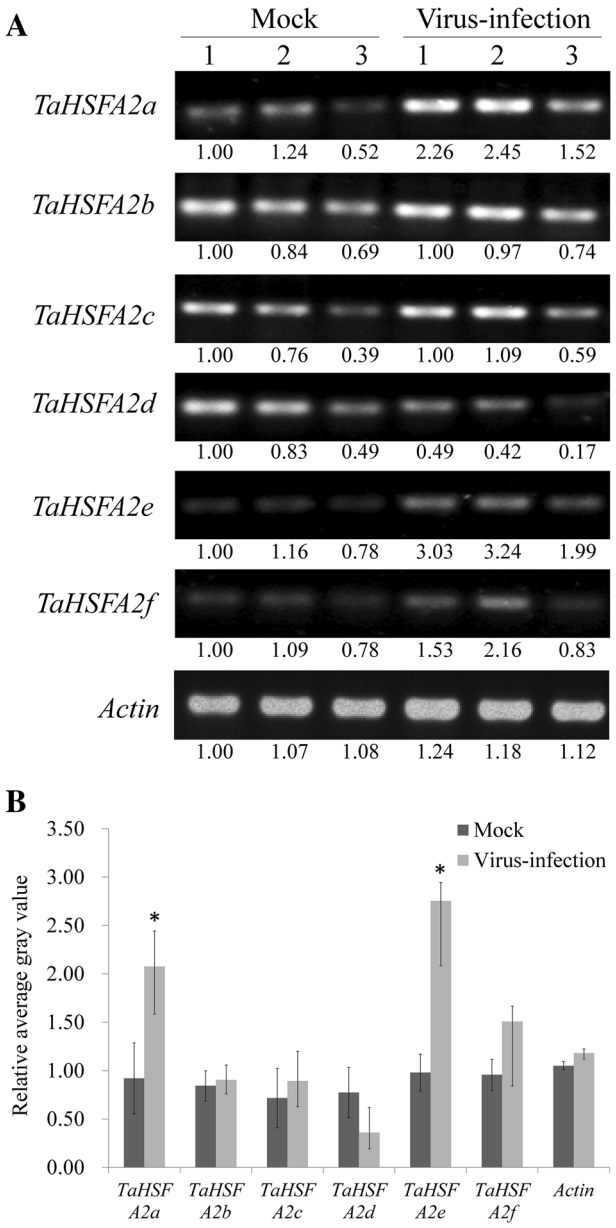

Fig. 4 Relative levels of *TaHSFA2a, TaHSFA2b, TaHSFA2c, TaHSFA2d, TaHSFA2e*, and *TaHSFA2f* in wild-type plants and virus-infected wheat. **a** The expression levels of *TaHSFA2* genes detected by semi-qPCR assay in a 2% agarose gel. Total RNAs were isolated from leaves infected by WYMV (virus infection). Healthy leaves were used as negative controls (mock). The *Actin* gene (bottom) was used as an internal control. The gray values were indicated below the bands analyzed by ImageJ. **b** Relative average gray values of *TaHSFA2* genes in mock and virus-infected plants. The gray value of DNA bands was analyzed as described above. Results were presented as means ± standard deviation from three replicates. Bars represent the standard errors of the means. A two-sample unequal variance directional *t* test was used to test the significance of the difference (*$P < 0.05$)

genome-wide microarray analysis, the transcript levels of *SlHSP20* genes were strongly influenced by TSWV [15]. The OsHSP20 in rice has been proved to be involved in the replication of RSV when interacted with a viral protein RdRp [16]. 163 TaHSP20 genes have been identified in wheat, and their expression was induced in various biotic and abiotic stresses [13]. Few efforts have been made to understand the roles of sHSPs during pathogen infection in wheat. In this study, we isolated a member of the sHSPs from wheat, TaHSP23.6 (Accession Number: AF104108), that interacted with the CP from WYMV in vitro and in vivo and showed greatly increased expression in wheat in response to WYMV but was difficult to detect in healthy plants, suggesting that TaHSP23.6 is involved in the process of WYMV infection.

Potyvirus CP proteins have diverse functions and target multiple host cellular pathways [33–35]. For an efficient infection, virus proteins need to recruit host factors for completing the above processes, including host HSPs proteins. The function of a potyvirus CP was regulated by interaction with a DnaJ-like protein (CPIP) and HSP70 from tobacco [33]. The HSP70 and its co-chaperone CPIP were identified as components of a membrane-associated viral ribonucleoprotein (RNP) complex and were required for potato virus A (PVA) replication and CP accumulation [34, 35]. Previously, the WYMV CP has been proved to interact with VPg, another multifunctional protein that functions in the translation and replication of viral genome [10]. In addition, the CP may be involved in cell-to-cell movement [11]. The association of some host factors with these processes is far from clear. In our study, TaHSP23.6 co-localized and interacted with WYMV CP in *N. benthamiana*. We hypothesize that TaHSP23.6 might contribute to the functional roles of the WYMV CP during virus infection by protein interaction that requires further determination.

Many host HSPs together with co-chaperones have been shown to interact with viral proteins and play important roles for different viruses in replication, viral assembly, movement, and other steps of the viral life cycle [12, 15, 19–22, 29–31]. HSP70 and HSP90 are involved in the establishment of TYLCV infection [23]. HSP18 and HSP70 mRNAs and proteins were induced with temperature-sensitive *cp* mutants of tobacco mosaic virus (TMV) at high temperature [36]. Using semi-qPCR analysis and Western blot assay, we revealed that different HSP family members are induced during WYMV infection. The transcript levels of *TaHSP70, TaHSP90*, and *TaHSP101* as well as *TaHSP23.6* were all increased in response to WYMV infection. We hypothesized that different HSP family members may also participate in the TaHSP23.6-CP interaction complex and be involved in various stages due to their multiple functions and their abundance inside cells. The possible crosstalk among these chaperones cannot be ruled out.

Transcription of HSP genes is controlled by regulatory proteins called HSFs [24]. HSF binding to HSEs leads to transcriptional regulation of HSPs. The heat-induced expression profiles of many HSP genes were paralleled by those of A2 and A6 subclass members that have been proved to be transcriptional activators that directly regulate several classes of *TaHSPs* in wheat [25]. In the current study, six TaHSFA2 members were determined and showed differential accumulations in response to WYMV infection. *TaHSFA2a* and *TaHSFA2e* were significantly up-regulated, suggesting A2a and A2e may act as positive regulators of TaHSPs during WYMV infection in wheat as described previously [25]. It is interesting to note that the member *TaHSFA2d* was decreased in response to WYMV infection. It has been shown that all six TYLCV proteins could capture tomato HSFA2 that suppressed the transcriptional activation of heat stress response genes [26]. The transcriptional patterns of a series of heat stress response genes were significantly decreased in TYLCV-infected tomatoes [26]. Therefore, we speculate that the decrease of *TaHSF2d* influenced by WYMV is "similar" to that observed in TYLCV-infected tomato. These data indicate that TaHSF members may have multiple regulatory roles in wheat adaptation to virus infection. TaHSFA6 transcription factor plays a role in regulation of a suite of heat stress protection genes, which have a significant impact on thermotolerance [37]. In this study, the levels of *TaHSFA6* members were also tested with no significant differences suggesting the relevance is absent with WYMV infection.

In conclusion, this study provides evidence that the TaHSP23.6 is involved with WYMV infection that probably plays multiple roles acting in conjunction with other HSPs family members. These results confirm the essential role of plant chaperones in WYMV infection. The promiscuous interactions and pleiotropic functions of sHSPs and the underlying molecular mechanisms are not completely understood. Further experiments to determine the TaHSP23.6 activity in WYMV infection are in progress.

Acknowledgements We would like to thank all the laboratory technicians for their contribution to this research. This work was supported by the National Natural Science Foundation of China (31701762), the Natural Science Foundation of Shandong (BS2015SW015), the Open Fund of State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control (2010DS700124-KF1703), and the National Key R&D Program of China (2017YFD0201700).

Author contributions SJ, FY, and XX conceived, designed, and performed the experiments; SJ, LJ, and YL helped perform the experiments; BW and MZ analyzed the date; SW was involved in the survey and sample collection; SW and XX contributed reagents/materials/analysis tools; and SJ wrote the manuscript. All authors approved the final draft of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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

Informed consent Consent to submit has been received explicitly from all co-authors, as well as from the responsible authorities—tacitly or explicitly—at the institute/organization where the work has been carried out before the work is submitted.

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