Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2017) 18(5), 649–661 DOI: 10.1111/mpp.12425

# Wheat transcription factor *TaWRKY70* is positively involved in high-temperature seedling plant resistance to *Puccinia striiformis* f. sp. *tritici*

JUNJUAN WANG[1], FEI TAO[1], FEI AN[1], YIPING ZOU[1], WEI TIAN[1], XIANMING CHEN[2], XIANGMING XU[3] AND XIAOPING HU[1,*]

[1]*State Key Laboratory of Crop Stress Biology for Arid Areas and College of Plant Protection, Northwest A&F University, Taicheng Road 3, Yangling, Shaanxi 712100, China*

[2]*Agricultural Research Service, United States Department of Agriculture and Department of Plant Pathology, Washington State University, Pullman, WA 99164-6430, USA*

[3]*East Malling Research, New Road, East Malling, Kent ME19 6BJ, UK*

## SUMMARY

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), is a devastating disease of wheat (*Triticum aestivum*) worldwide. Wheat high-temperature seedling plant (HTSP) resistance to *Pst* is non-race-specific and durable. WRKY transcription factors have been proven to play important roles in plant defence responses to attacks by several pathogens. However, there is no direct evidence as to whether WRKY transcription factors play a role in HTSP resistance to *Pst*. We isolated a WRKY gene, named *TaWRKY70*, from wheat cultivar Xiaoyan 6. The expression level of *TaWRKY70* was increased significantly when exposed to high temperatures (HTs) during the initial symptom expression stage of *Pst* infection. The expression of this gene increased in plants treated with ethylene (ET), salicylic acid (SA) and cold (4°C) stresses, but decreased in plants treated with methyl jasmonate (MeJA) and heat (40°C) stresses. Silencing of *TaWRKY70* led to greater susceptibility to *Pst* (in terms of the increase in length of uredinial pustules and the decrease in the number of necrotic cells) compared with non-silenced plants when exposed to HT during the initial symptom expression stage of *Pst* infection, coinciding with expression changes of the ET- and SA-responsive genes *TaPIE1* and *TaPR1.1*. In contrast, the expression level of the jasmonic acid (JA)-responsive gene *TaAOS* was not affected by *TaWRKY70*. These results indicate that *TaWRKY70* is positively involved in HTSP resistance, during which SA and ET signalling are probably activated.

**Keywords:** high-temperature seedling plant resistance, *Puccinia striiformis* f. sp. *tritici*, virus-induced gene silencing, WRKY70 transcription factor.

* *Correspondence*: Email: xphu@nwsuaf.edu.cn

## INTRODUCTION

Wheat stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), is a destructive disease, causing significant yield losses (Chen, 2005). Although application of fungicides can reduce yield loss, the growth of cultivars with an adequate level of durable resistance is the most effective and environmentally sustainable strategy for the management of *Pst* (Line and Chen, 1995).

Resistance to *Pst* induced by temperature changes is usually non-race-specific and sustainable; one type of such resistance is known as high-temperature adult plant (HTAP) resistance. Wheat cultivars with only HTAP resistance are susceptible to *Pst* in the seedling stage and at low temperature (LT), but become resistant as plants grow old and temperatures increase (Chen and Line, 1995; Qayoum and Line, 1985). Another less commonly reported type of resistance is high-temperature seedling plant (HTSP) resistance. Seedlings of cultivars with HTSP resistance are susceptible at LTs, but become resistant when exposed to high temperatures (HTs) for 24 h at the initial *Pst* symptom expression stage (8 days after inoculation) (Ma and Shang, 2000; Shang, 1998; Shang *et al.*, 1997; Wang et al., 2000). Xiaoyan 6 is a wheat cultivar with typical HTSP resistance to *Pst*, and HTSP resistance is present in many cultivars of Xiaoyan lines (Ma and Shang, 2000). However, the molecular mechanisms of HTSP resistance are poorly understood.

In general, plants have two layers of innate immunity against invading pathogens: pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Dangl *et al.*, 2013). Conserved microbial elicitors, which are PAMPs, are recognized by plant pattern recognition receptors (PRRs), resulting in the production of reinforced plant cell walls and antimicrobial compounds (Schwessinger and Ronald, 2012; Zipfel, 2009). A virulent pathogen secretes effectors to suppress plant PTI, whereas the plant detects these effectors by resistance (R) proteins, activating ETI, which induces the hypersensitive response (HR) in association with plant cell death (Dangl *et al.*, 2013). PTI and ETI are associated with the expression of various genes and reprogramming of metabolites, during which various phytohormones and

transcription factors (TFs), such as WRKY TFs, play important roles (Chandran *et al.*, 2014; Pandey and Somssich, 2009).

Phytohormones, especially salicylic acid (SA) and jasmonic acid (JA), play crucial roles in the plant defence response (Koornneef and Pieterse, 2008). Plant defences depending on SA are generally triggered by biotrophic pathogens (Glazebrook, 2005), whereas defences depending on JA are often activated by necrotrophic pathogens (Spoel *et al.*, 2007) and herbivorous insects (Zhang *et al.*, 2011). Ethylene (ET), first found to trigger fruit ripening and to influence senescence and abscission of plant organs (Spencer, 1956), often together with JA, regulates resistance to necrotrophic pathogens (Bari and Jones, 2009; Glazebrook, 2005), but there is evidence that these pathways may play some role in resistance to biotrophic pathogens (Berrocal-Lobo *et al.*, 2002; Robert-Seilaniantz *et al.*, 2011).

The rapid and transient production of reactive oxygen species (oxidative burst) not only acts as an antimicrobial agent, but also induces HR which is related to host cell necrosis in infected cells (Lamb and Dixon, 1997; Tenhaken *et al.*, 1995). Host cell death or programmed cell death is beneficial for plants as a defence response to biotrophic pathogens, because it is detrimental to biotrophic pathogens as they require living host cells to grow and reproduce (Williams and Dickman, 2008).

WRKY TFs constitute a large superfamily of TFs that are defined by the 60-amino-acid WRKY domain (Eulgem and Somssich, 2007) and can be divided into three groups (I, II and III) depending on the number of WRKY domains and the type of zinc-finger motif (Eulgem *et al.*, 2000). WRKY TFs are not only involved in normal plant development, including embryogenesis, senescence, seed coat and trichome development, leaf morphology, flowering initiation and hormonal signalling (Guo *et al.*, 2004; Johnson *et al.*, 2002; Lagace and Matton, 2004; Rinerson *et al.*, 2015; Xie *et al.*, 2005; Xu *et al.*, 2004; Zhang H *et al.*, 2004; Zhang *et al.*, 2004; Zou *et al.*, 2004), but also play pivotal roles in plant–pathogen interactions (Abbruscato *et al.*, 2012; Bahrini *et al.*, 2011; Chang *et al.*, 2013; Chujo *et al.*, 2007; Satapathy *et al.*, 2014). WRKY TFs are involved in the HR to microbial infection. For example, *CaWRKYd* (a member of the WRKY IIa group)-silenced hot pepper plants exhibit expression reduction for some HR-related genes and pathogenesis-related (PR) genes compared with control plants on *Tobacco mosaic virus* infection (Huh *et al.*, 2012). A homologue of the *WRKY5* gene is induced on *Yr39*-conditioned HTAP resistance (Coram *et al.*, 2008), suggesting that WRKY TFs are involved in HTAP resistance to *Pst*. WRKY TFs may also be induced by various plant defence-related hormone treatments. In *Arabidopsis*, the majority of group III WRKY factors are responsive to SA treatment (Kalde *et al.*, 2003). In wheat, nine and six WRKY TFs are regulated by exogenous SA and abscisic acid (ABA), respectively (Zhu *et al.*, 2013).

*WRKY70* plays diverse roles in response to different types of pathogen on model plants. In *Arabidopsis*, the overexpression of *AtWRKY70*, resulting in the constitutive expression of SA-induced PR genes, increases resistance to two bacterial pathogens, *Pseudomonas syringae* and *Erwinia carotovora*, and enhances SA-mediated resistance to the biotrophic fungus *Erysiphe cichoracearum*, but compromises JA-mediated resistance to the necrotrophic fungus *Alternaria brassicicola* (Li *et al.*, 2006). In contrast, loss of WRKY70 function renders plants susceptible to both of the above bacterial pathogens and the fungal pathogens *E. cichoracearum* and *Botrytis cinerea*, but with more resistance to the fungus *Al. brassicicola* (AbuQamar *et al.*, 2006; Li *et al.*, 2006; Wang *et al.*, 2006). T-DNA mutants have shown that *AtWRKY70* acts as a component of basal defence and gene-for-gene resistance to the oomycete *Hyaloperonospora parasitica*, and *AtWRKY70* is required for full function of the SA-dependent *R* gene RPP4 (Knoth *et al.*, 2007). In addition, some epigenetic results have shown that the activation of *AtWRKY70* is regulated by ATX1 (*Arabidopsis* homologue of trithorax) to establish nucleosomal histone H3K4 trimethylation marks, which mediate the regulation of the disease-responsive gene *PR1* (Alvarez-Venegas *et al.*, 2007), suggesting the indirect regulation of *WRKY70* on the expression of *PR1.1*.

However, to date, there is no evidence for the function of this group of TFs in HTSP resistance against *Pst*. We conducted a preliminary RNA-sequencing (RNA-seq) study to determine the differentially regulated genes during the HTSP resistance process, and identified several WRKY TFs that were up-regulated in HTSP resistance, including the WRKY70 homologue (most highly up-regulated), named *TaWRKY70*. Based on the preliminary data, we hypothesized that *TaWRKY70* is involved in HTSP resistance to *Pst*. The objectives of this study were to test the hypothesis and to determine the effects of stress factors/plant hormones on the expression of *TaWRKY70*.

## RESULTS

### Sequence analysis of *TaWRKY70*

Preliminary RNA-seq data analysis identified 24 WRKY TFs with differential expression during HT treatment post-*Pst* infection, among which the expression of a *TaWRKY70* homologous gene was the most highly up-regulated (more than eight times). We used rapid amplification of cDNA ends (RACE) to obtain a full-length, 1123-bp cDNA from *Pst*-infected leaves of wheat cv. Xiaoyan 6. The cDNA encodes a 303-amino-acid protein with a molecular mass of 32.5 kDa, and the protein was predicted to localize in the nucleus. This protein was found to have 35.0%, 98.0% and 95.0% identity with AtWRKY70 (NP_191199.1) in *Arabidopsis thaliana*, AetWRKY70 (EMT19878.1) in *Aegilops tauschii* and TuWRKY70 (EMS67413.1) in *Triticum urartu*, respectively. Therefore, according to the nomenclature for plant WRKYs and the alignment of this WRKY sequence with related sequences, we designated this gene as *TaWRKY70* (GenBank accession no.

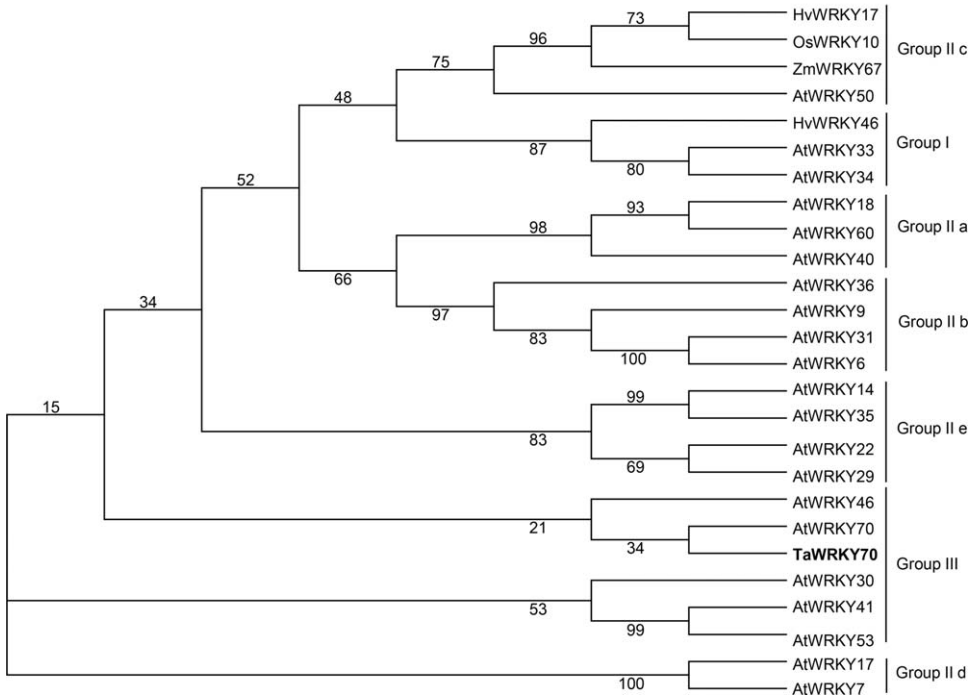


**Fig. 1** Dendrogram of *TaWRKY70* with other WRKYs from various plant species. The GenBank accession numbers of the WRKY proteins used for the construction of the phylogenetic tree are shown in Table S2 (see Supporting Information). At, *Arabidopsis thaliana*; Hv, *Hordeum vulgare*; Os, *Oryza sativa*; Ta, *Triticum aestivum*; Zm, *Zea mays*.

LC068769). This gene was mapped onto the long arm of wheat chromosome 1A. TaWRKY70 was clustered in a large clade composed of the group III WRKY family (Fig. 1). The alignment of TaWRKY70 with nine other homologues showed that all 10 proteins were highly conserved in their WRKY domains, which contain one conserved WRKYGQK at the N-terminus and one $C_2$–HC ($C–X_7–C–X_{23}–H–X_1–C$)-type zinc-finger motif at the C-terminus (Fig. S2, see Supporting Information). Based on the WRKY classification criteria (Eulgem *et al.*, 2000; Ulker and Somssich, 2004), *TaWRKY70* is a member of the group III WRKY family.

## Tissue-specific expression profiles of *TaWRKY70* in wheat

To determine the expression profiles of *TaWRKY70*, we examined the transcripts of *TaWRKY70* in various wheat tissues. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis was performed to quantify the expression of *TaWRKY70* in roots, stems and leaves. Its expression level was higher ($P < 0.01$) in leaves (1.0) than in roots (0.3) or stems (0.2) (Fig. 2).

## Expression levels of *TaWRKY70* in HTSP resistance to *Pst* and exposure to hormone and extreme temperature stresses

At the initial infection stage of *Pst* [<194 h post-inoculation (hpi)], the expression level of *TaWRKY70* in the LT sample was higher ($P < 0.05$) than that of the LT mock (without inoculation of *Pst*) from 48 to 192 hpi (11-fold at 192 hpi). From 192 hpi, HT treatment was employed, and the expression level of *TaWRKY70* in LT and LT mock was similar from 204 hpi onwards. The expression level of *TaWRKY70* on HT treatment was higher than that for the HT mock sample from 216 to 264 hpi [seven-fold at 216 hpi at which the expression level was highest ($P < 0.05$) among the LT, LT mock, HT and HT mock treatments] (Fig. 3a).

Subsequently, the expression patterns of *TaWRKY70* were investigated on treatment with five phytohormones: SA, ET,

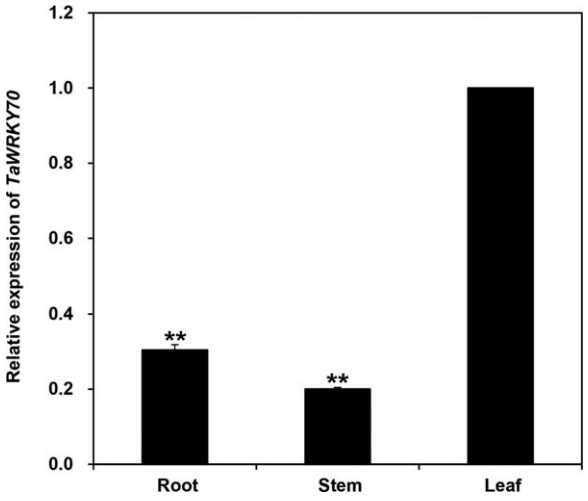


**Fig. 2** Expression profiles of *TaWRKY70* in different wheat tissues. The values are means of three independent biological replicates. Error bars indicate standard errors. **Significant difference (Student's *t*-test, $P < 0.01$) from the mean *TaWRKY70* relative expression level in the leaf.

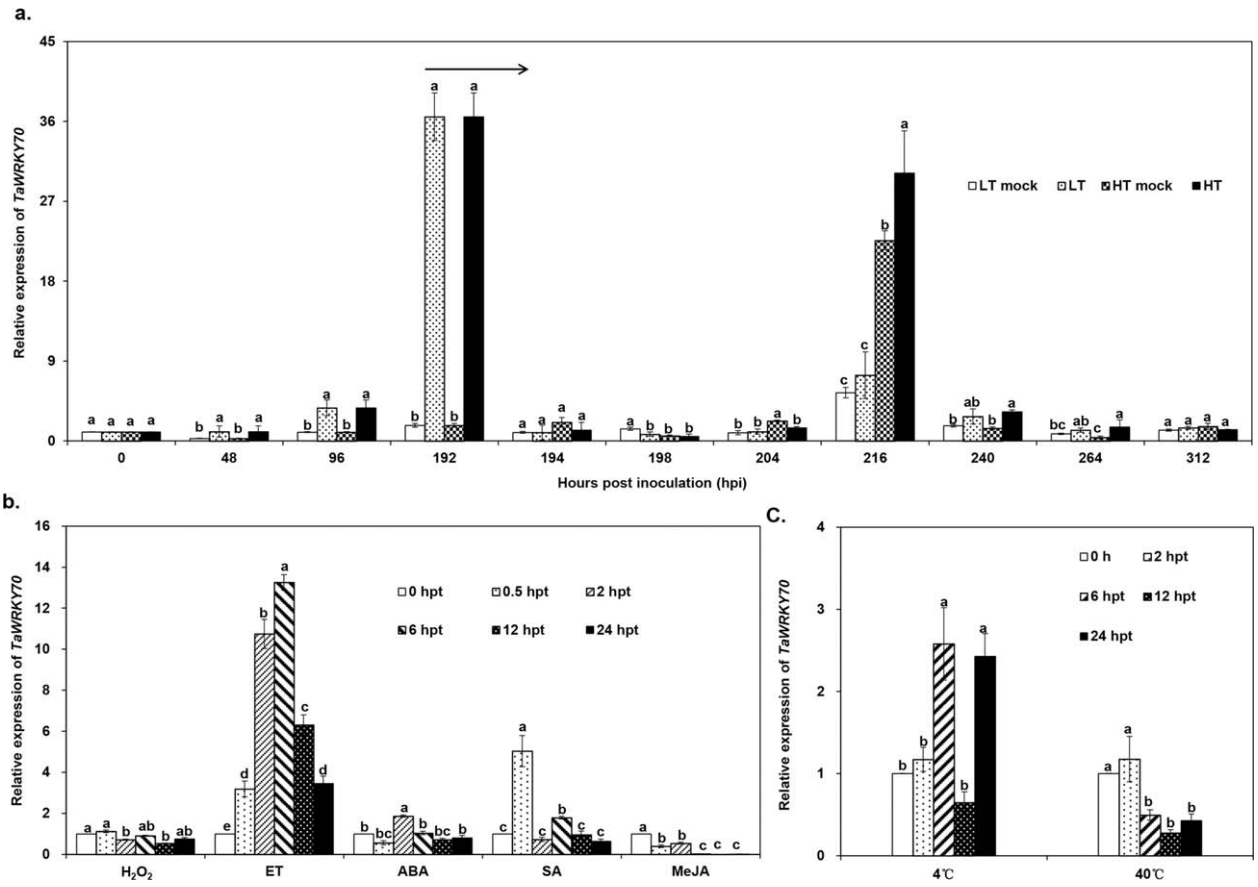


**Fig. 3** Expression patterns of *TaWRKY70* under different stress conditions. (a) Expression levels of *TaWRKY70* in response to low temperature (LT) (constant 15°C) and high temperature (HT) [15°C initially, then 20°C from 192 h post-inoculation (hpi) for 24 h, and back to 15°C again] treatments after inoculation with *Puccinia striiformis* f. sp. *tritici* (*Pst*). LT mock, low temperature without inoculation of *Pst*; HT mock, high temperature without inoculation of *Pst*. The expression levels were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis and were relative to the level observed at 0 hpi. The values are the means of three independent biological replicates. The error bars indicate standard errors. The arrow indicates the beginning of HT treatment. (b) Expression levels of *TaWRKY70* in response to hormone treatment. ABA, abscisic acid; ET, ethylene; $H_2O_2$, hydrogen peroxide; MeJA, methyl jasmonate; SA, salicylic acid; hpt, hours post treatment. (c) Expression levels of *TaWRKY70* in response to cold and heat stresses. The values are the means of three independent biological replicates. The error bars indicate standard errors. Analysis of variance (ANOVA) was conducted by Duncan's multiple range test ($P = 0.05$); the means of the *TaWRKY70* expression levels do not differ significantly if they contain at least one common lowercase letter between time points for each treatment.

methyl jasmonate (MeJA), ABA and hydrogen peroxide ($H_2O_2$). In response to ET treatment, the expression level of *TaWRKY70* increased transiently to 13.2-fold at 6 h post-treatment (hpt) and then rapidly decreased from 12 to 24 hpt (Fig. 3b). With SA treatment, *TaWRKY70* expression was up-regulated; its transcription level increased rapidly to a maximum of five-fold at 0.5 hpt and was then suppressed until 24 hpt (Fig. 3b). With ABA treatment, the expression level of *TaWRKY70* only reached 1.8-fold at 2 hpt (Fig. 3b). In contrast, $H_2O_2$ treatment did not induce the expression of *TaWRKY70*, and MeJA treatment suppressed the expression of this gene (Fig. 3b). Although cold (4 °C) treatment induced the transcripts of *TaWRKY70* at 6 and 24 hpt, heat (40 °C) resulted in a significant decrease in transcript level from 6 hpt onwards (Fig. 3c).

## Differences in the disease phenotype of *TaWRKY70*-silenced and *TaWRKY70*-induced plants

Mild chlorotic mosaic symptoms appeared on the fourth leaves at 9 days post-inoculation (dpi) in all plants inoculated with *Barley stripe mosaic virus* (BSMV), and strong photobleaching symptoms were observed in the phytoene desaturase (*PDS*) gene-silenced (BSMV:TaPDS inoculation) plants (Fig. 4a), suggesting that the virus-induced gene silencing (VIGS) system worked successfully.

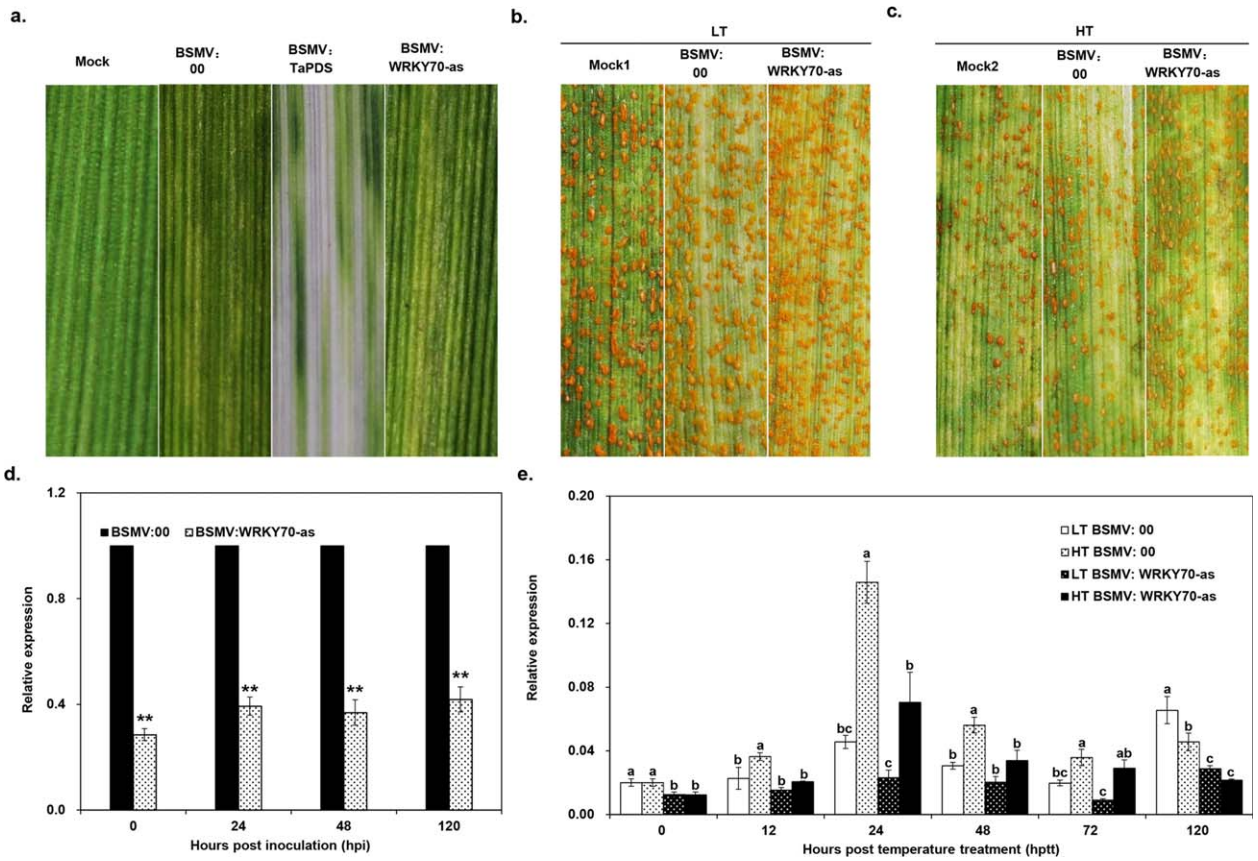


**Fig. 4** Functional analyses of *TaWRKY70* during high-temperature seedling plant (HTSP) resistance to *Puccinia striiformis* f. sp. *tritici* (*Pst*) using a *Barley stripe mosaic virus* (BSMV)-induced gene silencing (VIGS) system. (a) Mild chlorotic mosaic symptoms of BSMV at 9 days post-inoculation (dpi) (mock, plants treated with FES buffer). Disease symptoms on the fourth leaves pre-inoculated with BSMV-derived RNAs, challenged with *Pst* race CYR32 and then subjected to low-temperature (LT, 15 °C) (b) and high-temperature (HT, 20 °C) (c) treatments. 0 h post-temperature treatment (hptt), 192 h post-inoculation (hpi) from which HT was applied; disease symptoms were photographed at 14 dpi. Mock1 and Mock2, wheat plants were pre-inoculated with FES, and then inoculated with CYR32 and subjected to LT and HT treatments, respectively. (d) Efficiency of silencing of *TaWRKY70* by VIGS under LT treatment after *Pst* inoculation. **Student's *t*-test ($P < 0.01$) indicates significant differences in the mean *TaWRKY70* expression level between the BSMV:WRKY70-as-inoculated plants and BSMV:00-inoculated plants. (e) Induction of *TaWRKY70* in the fourth leaves pre-inoculated with BSMV:00 or BSMV:TaWRKY70-as under HT or LT treatments. The error bars represent standard errors. Analysis of variance (ANOVA) was conducted and followed by Duncan's multiple range test ($P = 0.01$); the means of the *TaWRKY70* expression level do not differ significantly if they contain at least one common lowercase letter.

The *TaWRKY70* transcripts in BSMV:TaWRKY70-as inoculated leaves were reduced by 71.5%, 60.7%, 63.1% and 58.2% at 0, 24, 48 and 120 hpi with *Pst*, respectively (Fig. 4d). The *TaWRKY70*-silenced leaves under HT treatment showed chlorotic stripes with abundant sporulation, which displayed increased susceptibility compared with leaves of the non-silenced control vector (BSMV:00) that exhibited necrotic or chlorotic stripes with intermediate sporulation (Fig. 4c). This was consistent with the lower expression levels of *TaWRKY70* in HT-treated *TaWRKY70*-silenced plants than in HT-treated BSMV:00 plants (Fig. 4e). However, *TaWRKY70*-silenced leaves subjected to HT treatment still showed fewer disease symptoms than *TaWRKY70*-silenced leaves subjected to LT treatment, which exhibited abundant sporulation almost completely covering the leaves without necrosis (Fig. 4b). This was consistent with the higher transcript level of *TaWRKY70* in *TaWRKY70*-silenced leaves on exposure to HT than LT (Fig. 4e).

## Pathogen development and host response in *TaWRKY70*-silenced and *TaWRKY70*-induced plants

Under LT treatment, *Pst* development and host response to *Pst* in *TaWRKY70*-silenced plants were observed microscopically (Fig. 5). Except for the increase ($P < 0.05$) in the number of haustorial mother cells at 120 hpi in BSMV:TaWRKY70-as plants compared with BSMV:00 control plants, no significant differences were detected in hyphal length or numbers of necrotic cells between the two vector plants challenged with *Pst* race CYR32 (Table 1).

When *TaWRKY70*-silenced plants were subjected to HT treatment, fungal development and host response were assessed

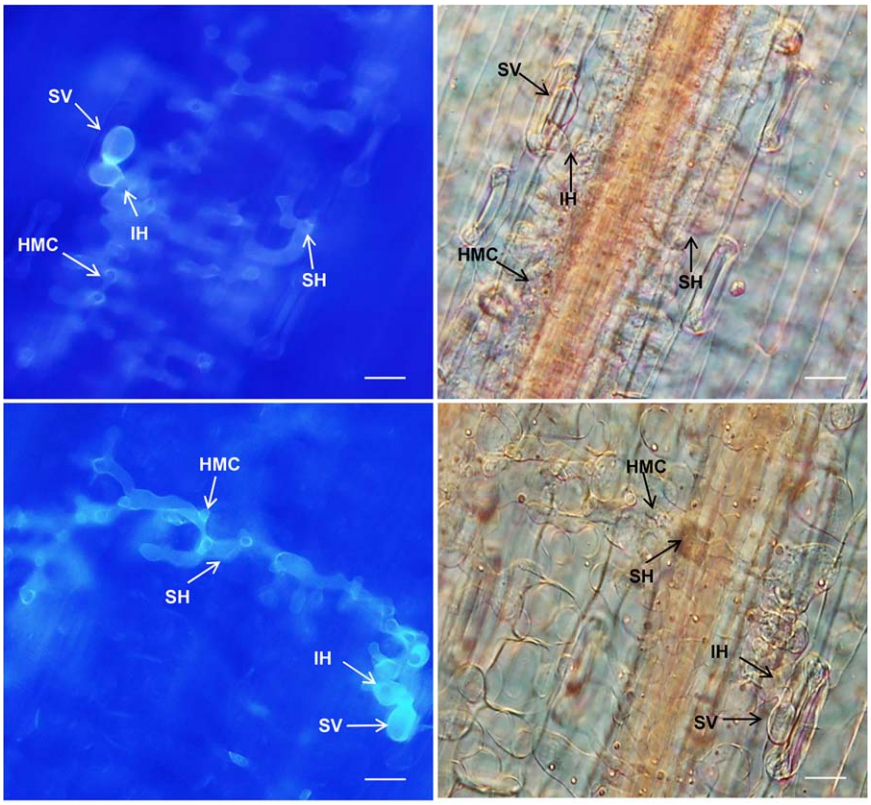


**Fig. 5** Histological observation of *Puccinia striiformis* f. sp. *tritici* (*Pst*) in the leaves of *TaWRKY70*-knockdown wheat plants under the low-temperature (LT, 15 °C) treatment. Photographs were obtained from *Barley stripe mosaic virus* (BSMV):00-infected (top panels) and BSMV:TaWRKY70-as-infected (bottom panels) leaves inoculated with *Pst* race CYR32 under an epifluorescence (left panels) or light (right panels) microscope at 120 h post-inoculation (hpi). HMC, haustorial mother cell; IH, initial hyphae; SH, secondary hyphae; SV, substomatal vesicle. Bars, 100 μm.

microscopically over time (Fig. 6a). The colony length on BSMV:00 vector leaves was reduced ($P < 0.01$) at 24 h post-temperature treatment (hptt) for HT compared with LT, but no changes were observed in *TaWRKY70*-silenced plants (Fig. 6b). The hyphae expanded rapidly from 24 hptt and pustules became visible at 72 hptt (at 10 dpi) under a fluorescent microscope, and abundant uredinia were observed at 120 hptt (at 12 dpi) (Fig. 6a). The uredinium length on *TaWRKY70*-silenced leaves was longer ($P < 0.01$) than on BSMV:00 vector leaves when subjected to HT treatment, but was shorter than that of LT-treated *TaWRKY70*-silenced leaves (Fig. 6c). In addition, the number of necrotic cells was greater ($P < 0.01$) for HT-treated BSMV:00 vector leaves than for HT-treated *TaWRKY70*-silenced leaves, but HT-treated *TaWRKY70*-silenced leaves generated a greater number of necrotic cells than LT-treated *TaWRKY70*-silenced leaves almost across all assessments, and the difference was greatest at 120 hptt (Fig. 6d).

As a result of the marked induction of the expression of *TaWRKY70* by ET and SA, and suppression by MeJA, we studied whether the expression of *TaWRKY70* induced by HT stimuli resulted in transcriptional changes in ET-, SA- and JA-mediated signalling. Before HT treatment (0 hptt), the expression level of *TaPR1.1* in *TaWRKY70*-silenced leaves was higher than that in non-silenced leaves (Fig. 7a). Thereafter, HT-treated, *TaWRKY70*-silenced leaves possessed a higher level of *TaPR1.1* transcripts

**Table 1** Histological observations of *Puccinia striiformis* f. sp. *tritici* (*Pst*) development and host responses to *Pst* in *TaWRKY70*-knockdown wheat plants under low-temperature (LT, 15 °C) treatment.

| | Hyphal length† | | Number of necrotic cells‡ | Number of haustorial mother cells§ |
|---|---|---|---|---|
| Treatment* | 48 hpi | 120 hpi | 120 hpi | 120 hpi |
| BSMV:00 | 38.54 ± 0.20a | 101.91 ± 1.44a | 0.24 ± 0.01a | 8.05 ± 0.08b |
| BSMV:TaWRKY70-as | 38.28 ± 0.41a | 108.83 ± 0.46a | 0.20 ± 0.02a | 9.20 ± 0.04a |

The significance of the differences was tested with one-way analysis of variance (ANOVA) (treatments with different lowercase letters indicate significant difference at $P = 0.05$). hpi, hours post-inoculation.

*Wheat leaves pre-infected with BSMV:00 or recombinant BSMV:TaWRKY70-as and then inoculated with *Pst* race CYR32.

†Average distance from the junction of the substomatal vesicle and the hyphal tip (calculated from the counts obtained for 30–50 infection sites).

‡,§Average number at an infection site (calculated from the counts obtained for 30–50 infection sites).

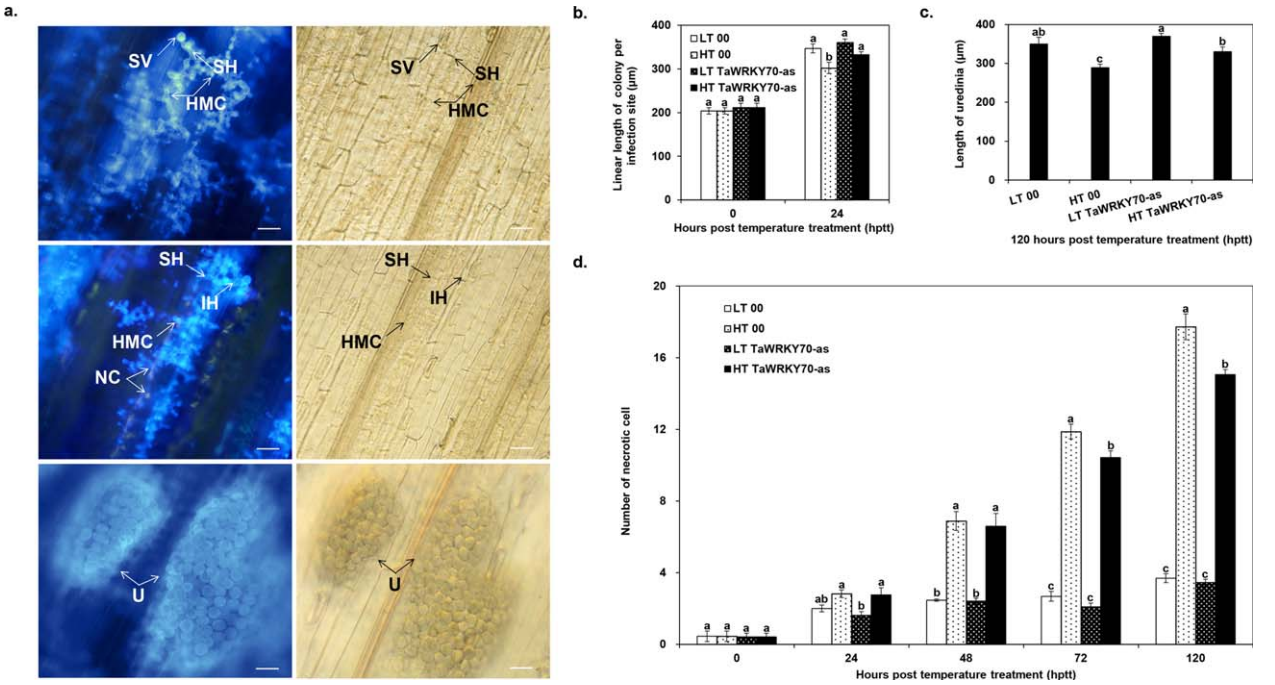


**Fig. 6** Differences in *Puccinia striiformis* f. sp. *tritici* (*Pst*) development and the wheat response between *TaWRKY70*-silenced and *TaWRKY70*-induced plants during the initial symptom expression stage of stripe rust development. (a) Fungal colonies under low-temperature (LT, 15 °C) (top panels) and high-temperature (HT, 20 °C) (centre panels) treatments, as well as uredinia (bottom panels), under epifluorescence (left panels) or light (right panels) microscopy. HMC, haustorial mother cell; IH, initial hyphae; NC, necrotic cell; SH, secondary hyphae; SV, substomatal vesicle; U, uredinia. Bars, 100 μm. Colony length (b), uredinium length (c) and numbers of necrotic cells (d). 00, plants inoculated with BSMV:00 vectors were used as a control; TaWRKY70-as, plants inoculated with BSMV:TaWRKY70-as vectors for the knockdown of *TaWRKY70*. The values are the means of three independent biological replicates. The bars show standard errors. Analysis of variance (ANOVA) was conducted by Duncan's multiple range test ($P = 0.01$); the same lowercase letter indicates that the level does not differ significantly.

than LT-treated leaves from 24 hptt, but showed almost no increase in *TaPR1.1* transcripts in comparison with HT-treated control vector leaves (Fig. 7a). The expression trend of *TaPIE1* showed a similar pattern to *TaPR1.1* (Fig. 7b). In contrast, the transcriptional abundance of *TaAOS* did not vary with treatment (Fig. 7c).

## DISCUSSION

This study shows that *TaWRKY70* is responsive to various abiotic and biotic stresses, and is associated with HTSP resistance to *Pst*. As a modulator, *AtWRKY70* acts as an activator of SA-mediated signal transduction, which is required for resistance to the biotroph *E. cichoracearum*, and as a repressor of JA-mediated signal transduction, which is required for resistance to the necrotroph *Al. brassicicola*, and thus it integrates signals from these mutually antagonistic pathways in *A. thaliana* (Li *et al.*, 2006). However, the relationships between *WRKY70* and SA or JA have not been documented in any cereal crop in the resistance response to fungal infections. The SA-related gene *TaPR1.1* was induced in HT-treated *TaWRKY70*-silenced plants relative to LT treatment. Unexpectedly, the ET-related gene *TaPIE1* was also induced, although its expression level was lower than that of *TaPR1.1*, whereas no difference was observed for the JA-related gene *TaAOS*. These results suggest that SA and ET signalling is probably involved in *TaWRKY70*-regulated HTSP resistance.

The WRKY superfamily TFs respond to various stresses (Liu *et al.*, 2014; Ramamoorthy *et al.*, 2008). In *Arabidopsis*, the majority of the group III WRKY factors, including *AtWRKY70*, respond to both pathogen infection and SA treatment (Kalde *et al.*, 2003). Like its family members, this study showed that *TaWRKY70* (a homologue of *AtWRKY70*) was responsive to SA treatment and *Pst* infection, indicating functional conservation of WRKY70 among different plant species. A large body of evidence indicates that WRKY TFs are implicated in diverse biological functions in plant–pathogen interactions. In rice, *OsWRKY53* and *OsWRKY22* play positive roles in resistance to fungal blast (Abbruscato *et al.*, 2012; Chujo *et al.*, 2007), and *OsWRKY62* is a negative regulator of *Xa21* (receptor-like kinase)-mediated defence against *Xanthomonas oryzae* pv. *oryzae* (Peng *et al.*, 2008). *ZmWRKY19* exhibits elevated expression in the resistant maize cultivar TZAR101 during its response to infection by *Aspergillus flavus* (Fountain *et al.*, 2015), and *TaWRKY10*, *TaWRKY15*, *TaWRKY17* and *TaWRKY56* respond specifically to wheat leaf rust (Satapathy *et al.*, 2014). Here, *TaWRKY70* was greatly induced by HT treatment post-

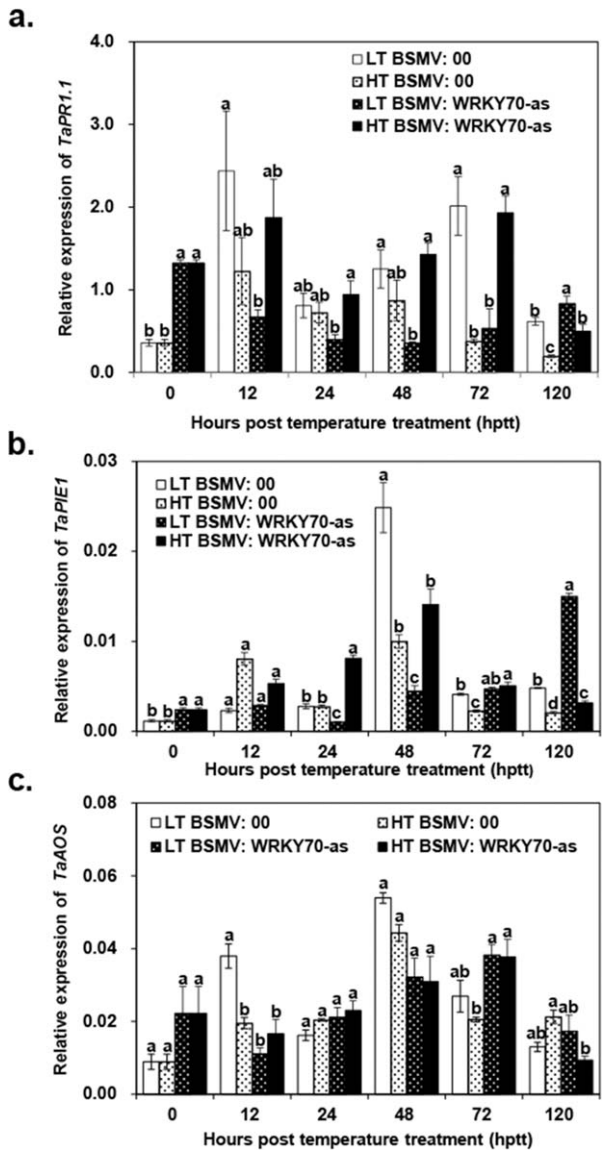


**Fig. 7** Transcriptional changes in the defence-related genes *TaPR1.1* (a), *TaPIE1* (b) and *TaAOS* (c) in *TaWRKY70*-induced [under high-temperature (HT, 20 °C) treatment] wheat leaves during the initial symptom expression stage of *Puccinia striiformis* f. sp. *tritici* development. The expression levels of these genes were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The presented transcript levels are relative to the reference gene *26S* (ATP-dependent 26S proteasome regulatory subunit). The bars show standard errors. Analysis of variance (ANOVA) was conducted by Duncan's multiple range test ($P = 0.01$) to analyse the gene expression differences among different vector-inoculated plants for each time point; the means of the gene expression levels do not differ significantly if they are indicated by the same lowercase letter.

inoculation of *Pst*, indicating the positive role of *TaWRKY70* in HT (abiotic) and *Pst* (biotic) double stresses. The overexpression of WRKY genes enhances plant resistance to pathogen infection. For example, *OsWRKY45*-overexpressing rice plants display strong resistance to fungal blast (Shimono *et al.*, 2007) and bacterial leaf blight (Shimono *et al.*, 2012) pathogens. Although the present study employed HT-induced expression rather than overexpression of *TaWRKY70*, this increased expression of *TaWRKY70* conferred enhanced resistance to *Pst*, which is similar to the often-observed phenotype of HTSP resistance (Shang *et al.*, 1997). The effect of temperature on *Pst* resistance genes has been reported (Chen, 2007, 2013). For example, *Yr36* was able to confer enhanced resistance when plants were grown at 12°C/18°C pre-inoculation and then transferred to 12°C/25°C post-inoculation, highlighting the importance of temperature change rather than prolonged exposure to a temperature threshold for the activation of resistance (Bryant *et al.*, 2014). Similarly, the HT profile used in this study was 15°C (constant)/20°C (24 h)/15°C (constant) post-inoculation, although it was different temperature regime from previous study, a common character of them is that a change in temperature could trigger resistant response. Plants continuously encounter a changing environment, and so they need to balance resources between growth and defence to achieve maximum productivity (Koga *et al.*, 2004; Mosher *et al.*, 2010). In *Arabidopsis*, a general trade-off mechanism has been shown for hormone-mediated growth which may antagonize immune responses (Albrecht *et al.*, 2012; Anderson *et al.*, 2004). Therefore, temperature change may trigger the reorganization of energy supplies between growth and defence in some temperature-sensitive cultivars, indirectly leading to deficient nutrients to *Pst*, a biotrophic fungus (Grof *et al.*, 2010; Viola and Davies, 1994). Meanwhile, some reports have suggested that temperature change could result in the accumulation of pathogen-inhibiting metabolites (Hu *et al.*, 2013). Thus, the enhanced resistance resulting from temperature change may occur via the augmentation of the growth of plants and the deterrence of colonization by pathogens. The biological basis for the enhanced resistance of *TaWRKY70* to *Pst* induced by HT (post-inoculation) is not known. The rice blast *R* gene *Pib* is induced at HTs, and it has been inferred that the increase in the *R* gene product confers an enhanced defence response (Wang *et al.*, 2001). The increased transcript amounts of *TaWRKY70* at HTs may account for the transcriptional reprogramming of downstream defence-related genes. Alternatively, protein–protein interactions involving TaWRKY70 may be affected by HTs. In *Arabidopsis*, the expression of *AtWRKY70* is induced by the application of SA and infection by several fungal pathogens (Kalde *et al.*, 2003), and it has been demonstrated that *AtWRKY70* activates the SA-linked signalling pathway in the defence response to biotrophic pathogens (Li *et al.*, 2006). Consistently, the transcript level of *TaWRKY70* was up-regulated by exogenous application of SA and ET, and was down-regulated by MeJA. Therefore, it is reasonable to speculate that *TaWRKY70* positively participates in the HT-induced resistance to *Pst* (post-inoculation), mainly through SA and ET signalling.

Studies in *Arabidopsis* have shown that a loss of WRKY70 renders plants susceptible to the biotrophic fungus *E. cichoracearum*

(Li *et al.*, 2006). In this study, we silenced *TaWRKY70* in wheat using VIGS. The increased *Pst* symptoms in *TaWRKY70*-silenced leaves compared with the control on exposure to HT suggested that a reduction in *TaWRKY70* transcripts may lead to increased susceptibility to *Pst* infection during HTSP resistance. Rapid programmed cell death of infected cells is characteristic of HR, as the plant defence response to pathogen infection, which suppresses further pathogen colonization of the host (Coll *et al.*, 2011). The *Yr36*-mediated HTAP resistance to *Pst* is correlated with hypersensitive cell death (Fu *et al.*, 2009). In addition, *Yr1*- and *Yr5*-mediated race-specific seedling resistance reactions have been found to be associated with a rapid cell death response and early retardation of *Pst* infection (Bozkurt *et al.*, 2010; Coram *et al.*, 2008). Consistently, the present results showed that the number of host necrotic cells was decreased and the length of uredinial pustules was increased in *TaWRKY70*-silenced compared with non-silenced leaves on HT treatment, suggesting that hypersensitive cell death is closely related to the resistance reaction to *Pst*. *TaWRKY70* involved in HTSP resistance responses to *Pst* may be associated with hypersensitive cell death.

*PR1.1* and *AOS*, considered as SA- and JA-responsive genes, respectively (Agrawal *et al.*, 2002; Mei *et al.*, 2006; Van Loon *et al.*, 2006), were induced differentially in HT-treated *TaWRKY70*-silenced plants. *PR1.1* was rapidly activated starting at 24 hptt, which may imply that *TaWRKY70*, like its orthologue in *Arabidopsis*, enhances SA-mediated signalling in the resistance response to biotrophic *Pst*. A weak accumulation of *OsAOS* mRNA in rice leaves inoculated with the blast pathogen during incompatible host–pathogen interactions was observed at 24 hpi, and this accumulation increased with increasing time after inoculation (Agrawal *et al.*, 2002). However, in the present study, the JA-related gene *TaAOS* was not much affected by HT treatment; this is not unexpected as this signalling pathway is specific to necrotrophic pathogens. Another possibility is that activation of JA signalling can severely affect plant growth in response to defence (Yang *et al.*, 2012). In *Arabidopsis*, ET potentiates SA-responsive *PR-1* expression (De Vos *et al.*, 2006; Lawton *et al.*, 1994), and ET acts in concert with SA as a positive regulator of cell death propagation (Bouchez *et al.*, 2007). *TaPIE1*, an ET biosynthesis-responsive gene that differs from *AtERF1* (*Arabidopsis* ethylene response factor 1) (Zhu *et al.*, 2014), was induced in HT-treated *TaWRKY70*-silenced plants, and the trend of the expression level exhibited by this gene was similar to that of *TaPR1.1*. Thus, *TaWRKY70* may also activate the ET-related signalling pathway.

The present study suggests a possible model by which *TaWRKY70* is involved in HTSP resistance to *Pst*. After *Pst* infection, high expression of *TaWRKY70* induced by HT appears to favour the induction of SA- and ET-responsive genes (*TaPR1.1* and *TaPIE1*), but not JA-responsive genes (*TaAOS*) (Fig. 8). In *A. thaliana*, several lines of evidence suggest that some SA-responsive WRKY TFs can bind to the cognate W or W-like boxes to activate the expression of their target genes (Maleck *et al.*, 2000; Yu *et al.*, 2001). The close inverse relationship between *AtWRKY70* and JA signalling is partly executed by NPR1 (Li *et al.*, 2006). ET, often together with JA, is a key regulator of SA-independent defence and triggers a subset of defence-related genes (Kunkel and Brooks, 2002; Penninckx *et al.*, 1998; Thomma *et al.*, 1998). Although our findings imply that the ET- and SA-related genes were positively regulated by *TaWRKY70* during the HTSP resistance response, the roles of the SA and ET signalling pathways in the wheat resistant response to *Pst* infection are still unclear because the regulatory mechanisms underlying the cross-talk between these signalling pathways are complicated. Therefore, the exact molecular mechanisms through which *TaWRKY70* regulates HTSP resistance to *Pst* requires further study.

## EXPERIMENTAL PROCEDURES

First, we conducted experiments to compare the expression levels of *TaWRKY70* under different temperature regimes at which HTSP resistance to *Pst* was or was not activated. To further confirm the role of *TaWRKY70* in HTSP resistance to *Pst*, the gene was silenced using VIGS technology. Then, we investigated the effects of abiotic stress factors (extreme temperatures) and plant hormones (SA, ET, ABA, $H_2O_2$ and MeJA) on the expression of *TaWRKY70*.

### Plant materials, growth conditions, HT induction and stress treatments

Wheat cultivar Xiaoyan 6 and *Pst* race CYR32 were used to study the wheat–*Pst* interaction. Seeds (10–15) were grown in a plastic pot ($10 \times 10 \times 10$ $cm^3$) filled with a potting mixture under rust-free conditions. The

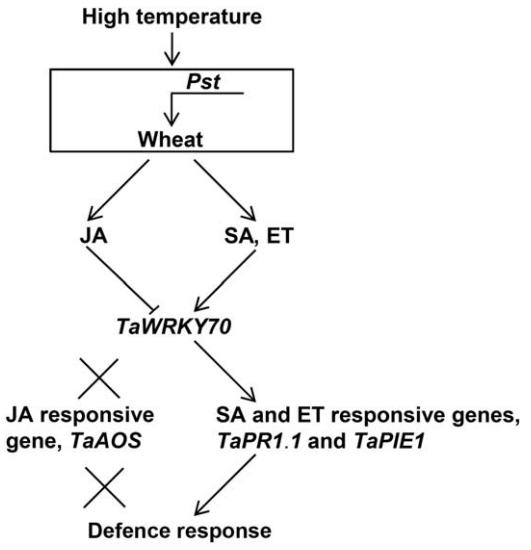


**Fig. 8** Model of the regulation of *TaWRKY70* in jasmonic acid (JA), ethylene (ET) and salicylic acid (SA) signalling during the high-temperature (HT)-induced wheat defence responses to *Puccinia striiformis* f. sp. *tritici* (*Pst*).

first leaves of the seedlings at the two-leaf stage (approximately 10–14 days after planting) were uniformly brushed with a mixture of *Pst* urediniospores and sterile water at a ratio of approximately 1 : 10–15 (v/v). The seedlings were then placed in a dew chamber in the dark for 24 h (temperature, 10°C; relative humidity, 90%–100%) and subsequently transferred to a growth chamber (Percival E-30B, Perry, IA, USA) and grown under 16 h of light at 15 ± 1°C [relative humidity, 60%–80%; supplemented with sodium lighting (505 μmol/$m^2$/s photon flux density)] and 8 h of dark at 12 ± 1°C (relative humidity, 60%–80%). In parallel, control plants were brushed with sterile water. In the initial symptom expression stage of rust development (8 dpi), the plants were divided into two groups for exposure to different temperature regimes. The first group was subjected to LT treatment, i.e. *Pst*-inoculated plants were incubated at a constant temperature (15 ± 1°C). The second group was subjected to HT treatment, i.e. *Pst*-inoculated wheat plants were incubated at 15 ± 1°C; at 8 dpi, they were transferred to a growth chamber set at 20 ± 1°C and incubated for 24 h; they were then moved back to and maintained thereafter at 15 ± 1°C, as the exposure to 20 ± 1°C for 24 h has been shown previously to activate HTSP resistance to *Pst* (Shang, 1998). The leaf tissues from LT and HT treatments were sampled at 0, 48, 96, 192, 194, 198, 204, 216, 240, 264 and 312 hpi; the 192-hpi time corresponded to the beginning of HT treatment. Leaves, stems and root tissues were also sampled from two-leaf-stage seedlings. Three biological replicates were used for each assay.

In addition, experiments were conducted to study wheat responses to extreme temperatures and to hormone treatments. For extreme temperature treatments, two-leaf-stage seedlings were incubated under cold (4°C) or hot (40°C) temperatures. For hormone treatments, seedlings were sprayed at 15°C with $H_2O_2$ (100 μM), MeJA (100 μM), ET (100 μM), SA (100 μM) or ABA (100 μM) (Zhang H *et al.*, 2004). In all of these treatments, seedlings in a similar state of growth (two-leaf stage) were used, and non-treated wheat seedlings were used as controls. All of the treated and non-treated seedlings were harvested at 0, 0.5, 2, 6, 12 and 24 h, frozen in liquid nitrogen and stored at −80°C. Three biological replicates were performed independently for each time point.

## Cloning and sequence analysis of *TaWRKY70*

Total RNA from leaf tissues was extracted using PureLink® Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA). After removal of genomic DNA contamination through DNase I treatment (Thermo Fisher, Waltham, MA, USA), 500 ng of poly(A)+ mRNA was converted into cDNA using RevertAid M-MuLV reverse transcriptase (Thermo Scientific, Waltham, MA, USA). Based on RNA-seq data, a set of RACE primers targeting the 5′ and 3′ ends was designed (Table S1, see Supporting Information). 5′ RACE was performed using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA), and 3′ RACE using the 3′-Full RACE Core Set with PrimeScript™ RTase (TaKaRa, Tokyo, Japan). Primers (Table S1) were designed to amplify the complete cDNA of *TaWRKY70* via RT-PCR. PCR products were extracted, combined with the pMD-18T plasmid (TaKaRa, Tokyo, Japan) and then sequenced. Amino acid and molecular weight predictions were conducted using EMBOSS (http://emboss.open-bio.org/wiki/Appdocs) and ExPASy (http://web.expasy.org/protparam/), respectively. The subcellular localization was predicted using Euk-mPLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/). Gene mapping was performed by EnsemblPlants (http://plants.ensembl.org/Triticum_aestivum/Tools/Blast?db = core). The amino acid sequence alignment of TaWRKY70 and its homologues was conducted using BLAST (http://www.ncbi.nlm.gov/blast) and DNAman software 5.2.2. A phylogenetic tree was constructed with MEGA4.0 (Tamura *et al.*, 2007) using a bootstrap test of phylogeny with a minimum evolution test and default parameters of 1000 replications.

## Plasmid construction

The γRNA-based vector derived from BSMV was constructed as described previously (Holzberg *et al.*, 2002). cDNA fragments derived from the coding sequence (161 bp, nucleotides 740–900) were used to construct the recombinant TaWRKY70-as plasmids. To guarantee the specificity of gene silencing, we employed BLASTn and BLASTx in the National Center for Biotechnology Information (NCBI) database to select the fragments that showed the highest polymorphism within the gene family and the lowest sequence similarities with other genes, and subsequently constructed the γRNA-based derivative plasmids BSMV:WRKY70-as. The primers used for vector construction are given in Table S1.

## BSMV-mediated *TaWRKY70* silencing

Plasmids used for gene silencing were constructed according to Holzberg *et al.* (2002). A cDNA fragment (161 bp) of *TaWRKY70* was amplified with a pair of primers (Table S1), and the wheat PDS gene *TaPDS* was then replaced with this fragment in BSMV:γ-PDS.

The preparation of infectious BSMV RNAs from each linearized plasmid was performed using an *in vitro* high-yield capped RNA transcription kit (mMESSAGE mMACHINE, Ambion, Austin, TX, USA). The BSMV inoculum was prepared by combining 2.5 μL of α, β and genetically modified γ transcripts with 42.5 μL of FES (viral inoculation buffer) (Pogue *et al.*, 1998), which was then inoculated onto the second leaves of wheat plants at the two-leaf stage by gently rubbing the surface with a gloved finger (Scofield *et al.*, 2005). BSMV:00 and BSMV:TaPDS were used as negative and positive controls for BSMV infection, respectively. Control inoculations were performed using 1 × FES. In each assay, 72 seedlings (12 pots, six seedlings per pot) were used and the experiment was conducted at least three times. After inoculation, the wheat plants were maintained in a growth chamber at 25 ± 1°C in the dark for 24 h, and then subjected to 16-h light/8-h dark cycles at 25 ± 1°C. The fourth leaves were further inoculated with fresh urediniospores of CYR32 on day 9 after virus inoculation, and the plants were maintained at 10 ± 1°C for 24 h in the dark and subsequently subjected to 16-h light (15 ± 1°C)/8-h dark (11 ± 1°C) cycles for *Pst* infection. Stripe rust symptoms and *Pst* sporulation on the fourth leaves were assessed at 14 dpi.

## qRT-PCR

Total RNA from different wheat organs and from plants subjected to various treatments was reversed into cDNA using the PrimeScript® RT Reagent Kit (TaKaRa, Tokyo, Japan), and qRT-PCR was then conducted to quantify *TaWRKY70* expression.

After a preliminary study (data not shown), wheat *26S* gene (ATP-dependent 26S proteasome regulatory subunit) (Unigene No. Ta22845) (Paolacci *et al.*, 2009) was used as the internal reference for each qRT-

PCR assay. All of the reactions were performed using UltraSYBR Mixture (Kangwei, Beijing, China) and conducted in an iQ$^{TM}$5 instrument (Bio-Rad, Hercules, CA, USA). The efficiency and specificity of the primer pairs are presented in Fig. S1 (see Supporting Information), and the primers are provided in Table S1. In all of the experiments, data were collected from three independent biological replicates, each consisting of at least three reactions, and negative controls without any templates were assessed to ensure the absence of contamination. The relative expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

### Analysis of the silencing efficiency and temperature-induced expression of *TaWRKY70*

The fourth leaves of plants that had been pre-inoculated with BSMV:00 and BSMV:WRKY70-as on the second leaves were sampled separately at 0, 24, 48 and 120 hpi after inoculation with CYR32. qRT-PCR was performed to determine the *TaWRKY70* silencing efficiency in each assay.

To clarify whether the expression level of *TaWRKY70* in *TaWRKY70*-silenced plants with *Pst* infection could be induced by HT treatment, plants from the LT treatment were divided into two groups (each group included *TaWRKY70*-silenced and BSMV:00 control plants): one group was immediately subjected to HT treatment at 8 days (192 h) after *Pst* inoculation, and the other group was maintained in the LT treatment constantly. Samples were harvested at 0, 12, 24, 48, 72 and 120 hptt from both LT and HT treatments to detect the temperature-induced expression of *TaWRKY70* via qRT-PCR. Stripe rust symptoms and *Pst* sporulation on the leaves were assessed at 14 dpi. In addition, to investigate the expression changes of SA-, JA- and ET-related genes accompanying the expression of *TaWRKY70* resulting from HT treatment, the transcript levels of *TaPR1.1* (AJ007348), *TaAOS* (AY196004) and *TaPIE1* (EF583940) in BSMV:00 and *TaWRKY70*-silenced leaves subjected to LT and HT treatments were compared at 0, 12, 24, 48, 72 and 120 hptt through qRT-PCR. Three biological replicates were performed independently for each time point.

### Histological observations of *TaWRKY70*-knockdown wheat plants under LT and HT treatments

*Pst* development and host response to inoculation were observed microscopically. Wheat leaves pre-infected with BSMV and subjected to LT treatment after *Pst* inoculation were sampled at 48 and 120 hpi to observe changes in *Pst* development and host response on *TaWRKY70*-silenced plants. During the initial symptom expression stage of *Pst* development (8 dpi), BSMV-pre-infected wheat leaves subjected to LT or HT treatments were sampled at 0, 24, 48, 72 and 120 h to examine changes in *Pst* development and host response, arising from the temperature-induced expression of *TaWRKY70*. Staining was performed as described by Wang *et al.* (2007). After fading and fixation, cleared wheat leaf segments were analysed to determine the hyphal length and number of haustorial mother cells under a microscope. Autofluorescence of pathogen-induced host necrotic cells was observed under a fluorescence microscope (excitation filter, 485 nm; dichromic mirror, 510 nm; barrier filter, 520 nm). Given the lateness of initial *Pst* development, linear lengths of the fungal colonies and uredinia were chosen for the assessment of fungal development. Colony length was measured from the substomatal vesicle to the apex of the longest hypha, and uredinium length as the length of its long shaft. In addition, plant cell death was defined as the presence of autofluorescence under a fluorescence microscope associated with an infection unit. About 30–50 infection sites on 8–10 wheat leaf segments (length, 1.5 cm) from 8–10 randomly selected wheat plants were examined. All of the microscopic observations were performed using an Olympus BX-51 microscope (Olympus Corporation, Tokyo, Japan), and the data were analysed using DP-BSW software.

### Statistical analyses

Analysis of variance and Tukey's test were conducted using the SAS program (SAS Institute Inc., Cary, NC, USA). The data relating to the number of necrotic cells and haustorial mother cells, as well as hyphal length, in BSMV:00 and BSMV:TaWRKY70-as vector plants under LT treatment were examined according to Student's *t*-test at $P < 0.05$ or $P < 0.01$ under the assumption of homogeneous variance. The data relating to hormone treatments, pre- with or without *Pst* inoculation, under treatments of LT or HT, cold and heat stresses, the number of necrotic cells, length of colony and uredinia, and the relative expression of *TaWRKY70*, *TaPR1.1*, *TaAOS* and *TaPIE1* in four conditions (HT BSMV:00, LT BSMV:00, HT BSMV:WRKY70-as, LT BSMV:WRKY70-as) were analysed using Duncan's multiple range test.

## ACKNOWLEDGEMENTS

We thank Dr Xiaojie Wang (Northwest A&F University) for providing the BSMV vectors. This work was supported by grants from the National Basic Research Program of China (no. 2013CB127700) and the National Natural Science Foundation of China (no. 31271985). The authors have no conflicts of interest to declare. The manuscript was edited by a native language speaker through American Journal Experts.

## REFERENCES

**Abbruscato, P., Nepusz, T., Mizzi, L., Del Corvo, M., Morandini, P., Fumasoni, I., Michel, C., Paccanaro, A., Guiderdoni, E., Schaffrath, U., Morel, J.B., Piffanelli, P. and Faivre-Rampant, O.** (2012) *OsWRKY22*, a monocot WRKY gene, plays a role in the resistance response to blast. *Mol. Plant Pathol.* **13**, 828–841.

**AbuQamar, S., Chen, X., Dhawan, R., Bluhm, B., Salmeron, J., Lam, S., Dietrich, R.A. and Mengiste, T.** (2006) Expression profiling and mutant analysis reveals complex regulatory networks involved in *Arabidopsis* response to Botrytis infection. *Plant J.* **48**, 28–44.

**Agrawal, G.K., Rakwal, R., Jwa, N.S., Han, K.S. and Agrawal, V.P.** (2002) Molecular cloning and mRNA expression analysis of the first rice jasmonate biosynthetic pathway gene allene oxide synthase. *Plant Physiol. Biochem.* **40**, 771–782.

**Albrecht, C., Boutrot, F., Segonzac, C., Schwessinger, B., Gimenez-Ibanez, S., Chinchilla, D., Rathjen, J.P., De Vries, S.C. and Zipfel, C.** (2012) Brassinosteroids inhibit pathogen-associated molecular pattern-triggered immune signaling independent of the receptor kinase BAK1. *Proc. Natl. Acad. Sci. USA*, **109**, 303–308.

**Alvarez-Venegas, R., Al Abdallat, A., Guo, M., Alfano, J.R. and Avramova, Z.** (2007) Epigenetic control of a transcription factor at the cross section of two antagonistic pathways. *Epigenetics*, **2**, 106–113.

**Anderson, J.P., Badruzsaufari, E., Schenk, P.M., Manners, J.M., Desmond, O.J., Ehlert, C., Maclean, D.J., Ebert, P.R. and Kazan, K.** (2004) Antagonistic interaction between abscisic acid and jasmonate–ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. *Plant Cell*, **16**, 3460–3479.

**Bahrini, I., Sugisawa, M., Kikuchi, R., Ogawa, T., Kawahigashi, H., Ban, T. and Handa, H.** (2011) Characterization of a wheat transcription factor, TaWRKY45, and its effect on Fusarium head blight resistance in transgenic wheat plants. *Breed. Sci.* **61**, 121–129.

**Bari, R. and Jones, J.D.** (2009) Role of plant hormones in plant defence responses. *Plant Mol. Biol.* **69**, 473–488.

**Berrocal-Lobo, M., Molina, A. and Solano, R.** (2002) Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. *Plant J.* **29**, 23–32.

**Bouchez, O., Huard, C., Lorrain, S., Roby, D. and Balague, C.** (2007) Ethylene is one of the key elements for cell death and defense response control in the Arabidopsis lesion mimic mutant vad1. *Plant Physiol.* **145**, 465–477.

**Bozkurt, T.O., McGrann, G.R.D., MacCormack, R., Boyd, L.A. and Akkaya, M.S.** (2010) Cellular and transcriptional responses of wheat during compatible and incompatible race-specific interactions with *Puccinia striiformis* f. sp. *tritici*. *Mol. Plant Pathol.* **11**, 625–640.

**Bryant, R.R., McGrann, G.R., Mitchell, A.R., Schoonbeek, H.J., Boyd, L.A., Uauy, C., Dorling, S. and Ridout, C.J.** (2014) A change in temperature modulates defence to yellow (stripe) rust in wheat line UC1041 independently of resistance gene *Yr36*. *BMC Plant Biol.* **14**, 10.

**Chandran, D., Rickert, J., Huang, Y., Steinwand, M.A., Marr, S.K. and Wildermuth, M.C.** (2014) Atypical E2F transcriptional repressor DEL1 acts at the intersection of plant growth and immunity by controlling the hormone salicylic acid. *Cell Host Microbe*, **15**, 506–513.

**Chang, C., Yu, D.S., Jiao, J., Jing, S.J., Schulze-Lefert, P. and Shen, Q.H.** (2013) Barley MLA immune receptors directly interfere with antagonistically acting transcription factors to initiate disease resistance signaling. *Plant Cell*, **25**, 1158–1173.

**Chen, X.M.** (2005) Epidemiology and control of stripe rust [*Puccinia striiformis* f. sp *tritici*] on wheat. *Can. J. Plant Pathol.* **27**, 314–337.

**Chen, X.M.** (2007) Challenges and solutions for stripe rust control in the United States. *Aust. J. Agric. Res.* **58**, 648–655.

**Chen, X.M.** (2013) High-temperature adult-plant resistance, key for sustainable control of stripe rust. *Am. J. Plant Sci.* **4**, 608–627.

**Chen, X.M. and Line, R.F.** (1995) Gene action in wheat cultivars for durable, high-temperature, adult-plant resistance and interaction with race-specific, seedling resistance to *Puccinia striiformis*. *Phytopathology*, **85**, 567–572.

**Chujo, T., Takai, R., Akimoto-Tomiyama, C., Ando, S., Minami, E., Nagamura, Y., Kaku, H., Shibuya, N., Yasuda, M., Nakashita, H., Umemura, K., Okada, A., Okada, K., Nojiri, H. and Yamane, H.** (2007) Involvement of the elicitor-induced gene *OsWRKY53* in the expression of defense-related genes in rice. *Biochim. Biophys. Acta*, **1769**, 497–505.

**Coll, N.S., Epple, P. and Dangl, J.L.** (2011) Programmed cell death in the plant immune system. *Cell Death Differ.* **18**, 1247–1256.

**Coram, T.E., Settles, M.L. and Chen, X.** (2008) Transcriptome analysis of high-temperature adult-plant resistance conditioned by *Yr39* during the wheat–*Puccinia striiformis* f. sp. *tritici* interaction. *Mol. Plant Pathol.* **9**, 479–493.

**Dangl, J.L., Horvath, D.M. and Staskawicz, B.J.** (2013) Pivoting the plant immune system from dissection to deployment. *Science*, **341**, 746–751.

**De Vos, M., Van Zaanen, W., Koornneef, A., Korzelius, J.P., Dicke, M., Van Loon, L.C. and Pieterse, C.M.** (2006) Herbivore-induced resistance against microbial pathogens in Arabidopsis. *Plant Physiol.* **142**, 352–363.

**Eulgem, T. and Somssich, I.E.** (2007) Networks of WRKY transcription factors in defense signaling. *Curr. Opin. Plant Biol.* **10**, 366–371.

**Eulgem, T., Rushton, P.J., Robatzek, S. and Somssich, I.E.** (2000) The WRKY superfamily of plant transcription factors. *Trends Plant Sci.* **5**, 199–206.

**Fountain, J.C., Raruang, Y.J., Luo, M., Brown, R.L., Guo, B.Z. and Chen, Z.Y.** (2015) Potential roles of WRKY transcription factors in regulating host defense responses during *Aspergillus flavus* infection of immature maize kernels. *Physiol. Mol. Plant Pathol.* **89**, 31–40.

**Fu, D., Uauy, C., Distelfeld, A., Blechl, A., Epstein, L., Chen, X., Sela, H., Fahima, T. and Dubcovsky, J.** (2009) A kinase-START gene confers temperature-dependent resistance to wheat stripe rust. *Science*, **323**, 1357–1360.

**Glazebrook, J.** (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43**, 205–227.

**Grof, C.P.L., Campbell, J.A., Kravchuk, O., Lambrides, C.J. and Albertson, P.L.** (2010) Temperature effect on carbon partitioning in two commercial cultivars of sugarcane. *Funct. Plant Biol.* **37**, 334–341.

**Guo, Y., Cai, Z. and Gan, S.** (2004) Transcriptome of *Arabidopsis* leaf senescence. *Plant Cell Environ.* **27**, 521–549.

**Holzberg, S., Brosio, P., Gross, C. and Pogue, G.P.** (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. *Plant J.* **30**, 315–327.

**Hu, B., Simon, J. and Rennenberg, H.** (2013) Drought and air warming affect the species-specific levels of stress-related foliar metabolites of three oak species on acidic and calcareous soil. *Tree Physiol.* **33**, 489–504.

**Huh, S.U., Choi, L.M., Lee, G.J., Kim, Y.J. and Paek, K.H.** (2012) *Capsicum annuum* WRKY transcription factor d (CaWRKYd) regulates hypersensitive response and defense response upon tobacco mosaic virus infection. *Plant Sci.* **197**, 50–58.

**Johnson, C.S., Kolevski, B. and Smyth, D.R.** (2002) TRANSPARENT TESTA GLABRA2, a trichome and seed coat development gene of Arabidopsis, encodes a WRKY transcription factor. *Plant Cell*, **14**, 1359–1375.

**Kalde, M., Barth, M., Somssich, I.E. and Lippok, B.** (2003) Members of the Arabidopsis WRKY group III transcription factors are part of different plant defense signaling pathways. *Mol. Plant–Microbe Interact.* **16**, 295–305.

**Knoth, C., Ringler, J., Dangl, J.L. and Eulgem, T.** (2007) *Arabidopsis* WRKY70 is required for full RPP4-mediated disease resistance and basal defense against *Hyaloperonospora parasitica*. *Mol. Plant–Microbe Interact.* **20**, 120–128.

**Koga, H., Dohi, K. and Mori, M.** (2004) Abscisic acid and low temperatures suppress the whole plant-specific resistance reaction of rice plants to the infection of *Magnaporthe grisea*. *Physiol. Mol. Plant Pathol.* **65**, 3–9.

**Koornneef, A. and Pieterse, C.M.** (2008) Cross talk in defense signaling. *Plant Physiol.* **146**, 839–844.

**Kunkel, B.N. and Brooks, D.M.** (2002) Cross talk between signaling pathways in pathogen defense. *Curr. Opin. Plant Biol.* **5**, 325–331.

**Lagace, M. and Matton, D.P.** (2004) Characterization of a WRKY transcription factor expressed in late torpedo-stage embryos of *Solanum chacoense*. *Planta*, **219**, 185–189.

**Lamb, C. and Dixon, R.A.** (1997) The oxidative burst in plant disease resistance. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 251–275.

**Lawton, K.A., Potter, S.L., Uknes, S. and Ryals, J.** (1994) Acquired-resistance signal-transduction in Arabidopsis is ethylene independent. *Plant Cell*, **6**, 581–588.

**Li, J., Brader, G., Kariola, T. and Palva, E.T.** (2006) WRKY70 modulates the selection of signaling pathways in plant defense. *Plant J.* **46**, 477–491.

**Line, R.F. and Chen, X.M.** (1995) Successes in breeding for and managing durable resistance to wheat rusts. *Plant Dis.* **79**, 1254–1255.

**Liu, B., Hong, Y.B., Zhang, Y.F., Li, X.H., Huang, L., Zhang, H.J., Li, D.Y. and Song, F.M.** (2014) Tomato WRKY transcriptional factor SlDRW1 is required for disease resistance against *Botrytis cinerea* and tolerance to oxidative stress. *Plant Sci.* **227**, 145–156.

**Livak, K.J. and Schmittgen, T.D.** (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(–Delta Delta C) method. *Methods*, **25**, 402–408.

**Ma, Q. and Shang, H.S.** (2000) High-temperature resistance of wheat cultivar Xiaoyan series to wheat stripe rust. *Acta Agric. Bor-Occid. Sin.* **9**, 39–42.

**Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L. and Dietrich, R.A.** (2000) The transcriptome of *Arabidopsis thaliana* during systemic acquired resistance. *Nat. Genet.* **26**, 403–410.

**Mei, C., Qi, M., Sheng, G. and Yang, Y.** (2006) Inducible overexpression of a rice allene oxide synthase gene increases the endogenous jasmonic acid level, PR gene expression, and host resistance to fungal infection. *Mol. Plant–Microbe Interact.* **19**, 1127–1137.

**Mosher, S., Moeder, W., Nishimura, N., Jikumaru, Y., Joo, S.H., Urquhart, W., Klessig, D.F., Kim, S.K., Nambara, E. and Yoshioka, K.** (2010) The lesion-mimic mutant cpr22 shows alterations in abscisic acid signaling and abscisic acid insensitivity in a salicylic acid-dependent manner. *Plant Physiol.* **152**, 1901–1913.

**Pandey, S.P. and Somssich, I.E.** (2009) The role of WRKY transcription factors in plant immunity. *Plant Physiol.* **150**, 1648–1655.

**Paolacci, A.R., Tanzarella, O.A., Porceddu, E. and Ciaffi, M.** (2009) Identification and validation of reference genes for quantitative RT-PCR normalization in wheat. *BMC Mol. Biol.* **10**, 1–27.

**Peng, Y., Bartley, L.E., Chen, X., Dardick, C., Chern, M., Ruan, R., Canlas, P.E. and Ronald, P.C.** (2008) OsWRKY62 is a negative regulator of basal and *Xa21*-mediated defense against *Xanthomonas oryzae* pv. *oryzae* in rice. *Mol. Plant.* **1**, 446–458.

**Penninckx, I.A.M.A., Thomma, B.P.H.J., Buchala, A., Metraux, J.P. and Broekaert, W.F.** (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. *Plant Cell*, **10**, 2103–2113.

**Pogue, G., Lindbo, J., Dawson, W. and Turpen, T.** (1998) Tobamovirus transient expression vectors: tools for plant biology and high-level expression of foreign proteins in plants. *Plant Mol. Biol. Manu.* **4**, 1–27.

**Qayoum, A. and Line, R.F.** (1985) High-temperature, adult-plant resistance to stripe rust of wheat. *Phytopathology*, **75**, 1121–1125.

**Ramamoorthy, R., Jiang, S.Y., Kumar, N., Venkatesh, P.N. and Ramachandran, S.** (2008) A comprehensive transcriptional profiling of the WRKY gene family in

rice under various abiotic and phytohormone treatments. *Plant Cell Physiol.* **49**, 865–879.

**Rinerson, C.I., Scully, E.D., Palmer, N.A., Donze-Reiner, T., Rabara, R.C., Tripathi, P., Shen, Q.X.J., Sattler, S.E., Rohila, J.S., Sarath, G. and Rushton, P.J.** (2015) The WRKY transcription factor family and senescence in switchgrass. *BMC Genomics*, **16**, 912.

**Robert-Seilaniantz, A., Grant, M. and Jones, J.D.** (2011) Hormone crosstalk in plant disease and defense: more than just jasmonate–salicylate antagonism. *Annu. Rev. Phytopathol.* **49**, 317–343.

**Satapathy, L., Singh, D., Ranjan, P., Kumar, D., Kumar, M., Prabhu, K.V. and Mukhopadhyay, K.** (2014) Transcriptome-wide analysis of WRKY transcription factors in wheat and their leaf rust responsive expression profiling. *Mol. Genet. Genomics*, **289**, 1289–1306.

**Schwessinger, B. and Ronald, P.C.** (2012) Plant innate immunity: perception of conserved microbial signatures. *Annu. Rev. Plant Biol.* **63**, 451–482.

**Scofield, S.R., Huang, L., Brandt, A.S. and Gill, B.S.** (2005) Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the *Lr21*-mediated leaf rust resistance pathway. *Plant Physiol.* **138**, 2165–2173.

**Shang, H.S.** (1998) High temperature resistance of wheat to stripe rust. *Sci. Agric. Sin.* **31**, 46–50.

**Shang, H.S., Wang, L.G., Lu, H.P. and Jing, J.X.** (1997) Characteristics of expression of high-temperature resistance to stripe rust in wheat. *Acta Phytophy. Sin.* **24**, 97–100.

**Shimono, M., Sugano, S., Nakayama, A., Jiang, C.J., Ono, K., Toki, S. and Takatsuji, H.** (2007) Rice WRKY45 plays a crucial role in benzothiadiazole-inducible blast resistance. *Plant Cell*, **19**, 2064–2076.

**Shimono, M., Koga, H., Akagi, A., Hayashi, N., Goto, S., Sawada, M., Kurihara, T., Matsushita, A., Sugano, S., Jiang, C.J., Kaku, H., Inoue, H. and Takatsuji, H.** (2012) Rice WRKY45 plays important roles in fungal and bacterial disease resistance. *Mol. Plant Pathol.* **13**, 83–94.

**Spencer, M.S.** (1956) Ethylene metabolism in tomato fruit. I. Relationship of ethylene evolution to fruit respiration and ripening. *Can. J. Biochem. Physiol.* **34**, 1261–1270.

**Spoel, S.H., Johnson, J.S. and Dong, X.** (2007) Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proc. Natl. Acad. Sci. USA*, **104**, 18 842–18 847.

**Tamura, K., Dudley, J., Nei, M. and Kumar, S.** (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**, 1596–1599.

**Tenhaken, R., Levine, A., Brisson, L.F., Dixon, R.A. and Lamb, C.** (1995) Function of the oxidative burst in hypersensitive disease resistance. *Proc. Natl. Acad. Sci. USA*, **92**, 4158–4163.

**Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. and Broekaert, W.F.** (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proc. Natl. Acad. Sci. USA*, **95**, 15 107–15 111.

**Ulker, B. and Somssich, I.E.** (2004) WRKY transcription factors: from DNA binding towards biological function. *Curr. Opin. Plant Biol.* **7**, 491–498.

**Van Loon, L.C., Rep, M. and Pieterse, C.M.** (2006) Significance of inducible defense-related proteins in infected plants. *Annu. Rev. Phytopathol.* **44**, 135–162.

**Viola, R. and Davies, H.V.** (1994) Effect of temperature on pathways of carbohydrate metabolism in tubers of potato (*Solanum-tuberosum L*). *Plant Sci.* **103**, 135–143.

**Wang, C.F., Huang, L.L., Buchenauer, H., Han, Q.M., Zhang, H.C. and Kang, Z.S.** (2007) Histochemical studies on the accumulation of reactive oxygen species ($O^{2-}$ and $H_2O_2$) in the incompatible and compatible interaction of wheat–*Puccinia striiformis* f. sp. *tritici*. *Physiol. Mol. Plant Pathol.* **71**, 230–239.

**Wang, D., Amornsiripanitch, N. and Dong, X.** (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. *PLoS Pathog.* **2**, e123.

**Wang, L.G., Shang, H.S., Lu, H.P. and Jing, J.X.** (2000) Studies on the histopathological features of high-temperature resistance cultivars of wheat infected with *Puccinia striiformis* f. sp. *tritici* West. *Acta Agric. Bor-Occid. Sin.* **28**, 22–26.

**Wang, Z., Yamanouchi, U., Katayose, Y., Sasaki, T. and Yano, M.** (2001) Expression of the *Pib* rice-blast resistance gene family is up-regulated by environmental conditions favoring infection and by chemical signals that trigger secondary plant defenses. *Plant Mol. Biol.* **47**, 653–661.

**Williams, B. and Dickman, M.** (2008) Plant programmed cell death: can't live with it; can't live without it. *Mol. Plant Pathol.* **9**, 531–544.

**Xie, Z., Zhang, Z.L., Zou, X.L., Huang, J., Ruas, P., Thompson, D. and Shen, Q.J.** (2005) Annotations and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. *Plant Physiol.* **137**, 176–189.

**Xu, Y.H., Wang, J.W., Wang, S., Wang, J.Y. and Chen, X.Y.** (2004) Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-delta-cadinene synthase-A. *Plant Physiol.* **135**, 507–515.

**Yang, D.L., Yao, J., Mei, C.S., Tong, X.H., Zeng, L.J., Li, Q., Xiao, L.T., Sun, T.P., Li, J., Deng, X.W., Lee, C.M., Thomashow, M.F., Yang, Y., He, Z. and He, S.Y.** (2012) Plant hormone jasmonate prioritizes defense over growth by interfering with gibberellin signaling cascade. *Proc. Natl. Acad. Sci. USA*, **109**, E1192–E1200.

**Yu, D., Chen, C. and Chen, Z.** (2001) Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. *Plant Cell*, **13**, 1527–1540.

**Zhang, H., Zhang, D., Chen, J., Yang, Y., Huang, Z., Huang, D., Wang, X.C. and Huang, R.** (2004) Tomato stress-responsive factor TSRF1 interacts with ethylene responsive element GCC box and regulates pathogen resistance to *Ralstonia solanacearum*. *Plant Mol. Biol.* **55**, 825–834.

**Zhang, P., Zhu, X., Huang, F., Liu, Y., Zhang, J., Lu, Y. and Ruan, Y.** (2011) Suppression of jasmonic acid-dependent defense in cotton plant by the mealybug *Phenacoccus solenopsis*. *PLoS One*, **6**, e22378.

**Zhang, Z.L., Xie, Z., Zou, X., Casaretto, J., Ho, T.H. and Shen, Q.J.** (2004) A rice WRKY gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells. *Plant Physiol.* **134**, 1500–1513.

**Zhu, X., Qi, L., Liu, X., Cai, S., Xu, H., Huang, R., Li, J., Wei, X. and Zhang, Z.** (2014) The wheat ethylene response factor transcription factor pathogen-induced ERF1 mediates host responses to both the necrotrophic pathogen *Rhizoctonia cerealis* and freezing stresses. *Plant Physiol.* **164**, 1499–1514.

**Zhu, X.L., Liu, S.W., Meng, C., Qin, L.M., Kong, L.N. and Xia, G.M.** (2013) WRKY transcription factors in wheat and their induction by biotic and abiotic stress. *Plant Mol. Biol. Rep.* **31**, 1053–1067.

**Zipfel, C.** (2009) Early molecular events in PAMP-triggered immunity. *Curr. Opin. Plant Biol.* **12**, 414–420.

**Zou, X., Seemann, J.R., Neuman, D. and Shen, Q.J.** (2004) A WRKY gene from creosote bush encodes an activator of the abscisic acid signaling pathway. *J. Biol. Chem.* **279**, 55 770–55 779.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Table S1** The primer sequences used in this study.

**Table S2** GenBank accession numbers of the WRKY proteins used for the generation of the dendrogram and alignments.

**Fig. S1** Specificity test of amplicons via quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and the efficiency (*E*) of the primer-specific polymerase chain reaction (PCR) amplification. (a) Agarose gel (2%) electrophoresis showing the amplification of a single product of the expected size for all tested genes. M represents the DL2000 DNA marker. (b) Dissociation curves with single peaks generated for all genes. (c) The efficiency of primer-specific PCR amplifications.

**Fig. S2** TaWRKY70 and homologues. The amino acid sequence of TaWRKY70 was aligned with those of the nine most closely matching proteins from a BLAST search. Identical amino acids are shown in black boxes, and similar amino acids are shown in grey boxes. The WRKYGQK peptide stretch is shown in red. The zinc-finger-like motifs in the domains are shown in blue.