Wheat Thioredoxin (*TaTrxh1*) Associates With RD19-Like Cysteine Protease *TaCP1* to Defend Against Stripe Rust Fungus Through Modulation of Programmed Cell Death

Beibei Shi,¹ Xinbei Zhao,^{1,2} Min Li,¹ Zihui Dong,¹ Qichao Yang,¹ Yang Wang,¹ Haifeng Gao,^{3,†} Brad Day^{4,5,†} and Qing Ma^{1,†}

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

² Institute of Plant Protection, Henan Academy of Agricultural Sciences/Key Laboratory of IPM of Pests on Crop (Southern North China), Ministry of Agriculture, Key Laboratory of Crop Pest Control of Henan, Zhengzhou, Henan 450002, China

³ Institute of Plant Protection, Xinjiang Academy of Agricultural Sciences/Key Laboratory of Integrated Pest Management on Crop in Northwestern Oasis, Ministry of Agriculture and Rural Affairs, Urumqi, Xinjiang 830091, China

⁴ Department of Plant, Soil and Microbial Sciences, Michigan State University, East Lansing, MI, U.S.A.

⁵ Plant Resilience Institute, Michigan State University, East Lansing, MI, U.S.A.

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Thioredoxins (Trxs) function within the antioxidant network through modulation of one or more redox reactions involved in oxidative-stress signaling. Given their function in regulating cellular redox, Trx proteins also fulfill key roles in plant immune signaling. Here, *TaTrxh1*, encoding a subgroup *h* member of the Trx family, was identified and cloned in wheat (*Triticum aestivum*), which was rapidly induced by *Puccinia striiformis* f. sp. *tritici* invasion and salicylic acid (SA) treatment. Overexpression of *TaTrxh1* in tobacco (*Nicotiana benthamiana*) induced programmed cell death. Silencing of *TaTrxh1* in wheat enhanced susceptibility to *P. striiformis* f. sp. *tritici* in different aspects, including reactive oxygen species accumulation and pathogen-responsive or -related gene expression. Herein, we observed that the cellular concentration of SA was significantly reduced in *TaTrxh1*-silenced plants, indicating that *TaTrxh1* possibly regulates wheat resistance to stripe rust through a SA-associated defense signaling pathway. Using a yeast two-hybrid screen to identify *TaTrxh1*-interacting partners, we further show that interaction with *TaCP1* (a RD19-like cysteine protease) and subsequent silencing of *TaCP1* reduced wheat resistance to *P. striiformis* f. sp. *tritici*. In total, the data presented herein demonstrate that *TaTrxh1* enhances wheat resistance against

P. striiformis f. sp. *tritici* via SA-dependent resistance signaling and that *TaTrxh1* interaction with *TaCP1* is required for wheat resistance to stripe rust.

Keywords: thioredoxin, cysteine protease, wheat stripe rust, programmed cell death, salicylic acid

Thioredoxins (Trxs) are present in all organisms and contain a highly conserved amino acid motif, Cys-Gly-Pro-Cys (Gelhay et al. 2005; Meyer et al. 2012), which has been demonstrated to be necessary and required for redox signaling. Specifically, this class of proteins functions through modifying a covalent mixed disulfide bond that exists in oxidized forms (i.e., Trx-S₂) and dithiol [i.e., TRX-(SH)₂] (Jacquot et al. 2002; Meyer et al. 2008). To date, at least 20 Trx genes have been reported in *Arabidopsis thaliana*, which are classified into six subfamilies (*f*, *h*, *m*, *o*, *x*, and *y*) (Meyer et al. 2002, 2012). Members of subfamilies *f*, *m*, *x*, and *y* and were localized to the chloroplast (Meyer et al. 2008, 2009), while members of subfamilies *h* and *o* showed localization patterns including the mitochondria, cytoplasm, and nuclei (Gelhay et al. 2005; Marchal et al. 2014; Meyer et al. 2008).

Given their highly ubiquitous and diverse cellular and tissue patterns of expression, it is reasonable to surmise that Trxs participate in numerous physiological processes in plants. For example, Trxs have demonstrable roles in seed development, wherein they function in the reduction of endosperm storage proteins, altering their sensitivity to hydrolysis (Thormählen et al. 2017; Wang et al. 2017; Zhou et al. 2013). Additionally, previous research has also shown that Trxm3, which is localized in the chloroplast, is required for processes associated with root growth and leaf development (Reichheld et al. 2010). As a function of stress response processes, Trxs have been shown to be required for robust signaling during abiotic stress response, including drought (Sahrawy et al. 1996), extreme temperatures (Park et al. 2009; Xie et al. 2009), and H₂O₂ perception (Spector et al. 1988).

Owing to their function in regulating cellular redox, Trxs also play a diverse role in the regulation of plant immune signaling (Mata-Pérez and Spoel 2019). For example, *Ralstonia*

B. Shi and X. Zhao contributed equally to this work.

†Corresponding authors: H. Gao; ghf20044666@163.com, B. Day; bday@msu.edu, and Q. Ma; maqing@nwsuaf.edu.cn

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solanacearum utilizes host Trxs to activate the glutathione degrading activity of RipAY (Mukaihara et al. 2016). This reaction results in the inhibition of pathogen-associated molecular pattern-triggered immunity (PTI) recognized by pattern recognition receptors that comprises basal level of disease resistance in a host (Dodds and Rathjen 2010; Jones and Dangl 2006). Moreover, Trx- ζ interacts with Cf-9, a tomato leucine-rich repeat resistance protein, which is a positive regulator of Cf-9-mediated hypersensitive response (HR) response in tomato (Jones and Dangl 2006; Macho and Zipfel 2015; Rivas et al. 2004).

The *h*-type Trx proteins represent the largest Trx family and are a signaling intermediate of antioxidant activities (Bashandy et al. 2010; Sun et al. 2010). For example, *OsTRXh1* regulates reactive oxygen species (ROS) accumulation in the apoplast and influences plant development and salt-stress responses (Zhang et al. 2011), while *CaTrxh1* plays a key role in the activation of HR-associated defense responses in pepper (Yi et al. 2007). In addition, an *h*-type Trx in tobacco *NtTRXh3* is involved in the resistance of tobacco to virus infection and abiotic oxidative stress (Sun et al. 2010). By regulating the conformational state of NPR1 through the reduction of the NPR1 (nonexpression of PR1) disulfide bond, *AtTrxh5* can promote the expression of defense-related genes induced by salicylic acid (SA) (Kneeshaw et al. 2014; Yun et al. 2016). Moreover, the expression of *AtTrxh5* is highly induced following the perception of pathogen elicitors, wounding, abscission, and senescence (Laloi et al. 2004; Reichheld et al. 2002; Tada et al. 2008). While several recent studies have provided evidence of a role for Trxs in pathogen resistance, the role of Trxs in conferring pathogen resistance in wheat has not been demonstrated, nor has it been shown that these proteins play a role in programmed cell death (PCD).

Wheat stripe rust, caused by *Puccinia striiformis* f. sp. *tritici*, is responsible for devastating yield reductions in wheat (*Triticum aestivum*). As an obligate biotrophic basidiomycete, *P. striiformis* f. sp. *tritici* produces urediospores on the leaves of susceptible wheat cultivars, ultimately leading to pathogen feeding and a resultant yield reduction (Singh et al. 2016). In response to *P. striiformis* f. sp. *tritici* infection, resistant wheat cultivars activate PCD-associated processes, resulting in the elicitation of tissue necrosis, a process hypothesized to restrict the invasion of *P. striiformis* f. sp. *tritici* (Chen et al. 2014). While emerging data has illuminated our understanding of the various processes that are activated in response to infection, additional studies describing the function of both pathogen-derived effectors and host-resistance factors are key in advancing our understanding of this complex host-pathogen interaction. Indeed, such approaches have been valuable in guiding breeding approaches for the development of new disease resistant cultivars (Eichmann and Hückelhoven 2008). From these, several key genes required for disease resistance signaling in response to *P. striiformis* f. sp. *tritici* have been identified, including the transcription factor *TabZIP1* (Zhang et al. 2008), *TaHSP70* (Duan et al. 2011), a metacaspase gene *TaMCA4* (Wang et al. 2012a), *TaNPSN* genes (Wang et al. 2014), and calcineurin B-like interacting protein kinase *TaCIPK10* (Liu et al. 2019).

Here, we describe the cloning of an *h*-type Trx, *TaTrxh1*, and its involvement in resistance to *P. striiformis* f. sp. *tritici* in wheat. In total, we demonstrate that overexpression of *TaTrxh1* induces cell death and ROS accumulation. Using a combination of in vivo-based interaction approaches, we demonstrate that a cysteine protease (TaCP1) is a downstream signaling protein of *TaTrxh1*. In total, the current work described herein provides evidence supporting a link between *TaTrxh1* and TaCP1 and the regulation of cell death following infection of wheat by *P. striiformis* f. sp. *tritici*.

RESULTS

Sequence analysis of wheat *h*-type Trx.

Based on publicly available sequence data (GenBank accession number AY072771.1), DNA primers (Supplementary Table S1) were designed for PCR-based cloning of a wheat (*Triticum aestivum*) *h*-type Trx. Using cDNA prepared from *T. aestivum* cv. Suwon11 (Su11), a 357-bp DNA sequence was obtained. This coding sequencing (CDS) encode a 118-amino acid protein with an estimated molecular mass of 12.69 kDa and an isoelectric point of 5.29. Blast analysis of the CDS of *TaTrxh* in URGI revealed that three copies of *TaTrxh* exist in *T. aestivum* cv. Chinese Spring, with 99, 95, and 94% nucleotide sequence identity, which were located on chromosomes 2A, 2B, and 2D, respectively (Supplementary Fig. S1A). The acquired *TaTrxh* clones (GenBank accession number AY072771.1) as well as four additional *h*-type Trx genes from *T. aestivum* (GenBank accession number AF438359.1, AF286593.2, EU706448.1, and AJ009762.1) were analyzed by multiple DNA and mitochondrial RNA (mRNA) sequence alignment approaches (Supplementary Fig. S1B and C). Using this approach, we observed that the acquired *TaTrxh* (GenBank accession number AY072771.1) had the closest relationship (i.e., sequence-based similarity) with *HvTrxh* (AY245454.1), with the next closest alignment being to *OsTrxh1* (Os07g0186000). Based on this, the protein was named wheat Trxh1 (*TaTrxh1*) (Supplementary Fig. S1D).

TaTrxh1 is significantly induced

by *P. striiformis* f. sp. *tritici* infection and SA treatment.

Trxs are involved in plant disease resistance signaling (Mukaihara et al. 2016). To analyze the transcriptional profiles (i.e., mRNA accumulation) of wheat resistance to stripe rust, accumulation of five *TaTrxh* mRNAs was evaluated in leaf samples collected at 0, 12, 24, 36, 48, 72, 96, and 120 h post-inoculation (hpi) with *P. striiformis* f. sp. *tritici*. As shown in Figure 1A, *TaTrxh1* expression was significantly induced at 96 and 120 hpi during an incompatible interaction, with a 3.3- and 5.3-fold increase, respectively, when compared with samples collected at 0 hpi. Interestingly, we did not observe a significant difference in *TaTrxh1* mRNA accumulation during a compatible interaction, indicating that *TaTrxh1* may regulate wheat resistance to stripe rust.

Plant hormones play an important role in numerous signal transduction processes. As a link to the current study, the activity of Trxs has been described has a key component of SA-mediated immune signal transduction cascades, suggesting a link between redox-based processes and hormone signaling in response to pathogen infection (Mata-Pérez and Spoel 2019). To investigate the role of *TaTrxh1* in immune signaling and to further determine the response of *TaTrxh1* following hormone perception, we treated wheat leaves with SA and methyl-jasmonate (MeJA) and evaluated the mRNA accumulation of *TaTrxh1*. As shown in Figure 1B, following treatment of plants with SA, the accumulation of *TaTrxh1* mRNA was significantly increased at 3 and 6 h posttreatment (hpt), with an observed 2.6- and 4.1-fold increase, respectively. *TaTrxh1* mRNA accumulation did not change significantly (in comparison with untreated samples) following MeJA treatment. Taken together, *TaTrxh1* could enhance the resistance of wheat to *P. striiformis* f. sp. *tritici* through SA-mediated signaling.

Overexpression of *TaTrxh1* in tobacco leaves can induce the cell death.

To further understand the mechanism or mechanisms underpinning the role of *TaTrxh1* in defense signaling in wheat following *P. striiformis* f. sp. *tritici* infection, the full CDS of

TaTrxh1 was cloned into the heterologous expression vector PVX106 and was expressed in tobacco (*Nicotiana benthamiana*) leaves using *Agrobacterium tumefaciens*. Our aim was to evaluate the overexpression cell-death activity of *TaTrxh1* and its ability to suppress PCD. To do this, we exploited the activity of BAX, a death promoting member of the Bcl-2 family of proteins that can trigger cell death, which has similar physiological characteristics to plant HRs when expressed in plants (Lacomme and Santa Cruz 1999). As shown in Figure 2, PVX106:*TaTrxh1*+Bax, PVX106+Bax, and PVX106:*TaTrxh1* expression zones showed the induction of necrosis, while no cell-death phenotype was observed in regions infiltrated with PVX106 alone or buffer-only controls.

Suppression of *TaTrxh1* reduces wheat resistance to *P. striiformis* f. sp. *tritici*.

Virus-induced gene silencing (VIGS) is a common approach tool for the evaluation of the function and activity of plant disease resistance genes (Bennypaul et al. 2012). Based on the observation that the expression of *TaTrxh1* increased when wheat was challenged with *P. striiformis* f. sp. *tritici*, we

Fig. 1. Expression time course of *TaTrxh1* in wheat leaves in response to *Puccinia striiformis* f. sp. *tritici* infection and salicylic acid (SA) treatment. **A**, The accumulation of *TaTrxh1* mRNA after *P. striiformis* f. sp. *tritici* infection. The Su11 leaves inoculated with *P. striiformis* f. sp. *tritici* race CYR23 (incompatible interaction) and race CYR31 (compatible interaction). **B**, SA treatment enhances *TaTrxh1* expression. Wheat seedlings were treated with 100 mM methyl jasmonate (MeJA) and 2 mM SA. The asterisk (*) indicates a statistically significant difference ($P < 0.05$), using Student's *t* test, while 0 h was normalized as 1, and there were three independent biological replicates. All data were determined by real-time quantitative PCR with wheat *TaEF-1a* as a reference gene. Wheat leaves treated with distilled water were included as mock control.

utilized VIGS to silence *TaTrxh1* in wheat, to evaluate its in-plant contribution in response to *P. striiformis* f. sp. *tritici* infection. As shown in Figure 3A, expression of BSMV: γ -*TaPDS* (BSMV vector carrying a segment from wheat phytoene desaturase gene) was used as a positive control and induced a signature photobleaching response on inoculated plants (Wang et al. 2012a and b). Plants inoculated with BSMV (BSMV: γ /BSMV: γ -*TaTrxh1*) yielded mild chlorotic mosaic symptoms, indicating the efficacy of gene silencing. As shown, obvious mild chlorotic mosaic symptoms were observed on the fourth leaves in BSMV: γ and BSMV: γ -*TaTrxh1* plants at 10 days postinoculation (dpi), while leaves inoculated with BSMV: γ -*TaPDS* displayed signs of photobleaching (Fig. 3A). Next, to evaluate the role of BSMV: γ -*TaTrxh1* in wheat resistance to stripe rust, the silencing efficiency of *TaTrxh1*-infected plants was tested using real-time quantitative PCR (RT-qPCR) assay at 24, 48, and 120 hpi with avirulent *P. striiformis* f. sp. *tritici* CYR23 and virulent *P. striiformis* f. sp. *tritici* CYR31 on the fourth leaves of inoculated wheat. As shown in Figure 3C and D, the accumulation of *TaTrxh1* was reduced approximately 70 to 90% compared with BSMV: γ control plants, suggesting that *TaTrxh1* was silenced using the BSMV system.

To further investigate this mechanism, we evaluated the induction of cell-death phenotypes in CYR23-inoculated BSMV: γ , BSMV: γ -*TaTrxh1* and mock-inoculated plants at 15 dpi. As shown, the necrotic area of BSMV: γ -*TaTrxh1* plants was more pronounced than in control plants, with a reduced number of uredia observed on BSMV: γ -*TaTrxh1* plants (Fig. 3B). As shown, all leaves showed considerably pronounced uredia phenotypes after inoculating with *P. striiformis* f. sp. *tritici* CYR31, while the *TaTrxh1*-silenced plants produced more uredia. As a final step to investigate the host response in *TaTrxh1*-silenced plants after *P. striiformis* f. sp. *tritici* infection, two pathogenesis-related (PR) genes, *TaPR1* and *TaPR3*, were evaluated using RT-qPCR. As shown in Figure 3E, both mRNAs were significantly reduced compared with control plants. Based on the sum of this series of data, we conclude that *TaTrxh1* plays a positive-regulatory role in resistance signaling to stripe rust.

TaTrxh1 silencing decreases ROS accumulation.

To further define pathogen-induced defense changes as a function of *TaTrxh1* activity following *P. striiformis* f. sp. *tritici* infection, we next evaluated the production of H_2O_2 following *P. striiformis* f. sp. *tritici* infection. As a point of reference, the formation of a substomatal vesicle was considered a successful infection site. As shown in Figure 4A and B, the area of H_2O_2 generation was significantly reduced at 120 h in *TaTrxh1*-silenced plants after inoculating with CYR23, as compared with control (i.e., unsilenced) plants. Concomitant with this, and not unsurprisingly, the expression of *TaCAT* (catalase) and *TaSOD* (superoxide dismutase), both of which are involved in ROS signaling, were induced (Fig. 4C). These data support the hypothesis that the function of *TaTrxh1* in wheat defense against *P. striiformis* f. sp. *tritici* is ROS-dependent. As an additional measure of the role of *TaTrxh1* in wheat defense against *P. striiformis* f. sp. *tritici*, we also evaluated the histology of *P. striiformis* f. sp. *tritici* race CYR23-infected wheat. Using this approach, we observed that hyphal length and the leaf area of pathogen colonization were significantly increased in silenced plants (Fig. 4D to H), further confirming that *TaTrxh1* is required for robust resistance to *P. striiformis* f. sp. *tritici*.

Effects of *TaTrxh1* silencing on SA and JA accumulation.

The SA- and JA-dependent signaling pathways are two important disease resistance-signaling pathways in plants. As

shown above, we observed that *TaTrxh1* mRNA accumulation was induced following exogenous application of SA (Fig. 1B). We also detected that the expression of *TaPR1* and *TaRP3* were suppressed in *TaTrxh1*-silenced plants. *TaPR1* and *TaRP3* are marker genes in the SA signaling pathway and can be induced by SA. Thus, we speculated that *TaTrxh1* was involved in SA signaling. To confirm our view, we collected samples of the control and *TaTrxh1*-silenced plants at 18 and 24 h after inoculating with *P. striiformis* f. sp. *tritici* race CYR23 to quantify SA and JA content. As shown in Figure 5, the concentration of SA was significantly reduced in *TaTrxh1*-silenced plants at 24 hpi, with an approximate 40% reduction in SA, as compared with control plants. Consistent with our observations above, we did not observe a significant change in the level of JA. Additionally, the SA biosynthesis pathway-related genes (i.e., *TaICS1* [isochorismate synthase 1] and *TaPAL* [phenylalanine ammonia-lyase]) and SA-regulated gene *TaNPR1* were evaluated using RT-qPCR. As shown in Supplementary Fig. S2, the expression of *TaICS1* (120 hpi), *TaPAL* (24 and 120 hpi), and *TaNPR1* (48 and 120 hpi) were significantly reduced in *TaTrxh1*-silenced plants after inoculating with CYR23 compared with control plants, which further indicated that *TaTrxh1* is involved in SA signaling following *P. striiformis* f. sp. *tritici* infection. Taken together, these data support the hypothesis that *TaTrxh1* regulates wheat resistance to stripe rust through the SA signaling pathway.

TaTrxh1 interacts with TaCP1, a wheat RD19-like cysteine protease.

As a means to investigate the mechanism underpinning the *TaTrxh1*-induced cell-death response in tobacco, we used *TaTrxh1* as a bait protein to screen for interacting proteins, using the yeast two-hybrid system. Using this approach, we screened a wheat yeast two-hybrid for *TaTrxh1*-interacting proteins and, through this, developed a candidate list of approximately 40 *TaTrxh1*-interacting proteins (Supplementary Table S2). From this list, we focused on the characterization of a partial cDNA clone encoding cysteine proteinase 1 (CP1), which is a key enzyme that regulates PCD and plays roles in numerous aspects of cell signaling in plants (Van Hautegeem et al. 2015). Using the sequence derived from these clones, we isolated the full-length CDS and found that it is predicted to encode a papain-like cysteine proteinase (PLCPs) (Richau et al. 2012). Evolutionary analysis showed that this cysteine proteinase had the closest relationship with RD19C from among 30 papain-like cysteine proteases found in *Arabidopsis* (Supplementary Fig. S3A). The derived *TaCP1* clone was predicted to possess a conserved catalytic triad, Cys-His-Asn, with an

additional two specific pairs of highly conserved cysteine (Cys) residues found in RD19A-like proteases from among four *Arabidopsis* RD19A-like proteins analyzed (Supplementary Fig. S3B).

To further evaluate the results of the yeast two-hybrid analysis, a full-length clone of *TaCP1* was cloned into the yeast expression vector pGADT7. *TaTrxh1* and *TaCP1* were cotransformed into yeast for further evaluation. As shown in Figure 6A, we observed that yeast containing both *TaTrxh1* and *TaCP1* grew on synthetic defined (SD)-Trp-Leu-His and SD-Trp-Leu-His-Ade media; further confirmation of this interaction was obtained in combination with the development of blue staining in the presence of X- α -galactosidase (X- α -Gal). The negative control group showed no significant growth. The interaction strength of *TaTrxh4* and *TaCP1* was further confirmed by spotting yeast dilutions (Fig. 6A). Lastly, bi-molecular fluorescence complementation (BiFC) was used to confirm the interaction between *TaTrxh1* and *TaCP1* in plant cells, wherein *pSPYCE::TaTrxh1* and *pSPYNE::TaCP1* vector were transformed in *A. tumefaciens* for expression in *N. benthamiana*. As shown in Figure 6B, only the *TaTrxh1*-cYFP/nYFP-*TaCP1* showed a strong yellow fluorescence signal, indicating that *TaTrxh1* interacts with *TaCP1* in planta.

TaCP1 positively regulates wheat resistance to P. striiformis f. sp. tritici.

Wang et al. (2012a) previously reported that *TaMCA4*, a wheat metacaspase, is induced by *P. striiformis* f. sp. *tritici* and functions in PCD development, demonstrating the importance of cysteine proteinases in wheat immunity to pathogen infection. Similarly, Bernoux et al. (2008) showed that *Arabidopsis* RD19-mediated resistance to *Ralstonia solanacearum* is conferred by the interaction with the R protein PopP2. To evaluate the activity of *RD19* in wheat immunity to *P. striiformis* f. sp. *tritici*, we quantified the mRNA accumulation of *TaCP1* accumulation following inoculation with *P. striiformis* f. sp. *tritici* races CYR23 and CYR31. As shown in Figure 7A, we observed that *TaCP1* mRNA accumulation was induced following *P. striiformis* f. sp. *tritici* infection. Unsurprisingly, and consistent with our observations, described above, we also observed that the expression of *TaCP1* mRNA was significantly different in both compatible and incompatible interactions, with expression in the incompatible interaction being 14.0- and 2.6-fold higher compared with the compatible interaction at 12 and 96 hpi, respectively.

Using the BSMV-VIGS system, we demonstrate the *TaCP1* is required for resistance to *P. striiformis* f. sp. *tritici*, using a 230-bp segment of *TaCP1* inserted into BSMV: γ . After

Fig. 2. Overexpression of *TaTrxh1* in tobacco (*Nicotiana benthamiana*) leads to cell death. **A**, Tobacco leaves were infiltrated with *Agrobacterium tumefaciens*. After 24 h, Bax was infiltrated at PVX106:*TaTrxh1*+Bax and PVX106+Bax sites. Symptoms on leaves of *N. benthamiana* were assessed 5 days after infiltration. **B**, The same leaves were placed in 100% ethanol for decolorizing.

successful virus inoculation, mild chlorotic mosaic symptoms and photobleaching were observed on BSMV:γ-as and BSMV:γ-*TaPDS*, respectively. At 10 dpi (Fig. 7B), urediospores of *P. striiformis* f. sp. *tritici* race CYR23 (avirulent) were inoculated on the fourth leaves of BSMV:γ-as, which all had cell-death phenotypes, with the necrotic area of BSMV:γ-*TaCP1* being larger than negative and blank controls, and a small amount of uredia were produced at 15 dpi (Fig. 7C). The silencing efficiency of *TaCP1* was approximately 78 to 86% (Fig. 7D).

To further understand how *TaCP1* participates in wheat resistance, a histological examination of *P. striiformis* f. sp. *tritici* growth was performed in both control and silenced plants (Supplementary Fig. S4) by analyzing the number of hyphal branches,

the length of hyphae, and the area of pathogen colonization and the results showed all were increased significantly in silenced plants compared with control plants at 120 hpi (Fig. 7E to G). The expression of *TaPR1* and *TaPR3* were all reduced significantly at 24 to 120 hpi in BSMV:γ-*TaCP1* plants when challenged with the avirulent race CYR23 (Fig. 7H). In addition, the expression of *TaSOD* and *TaCAT* were significantly induced at 120 and 48 hpi, respectively (Fig. 7I). Therefore, *TaCP1* was a positive regular in wheat resistance.

DISCUSSION

The modulation of plant immune response signaling processes is intimately associated with the rapid production and clearing

Fig. 3. Suppression of *TaTrxh1* reduces wheat resistance to *Puccinia striiformis* f. sp. *tritici*, as indicated by the barley stripe mosaic virus (BSMV)-induced gene silencing. **A**, Photo-bleaching and mild chlorotic mosaic symptoms were observed on the fourth leaves of BSMV:γ- *TaPDS*, BSMV:γ, and BSMV:γ-*TaTrxh1*, respectively, at 10 days postinoculation (dpi), while there were no symptoms on the mock leaves treated with FES buffer. **B**, Fourth leaves pre-inoculated with BSMV:γ and BSMV:γ-*TaTrxh1* were challenged with urediospores of *P. striiformis* f. sp. *tritici* race CYR23 or CYR31. Typical leaves were photographed at 15 dpi. **C**, and **D**, The relative expression of *TaTrxh1* was decreased in *TaTrxh1*-silenced plants inoculated with CYR23 and CYR31, respectively. **E**, The expressions of resistance-related genes in *TaTrxh1* silenced plants were significantly reduced compared with BSMV:γ after inoculation with *P. striiformis* f. sp. *tritici* race CYR23. *TaPR1*: pathogenesis-related protein (AJ007348.1), *TaPR3*: chitinase 3 (AB029936.1). $2^{-\Delta\Delta Ct}$ method was used and each sample had three independent biological repeats. Asterisks (*) indicate a significant difference ($P < 0.05$), using Student's *t* test. *TaEF-1a* mRNA accumulation was monitored as a control.

of reactive oxygen (Vieira Dos Santos and Rey 2006). As a key regulator of this process, Trxs function as critical components of this highly conserved defense mechanism and are required for the modulation of the redox status of components involved

in pathways linked to oxidative-stress and gene expression regulation. Here, we present the identification and characterization of TaTrxh1, a positive regulator of biotic stress signaling in wheat. In total, the data described in the current study support

Fig. 4. Response of silenced plants after inoculation with the stripe rust fungus. **A**, H₂O₂ accumulation observations at infection sites pretreated with BSMV:γ and BSMV:γ-TaTrxh1 and infected with CYR23. **B**, The area of H₂O₂ stained by 3,3'-diaminobenzidine was measured. **C**, TaSOD and TaCAT mRNA accumulations were significantly induced in silenced plants. **D**, Expansion of stripe rust race CYR23 as observed in TaTrxh1-silenced plants. **E**, Hyphal branch number, **F**, hyphal length, and **G**, colony area averages were calculated. Averages were calculated from 50 infection sites wherein the formation of a subtomatal vesicle was observed, which is a marker for the induction of success fungal penetration. Asterisks (*) indicate significant difference ($P < 0.05$) from BSMV:γ, using Student's *t* tests. HMC = haustorial mother cell, IH = infection hypha, and SV = subtomatal vesicle. Bar = 20 μm.

a role for TaTrxh1 as a SA-dependent regulator of *P. striiformis* f. sp. *tritici*-induced defense signaling in wheat and, in combination with TaCP1, is required for robust immune signaling activation.

Previous work demonstrated that Trxs are required for both PTI and effector-triggered immunity (Arsova et al. 2010; Mukaihara et al. 2016; Rivas et al. 2004). Indeed, each of these studies, including additional work describing the expression of *AtTrxh5* in *Arabidopsis* (Reichheld et al. 2002), *CaTrxh1* in pepper (Yi et al. 2007), *NtTRXh3* in tobacco (Sun et al. 2010), and *OsTRXh1* in rice (Zhang et al. 2011) correlated the induced mRNA accumulation of Trx with pathogen perception and the subsequent activation of robust immune signaling processes.

Fig. 5. Quantification of salicylic acid (SA) and jasmonic acid (JA) in *Puccinia striiformis* f. sp. *tritici* race CYR23-inoculated *TaTrxh1*-silenced plants. **A**, SA accumulation was significantly reduced. **B**, Accumulation of jasmonic acid (JA) remained unchanged at 18 and 24 h postinoculation. Each sample had at least two independent biological repeats. An asterisk (*) indicates a significant difference ($P < 0.05$).

Here, we demonstrate that *TaTrxh1* was also rapidly induced in wheat following pathogen inoculation. As a further step in defining the defense signaling processes associated with *P. striiformis* f. sp. *tritici*-based immune signaling in wheat, we leveraged one of the key activities of Trxs to define the breadth and specificity of the mechanism or mechanisms that underpin their role in immune signaling—namely, their role in buffering host tolerance to oxidative stress. The HR cell death is a local defense response induced, in large part, by the production and accumulation of reactive oxygen (Coll et al. 2011). As a function of the regulatory processes, associated ROS production in rice, for example, is dependent upon the activity of *OsPHO2*, whose activity is regulated in a redox-dependent manner by Trxs (Cao et al. 2014). To determine if *TaTrxh1* functions in a ROS-based defense mechanism following *P. striiformis* f. sp. *tritici* infection, we show that silencing of *TaTrxh1* resulted in a significant reduction in *P. striiformis* f. sp. *tritici*-induced ROS production, with a concomitant increase in the expression of *TaCAT* and *TaSOD*, which are required for ROS scavenging. Conversely, overexpression of *TaTrxh1* in tobacco (*N. benthamiana*) leaves led to the induction of a localized cell-death response, demonstrating that *TaTrxh1* is involved in ROS production and accumulation, as well as the activation of PCD signaling in response to pathogen infection.

SA-based signaling plays a critical role in plant immune activation in response to biotrophic pathogens. SA biosynthesis in plants occurs through two pathways, the phenylalanine pathway and the ICS1 pathway. And the expression of over 98% of SA-regulated genes is dependent on NPR1 (Qi et al. 2018). As an indicator of this dependency, it has been reported that *PR* genes are induced by SA, with *PR1* being a critical marker for the activation of this pathway (Fu and Dong 2013). In recent years, emerging data has demonstrated a role for Trxs in the local and systemic immune signaling processes under the control of SA (Mata-Pérez and Spoel 2019). For example, work by Tada et al. (2008) suggests that *AtTrxh5* promotes SA-induced immune gene expression by regulating NPR1 conformational status; this is achieved by the reduction of NPR1 disulfide bonds, leading to a release of NPR1 monomers for translocation into the nucleus. Subsequent work showed that this activity further correlated with the localization of *AtTrxh5*, which was observed in both the cytoplasm and nucleus, an observation consistent with the movement of NPR1 (Kneeshaw et al. 2014). In wheat defense signaling following *P. striiformis* f. sp. *tritici* infection, previous work described a role for SA (Liu et al. 2019). Herein, the observation of an increase in *TaTrxh1* mRNA accumulation following SA treatment, supports the hypothesis that *TaTrxh1* is involved in SA signaling following *P. striiformis* f. sp. *tritici* infection. Indeed, our data demonstrate that during the interaction between *TaTrxh1*-silenced plants and *P. striiformis* f. sp. *tritici*, *TaICS1*, *TaPAL*, *TaNPR1*, *TaPR1*, and *TaPR3* mRNAs were significantly reduced following *P. striiformis* f. sp. *tritici* infection, while SA accumulation was reduced following *P. striiformis* f. sp. *tritici* inoculation. These data suggest that the function of *TaTrxh1* in wheat resistance to *P. striiformis* f. sp. *tritici* may depend upon the generation of the SA signal.

The ability to both recognize and reduce target proteins underpins the primary function of Trxs in the regulation of the cellular redox environment. Based on this, role, it is reasonable to assume that the interaction between Trx and one or more target proteins likely occurs at the Cys-rich disulfide bridge of the oxidized target protein. Following this initial interaction, the subsequent formation of a mixed disulfide bridge results in the release of the reduced protein, and the generation of an oxidized Trx (Mata-Pérez and Spoel 2019). In this study, we

screened CP1 for its ability to interact with TaTrxh1, using a yeast two-hybrid-based assay and an in-planta BiFC approach. Through this, we demonstrate that TaTrxh1 interacted with TaCP1. This is significant, as TaCP1 is classified as a member

of the RD19A-like subfamily of PLCPs, with the closest homology to *Arabidopsis* RD19C, previously demonstrated to function in the regulation of processes associated with disulfide bridge formation (Richau et al. 2012). In support of observations

Fig. 6. TaTrxh1 interacts with TaCP1. **A**, Initial validation of TaTrxh1-TaCP1 interactions via the yeast two-hybrid system. Yeast strain Y2HGold containing TaTrxh1 and TaCP1 were grown SD-Leu-Trp-His and SD-Trp-Leu-His-Ade selective medium and were stained with X-α-Gal solution. The negative control groups had no significant strain growth or color reaction. **B**, Bimolecular fluorescence complementation showed that TaTrxh1 interacts with TaCP1 in leaf cells of *Nicotiana benthamiana*. TaTrxh1 and TaCP1 were coexpressed in *N. benthamiana* by agroinfiltration. Yellow fluorescence signals were observed at 48 to 72 h postinoculation (bars = 20 μm).

Fig. 7. *TaCP1* plays a positive role in wheat resistance to *Puccinia striiformis* f. sp. *tritici*. **A**, Transcriptional level of *TaCP1* as a function of *P. striiformis* f. sp. *tritici* infection. Asterisks (*) indicate significant difference ($P < 0.05$) between *P. striiformis* f. sp. *tritici* races CYR23- (incompatible interaction) and CYR31 (compatible interaction)-infected Suwon11 at the same timepoint, using Student's *t* test. **B**, Barley stripe mosaic virus with a specific fragment of *TaCP1* was pretreated on the second leaves of wheat. After 10 days, photobleaching and mild chlorotic mosaic symptoms were observed on the fourth leaves. **C**, Functional characterization of *TaCP1* using barley stripe mosaic virus (BSMV)-induced gene silencing. Urediospores of CYR23 were inoculated onto silenced plants and were evaluated at 15 days postinoculation. Representative leaves are shown. **D**, Effective silencing of *TaCP1*. **E**, Number of hyphal branches, **F**, hyphal length, and **G**, colony area were all recorded from 50 infection sites. **H** and **I**, Transcript analysis of defense-related genes after *P. striiformis* f. sp. *tritici* inoculation in silenced leaves. *TaPR1* = pathogenesis-related protein (AJ007348.1), *TaPR3* = chitinase 3 (AB029936.1), *TaSOD* = superoxide dismutase (AK453889.1), and *TaCAT* = catalase (AF475100.1). The $2^{-\Delta\Delta Ct}$ method was used and each sample had three independent biological repeats. Asterisks (*) indicate significant difference ($P < 0.05$), using Student's *t* test. *TaEF-1a* was used as a control.

presented herein, PLCPs are crucial enzymes functioning in the regulation of PCD and pathogen resistance. For example, in *Arabidopsis*, *AtRD19* has been demonstrated as a required signaling protein associated with PopP2-mediated plant immunity (Bernoux et al. 2008). Among all identified RD19 homolog proteins in wheat, TaCP1 was the only one confirmed to have a function associated with resistance signaling in response to *P. striiformis* f. sp. *tritici*. Indeed, our data demonstrate that TaTrxh1 specifically interacts with TaCP1 in planta and that this interaction positively regulates cell-death elicitation following *P. striiformis* f. sp. *tritici* infection (Fig. 8).

In summary, we identified an *h*-type Trx, TaTrxh1, as a positive regulator of immunity in wheat. Unlike previously reported Trxs, TaTrxh1 can directly induce the activation of cell-death signaling, a function that likely underpins the downstream activation of disease resistance through its interaction with TaCP1. In total, the data presented herein provide direct evidence that TaTrxh1 functions in an SA-dependent manner to regulate immune signaling, and moreover, through its interaction with TaCP1, it plays a key, indispensable, role in the activation of robust immunity to the stripe rust pathogen *P. striiformis* f. sp. *tritici*. These data lay a solid foundation from which future mechanistic studies will emerge to describe the role of cellular redox and PCD in response to fungal pathogenesis in plants.

MATERIALS AND METHODS

Plant and pathogen material, treatment, and growth.

The wheat cultivar Su11 plants that carried the *YrSu* resistance gene were grown at 25°C, with a day and night cycle of 16 and 8 h, respectively, and were used for all experiments described here. *P. striiformis* f. sp. *tritici* race CYR23 (avirulent) was used as an incompatible pathogen, while race CYR31 (virulent) was used as a compatible interaction (Cao et al. 2003).

The urediospores of *P. striiformis* f. sp. *tritici* (CYR23 and CYR31, individually) were grown on wheat cv. Mingxian169 leaves. The inoculated plants were placed in a dark incubator with a temperature of 10°C and a relative humidity of 90% for 24 h. After 24 h, inoculated plants were moved to an incubator at 16°C, under a day and night cycle of 16 and 8 h, respectively. After 12 dpi, the fresh urediospores of stripe rust were collected (Kang and Li 1984).

To evaluate the expression levels of *TaTrxh1* and *TaCP1* in response to *P. striiformis* f. sp. *tritici* infection, Su11 seedlings were inoculated with urediospores of CYR23 and CYR31, and leaves were harvested at 0, 12, 24, 36, 48, 72, 96, and 120 hpi. For hormone treatment, 100 µM MeJA and 2 µM SA were sprayed on Su11 leaves, and samples were taken at 0, 0.5, 3, 6, 12, and 24 hpt. All leaves were treated with distilled water as a control, and each treatment was repeated three times (Zhang et al. 2017).

Tobacco (*N. benthamiana*) was grown at 25°C under a day and night cycle of 14 and 10 h, respectively. After 4 to 6 weeks, at the six-leaf stage, leaves were inoculated with *A. tumefaciens* GV3101 containing one of several PVX106-derived vectors for protein overexpression.

Escherichia coli DH5α was grown in Luria-Bertani (LB) medium at 37°C. *A. tumefaciens* GV3101 was grown in LB media at 28°C. *Saccharomyces cerevisiae* Y2H Gold was grown on YPDA (1% yeast extract, 2% peptone, 2% dextrose, 0.02% adenine) medium at 30°C.

DNA cloning and sequence analysis.

Full-length DNA sequences with 347 bp were isolated from wheat cv. Su11 by homology-based cloning, using wheat *Trxh* as a template (GenBank accession number AY072771.1). The

open reading frame (ORF) sequence of *TaTrxh1* was analyzed using the National Center for Biotechnology Information (NCBI) ORF Finder and URG1 BLASTN. The protein sequence of *TaTrxh1* was analyzed using Protparam. Multiple sequence alignments were performed using DNAMAN6.0. A phylogenetic tree was constructed with the MEGA 6.0 software (Sun et al. 2019).

RT-qPCR analysis.

Total RNA was isolated using Biozol total RNA extraction kit (Bioflux). After evaluation of quality, 2 µg of RNA from each sample was subjected to first strand cDNA synthesis using FastKing cDNA Kit (with gDNase) (Tiangen). Primer Premier 6.0 software was used to design the DNA primers for RT-qPCR (Supplementary Table S1). DNA primers were evaluated using the NCBI Primer-BLAST tool to determine target site specificity. The total reaction system and RT-qPCR protocol was as recommended for the UltraSYBR mixture system (KangWei). After the reaction, the $2^{-\Delta\Delta Ct}$ method was used for quantitation, with the *TaEF-1a* gene (GenBank accession number Q0303033) as control gene. After repeating the reaction in triplicate, the SPSS 23.0 software was used to determine statistical significance ($P < 0.05$) (Livak and Schmittgen 2001).

Fig. 8. A hypothetical model of the function of TaTrxh1 that positively regulates cell death at the site of *Puccinia striiformis* f. sp. *tritici* (*Pst*) infection. In plants, TaTrxh1 directly associates with TaCP1, which may regulate the redox reaction by reduction of the disulfide bond and positively regulates cell death mediated by nucleotide binding-leucine-rich repeat (NB-LRR) recognition of the invading *P. striiformis* f. sp. *tritici*. In the process, the accumulation of reactive oxygen species (ROS) is induced and TaTrxh1 and TaCP1 can regulate TaSOD and TaCAT to scavenge ROS. In addition, TaTrxh1 may regulate wheat resistance to *P. striiformis* f. sp. *tritici* through salicylic acid (SA) signal.

Overexpression of TaTrxh1 protein in *N. benthamiana*.

The full ORF of *TaTrxh1* was cloned into the PVX106 vector via *Sall* digestion and ligation. After vector construction, PVX106: *TaTrxh1* was transformed into *A. tumefaciens* GV3101. Cultures of *A. tumefaciens* were centrifuged and the resultant bacterial pellets were resuspended in infiltration buffer (10 mM morpholineethane sulfonic acid, pH 5.6) to a final optical density at 600 nm of 0.6. With *A. tumefaciens* infecting *N. benthamiana*, the Bax site was infiltrated with Bax at 0 hpi and was the positive control for PVX106: *TaTrxh1*, PVX106 vector was the blank control, and infiltration buffer was the negative control. The PVX106+ Bax site was infiltrated with PVX106 earlier and challenged with Bax at 24 hpi, which was the positive control for PVX106: *TaTrxh1*+Bax to determine whether *TaTrxh1* could suppress PCD. The cell-death symptoms caused by Bax were monitored at 5 dpi, which were then decolorized with 100% alcohol and were photographed. The experiment was repeated at least two times.

BSMV-mediated gene silencing.

The special segment of *TaTrxh1* (Supplementary Fig. S1B) was constructed into BSMV: γ vector with *NotI* and *PacI* restriction enzyme for gene silencing. The BSMV RNAs were transcribed in vitro from linearized plasmids BSMV: γ -gene, γ -*TaPDS* (*phytoene desaturase*), BSMV: γ , BSMV: α , BSMV: β according to manufacturer instructions (RiboMAX Large Scale RNA Production Systems-T7). The BSMV RNAs (α , β , and γ/γ genes) were mixed with FES buffer and were inoculated on the second leaf of two-leaf Su11. The plants were then put in a dark incubator with temperature of $23 \pm 1^\circ\text{C}$ and humidity of 90% for 24 h, with an adjusted day and night cycle of 16 and 8 h, respectively (Wang et al. 2012b). Ten days after virus inoculation, fresh CYR23 and CYR31 urediniospores were inoculated onto the fourth leaf, and the leaves were sampled at 0, 24, 48, and 120 hpi for isolating total RNA, were sampled at 24, 48, and 120 hpi for histological observation, and were sampled at 18 and 24 hpi for quantification of SA and JA. At 15 dpi with *P. striiformis* f. sp. *tritici*, phenotypes were observed and photographed. The experiment was repeated three times.

We measured the expression of *TaTrxh1* and *TaCP1* in silenced plants to determine silencing efficiency. In addition, to analyze effects of gene expression on plant resistance at the molecular level, we designed the RT-qPCR primers to *TaPR1* (pathogenesis-related protein), *TaPR3* (chitinase 3), *TaSOD*, *TaCAT*, *TaICS1*, *TaPAL*, and *TaNPR1* and then measured the expression of these genes.

Histological observation of *P. striiformis* f. sp. *tritici* development in gene-silenced plants.

The samples at each timepoint (0, 24, 48, and 120 hpi) were stained using 1 mg of DAB (3,3-diaminobenzidine) per milliliter (Amresco) to detect the production and accumulation of H_2O_2 . Following staining, leaves were decolorized using 100% ethanol and were stained with WGA AF488 to observe *P. striiformis* f. sp. *tritici* development; this included evaluating the number of hyphal branches, hyphal length, and colony area. Using a NIKON-80i microscope, 50 infection points were randomly inspected for each treatment. The development of a substomatal vesicle was regarded as successful *P. striiformis* f. sp. *tritici* infection (Ayliffe et al. 2011). The proportions of H_2O_2 areas, number of hyphal branches, hyphal length, and colony area were observed by microscopy and were calculated using DP-BSW software (Olympus LifeScience). The SPSS (ver. 23.0) software was used to determine statistical significance ($P < 0.05$).

Quantification of SA and JA by liquid chromatography-tandem mass spectrometry (LC-MS) in *TaTrxh1*-silenced plants.

After *TaTrxh1* silencing, the silenced and control plants were inoculated with *P. striiformis* f. sp. *tritici* CYR23. At 18 and

24 hpi, 500 mg of leaf tissue was sampled, was frozen in liquid nitrogen, and was stored at -80°C . Frozen samples were ground with a mortar and pestle under liquid nitrogen and 2 ml of methanol/water/acetic acid (90:9:1, vol/vol/vol) and were centrifuged for 1 min at 10,000 rpm. The extraction was repeated twice with 400 μl to collect the supernatant. The resultant was then dried under nitrogen, and 200 μl of methanol was added to resuspend the dried sample. After filtering through a 0.2 μm polytetrafluoroethylene membrane, the samples were detected by LC-MS, and SA and JA were quantified based on standard curves (Zhang et al. 2017).

Yeast two-hybrid and BiFC analysis.

The full ORF of *TaTrxh1* was cloned into pGBKT7 (BD) vector. Then, the plasmid was transferred into Y2H Gold following the Yeast Protocols Handbook (Takara). The cDNA library constructed with wheat infected by *P. striiformis* f. sp. *tritici* was transformed into the yeast containing pGBKT7-*TaTrxh1*, to screen candidate interaction proteins. After selection on SD-Leu-Trp-His and SD-Leu-Trp-His+X- α -Gal medium, PCR was carried out on the positive clones and the products of PCR reaction were sent to Sangon Biotech to obtain DNA sequences. The results of those DNA sequences were analyzed and compared using the NCBI BLAST tool.

The candidate protein sequence of *TaCP1* was cloned into pGADT7 (AD), and the pairs of recombinant plasmids of pGADT7-*TaCP1* and pGBKT7-*TaTrxh1* were cotransformed into Y2H Gold. Meanwhile, the negative controls (pGBKT7+pGADT7, pGBKT7-*TaTrxh1*+pGADT7, pGBKT7+pGADT7-*TaCP1*) were also cotransformed into Y2H Gold.

To verify the screening results of yeast two-hybrid, a published BiFC procedure was used (Waadt et al. 2008). The ORF sequence *TaTrxh1* and *TaCP1* were constructed on pSPYCE and pSPYNE, respectively. The constructed vector was transformed into GV3101 to coexpress protein in 4-week-old *N. benthamiana* including pSPYCE-*TaTrxh1*+pSPYNE-*TaCP1*, pSPYCE+pSPYNE-*TaCP1*, and pSPYCE-*TaTrxh1*+pSPYNE. From 48 to 72 h after coagroinfection, yellow fluorescence was observed using confocal laser scanning microscopy (Olympus FV1000MPE) with the FV10-ASW 3.1 software suite. The assay was repeated in triplicate.

AUTHOR-RECOMMENDED INTERNET RESOURCES

NCBI ORF Finder: <https://www.ncbi.nlm.nih.gov/orffinder>
ProtParam: <https://web.expasy.org/cgi-bin/protparam/protParam>
URGI BLASTN: <https://urgi.versailles.inra.fr/blast>

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