

# A novel wheat NAC transcription factor, *TaNAC30*, negatively regulates resistance of wheat to stripe rust

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**Abstract** NAC transcription factors are widespread in the plant kingdom and play essential roles in the transcriptional regulation of defense responses. In this study, we isolated a novel NAC transcription factor gene, *TaNAC30*, from a cDNA library constructed from wheat (*Triticum aestivum*) plants inoculated with the stripe rust pathogen *Puccinia striiformis* f. sp. *tritici* (*Pst*). *TaNAC30* contains a typical NAM domain and localizes to the nucleus. Yeast one-hybrid assays revealed that *TaNAC30* exhibits transcriptional activity and that its C-terminus is necessary for the activation of transcription. Expression of *TaNAC30* increased when host plants were infected with a virulent race (CYR31) of the rust fungus *Pst*. Silencing of *TaNAC30* by

virus-induced gene silencing inhibited colonization of the virulent *Pst* isolate CYR31. Moreover, detailed histological analyses showed that silencing of *TaNAC30* enhanced resistance to *Pst* by inducing a significant increase in the accumulation of H<sub>2</sub>O<sub>2</sub>. Finally, we overexpressed *TaNAC30* in fission yeast and determined that cell viability was severely reduced in *TaNAC30*-transformed cells grown on medium containing H<sub>2</sub>O<sub>2</sub>. These results suggest that *TaNAC30* negatively regulates plant resistance in a compatible wheat-*Pst* interaction.

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## INTRODUCTION

Plants have evolved complex defense mechanisms to recognize and defend against invading pathogens (Pedley and Martin 2005). These complex response mechanisms are primarily regulated by controlling the expression of pathogen-responsive genes (Tsuda and Somssich 2015), generally through activities of transcription factors (Duan et al. 2013). A wide range of transcription factor (TF) families have been identified in plants. Among these, members of the NAC, WRKY and MYB families have been implicated in the control of plant defense responses (Gao et al. 2016; Shan et al. 2016; Birkenbihl et al. 2017).

NAC (NAM, ATAF and CUC) TFs are widespread in plants and are grouped into different families based on the presence of conserved DNA binding domains.

NAC TFs contain a highly conserved DNA-binding domain of 160 amino acids (aa) at the N-terminus. By contrast, the C-terminal region of NAC proteins is variable, consistent with their diverse transcriptional activation activities (Olsen et al. 2005). NAC TFs are involved in various biological processes, including development, metabolism of reactive oxygen species, senescence, secondary cell wall biosynthesis and cell death (Shang et al. 2013; Nakano et al. 2015; Zhao et al. 2015; Baranwal and Khurana 2016; Christiansen et al. 2016; Lee et al. 2017).

Genetic and molecular studies have indicated that NAC family proteins play important roles in regulating plant defense responses against pathogens. For example, *Arabidopsis thaliana* ATAF1 is a negative regulator of defense responses against necrotrophic fungal and bacterial pathogens (Wang et al. 2009). *Arabidopsis*

NAC019 and NAC055 regulate JA-induced expression of defense genes (Bu et al. 2008). The banana (*Musa acuminata*) NAC TF MaNAC5 cooperates with MaWRKY TFs to enhance the expression of pathogenesis-related genes in the response to *Colletotrichum musae* (Shan et al. 2016), and tomato (*Solanum lycopersicum*) NAC1 is involved in regulating resistance to *Pseudomonas* infection (Miao et al. 2016). Moreover, NAC family genes are differentially expressed in rice (*Oryza sativa*) during infection with *Rice dwarf virus*, *Rice black-streaked dwarf virus*, *Rice grassy stunt virus*, *Rice ragged stunt virus* and *Rice transitory yellowing virus* (Nuruzzaman et al. 2015).

Wheat (*T. aestivum* L.) is an important crop plant, with a large production area, worldwide (Cao et al. 2012). Wheat yield and grain quality are strongly affected by a wide variety of environmental factors, such as water availability, temperature, and the presence or absence of pathogens. In recent years, wheat stripe rust has become the largest biotic limitation to wheat production, and it currently threatens the global food supply (Liu et al. 2016; Schwessinger 2017). Wheat stripe rust is caused by the obligate biotrophic fungus *Puccinia striiformis* f. sp. *tritici* (Pst). We previously reported that several wheat NAC genes are involved in wheat–Pst interactions. For example, expression of TaNAC8 responds to infection by Pst and to abiotic stresses (Xia et al. 2010). Also, TaNAC21/22, a target of the microRNA tae-miR164, plays a negative role in regulating the resistance of the host plant to stripe rust (Feng et al. 2014).

In this study, we performed a detailed characterization of the novel wheat TF, TaNAC30. TaNAC30 localizes to the nucleus and may function as a transcriptional activator. Functional characterization supported our hypothesis that TaNAC30 is a negative regulator of resistance to Pst in wheat.

## RESULTS

### Cloning and sequence analysis of TaNAC30

Using a complementary DNA (cDNA) library constructed from wheat plants inoculated with the virulent race CYR31 of the stripe rust pathogen *Puccinia striiformis* f. sp. *tritici* (Pst), we earlier identified a transcript encoding a putative NAM domain protein

(Ma et al. 2009). The corresponding gene comprises a 909 bp open reading frame (ORF), encoding a 302 aa protein with a predicted molecular weight of 33.38 kDa. The N-terminus of the predicted protein contains a 142 aa NAM domain, and the more variable transcriptional regulation region appears to be located in the C-terminus of the predicted protein (Figure 1A).

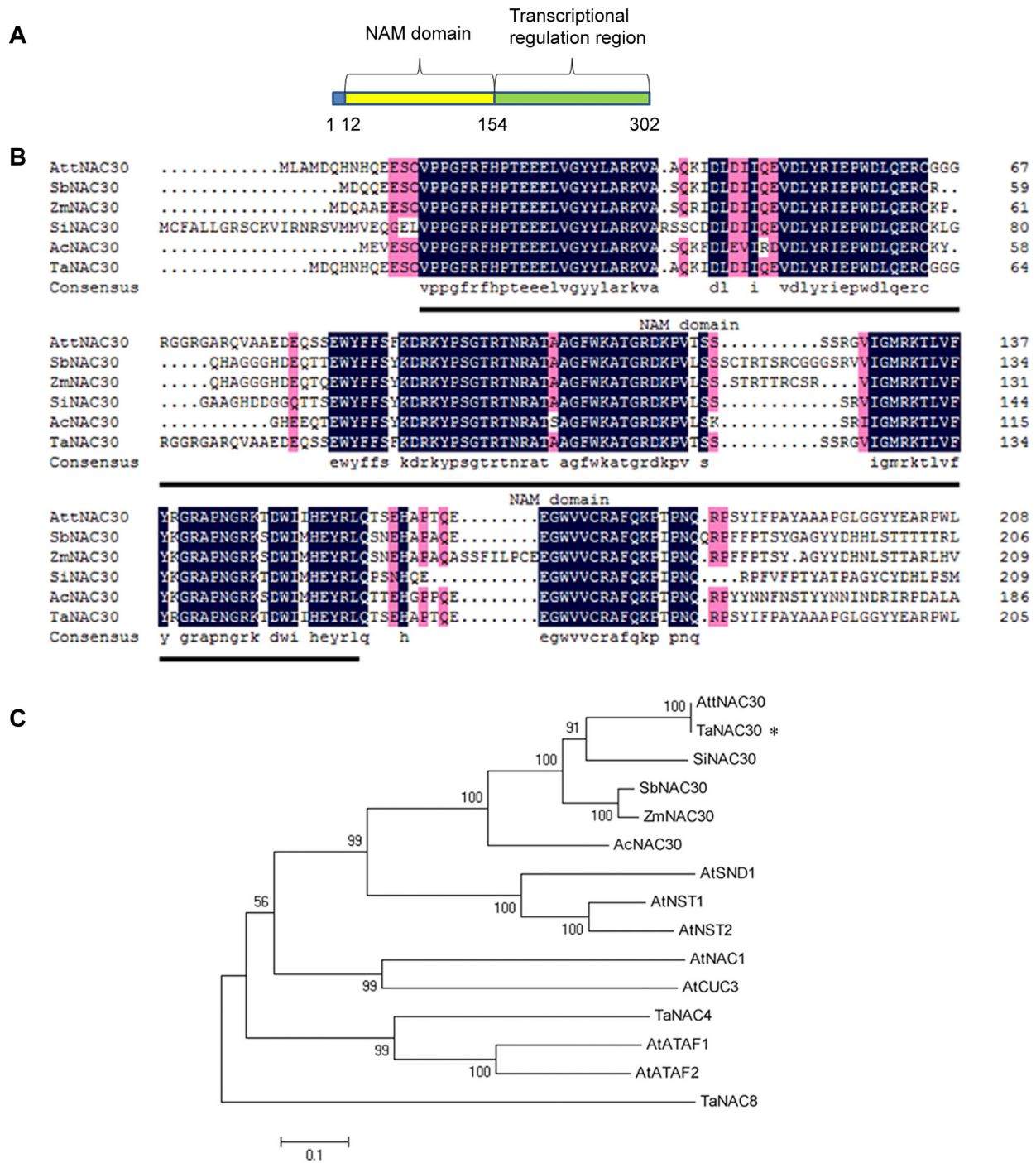
Searches of a plant TF database ([http://planttfdb\\_v3.cbi.pku.edu.cn/](http://planttfdb_v3.cbi.pku.edu.cn/)) revealed that the predicted gene belongs to the wheat NAC family (Tae062585). To identify the subfamily of plant NAC TFs, we performed homology searches against protein sequences from other plants obtained from the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). BLASTP analysis revealed that the deduced protein shares high similarity (99.02%) with AttNAC30 in *Aegilops tauschii* subsp. *tauschii*. Further analysis confirmed its relatedness to other plant NAC30 proteins, such as *Sorghum bicolor* SbNAC30, *Zea mays* ZmNAC30, and *Setaria italica* SiNAC30 (Figure 1B, C). Therefore, this gene was designated TaNAC30.

### TaNAC30 localizes to the nucleus

The deduced TaNAC30 protein sequence contains a putative nuclear localization signal, suggesting it is localized to the nucleus. To confirm this prediction, we conducted subcellular localization experiments using *Nicotiana benthamiana* leaf cells. When *N. benthamiana* leaves were transformed with the pCAMBIA-1302-TaNAC30-GFP construct, which encodes a fusion of TaNAC30 to green fluorescent protein (GFP), GFP fluorescent signals were detected in the nucleus, whereas in the control (GFP), fluorescent signals were uniformly distributed in both the cytoplasm and nucleus (Figure 2). These results demonstrate that TaNAC30 is located in the nucleus.

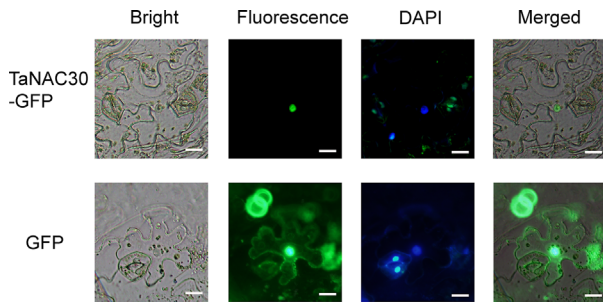
### The C-terminal domain of TaNAC30 has transactivation activity in yeast

To examine the transcriptional activity of TaNAC30, we tested whether TaNAC30 fused to a DNA-binding domain (BD) could activate *His3* and *LacZ* reporter genes downstream of the BD binding site. We also tested N- and C-terminal fragments of TaNAC30. To this end, plasmids pBD-TaNAC30, pBD-TaNAC30-N (1–154 aa), and pBD-TaNAC30-C (155–302 aa) and the negative control (pGBKT7), all containing the *Trp* gene,



**Figure 1. TaNAC30 harbors a conserved N-terminal NAM domain**

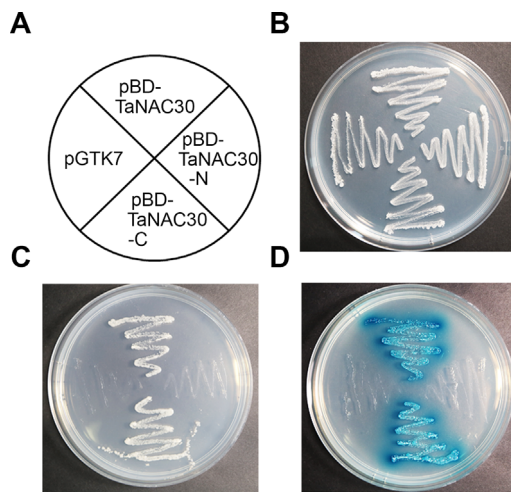
(A) Structure of TaNAC30. (B) Alignment of TaNAC30 with other NAC transcription factors. The black line indicates the conserved NAM domain and the consensus sequence is shown in black. (C) Phylogenetic analysis of TaNAC30. Different NAC transcription factors from *Aegilops tauschii* subsp. *tauschii* (Att), *Sorghum bicolor* (Sb), *Zea mays* (Zm), *Setaria italic* (Si), *Ananas comosus* (Ac), *Triticum aestivum* (Ta) and *Arabidopsis thaliana* (At) were used for the phylogenetic analyses (B and C). The accession numbers are as follows: AcNAC30 (XP\_020089105.1), AmNAC30 (ONM15671.1), AtATAF1 (OAP14514.1), AtATAF2 (OAO95967.1), AtNAC1 (NP\_175997.1), AtNST1 (OAP10209.1), AtNST2 (NP\_191750.1), AtSND1 (OAP13445.1), AtCUC3 (Q9S851.1), AttNAC30 (XP\_020184116.1), SbNAC30 (XP\_002448830.1), SiNAC30 (XP\_004959950.1), TaNAC4 (ADD10666.1), TaNAC8 (ADD10614.1).



**Figure 2. TaNAC30 localizes to the nucleus**

TaNAC30-GFP fusion protein and green fluorescent protein (GFP) (control) were transiently expressed in *Nicotiana benthamiana*. Bars = 20  $\mu$ m. Similar results were obtained from three biological replicates.

were separately transformed into yeast strain AH109. This strain containing the different recombinant plasmids grew well on selective dropout/tryptophan (SD/-Trp) medium, indicating that the four vectors were successfully transformed into the yeast cells (Figure 3A, B). Yeast cells containing pBD-TaNAC30 or



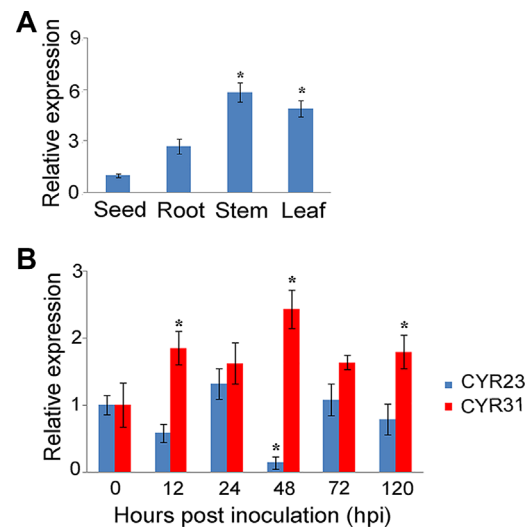
**Figure 3. The C-terminal part of TaNAC30 positively regulates transcription**

(A) The diagram indicates the corresponding vector for the assay. (B) Growth of transformants on selective dropout/tryptophan (SD/-Trp) medium at 30°C for 3 d. (C) Growth of transformants on selective dropout/tryptophan-histidine-adenine (SD/-Trp-His-Ade) medium. (D)  $\alpha$ -galactosidase assay on SD/-Trp-His-Ade medium containing 5-bromo-4-chloro-3-indoxyl- $\beta$ -D-galactopyranoside (X- $\beta$ -Gal). TaNAC30-N (1–462 bp), TaNAC30-C (463–909 bp). Similar results were obtained from three biological replicates.

pBD-TaNAC30-C grew well on SD medium in the absence of tryptophan, histidine and adenine (Trp-/His-/Ade-), whereas cells harboring pGBK7 or pBD-TaNAC30-N failed to grow on this medium (Figure 3C). In an  $\alpha$ -galactosidase activity assay, transformants harboring pBD-TaNAC30 or pBD-TaNAC30-C turned blue on SD medium (Trp-/His-/Ade-) containing X-Gal, suggesting that TaNAC30 and pBD-TaNAC30-C successfully activated the transcription of the *His3* and *LacZ* reporter genes (Figure 3D). These results support the hypothesis that the C-terminal region of TaNAC30 positively regulates transcription of the reporter genes.

### Transcriptional responses of TaNAC30 to Pst

To test whether TaNAC30 showed tissue-specific expression, we analyzed the transcript levels of TaNAC30 by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) in a variety of samples, including seeds, roots, stems and leaves. As shown in Figure 4A, TaNAC30 mRNA was detected in all tissue types, with the highest transcript levels detected in stem and leaf tissue.



**Figure 4. Quantitative reverse transcription polymerase chain reaction analysis of relative transcript levels of TaNAC30**

(A) Transcript analysis of TaNAC30 in different wheat tissues. (B) Transcript analysis of TaNAC30 in wheat leaves (Su11) inoculated with *Pst* race CYR31 (virulent) or CYR23 (avirulent), respectively. Samples were collected from leaves, stems, roots, and seeds. Mean expression values were calculated from three independent replicates.

We also examined *TaNAC30* transcript levels in *Pst*-infected wheat leaves. *TaNAC30* transcript level increased as early as 12 h post-inoculation (hpi) and exhibited an approximately 2.4-fold peak response at 48 hpi in the compatible interaction (Figure 4B). *TaNAC30* transcript levels were downregulated at 48 hpi in the incompatible interaction. At 48 hpi, relative *TaNAC30* transcript levels were approximately 17-fold higher in the compatible than in the incompatible interaction. These results support our hypothesis that *TaNAC30* acts as a negative regulator of plant defense responses.

### Knockdown of *TaNAC30* increases resistance to *Pst*

To further characterize the function of *TaNAC30* in stripe rust resistance, we used a virus-induced gene silencing (VIGS) system with *Barley stripe mosaic virus* (BSMV), which has been successfully used to study gene function in plants (Liu et al. 2012). The BSMV:*TaNAC30* plants displayed mild chlorotic mosaic symptoms and no other obvious defects, at 10 d post-inoculation (dpi) with *Pst*. As a control to confirm that VIGS was functioning correctly, we silenced the wheat phytoene desaturase (*PDS*) gene, via inoculation with the recombinant virus BSMV:*TaPDSas*, causing severe chlorophyll photobleaching at 10 dpi. The fourth leaves of wheat cultivar Suwon 11 (Su11) plants were inoculated with avirulent *Pst* race CYR23 and virulent race CYR31 at 10 dpi with BSMV. We detected masses of urediniospores on CYR31-infected control seedlings (Mock-CYR31) and seedlings that were previously infected with the control BSMV: $\gamma$  (BSMV: $\gamma$ -CYR31). However, we detected only limited urediniospore production on leaves treated with BSMV:*TaNAC30* inoculated with CYR31 (BSMV:*TaNAC30*-CYR31) at 14 dpi with the rust fungus (Figure 5A). Moreover, there was no phenotypic change in the incompatible interaction (BSMV:*TaNAC30*-CYR23) compared to the controls (Mock-CYR23; BSMV: $\gamma$ -CYR23), which exhibited no sporulation.

We next performed qRT-PCR to confirm that the VIGS system was functional by determining the silencing efficiency of *TaNAC30*. Compared to leaves inoculated with BSMV: $\gamma$ , *TaNAC30* transcript levels were reduced by 62%, 72% and 77% at 24, 48 and 120 hpi, respectively, in BSMV:*TaNAC30* leaves inoculated with CYR31 (Figure 5B). In the incompatible interaction (CYR23), the expression of *TaNAC30* was reduced by 64%–66% (Figure 5B).

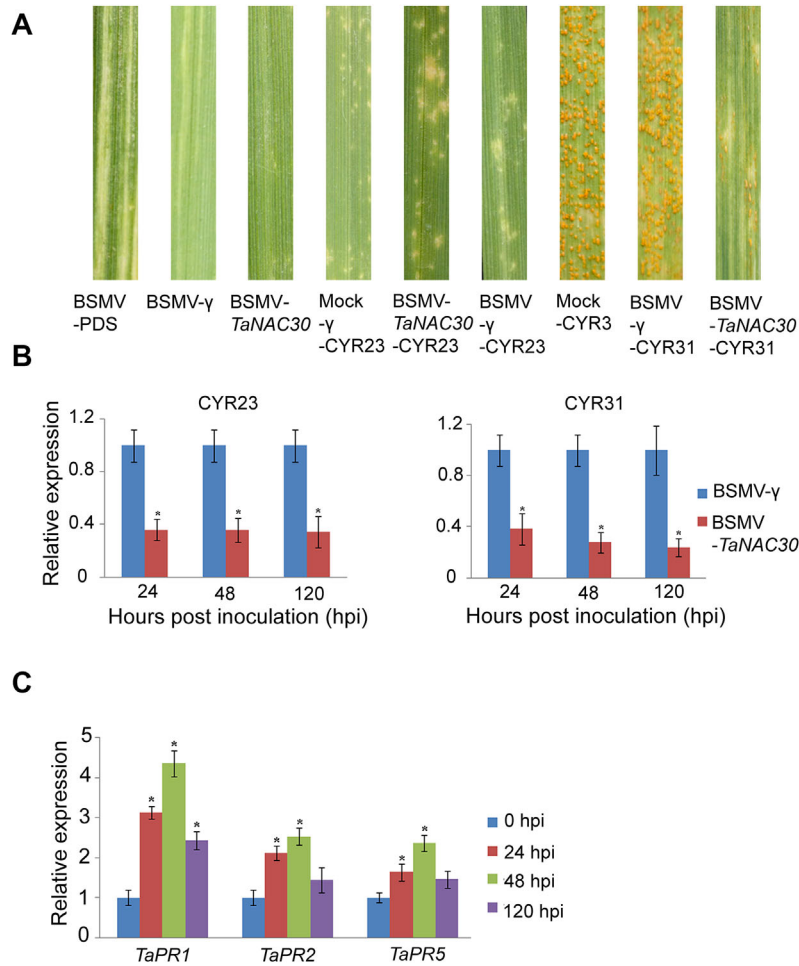
To determine whether the transcript levels of defense-related genes in *TaNAC30*-knockdown leaves were affected by infection with CYR31, we selected several wheat *Pathogenesis-related* (*PR*) genes for qRT-PCR analysis. *PR1* transcript levels at 24, 48 and 120 hpi were significantly higher in BSMV:*TaNAC30* leaves inoculated with CYR31 compared to the control (Figure 5C). *PR4* and *PR5* transcript levels showed similar patterns, but the increase was only significant at 24 hpi and 48 hpi. It is worth noting that, for all three genes analyzed, the transcript levels were lower at 120 hpi than at 48 hpi. These findings strongly support our hypothesis that *TaNAC30* is a negative regulator of wheat defense responses.

To further verify the phenotypes of leaves treated with BSMV:*TaNAC30* inoculated with CYR31, we performed detailed histological analyses (Table 1, Figure S1). Hyphal length and hyphal branch number in *Pst* were significantly reduced ( $P < 0.05$ ) in BSMV:*TaNAC30* knockdown leaves versus the control at 24 and 48 hpi. Moreover, *Pst* colony size was significantly reduced ( $P < 0.05$ ) in wheat leaves pre-inoculated with BSMV:*TaNAC30* at 120 hpi compared to the control.

To further analyze the host resistance response, we measured  $H_2O_2$  accumulation during the compatible interaction by 3,3-diaminobenzidine (DAB) staining. Many mesophyll cells in contact with secondary hyphae had abundant levels of  $H_2O_2$  in *TaNAC30*-knockdown seedlings at 48 hpi and 120 hpi with the CYR31 isolate (Figure 6A). However, no  $H_2O_2$  was detected in mesophyll cells of control (BSMV: $\gamma$ -inoculated) plants. Moreover, the area containing  $H_2O_2$  was significantly larger in *TaNAC30*-knockdown seedlings compared to control plants at 48 hpi and 120 hpi (Figure 6B). These results further support the role of *TaNAC30* in plant defense responses.

### Overexpression of *TaNAC30* in fission yeast decreases resistance to $H_2O_2$

To investigate the possible role of *TaNAC30* in  $H_2O_2$  sensitivity, we heterologously overexpressed *TaNAC30* in fission yeast (*Schizosaccharomyces pombe*) using the pREP3X vector (Forsburg 1993). Yeast cells expressing *TaNAC30* were cultured in induction (-Thiamine) or repression (+Thiamine) medium for 20 h. We examined the effects of *TaNAC30* on the survival of yeast cells exposed to  $H_2O_2$ . As shown in Figure 7, cell viability was severely reduced in *TaNAC30*-transformed cells



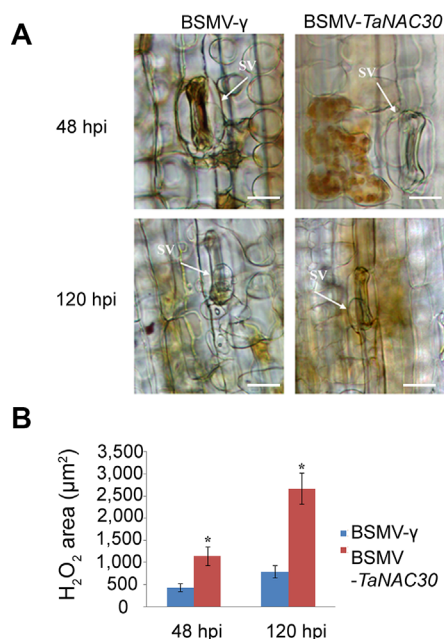
**Figure 5. Functional characterization of *TaNAC30* after virus-induced gene silencing**

(A) Phenotypic changes of fourth leaves in plants pre-inoculated with positive control vector (*Barley stripe mosaic virus* phytoene desaturase (BSMV-PDS)), or empty BSMV vector (BSMV: $\gamma$ ) at 14 d post-virus treatment. Phenotypes for the fourth leaves inoculated with *Puccinia striiformis* f. sp. *tritici* (*Pst*) races CYR23, or CYR31 at 14 d post-infection (dpi). (B) Relative transcript levels of *TaNAC30* in *TaNAC30* knock-down leaves. RNA samples were isolated from *TaNAC30* knock-down leaves inoculated with *Pst* races CYR23, or CYR31. (C) Transcriptional changes in pathogenesis-related protein genes in *TaNAC30*-knockdown wheat seedlings. Similar results were obtained from three biological replicates.

**Table 1. Histological analysis of *TaNAC30* knock-down leaves responding to infection by *Puccinia striiformis* f. sp. *tritici* (*Pst*) race 31**

Treatment	Hyphal length ( $\mu\text{m}$ )		Hyphal branches		Colony size ( $\times 1,000 \mu\text{m}^2$ )
	24 hpi	48 hpi	24 hpi	48 hpi	120 hpi
BSMV- $\gamma$	34.21a $\pm$ 2.15	59.87a $\pm$ 2.18	2.46a $\pm$ 0.19	3.16a $\pm$ 0.24	2.52a $\pm$ 0.16
BSMV- <i>TaNAC30</i>	29.56b $\pm$ 1.89	41.36b $\pm$ 2.12	2.01b $\pm$ 0.15	2.67b $\pm$ 0.21	1.48b $\pm$ 0.17

Leaves were inoculated with BSMV- $\gamma$ , or BSMV-*TaNAC30*, followed by inoculation with *Pst* CYR31. hpi, hours post-inoculation; BSMV, *Barley stripe mosaic virus*. *Pst* hyphal branches, hyphal length and uredinial area were measured for at least 50 infection sites. Numbers within the same column marked with different superscript letters are significantly different according to one-way analysis of variance ( $P < 0.05$ ). Results were obtained in three independent biological replicates.



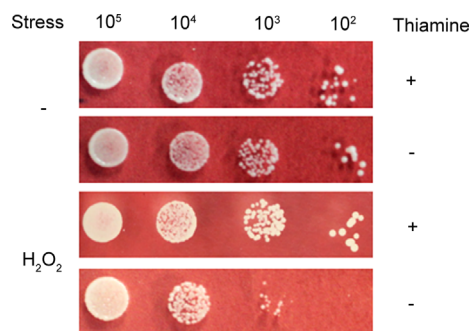
**Figure 6. H<sub>2</sub>O<sub>2</sub> accumulation in TaNAC30-knockdown seedlings challenged with *Pst* race CYR31**

(A) Histochemical localization of H<sub>2</sub>O<sub>2</sub> at infection sites. Inoculated wheat leaves were stained with 3,3-diaminobenzidine (DAB) to detect the production of H<sub>2</sub>O<sub>2</sub>. Barley stripe mosaic virus (BSMV): $\gamma$  and BSMV:TaNAC30 at 48 h post-infection (hpi) (bars = 20  $\mu\text{m}$ ); BSMV: $\gamma$  and BSMV:TaNAC30 at 120 hpi (bars = 50  $\mu\text{m}$ ). SV, substomatal vesicle. (B) Significant increase in H<sub>2</sub>O<sub>2</sub> accumulation in TaNAC30-knockdown plants. Three biological replications were performed.

grown on induction medium + H<sub>2</sub>O<sub>2</sub> compared with cells grown on repression medium + H<sub>2</sub>O<sub>2</sub>. Therefore, TaNAC30 decreases H<sub>2</sub>O<sub>2</sub> resistance in yeast cells.

## DISCUSSION

NAC TFs, comprising one of the largest TF families, are thought to function as key regulators of stress responses in plants. More than 100 NAC proteins have been identified in every plant species in which genomes have been completely sequenced to date. For example, there are 105 redundant putative NAC genes in *A. thaliana* and 140 putative NAC or NAC-like genes in rice (Nuruzzaman et al. 2010). In the plant TF database (3.0), 134 NAC or NAC-like genes were predicted in wheat. However, currently, only a few of these NAC genes have been functionally



**Figure 7. Overexpression of TaNAC30 in fission yeast decreases resistance to H<sub>2</sub>O<sub>2</sub>**

Yeast cells expressing TaNAC30 were spotted on solid medium containing 0.5 mmol/L H<sub>2</sub>O<sub>2</sub>. Similar results were obtained from three biological replicates.

characterized (Feng et al. 2014). In the present study, we characterized the NAC TaNAC30 from wheat leaves infected with *Pst* race CYR31. The deduced TaNAC30 protein is predicted to contain a NAM domain at its N-terminus. However, the C-terminal region of TaNAC30 is not conserved and appears to be necessary for transactivation. In subcellular localization experiments, a TaNAC30-GFP fusion protein localized to the nucleus. Our findings suggest that TaNAC30 is a novel wheat NAC gene encoding a transcriptional activator that functions in the nucleus.

NAC TFs play central roles in multiple stress responses, including biotic stress responses (McGrann et al. 2015). Some NAC TFs appear to positively regulate the plant defense responses. By contrast, other NAC TFs are negative regulators of resistance responses to pathogens (Shan et al. 2016). Also, some potato NAC genes are upregulated by bacterial infection (Feng et al. 2014), and *Arabidopsis* ATAF1 is a negative regulator of defense responses against necrotrophic fungal and bacterial pathogens (Wang et al. 2009).

In the present study, we show that TaNAC30 was upregulated during a compatible interaction with *Pst*. We then used a VIGS system to determine the role of TaNAC30 in wheat-*Pst* interactions. Knockdown of TaNAC30 inhibited uredinial development of the compatible *Pst* race CYR31 in the wheat cultivar Su11. Histological observations were consistent with these phenotypes. Hyphal length, hyphal branch number, and colony size of *Pst* were reduced in

TaNAC30-knockdown leaves compared to wild type. Taken together, these results support the notion that TaNAC30 is a negative regulator of the wheat-Pst interaction.

NAC TFs control downstream molecular pathways to help protect plants from various stress conditions (Bu et al. 2008). NAC TFs function as negative regulators of pathogen resistance by suppressing defense-related gene expression (Bu et al. 2008; Shan et al. 2016). For example, ATAF2-overexpressing plants showed higher susceptibility to the soil-borne fungal pathogen *Fusarium oxysporum* than wild type, due to the repression of a number of PR genes (Delessert et al. 2005). In the present study, we analyzed the expression of PR genes in TaNAC30-knockdown seedlings and established that *TaPR1*, *TaPR2* and *TaPR5* were significantly upregulated in these plants, relative to the control. Based on this result, we suggest that the induced expression of PR genes inhibits uredinial development in TaNAC30-knockdown seedlings.

During defense responses, one of the most efficient, rapid immune responses is the hypersensitive response (HR). H<sub>2</sub>O<sub>2</sub> regulates various aspects of disease resistance and functions as a threshold trigger for the HR (Do et al. 2003). H<sub>2</sub>O<sub>2</sub> generation during the infection stage is associated with the occurrence of the HR and the resistance response (Wang et al. 2007). NAC TFs are involved in H<sub>2</sub>O<sub>2</sub>-mediated signaling, with 15 NAC genes known to be H<sub>2</sub>O<sub>2</sub>-responsive in *Arabidopsis* (Balazadeh et al. 2010). ORS1 (encoding an H<sub>2</sub>O<sub>2</sub>-responsive NAC TF) is rapidly and strongly induced by H<sub>2</sub>O<sub>2</sub> treatment (Salma et al. 2011). In the current study, TaNAC30-knockdown plants exhibited a sharp increase in H<sub>2</sub>O<sub>2</sub> accumulation after infection with *Pst* race CYR31. We also overexpressed TaNAC30 in *S. pombe*, finding that cell viability was severely reduced in TaNAC30-transformed yeast cells grown on induction medium + H<sub>2</sub>O<sub>2</sub> compared to the control. These results suggest that TaNAC30 is involved in regulating H<sub>2</sub>O<sub>2</sub> accumulation.

In conclusion, TaNAC30 is a novel wheat NAC gene in which its product functions as a transcriptional activator in the plant nucleus. TaNAC30 is induced during wheat-Pst compatible interactions and negatively regulates Pst resistance in wheat by inhibiting the expression of PR genes and H<sub>2</sub>O<sub>2</sub> accumulation.

## MATERIALS AND METHODS

### Plant materials and inoculation

Suwan11 (Su11) is a Chinese wheat cultivar that is highly resistant to *Pst* race CYR23 (avirulent) and susceptible to race CYR31 (virulent). The procedures and conditions used for wheat seedling (Su11) culture, inoculation and incubation were previously described (Kang et al. 2002). Inoculated and control leaves were harvested at 0, 12, 24, 48, 72 and 120 hpi and immediately frozen in liquid nitrogen. Three independent biological replicates were performed per treatment.

### Sequence analysis, alignment and protein structure prediction

The conserved domains of TaNAC30 and other proteins were deduced using NCBI conserved domain searches (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Phylogenetic trees were generated using MEGA5 (<http://www.megasoftware.net>) (Tamura et al. 2011). Bootstrap values were calculated from 1,000 bootstrap replicates. Multiple alignments were created using CLUSTALW (Thompson et al. 1994), with minor manual adjustments as necessary to optimize the alignment.

### RNA extraction and qRT-PCR analysis

Total RNA was extracted from plant tissue using a PureLink RNA Mini Kit (Invitrogen, Beijing, China), following the recommended protocol. All qRT-PCR experiments were performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The reactions were performed in a total volume of 25 µL. The PCR conditions were as follows: 95°C for 1 min, 45 cycles of 95°C for 10 s, 55°C for 30 s, and 72°C for 1 min, followed by 72°C for 5 min. To standardize the results, the wheat elongation factor gene *TaEF-1α* was used as an endogenous control for qRT-PCR analysis. The primers used in qRT-PCR are listed in Table S1. Relative gene expression levels were calculated using the comparative 2<sup>-ΔΔCT</sup> method (Livak and Schmittgen 2001).

### Subcellular localization

The oligonucleotides used for plasmid construction and the constructs used in this study are listed in Table S1. TaNAC30 was cloned from cDNA from wheat cultivar Su11 using FastPfu DNA Polymerase (TransGen Biotech, Beijing, China). The amplicon was digested with the appropriate restriction enzymes and ligated into plant



binary expression vector pCAMBIA-1302. *TaNAC30* in the pCAMBIA-1302 backbone was used as a template to amplify the gene using specific primers with *Sall* and *BamHI* restriction enzyme sites. To examine the subcellular localization of *TaNAC30*, transient expression analysis was performed using *N. benthamiana* leaves. A *TaNAC30*-GFP fusion vector was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Cells carrying the different recombinant vectors (empty vector and *TaNAC30*-GFP) were cultured to an optical density of 0.8 at 600 nm (OD<sub>600</sub>) and used to infiltrate the leaves of 4-week-old *N. benthamiana* plants. Infiltrated seedlings were transferred to a growth chamber at 25°C with a 16 h light photoperiod. Leaf tissue samples were harvested at 2 d post-infiltration. GFP signals were detected under an Olympus BX-51 microscope (Olympus Corp., Tokyo, Japan). Each experiment was repeated at least three times, with each assay consisting of at least three plants.

#### Transcriptional activation analysis in yeast

*TaNAC30* was examined for the presence of an activation domain using a yeast assay system as previously described (Fujita et al. 2004). The recombinant plasmids pBD-*TaNAC30*, pBD-*TaNAC30*-N (1–462 bp), and pBD-*TaNAC30*-C (463–909 bp) and the negative control plasmid pGKT7 were individually transformed into yeast strain AH109 (Clontech, Mountain View, CA, USA). Transformed cells were streaked onto SD/Trp- and SD/Trp-/His-/Ade- medium. The growth status of the transformants was evaluated after incubation at 30°C for 3 d. The  $\alpha$ -galactosidase activity assays were performed using X- $\alpha$ -Gal as a substrate.

#### BSMV-mediated *TaNAC30* gene silencing

At the two-leaf stage, wheat seedlings were infected with BSMV as described by Hein et al. (2005). The seedlings were inoculated with each of three viruses (BSMV: $\gamma$ , BSMV:*TaPDS* and BSMV:*TaNAC30*), followed by incubation at 25°C and subsequent examination for symptoms at regular intervals. BSMV:*TaPDS* was used as a positive control. Ten days after virus inoculation, the fourth leaves of seedlings were inoculated with urediniospores of *Pst* race CYR23 (avirulent) or CYR31 (virulent). The fourth leaves were sampled at 0, 24, 48 and 120 hpi with *Pst* for RNA isolation and cytological observation. The silencing efficiency of *TaNAC30* was

calculated based on qRT-PCR data. Symptoms of wheat stripe rust were recorded and photographed at 14 dpi.

Plasmids used for gene silencing were based on previously described constructs (Holzberg et al. 2002). RNA-derived clones (BSMV-*TaNAC30*) were generated using BSMV-*TaPDS* in which the wheat phytoene desaturase (*TaPDS*) gene fragment was replaced with specific *TaNAC30* sequences. Following digestion with the restriction enzymes *NotI* and *PacI*, the amplicon was directionally ligated into the *NotI*/*PacI* sites of the BSMV: $\gamma$  vector.

#### Histological analysis of fungal growth and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) accumulation

Plant samples were harvested and decolorized as previously described (Wang et al. 2007). A staining procedure for wheat germ agglutinin (WGA) conjugated to the fluorophore Alexa 488 (Invitrogen, Carlsbad, CA, USA) was used to obtain high-quality images of developing uredia. Cleared wheat leaf segments were examined under an Olympus BX-51 microscope (Olympus Corporation, Tokyo, Japan). At least 50 infection sites were examined per treatment. Hyphal lengths and infection area were measured using DP-BSW software (Olympus Corporation, Tokyo). SPSS software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

H<sub>2</sub>O<sub>2</sub> was detected, as previously described (Thordal-Christensen et al. 1997). Inoculated wheat leaves were cut and the ends were immersed in a solution containing 1 mg/mL DAB (Amresco, Solon, OH, USA) dissolved in HCl-acidified distilled water (pH 3.8). Leaves were incubated for 8 h to allow for the uptake of DAB and reaction with H<sub>2</sub>O<sub>2</sub>.

#### Overexpression of *TaNAC30* in *Schizosaccharomyces pombe*

The *TaNAC30* amplicon and the pREP3X vector were digested with restriction enzymes *Sall* and *BamHI*. The recombinant vector pREP3X-*TaNAC30* was transformed into *S. pombe* by electroporation. Thiamine was used as a repressor for the *nmt1*-promoter. Transformed cells with a starting optical density at 600 nm (OD<sub>600</sub>) of 0.2 were cultured in induction (-Thiamine) or repression (+Thiamine) medium for 20 h at 30°C (Li et al. 2010). To assay sensitivity to various environmental stimuli, cells were removed from logarithmic cultures after 24 h of growth, collected by centrifugation, washed twice with sterile distilled water, and diluted to a density of 10<sup>5</sup>, 10<sup>4</sup>,

$10^3$  and  $10^2$  cells/mL. Cell growth was assayed on solid yeast medium containing 0.5 mmol/L  $H_2O_2$  (Soto et al. 1999).

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## AUTHOR CONTRIBUTIONS

B.W. and J.P.W. carried out most of the experiments. B.W. and Z.S.K. wrote the manuscript. N.S. performed qRT-PCR and analyzed the data. B.W. and N.W. grew the plant samples, N.W. collected all the phenotypic data, J.Z. and Z.S.K. revised the manuscript. All authors read and approved the final manuscript.

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## SUPPORTING INFORMATION

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**Figure S1.** Histological observation of fungal growth in TaNAC30-knockdown seedlings challenged with virulent *Puccinia striiformis* f. sp. *tritici* race CYR31

Barley stripe mosaic virus (BSMV): $\gamma$  and BSMV:TaNAC30 at 24 hpi and 48 hpi (bars = 20  $\mu\text{m}$ ); BSMV: $\gamma$  and BSMV:TaNAC30 at 120 hpi (bars = 100  $\mu\text{m}$ ). H, haustorium; HMC, haustorial mother cell; SV, substomatal vesicle;

IH, infection hypha. All results were obtained from 50 infection sites, and three biological replications were performed.

[Table S1. Primers used in this study](#)



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