TaCIPK10 interacts with and phosphorylates TaNH2 to activate wheat defense responses to stripe rust

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Received 10 September 2018; revised 14 November 2018; accepted 14 November 2018.
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Summary
Calcineurin B-like interacting protein kinase (CIPKs) has been shown to be required for biotic stress tolerance of plants in plant-pathogen interactions. However, the roles of CIPKs in immune signalling of cereal crops and an in-depth knowledge of substrates of CIPKs in response to biotic stress are under debate. In this study, we identified and cloned a CIPK homologue gene TaCIPK10 from wheat. TaCIPK10 was rapidly induced by Puccinia striiformis f. sp. tritici (Pst) inoculation and salicylic acid (SA) treatment. In vitro phosphorylation assay demonstrated that the kinase activity of TaCIPK10 is regulated by Ca\(^{2+}\) and TaCBL4. Knockdown TaCIPK10 significantly reduced wheat resistance to Pst, whereas TaCIPK10 overexpression resulted in enhanced wheat resistance to Pst by the induction of defense response in different aspects, including hypersensitive cell death, ROS accumulation and pathogenesis-relative genes expression. Moreover, TaCIPK10 physically interacted with and phosphorylated TaNH2, which was homologous to AtNPR3/4. Silencing of TaNH2 in wheat resulted in enhanced susceptibility to the avirulent Pst race, CYR23, indicating its positive role in wheat resistance. Our results demonstrate that TaCIPK10 positively regulate wheat resistance to Pst as molecular links between of Ca\(^{2+}\) and downstream components of defense response and TaCIPK10 interacts with and phosphorylates TaNH2 to regulate wheat resistance to Pst.

Keywords: wheat, TaCIPK10, resistance, phosphorylation, TaNH2, Puccinia striiformis f. sp. tritici.

Introduction
Wheat stripe rust, caused by Puccinia striiformis f.sp. tritici (Pst), an obligate biotrophic pathogenic, poses a tremendous threat to the production of wheat worldwide (Wan et al., 2004). Two strategies are used to control rust in wheat: chemical control and genetic control. Genetic control is the most cost-effective strategy to reduce the threat of the disease because of its environmental and economic factors (Ellis et al., 2014). Normally, two general classes of genes based on their phenotypic effects are used for genetic control: pathogen race- or strain-specific resistance (R genes) and adult plant resistance (APR) genes (Chen, 2005). R genes usually mediate resistance during all plant developmental stages, whereas APR genes function mainly during the adult stage. A series of defense responses have been confirmed to be involved in wheat all-stage resistance against Pst infection, including cell wall apposition, papilla formation and accumulation of several antifungal compounds, such as lignin, callosates, plant hydrolases and reactive oxygen species (ROS; Wang et al., 2007; Zhang et al., 2008). In addition, many permanently or temporarily named Yr genes and quantitative trait loci (QTL) have been identified to confer APR to stripe rust (Zhou et al., 2014; Lu et al., 2014). Besides these two classes of genes, numerous genes in wheat have been extensively reported to be involved in the interaction between wheat and Pst (Kang et al., 2017). However, as an obligate biotrophic basidiomycete, Pst cannot be cultured in vitro. Consequently, studies on the mechanism of wheat resistance to Pst are challenging.

Normally, plants establish a series of immune events in response to pathogen infection, including ion fluxes across the plasma membrane, an increase in the intracellular Ca\(^{2+}\) concentration, accumulation of ROS, biosynthesis of salicylic acid (SA), cell-wall strengthening, etc. (Boller and Felix, 2009; Tsuda and Katagiri, 2010). Among these signalling processes, SA has been demonstrated to be a critical modulator of multiple levels of immunity, protecting plants from a wide range of phytopathogens by mediating immune responses at both local and systemic levels (Vlot et al., 2009). The mechanism of the mode of action of SA has been studied in-depth in model plant Arabidopsis. SA signalling through NPR1 (nonexpresser of PR protein 1) is required to establish systemic acquired resistance (SAR). In the absence of pathogen attack, NPR1 is present in the cytoplasm as disulfide-bound oligomers. NPR3 and NPR4, the paralogues of NPR1, directly bind SA and function as substrate adaptors that regulate proteasome-mediated degradation of NPR1 (Fu et al., 2012). However, the research on mechanism of NPR1 homologue genes is still very shallow. In rice, overexpression of the rice NPR1 homologues NH1 and NH3 result in enhanced resistance to Xanthomonas oryzae pv. oryzae (Xoo) and Magnaporthe oryzae (Yuan et al., 2007; Bai et al., 2011), suggesting that OsNH1 and OsNH3 play a positive role in rice resistance. Another NPR1-like gene, OsNPR2/NH2, was shown to respond to infection by the bacterial blight pathogen, Xoo, and the rice blast fungus, M. oryzae (Yuan et al., 2007).

SA-mediated plant immunity has been confirmed to be regulated by calcium signals. In Arabidopsis, the calmodulin-binding transcription activation CAMTA3 (also called AtSR1) directly binds the promoter of EDS1 to repress the expression of EDS1, which is known to be required for NLR-mediated cell death and activation of SA biosynthesis (Yuan et al., 2018). Calcium functions as a second messenger and regulates a plethora of inter- and extracellular signalling processes. The transient changes in Ca\(^{2+}\) concentration is sensed and decoded by sensor relay and responder proteins, such as calmodulins (CaMs), calcium-dependent protein kinases (CDPK or CPKs) and calcineurin B-like proteins.
proteins (CBLs). Unlike CaM and CDPK, which interact with a large number of target proteins, CBLs specifically interact with a family of protein kinases referred to as CBL-interacting protein kinases (CIPKs), which belong to the Snf1-RELATED KINASE3 family (Suc nonfermenting 1-related kinases, group 3; SnRK3). It has been shown that the protein kinase of CIPKs is regulated by CBLs binding Ca\(^{2+}\) through their four EF hands (Halfter et al., 2000; Kurusu et al., 2010). Bioinformatic analyses identified ten CBLs and 26 CIPKs in Arabidopsis and seven CBLs and 32 CIPKs in wheat (Hashimoto et al., 2012; Sun et al., 2015). Different CBLs interact specifically with different CIPKs, resulting in a vast array of potential CBL/CIPK module combinations and versatility of Ca\(^{2+}\) responsiveness to this signalling system. Each potential CBL/CIPK module generates specificity in the signalling pathway and functions for crosstalk or overlap in signalling pathways (Thorude-Kennedy et al., 2015). Most CBL-CIPK components have been thoroughly studied in the regulation of different abiotic stress-triggered signalling pathways (Cheong et al., 2003; Guo et al., 2002; Pandey et al., 2004, 2005), but the function of the CBL-CIPK system in response to biotic stresses is still an under-studied area of research. In Arabidopsis, NPR1 is phosphorylated and activated by PKS5/CIPK11 to induce WRKY38 and WRKY62 after bacterial inoculation, suggesting that crosstalk occurs between CBL/CIPK modules and SA signalling pathways during biotic stress responses (Xie et al., 2010). To date, the relationship of Ca\(^{2+}\) signals and NPR1-like genes in stress responses in crop plants is still unclear. Therefore, characterization of CIPK and NPR1-like genes in wheat is a worthwhile goal, especially towards elucidating mechanisms of pathogen resistance.

In this study, we identified and functionally characterized one CIPK gene, TaCIPK10, and assessed its important roles in wheat resistance to Pst. Additionally, we demonstrated that TaCIPK10 kinase activity is regulated by conjoined TaCBL4 protein and Ca\(^{2+}\). Concurrently, TaNH2, an Arabidopsis NPR3/4 orthologue, which was confirmed to interact with and be phosphorylated by TaCIPK10, plays a positive role in wheat resistance. Our results demonstrated that TaCIPK10 transmit the Ca\(^{2+}\) signals to positively regulate wheat resistance to Pst and TaNH2 may functions as target of TaCIPK10 in wheat response to Pst infection. Our results provide new insights towards understanding the roles of CBL-CIPK and the NPR family in plant resistance.

Results

TaCIPK10 is significantly induced by Pst infection and SA treatment

A total of 32 TaCIPK genes were identified in the wheat genome database (Sun et al., 2015). In rice, OsCIPK14/15 was confirmed to be involved in plant immunity (Kurusu et al., 2010). To investigate whether CIPKs participate in wheat responses to Pst, the transcript levels of four TaCIPK genes (TaCIPK2, 10, 14, 15), which located in the same cluster with OsCIPK14/15 (Sun et al., 2015), were determined by qRT-PCR in leaves of wheat seedlings following Pst infection. The results showed that transcript levels of all selected TaCIPKs were affected by Pst infection (\(P < 0.01\) and fold change \(>2\); Figure 1a and Figure S1), suggesting that TaCIPKs participate in the interaction between wheat and Pst (Figure S1).

As shown in previous studies, the SA-mediated defense signalling pathway is activated following infection by biotrophic pathogens (Vlot et al., 2009). Thus, we measured SA concentrations during wheat resistance to Pst. The results indicated that SA concentrations were significantly increased during the interaction between wheat and Pst, suggesting that the SA signalling pathway is involved in wheat resistance to Pst (Figure S2). In order to explore whether the mechanisms of TaCIPKs in wheat resistance are related to SA, transcript levels of these TaCIPKs were determined in leaves of wheat seedlings following exogenous application of SA. The results showed that transcript levels of TaCIPK10 are significantly enhanced fivefold at 2 hpt (Figure 1b), while those of other CIPKs were not significantly induced (Figure S1). Based on these observations, TaCIPK10 was selected for further analysis during the response of wheat to Pst infection.

Sequence analysis and subcellular localization of TaCIPK10

According to the sequences identified in the wheat genome, the full-length cDNA of TaCIPK10, encoding a putative protein composed of 438 amino acid residues with a molecular weight of 49.9 kDa and an isoelectric point (pI) of 9.0, was obtained from wheat cv. Suwon11 by RT-PCR. BlastN analysis of the wheat...
genome identified three copies of TaCIPK10 with 96% nucleotide sequence identity, which are located on chromosomes 4A, 4B and 4D (Figure S3a). TaCIPK10 obtained from Suwon11 was on chromosome 4D and contained a few amino acid variations relative to other predicted amino acid sequences on chromosome 4A and 4B (Figure S3b). In silico sequence analysis revealed that TaCIPK10 contains a kinase catalytic domain and a regulatory domain. The activation loop containing three conserved phosphorylatable residues (threonine, serine and tyrosine) were found in the kinase catalytic domain, while the NAF motif (also known as FISL motif), mediated CIPK binding to CBL, in the regulatory domain (Figure S4a). Based on the amino acid sequences of CIPKs with assigned functions, TaCIPK10 shared the same clade with other CIPK10 protein sequences from monocot plants (Figure S4b).

**TaCIPK10 interacts with several TaCBLs**

Seven CBL homologues in wheat cv. Suwon11 were investigated for interaction preferences with TaCIPK10 by the yeast two-hybrid (Y2H) system. The results showed that TaCIPK10 interacts with six TaCBLs (TaCBL1.1, TaCBL1.2, TaCBL2, TaCBL3, TaCBL4 and TaCBL9) but not with TaCBL6 (Figure 2a). The NAF motif in the CIPK family had been reported to interact with CBLs and possess auto-inhibitory function (Pandey et al., 2014). As expected, TaCIPK10-ΔNAF, a mutant protein deficient in the FISL/NAF motif, failed to interact with TaCBLs in yeast (Figure 2b), suggesting the highly conserved functions of FISL/NAF motif in CIPKs among plant species. The strongest interaction of TaCIPK10 with TaCBL4 was confirmed by β-galactosidase activity (Figure 2c). In addition, protein expression in the Y2H system was confirmed by Western blot using Myc- and HA-antibodies (Figure S6).

**The protein kinase activity of TaCIPK10 is regulated by TaCBL4 and Ca²⁺**

As a Ser/Thr protein kinase, the kinase activity of TaCIPK10 was characterized. Three site-directed mutants of TaCIPK10 were generated following previous studies (Pandey et al., 2014) of other CIPKs that conferred enhanced kinase activity (Figure 3a). A TaCIPK10 mutant version lacking the FISL/NAF motif was generated (as that in the experiment of TaCIPK10 interacts with several TaCBLs; Figure 3a). In addition, an inactive version, mutating a conserved lysine (K) to asparagine (N) at position 42, was generated as a negative control. As shown in Figure 3b,c, the autophosphorylation and phosphorylation activity of wild-type TaCIPK10 was almost undetectable, whereas the other TaCIPK10 variants showed significantly increased kinase and autophosphorylation activity compared to the wild type. TaCIPK10-K42N had no activity (Figure 3b,c). In addition, TaCIPK10 and its mutants with expected molecular sizes were detected by Western blot using anti-His antibody (Figure 3d). To test if TaCIPK10 kinase activity was regulated by Ca²⁺ and TaCBLs, in vitro transphosphorylation assays were performed with MBP as substrate in the presence or absence of Ca²⁺. TaCBL4 and TaCBL6 were selected as the regulator of TaCIPK10 kinase activity because TaCIPK10 strongly interacted with TaCBL4 but not with TaCBL6. As shown in Figure 3e, the kinase activity of TaCIPK10 was significantly increased only in the presence of TaCBL4 and Ca²⁺, suggesting that the kinase activity of TaCIPK10 is modulated by TaCBLs in a Ca²⁺-dependent manner.

![Figure 2](image_url) TaCIPK10 interacts with TaCBLs in a yeast two-hybrid system. (a, b) TaCIPK10 (a) or TaCIPK10Δ (b) was cloned into pGADT7 vector and TaCBLs were cloned into the pGADT7 vector. Cells of yeast strain AH109 harbouring the indicated plasmid combinations were grown on either the non-selective (SD-LW) or selective (SD-LWβ-gal) medium containing 20 µg/mL X-gal. Positive control, the interaction between SV40 large T-antigen (T) and murine p53 (53) T-AD+53-BD. Negative control, the interaction between SV40 large T-antigen (T) and human lamin C (Lam), T-AD=Lam-BD. (c) Quantitative β-galactosidase activity of combinations between TaCIPK10 and TaCBLs or TaCIPK10Δ and TaCBLs. N.D., Not detected. Data represent the mean of three biological replicates ±SE.
Suppression of TaCIPK10 reduces wheat resistance to Pst

To characterize the function of TaCIPK10 in wheat defense response to Pst, BSMV-VIGS, a rapid and effective approach for analysing gene function in wheat and barley (Scofield et al., 2005), was used. Three specific fragments of TaCIPK10 cDNA were selected for the silencing experiment (Figure S3a). The mild chlorotic mosaic symptoms appeared on BSMV-inoculated plants, and BSMV:TaPDS-as infected plants exhibited strong photobleaching at 10 dpi (Figure 4a), indicating that the BSMV-mediated gene silencing system functioned correctly. The fourth leaf of all wheat plants was then inoculated with fresh urediospores of Pst race CYR23 or CYR31. The transcript level of TaCIPK10 in silencing plants was much lower than that in control plants during the interaction between TaCIPK10-knockdown plants and Pst (P < 0.01 and fold change <0.5), suggesting that TaCIPK10 was silenced successfully by BSMV system (Figure 4b). The transcript accumulation of each copy on chromosomes 4A, 4B and 4D could not be determined by qRT-PCR because of high similarity and identity among them (Figure S3a). Meanwhile, sequence alignment among three TaCIPKs and TaCIPK10 which located in the same clade did not contain consecutive 21- to 24-nucleotide sequences, indicating the specificity of virus-induced gene silencing (VIGS) fragments used in this study (Figure S7). At 14 dpi with Pst, conspicuous HR symptoms appeared on all leaves after inoculation with the incompatible strain, CYR23 (Figure 4c). A few fungal uredia surrounding the necrotic areas were observed on leaves that were pre-infected with BSMV:TaCIPK10-1/2/3as (Figure 4c). In contrast, all leaves infected with the compatible strain, CYR31, exhibited normal disease development (Figure 4c). To determine whether transcript accumulation of defense-related genes was affected by TaCIPK10, the transcript levels of three pathogenesis-related (PR) genes and three ROS-related genes were measured. The expression of TaPR1, TaPR2 and TaPR5 was significantly reduced during the interaction between TaCIPK10 and mutant versions were confirmed by Western blot using anti-His antibodies. (c) The kinase activity of TaCIPK10 is regulated by TaCBL4 in a Ca2+-dependent manner. TaCIPK10 was incubated with MBP, γ-32P ATP and Ca2+ or EDTA in kinase buffer in presence or absence of TaCBL4-His and TaCBL6-His fusion proteins.

Figure 3 TaCIPK10 is an active kinase and regulated by TaCBL4 and Ca2+. (a) Schematic diagrams of TaCIPK10 mutants and deletions. AD, activation domain. NAF, the motif for interacting with CBLs. Mutagenized residues and deleted domain are marked. WT, the full length of TaCIPK10. Single amino acid was substituted for Lysine-42 (K42) to Asparagine (N) and Serine-157 (S157). Threonine-171 (Y171), Tyrosine-173 (Y173) to Aspartic acid (D) and designated as K42N, S157D, Y171D and Y173D, respectively. (b) For the autophosphorylation assays, TaCIPK10 and mutant versions were incubated with γ-32P ATP and kinase buffer. The reaction products were analysed by SDS-PAGE gels and autoradiography. (c) For the phosphorylation assays, the proteins were incubated with 3 mg MBP in the presence of γ-32P ATP and kinase buffer. (d) Equimolecular levels of TaCIPK10 and mutant versions were confirmed by Western blot using anti-His antibodies. (e) The kinase activity of TaCIPK10 is regulated by TaCBL4 in a Ca2+-dependent manner. TaCIPK10 was incubated with MBP, γ-32P ATP and Ca2+ or EDTA in kinase buffer in presence or absence of TaCBL4-His and TaCBL6-His fusion proteins.
infected plants at 24 and 48 hpi (Figure S10b,c). However, the number of haustorial mother cells in leaves of TaCIPK10-knockdown plants was significantly increased over that in BSMV:γ infected plants (Figure S10d), as was infection area at 120 hpi (Figure S10e). Taken together, the results indicate that silencing TaCIPK10 diminishes the wheat defense response against Pst. Overexpression of TaCIPK10 enhances wheat resistance to Pst.

To further confirm the defense role of TaCIPK10 in wheat, overexpression transgenic wheat plants were produced by co-bombardment with TaCIPK10 expression vector pWMB003-TaCIPK10 and pAH20 into 2000 immature embryos of the wheat cultivar Xinong1376 (XN1376). Twelve positive transgenic plants were identified from 12 regenerated T0 plants by PCR using specific primers (Figure S11a,b). The expression level of TaCIPK10 was up-regulated in all positive T0 generation plants compared to wild type and the negative plants (Figure S11c). Two lines with the highest expression level, TaCIPK10-T0-63 and TaCIPK10-T0-95, were selected for further experiments. Two T1 lines, TaCIPK10-T1-63 and TaCIPK10-T1-95, derived from relevant T0 generations, expressed enhanced resistance to Pst race CYR32 (Figure S11d,e), with an induction of TaCPK10.
transcription as high as 10-fold compared to wild type (Figure S11e,g). At T2 generation, eight and nine positive plants from TaCIPK10-T2-63 and TaCIPK10-T2-95, respectively, were identified by PCR and qRT-PCR (Figure 5a,b). The transcript accumulation of TaCIPK10 was 3.4–19.4-fold higher than that in XN1376 (Figure 5b). Fourteen days after Pst inoculation, all positive plants expressed increased resistance with a significant reduction in sporulation compared to wild type and negative plants. By applying the McNeal's uniform scale system (McNeal et al., 1971), we observed that the host response of wild type and negative control inoculated with Pst ranged from 7 to 8, showing abundant sporulation with few necrotic/chlorotic stripes and indicating high susceptibility of wheat. By contrast, the response of transgenic plants was in the 1–4 range, indicating high or medium resistance (Table S1). The biomass analysis showed that the biomass of Pst in positive plants was significantly reduced by 30%–65% (Figure 5c). In addition, the PR genes (TaPR1, TaPR2 and TaPR5) and TaNOX showed higher expression levels than that in the wild type, while the TaCAT and TaSOD were down-regulated during the interaction between Pst.

**Figure 5** Overexpression of TaCIPK10 enhances wheat resistance to Pst. (a) Foliar parts of lines TaCIPK10-T2-L63 and TaCIPK10-T2-L95 inoculated with avirulent Pst isolate CYR32. (b) Relative transcript levels of TaCIPK10 in the leaves of lines TaCIPK10-T2-L63 and TaCIPK10-T2-L95 inoculated with Pst. The transcript level of TaCIPK10 in non-transformed plants was standardized as 1. (c) Fungal and wheat biomass ratio measured via total DNA content at 14 dpi by absolute quantification using the internal reference genes PsEF and TaEF, respectively. (d, e) Relative transcript levels of TaPR1, TaPR2, TaPR5, TaSOD, TaCAT and TaNOX in TaCIPK10 overexpression lines TaCIPK10-T2-L63 and TaCIPK10-T2-L95 inoculated with CYR32. The wild type was used as control. The transcript level of genes in control plants at time 0 was standardized as 1. Data represent the mean of three biological replicates ±SE. Asterisks indicate significant differences between that in transformed plants and control at the same time points using Student's t-test (P < 0.01).
two positive lines and CYR32 (Figure 5d,e). Overexpression of TaCIPK10 stimulates wheat resistance response expressed as induction of necrotic area, increased $\text{H}_2\text{O}_2$ accumulation and decreased infection area of Pst at 14 hpi compared to the control (Figure S12). Moreover, the growth of TaCIPK10 overexpression transgenic lines showed no obvious difference relative to control plants (Figure S13). These results indicate that elevated expression of TaCIPK10 induces the accumulation of defense-related gene transcripts, which enhanced the wheat resistance to Pst.

TaCIPK10 interacts with and phosphorylates an Arabidopsis NPR3/4 orthologue protein in wheat

AtNPR1, a key regulator in the SA signalling pathway, was confirmed as the target of AtCIPK11 (AtPKSS) in a previous study (Xie et al., 2010), suggesting that CIPK families are related to NPR1 homologue. Therefore, we speculated that the function of TaCIPK10 resistance to Pst is related to the homologue of AtNPR1 in wheat. To confirm our speculation, all NPR1 homologues were systematically identified from Triticum aestivum by a genome-wide search, protein sequences from Arabidopsis and rice NPR family genes as query. In total, 16 NPR1-type genes were identified in the wheat genome and were designated as TaNH1 to TaNH5 based on their relationship with the respective genes in rice (Figure S14a). Phylogenetic analysis indicated that all NPR1-type proteins from wheat, rice and Arabidopsis cluster in three major groups (Figure S14a). TaNH1 was located in the same group with Arabidopsis NPR1. TaNH2 and TaNH3 showed high similarity with AtNPR3 and AtNPR4. Additionally, TaNH4 and TaNH5 were similar to Arabidopsis NPR5 and NPR6 (BOP1 and BOP2), which are mainly involved in regulating plant development (Canet et al., 2010). Sequence analysis showed that TaNH1, TaNH2 and TaNH3 contained an ankyrin repeat (AKR) domain, a broad complex, tramtrack, and bric-a-brac/poxivirus and zinc-finger (BTB/POZ) domain and NPR1-like domains (Figure S14b). It has been reported that the AKR domain of NPR1 is required for interacting with CIPK11 in Arabidopsis (Xie et al., 2010). Thus, we investigated the interaction between the AKR domain of TaNH1, TaNH2 or TaNH3 and TaCIPK10 by the Y2H system. Interestingly, TaCIPK10 interacted only with the AKR domain of TaNH2 (Figure S14a,c). Furthermore, the full-length of TaNH2 showed a strong interaction with TaCIPK10. The TaNH2 mutant version, lacking the AKR domain and designated as TaNH2-ΔAKR, failed to interact with TaCIPK10 (Figure 6a). As shown in Figure 6b, TaCIPK10 is pulled down by full-length of TaNH2 and TaNH2-ΔAKR (AKR domain of TaNH2), but not by TaNH2-ΔAKR. In addition, in planta interaction of TaCIPK10 with TaNH2 was tested by co-immunoprecipitation (Co-IP) using tobacco (Nicotiana benthamiana) cells transiently co-expressing TaCIPK10 fused with Flag tag and TaNH2 or its derivatives fused with GFP. As expected, TaNH2 and TaNH2-ΔAKR co-immunoprecipitated with TaCIPK10, whereas no co-IP was detected with TaNH2-ΔAKR (Figure 6c). Our data confirmed that TaCIPK10 interacts with TaNH2, and that the AKR motif is required for their interaction. In addition, the results of in vitro phosphorylation assays showed that TaCIPK10 phosphorylates TaNH2 (Figure 6d).

TaNH2 plays a positive role in wheat resistance against Pst

To characterize the function of TaNH2 in wheat resistance, the transcript accumulation of TaNH2 following biotic stress and SA treatment was investigated. As shown in Figure S10a, transcript abundance of TaNH2 was significantly induced by Pst, peaking at 12 hpi during the incompatible and compatible interactions (Figure S16a). In addition, the expression level of TaNH2 in the incompatible interaction was higher than that in the compatible interaction. Meanwhile, SA treatment elicited the significant induction of TaNH2 as early as 2 hpi (Figure S16b).

To further confirm the function of TaNH2 during the interaction between wheat and Pst, TaNH2 was knocked down by VIGS. Three fragments, showing high conservation among all copies of TaNH2 in the wheat genome database, were cloned and inserted into the virus plasmid, designated as BSMV:TaNH2-1as, BSMV:TaNH2-2as and BSMV:TaNH2-3as, respectively (Figure S17). After BSMV-inoculated plants displayed mild chlorotic mosaic symptoms at 10 dpi, the fourth leaf was inoculated with freshurediospores of Pst race CYR23 and CYR31. By 14 dpi, large numbers of Pst uredia were produced around the necrotic spots on the leaves infected with BSMV:TaNH1/2/3as (Figure 7a). By contrast, all leaves inoculated with CYR31 produced numerous uredia (Figure 7a). The reduction in the TaNH2 transcript level was more than 50% at each time point after CYR23 and CYR31 infection (Figure 7b,c). The expression of TaPR1, TaPR2 and TaPR5 were all reduced significantly at 24–120 hpi in TaNH2-knockdown plants when challenged with the avirulent race CYR23 (incompatible interaction; Figure 7d). In addition, TaCAT and TaSOD were significantly induced, while TaNOX showed the opposite expression pattern in TaNH2-knockdown plants against Pst. The histological changes associated with enhanced susceptibility of TaNH2-knockdown plants to Pst were observed (Figure S18). The areas of cell death and $\text{H}_2\text{O}_2$ accumulation were significantly lower at 120 hpi than were observed in BSMV-γ-treated leaves (Figure S18a,b). Additionally, the numbers of haustoria and haustorial mother cells, the hyphal lengths and infection area were significantly increased in all TaNH2-knockdown plants inoculated with CYR23 (Figure S18c–f). Therefore, knocking down TaNH2 reduces wheat resistance to Pst.

Discussion

As a second messenger, $\text{Ca}^{2+}$ undergoes transient changes in concentrations in response to various stimuli and regulates numerous inter- and extra-cellular signalling processes (Ghosh and Greenberg, 1995). However, the function of $\text{Ca}^{2+}$-regulated signals in immune signalling of crop plants is largely unknown. In this study, we identified and characterized a SA-induced CIPK, TaCIPK10, which is regulated by $\text{Ca}^{2+}$-sensor proteins, TaCBLs. A series of complementary cellular- and genetics-based assays demonstrated that TaCIPK10 performs a crucial role in wheat resistance to Pst and TaNH2 functions as the interact partner of TaCIPK10 to regulate wheat defense response. Our results suggested that $\text{Ca}^{2+}$-regulated signal play an important role in wheat resistance and NPR1-like protein is involved in $\text{Ca}^{2+}$-regulated signal.

TaCIPK10 positively regulate wheat resistance to Pst as the downstream of $\text{Ca}^{2+}$ signal

In Arabidopsis and rice, the protein kinase activity of CIPKs have been shown to depend on CBLs and external $\text{Ca}^{2+}$ (Halfter et al., 2000; Kurusu et al., 2010). Similarly, the kinase activity of TaCIPK10 is regulated by TaCBL4 and $\text{Ca}^{2+}$. These results suggest that the regulation of CIPKs protein kinase activity is very
conservative among all plants. Moreover, the influx of Ca\(^{2+}\) from the intercellular space to the cytoplasm was promoted as early as 12 hpi during the incompatible interaction between wheat and Pst (Yin et al., 2015). TaCIPK10 expression was peaked at 12 hpi in the incompatible interaction (Figure 1b). All these results suggested that TaCIPK10 may be activated by Pst infection through TaCBLs binding to Ca\(^{2+}\) and inactivated at a resting cytosolic Ca\(^{2+}\) level. In addition, TaCIPK10 overexpression enhanced wheat resistance to Pst for the induction of a wide range of defense response, including hypersensitive cell death, ROS generation and PR gene expression. On the other hand, TaCIPK10-knockdown plants significantly reduced wheat resistance to Pst (Figures 4 and 5). Our results confirmed that TaCIPK10 play a positive role in wheat resistance. In Arabidopsis, cipk23 mutant exhibited severe sensitivity to low-K conditions (Xu et al., 2006). Furthermore, ATCIPK23 was identified to be involved in Ca\(^{2+}\)-dependent regulation of low-K\(^{+}\) tolerance and adaptation by detailed biochemical, electrophysiological and genetic-based approaches (Li et al., 2006). Thus, our results suggested that TaCIPK10 positively regulate wheat resistance to Pst as molecular links between of Ca\(^{2+}\) and downstream components of defense response.

The function of TaCIPK10 in wheat resistance to stripe rust may depend on SA signal

Salicylic acid has confirmed to be synthesized by plants in response to challenge by a diverse range of pathogens (Vlot et al., 2009). Similarly, SA was induced by Pst infection in wheat, which suggested that SA is involved in wheat resistance to Pst (Figure S2). With induction of TaCIPK10 expression under SA treatment, we suggested that TaCIPK10 is involved in SA signal. During the interaction between TaCIPK10-overexpression transgenic lines and Pst, the PR genes were significantly induced by Pst infection compared to control (Figure 5), while exhibiting the opposite expression pattern in TaCIPK10-knockdown plants. PRs have been shown to act as disease resistance factors and can be induced by SA (Loon et al., 2006). Particularly, PR1 is the marker gene in SA-dependent responses (Fu and Dong, 2013). Moreover, ATNPR3 and ATNPR4 have been shown to regulate SA-dependent immunity in Arabidopsis (Fu et al., 2012). Among all identified
Figure 7 TaNH2 positively regulates wheat resistance to Pst. (a) Wheat leaves of TaNH2-knockdown plants were inoculated with Pst isolates CYR23 and CYR31 and photographed at 14 dpi. (b, c) Relative expression of TaNH2 was decreased in TaNH2-knockdown plants inoculated with CYR23 and CYR31. CK indicates BSMV:γ. The quantitative RT-PCR values were normalized to those for TaEF-1α, and are presented as fold changes relative to that in plants with BSMV:γ treatment at each time point. The transcript level of TaCIPK10 after Pst inoculation in the wheat leaves with BSMV:γ treatment at each time points was standardized as 1. (d, e) Relative transcript levels of TaPR1, TaPR2, TaPR5, TaSOD, TaCAT and TaNOX were measured by qRT-PCR during the interaction between TaNH2-knockdown plants and CYR23. The quantitative RT-PCR values were normalized to those for TaEF-1α, and are presented as fold changes relative to that in plants with BSMV:γ treatment at time 0. The transcript level of genes in control plants at time 0 was standardized as 1. All results were calculated by the comparative threshold method. All data represent the mean of three biological replicates ±SE. Asterisks indicate significant differences between that in TaNH2-knockdown plants and control plants using Student’s t-test (P < 0.01).
NPR1 homologue proteins in wheat, TaNH2, which shared high similarity with AtNPR3/4, was confirmed to interact with and be phosphorylated by TaCIPK10. Our results suggested that the function of TaCIPK10 in wheat resistance to stripe rust may depend on SA signal.

In rice, OsCIPK14/15 was confirmed to regulate MAMP (Microbe-Associated Molecular Pattern) induced hypersensitive cell death, phytoalexin production and defense gene expression (Kurusu et al., 2010). Moreover, tomato CBL10-CIPK6 complex contributes to ROS generation during effector-triggered immunity in the interaction of Pseudomonas syringae pv tomato DC3000 (Drerup et al., 2013). Another CBL-CIPK complex in wheat, TaCB4-TaCIPK5, has been confirmed to positively contribute to wheat resistance in ROS-depend manner (Liu et al., 2018). Together with our result in this study, we suggested that CIPKs in plant kingdom not only participate in abiotic stress, but also play a crucial role in plant response to biotic stress.

TaNH2 may be involved in Ca²⁺-regulated signal during the interaction between wheat and *Pst*

The structure of proteins is regulated by their phosphorylation, normally accompanied by modulating protein activity and altered interaction partners or subcellular localization (Schulze, 2010). In *Arabidopsis*, Ser-589 of AtNPR1 being phosphorylated by NF1-related kinase 2.8 (SnRK2.8) was found to be required for nuclear import of AtNPR1 during SAR (Lee et al., 2015), indicating that AtNPR1 is involved in SnRK2.2-mediated plant immunity. In our study, TaCIPK10 phosphorylated TaNH2 and both TaNH2 and TaCIPK10 play a positive role in wheat resistance to *Pst*. Thus, we suggested that TaNH2 functions as the interact partner of TaCIPK10 to regulate wheat resistance to *Pst*. In *Arabidopsis*, SOS1, a Na⁺/H⁺ antiporter, phosphorylated and activated by SOS2/CIPK24-SOS3/CBL4 complex at plasma membrane, indicating that SOS1 plays very important role in transducing Ca²⁺ signal for providing salt tolerance (Shi et al., 2000; Zhu, 2002). Moreover, AtCIPK26 after interacting with the calcium sensors AtCBL1 or AtCBL9 enhances the activity of the NADPH oxidase RBOHF via phosphorylation, demonstrating AtRBOHF participated in Ca²⁺ induced ROS generation (Drerup et al., 2013). As TaCIPK10 was regulated by Ca²⁺, we suggested that TaNH2 is involved in Ca²⁺-regulated signal during the interaction between wheat and *Pst*.

The mechanism of NPR1 homologues mediated disease resistance in wheat is different to that in *Arabidopsis*

In our study, the expression of TaNH2 was found to be induced by *Pst* infection. As a previous study shown (Wang et al., 2010), the up-regulation of host genes during infection may indicate the exploitation of cellular resources and/or the activation of defense responses, suggesting that TaNH2 is involved in wheat resistance. In addition, TaNH2 was shown to positively regulate wheat resistance characterized by decreased necrotic area, reduced ROS accumulation and increased urea production in TaNH2-knockdown plants (Figure 7 and Figure S15). Similarly, OsNH3/OsNPR3, which located in the same group with TaNH2, has been confirmed to play a positive role in rice disease resistance to bacterial blight (Bai et al., 2011). However, AtNPR3/4, which shared higher similarity with TaNH2, has been shown to play a negative role in plant immunity (Fu et al., 2012). Additionally, The NPR1 paralogues NPR3 and NPR4 are involved in the degradation of NPR1 in a SA concentration-dependent manner, which is required for SAR establishment in *Arabidopsis* (Fu et al., 2012). In wheat, initial pathogen infection can induce plant resistance to secondary pathogen infection, but not systemic (Wang et al., 2018). These different biological processes caused by NPR1 homologue genes in wheat and *Arabidopsis* seem to be achieved by activating different signal pathways. All these different results indicate that the mechanism of NPR1 homologues mediated disease resistance in cereal plants is different to that in *Arabidopsis*.

To our knowledge, this is the first direct evidence demonstrating that the NPR1 homologue gene is involved in Ca²⁺-regulated signal during the interaction between wheat and *Pst* (Figure S19). Additionally, the overexpression transgenic wheat, which was used for investigating the function of TaCIPK10 in wheat resistance, has potential as an alternative material in conventional breeding for creating environmentally friendly and durable resistant varieties. In conclusion, the concentration of Ca²⁺ in plant cells is affected by *Pst* infection to secure the kinase activity of TaCIPK10, and TaNH2 functions as a phosphorylation substrate of TaCIPK10 for transmitting Ca²⁺ signals that are important in conferring wheat resistance. We suggest that TaNH2 participate in Ca²⁺-regulated signal in wheat defense response. Indeed, our findings support the notion that NPR1-like genes and the genes involved in CBL-CIPK pathway co-regulate wheat resistance against *Pst*.

**Experimental procedures**

**Plant material, fungal pathogens and treatments**

Wheat (*T. aestivum L.*) genotype Suwon 11 and isolates of *Pst* CYR23 (avirulent) or CYR31 (virulent) were used in this study. Suwon 11, carrying the YrSu resistance gene, is resistant to CYR23 infection, but is highly susceptible to CYR31 (Cao et al., 2002). The second leaves inoculated with CYR23, CYR31 or sterile distilled water (control) were harvested at 0, 6, 12, 18, 24, 48, 72 and 120 h postinoculation (hpi) for RNA extraction. Time points were selected based on a previous study (Wang et al., 2007). For biological stress treatments, wheat seedlings were grown, inoculated and maintained as described previously (Tang et al., 2015). For chemical treatments, 10-day-old wheat seedlings were sprayed with 2 mM salicylic acid (SA) dissolved in 0.1% (v/v) ethanol. Mock control plants were sprayed with 0.1% ethanol. Samples were collected at 0, 4, 6, 8, 12 and 24 h posttreatment (hpt) for RNA extraction and qRT-PCR. XN1376, an early maturing winter wheat variety, was used for wheat transformation and expressed high susceptibility to *Pst* race CYR32. Methods of gene cloning, sequence analysis DNA and RNA extraction, cDNA synthesis and quantitative real-time PCR were performed following a previous study (Liu et al., 2018). To measure fungal biomass, the relative quantification of the wheat gene *TaEF-1a* and the *Pst* gene *PstEF1* (Yin et al., 2009) was assessed. The relative quantities of the PCR product of *PstEF1* and *TaEF-1a* in infected samples were calculated using the gene-specific standard curves to quantify the *Pst* and wheat DNA, respectively.

**Yeast two-hybrid assays**

For interaction analysis of TaCIPK10 and TaCBLs or TaNH2, the MatchMaker yeast two-hybrid system was performed (Takara, Dalian, China). The coding sequences of TaCBLs, TaNH2, TaNH2-DAKR and the AKR motif of TaNH1/2/3 were subcloned into pGADT7 (activation domain, AD), the coding sequences of TaCIPK10 and TaCIPK10.1 were subcloned into pGBK7 (DNA-
binding domain, BD) vector. The pairs of recombinant plasmids of AD and BD were co-transformed into yeast strain AH109 following the Yeast Protocols Handbook (Takara, Dalian, China). Protein expression in yeast was confirmed by Western blots following a previous study (Wang et al., 2016).

Site-directed mutagenesis and deletion mutants
For site mutagenesis and creation of deletion mutants, the overlap-PCR approach was used. First-round PCR products were amplified with the following oligonucleotides: TaCIPK10(K42N)-S/AS for TaCIPK10(K42N), TaCIPK10(S157D)-S/AS for TaCIPK10(S157D), TaCIPK10(T171D)-S/AS for TaCIPK10(T171D), TaCIPK10(Y178D)-S/AS for TaCIPK10(Y178D), TaCIPK10(M125A)-S/AS for TaCIPK10(M125A) (Table S2). The PCR products were used as templates for amplification with TaCIPK10-32a-S/AS. Each final PCR fragment was cloned into pGEM-T Easy Vector (Promega, Madison, WI) for sequencing.

Preparation and purification of recombinant proteins
The prokaryotic expression constructs were created by cloning the PCR-amplified ORF of wheat CIPK10, NH2 or their mutants into BamHII and EcoRI sites of pET-32a or pGEX-4t-1 expression vector containing His-Tag or GST-Tag sequence. Escherichia coli BL21 (DE3) strain cells transformed with the resulting vectors were grown in 10-mL Luria-Bertani (LB) medium at 37 °C overnight, and the fresh cultures of E. coli carrying the foreign-genes were subcultured until the A600 reached 0.8 in 200-mL LB. His-tag fusion proteins were induced by adding 0.5 mM Isopropyl β-D-Thiogalactoside (IPTG) at 25 °C overnight. The cells were harvested by centrifugation, resuspended in cold lysis buffer [100 mM Tris (pH 7.5), 150 mM NaCl, and 10 mM Imidazole] and lysed by ultrasonic fragmentation in accordance with the supplier’s instructions of ultrasonic instrument (SCIENTZ, Ningbo, China; Amplitude 40%, Pulse on 4 s, Pulse off 6 s, total time 15 min). The cell lysate was centrifuged at 20 000 g for 30 min at 4 °C. The supernatant was applied onto a Ni-NTA agarose (QIAGEN, Düsseldorf, Germany) according to the manufacturer’s instructions. Purified proteins were eluted with 1-mL of wash buffer (100 mM Tris (pH 7.5), 150 mM NaCl, and 200 mM Imidazole) and used for the kinase activity assay.

Protein kinase assay
To assay autophosphorylation activity, purified TaCIPK10 and mutant version proteins were incubated in kinase buffer [10 μM of (γ-32P) ATP, 20 mM Tris-HCl pH 7.5, 1 mM DTT, 20 mM MgCl2, 50 μM ATP and either 5 mM CaCl2 or EDTA] for 60 min at 37 °C in a final volume of 25 μL. The reaction was stopped by addition of 5 μL of 4 x Laemml loading buffer, boiled immediately for 5 min, and analysed on 12% SDS-PAGE gels. Gels were stained with Coomassie Brilliant Blue, dried and visualized by autoradiography. TaCIPK10 kinase transacitivity was determined in the presence of 100 ng of His-TaCBL4 or His-TaCBL6 fusion protein and 3 μg of MBP (Roche) were added to the kinase reaction mixture and incubated at 30 °C for 60 min. Kinase activity was visualized by autoradiography. For phosphorylation of TaNH2 or its mutants, the reactions were initiated by mixing 500 ng of TaCIPK10 or TaCIPK10-K42N and 1 μg TaNH2 or its mutants in 25 μL kinase buffer in the presence of 100 ng of His-TaCBL4. The details of preparation and purification of recombinant proteins are described in the Supporting Information.

Virus-induced gene silencing
Three specific cDNA fragments of TaCIPK10 or TaNH2 with Ntot and PacI restriction sites were obtained by reverse transcription PCR for VIGS analysis as previously described (Holzberg et al., 2002). In addition, we confirmed that the fragments showed no similarity with any other genes by BLAST analysis of the wheat genome databases (https://urgi.versailles.inra.fr/blast/). The capped in vitro transcripts of BSMV RNA were prepared from linearized plasmids containing the tripartite BSMV genome using the Message T7 in vitro transcription kit (Ambion, Austin, TX; Promega, Shenzhen, China) following the manufacturer’s instructions. The more details of VIGS assay, host response detection and the methods of histological observation for fungal growth were performed according to a previous study (Liu et al., 2018).

Plant transformation
The TaCIPK10 ORF sequence was subcloned into the Smal site of the modified expression vector pWMB003, resulting in the transformation vector pWMB003-TaCIPK10. The herbicide tolerance gene, Bar, was used as a selectable marker gene in vector pAH'C20. The vector pAH'C20 and pWMB003-TaCIPK10 were co-transformed into wheat calli by particle bombardment. The methods of regeneration and selection were carried out according to a previous study (Qi et al., 2017). The second leaf of T2 generation wheat was inoculated with urediospores of CYR32. These leaves were collected at 0, 24, 48 and 120 hpi for RNA isolation and histological observation.

GST pull down and Co-immunoprecipitation (Co-IP) assay
The coding sequences of TaNH2, TaNH2- AKR and TaNH2-ΔAKR were subcloned into BamHII and EcoRI sites of pGEX-4t-1 expression vector containing the GST-Tag sequence. GST fusion proteins of TaNH2 or derivatives were expressed in E. coli (BL21) with induction by 0.5 mM IPTG at 25 °C overnight and purified by standard techniques with glutathione-sepharose following the previous study (Harper and Speicher, 2001). Equal amounts of GST-tagged proteins were mixed with TaCIPK10-His and incubated at 4 °C for 2 h followed by washing and protein blots to detect the recovered TaCIPK10-His levels using the GST Protein Interaction Pull-Down Kit (Thermos, Shanghai, China) according to the manufacturer’s instructions. Anti-His (Sungene, Tianjing, China) at 1:5000 dilution and anti-GST (Sungene) at 1:5000 dilution were used for the Western blots. The way of Co-ip assay were performed as described in a previous study (Liu et al., 2018).

Statistical analysis
Microsoft Excel software was used to calculate mean values and standard errors. Student’s t-test performed by the SPSS 16.0 (SPSS Inc., Chicago, IL) statistical software was used to determine the significant differences between control and treatment or between time-course points. The significant change with unequal variance was measured by probability (P) value <0.01.

Accession numbers
The nucleotide sequences used in this study had the following Accession numbers: TaEF-1 ORF sequence was subcloned into the Smal site of the modified expression vector pWMB003, resulting in the transformation vector pWMB003-TaCIPK10. The herbicide tolerance gene, Bar, was used as a selectable marker gene in vector pAH'C20. The vector pAH'C20 and pWMB003-TaCIPK10 were co-transformed into wheat calli by particle bombardment. The methods of regeneration and selection were carried out according to a previous study (Qi et al., 2017). The second leaf of T2 generation wheat was inoculated with urediospores of CYR32. These leaves were collected at 0, 24, 48 and 120 hpi for RNA isolation and histological observation.

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Accession numbers
The nucleotide sequences used in this study had the following GenBank accession numbers: TaEF-1 ORF (elongation factor-1, Q03033), TaCBL1.1 (KU736847), TaCBL2 (KU736848), TaCBL3 (KU736849), TaCBL4 (KU736850), TaCBL6 (KU736851), TaCBL9 (KU736852), TaCIPK10 (KU736856), TaNH2 (KU736846), TaNH2-ΔAKR (KU736848), TaNH2-ΔAKR (KU736848), TaNH2-ΔAKR (KU736848), TaNH2-ΔAKR (KU736848), TaNH2-ΔAKR (KU736848), TaNH2-ΔAKR (KU736848), TaNH2-ΔAKR (KU736848), TaNH2-ΔAKR (KU736848).
(KU736862.1), TaPR1 (pathogenesis-related protein 1, AF384143), TaPR2 (β-1,3-glucanase, DQ090946), TaPR5 (thau-matin-like protein; FG618781), TaSOD (superoxide dismutase, CB307850), TaCAT (catalase, X94352) and TaNOX (AY561153).

Acknowledgements

We thank Professor Yang Zhao (Shanghai Center for Plant Stress Biology, CAS, China) for providing technical support on in vitro phosphorylation assay and Professor Larry Dunkle (USDA-Agricultural Research Service at Purdue University, USA), Professor Brad Day (Department of Plant, Soil and Microbial Sciences at Michigan State University, USA) for critical reading of the manuscript.

Funding information

National Key R&D Program of China (2018YFD0200400), Natural Science Basic Research Plan in Shaanxi Province of China (2017JM3007) and National Natural Science Foundation of China (31620103913).

Author contributions

Z. K., J. G. (Jun Guo), P. L., J. J. G. (Jia Guo) and Y. D. designed experiments. P. L., R. Z., J. Z., C. L., T. Q. and Y. D. and performed the experiments. J. G. (Jia Guo) did the phylogenetic analysis. P. L. and J. G. (Jun Guo) wrote the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Transcript profiles of selected TaCIPKs in wheat leaves in response to SA treatment and Pst inoculation.

Figure S2 Infection of Pst race CYR23 (incompatible interaction) or CYR31 (compatible interaction) differentially increased the endogenous level of SA in wheat leaves of cultivar Suwon11.

Figure S3 Multiple sequence alignments of the coding sequences for the three TaCIPK10 copies.

Figure S4 TaCIPK10 protein structure and phylogenetic analysis.

Figure S5 Subcellular localization of TaCIPK10 in wheat protoplasts.

Figure S6 Western blot analysis of protein expression in yeast two-hybrid assays.

Figure S7 Multiple sequence alignment of TaCIPK10 and three TaCIPK members.

Figure S8 Relative transcript levels of TaPR1, TaPR2, TaPR5, TaSOD, TaCAT and TaNOX in TaCIPK10-knockdown plants inoculated with avirulent race, CYR23.

Figure S9 Host response in TaCIPK10-knockdown plants inoculated with Pst avirulent race CYR23.

Figure S10 Growth of Pst avirulent race, CYR23, was increased in TaCIPK10-knockdown plants.

Figure S11 Functional analysis of TaCIPK10 overexpression transgenic wheat.

Figure S12 Histological observations of positive TaCIPK10 overexpression lines infected with virulent race CYR32 at 120 hpi.

Figure S13 TaCIPK10 overexpression transgenic lines did not affect the growth of wheat.

Figure S14 TaCIPK10 interacted only with AKR motif of TaNH2.

Figure S15 Schematic diagrams of TaNH2.

Figure S16 TaNH2 was significantly induced by SA treatment and Pst inoculation.

Figure S17 Sequence alignment of the coding sequences for the three TaNH2 copies in the wheat genome database and TaNH2 cloned from Suwon 11.

Figure S18 Host response and histological observations of fungal growth in TaNH2-knockdown plants challenged by avirulent CYR23.

Figure S19 Schematic presentation of possible molecular mechanism of TaCIPK10-mediated resistance processes relative to NPR1-like gene in wheat.

Table S1 Disease severity scale.

Table S2 Primers used in this study.