A novel cysteine-rich receptor-like kinase gene, TaCRK2, contributes to leaf rust resistance in wheat

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Abstract
Leaf rust, caused by Puccinia triticina, is one of the most destructive fungal diseases in wheat production worldwide. The hypersensitive reaction (HR) is an important defence response against P. triticina infection. In this study, the physiological races 165 and 260 of P. triticina were combined with a line derived from the bread wheat cultivar Thatcher with the leaf rust resistance locus Lr26 to form compatible and incompatible combinations, respectively. Based on an RNA-Seq database of the interaction systems, a new wheat cysteine-rich receptor-like kinase gene, TaCRK2, is specifically induced and up-regulated in the incompatible combination. We identified that TaCRK2 was regulated in a Ca2+-dependent manner. Knockdown of TaCRK2 by virus-induced gene silencing and RNAi leads to a dramatic increase in HR area and the number of haustorial mother cells at the single infection site. In addition, urediniospores, a P. triticina-specific pathogenic marker in compatible combinations, were observed on leaf surfaces of silenced plants at approximately 15 days after inoculation in the incompatible combination. Moreover, transcription levels of TaPR1, TaPR2, and TaPR5 were obviously reduced in TaCRK2-silenced plants. TaCRK2 overexpression in Nicotiana benthamiana induced strong HR-like cell death. Finally, transient expression of green fluorescent protein fused with TaCRK2 in N. benthamiana indicated that TaCRK2 localizes in the endoplasmic reticulum. Thus, TaCRK2 plays an important role in the resistance to P. triticina infection and has a positive regulation effect on the HR cell death process induced by P. triticina.

Keywords
cysteine-rich receptor-like protein kinase, hypersensitive reaction, Puccinia triticina, VIGS, wheat
INTRODUCTION

Plants are continuously threatened by various pathogens throughout their life cycles, and they have evolved sophisticated natural immune systems to actively resist infection against them. Plants have large numbers of intracellular innate immune receptors that actively perceive pathogens and induce cellular defence. The plant immune response usually consists of a two-layered system. The primary defence system is that plants respond to pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), a process termed PAMP-triggered immunity (PTI) (Macho and Zipfel, 2014; Zipfel, 2014). PTI activates a range of disease-resistance responses in plants, including callose deposition, extracellular reactive oxygen species (ROS) burst, kinase activation, pathogenesis-related (PR) protein expression, and small RNA synthesis (Boller and Felix, 2009; Nicaise et al., 2009; Thomma et al., 2011; Zvereva and Pooggin, 2012), thereby preventing invasion of pathogens in the host plants. However, evolutionarily adapted pathogens have independently evolved large arsenals of effectors (virulence proteins), such as the type III secretion system (TTSS) effectors from bacterial pathogens (Asai and Shirasu, 2015). Effectors are delivered by various means into the apoplast or the host cell cytoplasm, where they target and manipulate key pathways of the host cellular machinery to suppress PTI, thus leading to effector-triggered susceptibility (Dou and Zhou, 2012; Wessler et al., 2014). To defend themselves against adapted pathogens, plants evolved a second layer of defence, referred to as effector-triggered immunity (ETI), which is usually associated with a rapid localized cell death at the infection site, the hypersensitive reaction (HR), which can inhibit further pathogen proliferation in some cases (Greenberg, 1996; de Wit, 2007; Mur et al., 2008; Stam et al., 2014; Schoonbeek et al., 2015; Salguero-Linares and Coll, 2019). The HR is a rapid plant-initiated cell death that is associated with the recognition of avirulence products by the corresponding resistance genes. Additionally, the HR helps plants defend themselves against pathogens by killing plant cells at the infection sites to limit pathogen growth.

Receptor-like protein kinases (RLKs), the largest known family of protein kinases, are reported to play essential roles in response to pathogen infection. Typical RLKs contain an extracellular ligand-binding domain, a transmembrane domain, and an intracellular protein kinase domain (Stone and Walker, 1995). The primary distinguishing feature of individual RLKs is the extracellular domain, which binds a specific ligand and allows RLKs to respond to different signals (Walker, 1994; Braun and Walker, 1996; Yoshimura et al., 1998; Hervé et al., 1999). The cysteine-rich receptor-like kinase (CRK) family is a large subgroup of plant RLKs. The extracellular domains of typical CRKs contain two unknown function 26 (DUF26) domains consisting of a Cx_{4}Cx_{4}C motif. The DUF26 domain has antifungal activity (Savano et al., 2007) and plays a crucial role in salt stress resistance (Zhang et al., 2009). Most CRKs are regulated by ROS, the common signalling molecules produced in response to various stresses in plants, suggesting that they play essential roles in multiple stress responses.

To date, more than 40 CRK members have been identified in the model plant Arabidopsis. However, only a few of these have been functionally characterized (Wrzaczek et al., 2010). AtCRK4, AtCRK5, AtCRK13, AtCRK19, AtCRK20, and AtCRK36 can induce HR cell death in Arabidopsis (Chen et al., 2003, 2004; Achariya et al., 2007; Ederli et al., 2011; Yeh et al., 2015; Lee et al., 2017). Moreover, the crk5 mutant plants displayed enhanced cell death in response to UV radiation (Burdia et al., 2015). Suppression of AtCRK36 expression increased sensitivity to abscisic acid (ABA) and osmotic stress in Arabidopsis (Tanaka et al., 2012). Both AtCRK6 and AtCRK7 mediate responses to extracellular oxidative stress (Idänheimo et al., 2014). SCR96 is a small cysteine-rich secretory protein of Phytophthora cactorum, can trigger plant cell death in the Solanaceae, and is important for pathogenicity and oxidative stress tolerance (Chen et al., 2016).

In crop species, barley HvCRK1 was identified as a negative regulator of resistance to powdery mildew infection (Rayapuram et al., 2012). OsCRK1 was demonstrated as a positive regulator in rice resistance to Xanthomonas oryzae (Zhang, 2015). The expression of TaCRK1 was enhanced when the disease-resistant wheat variety CI12633 was infected by Rhizoctonia solani. Moreover, ABA treatment also increased its expression. However, gene silencing by virus-induced gene silencing (VIGS) did not affect the resistance of wheat to R. solani, presumably due to the existence of functional redundancy among the genes in this gene family (Yang et al., 2013). These results indicate that CRKs play crucial roles in plant responses to biotic and abiotic stresses. In-depth studies of CRKs in plants have positive effects in revealing the mechanisms of plant disease resistance and signal transduction.

Our laboratory has long been engaged in research on the mechanism of resistance of wheat to Puccinia triticina, mainly focusing on the mechanism of HR. We have reported that Ca^{2+}, as a secondary messenger, mediates the HR process induced by P. triticina in the incompatible combination (Hou et al., 2007; Liu et al., 2010; Qiao et al., 2015). We set up an RNA-Seq database of wheat cultivar Thatcher (Tc) or Tc Lr26 infected by P. triticina and a database of differential gene expression regulated by calcium ions (Liu et al., 2019). We found a unigene that was up-regulated on P. triticina inoculation and it was robustly inhibited by the chelating agent ethylene glycol tetraacetic acid (EGTA) in the incompatible combination but not expressed in the compatible combination. We thus speculated that this gene might be related to Ca^{2+} signalling and resistance against P. triticina.

In this work, the coding sequence of the unigene was obtained by using the rapid amplification of cDNA ends (RACE) technique. The gene and its expressed protein were analysed using tools of bioinformatics and was named as TaCRK2 (Triticum aestivum cysteine-rich receptor-like kinase 2). Quantitative reverse transcription PCR (RT-qPCR) analysis showed that TaCRK2 was up-regulated in the incompatible combination between Tc Lr26 and P. triticina race 260. In addition, EGTA strongly inhibited the expression of TaCRK2 at the transcriptional level. The subcellular localization of TaCRK2 was in the endoplasmic reticulum (ER). Furthermore, knockdown of TaCRK2 in wheat using VIGS and RNA interference (RNAi) reduced plant disease resistance to P. triticina race 260, and the urediniospores could be observed on the surface of the plant leaves. Taken together, these data suggest that TaCRK2 is involved in the positive regulation of wheat resistance to P. triticina.
2 | RESULTS

2.1 | TaCRK2 encodes a novel cysteine-rich receptor-like protein kinase

To identify wheat genes in response to *P. triticina* infection, RNA-Seq analysis was used to compare transcriptome differences between the *P. triticina*-resistant line Tc Lr26 and *P. triticina*-susceptible cultivar Tc following *P. triticina* inoculation. We identified a unigene that was up-regulated on *P. triticina* inoculation. The complete coding sequence (CDS) of the gene (1,482 bp) was obtained from a cDNA library of *P. triticina*-inoculated wheat by amplifying cDNA ends (RACE) (EMBL-EBI accession MK424819). Bioinformatics analysis of the domain architecture of the deduced primary sequence identified a predicted signal peptide (SP) and two cysteine-rich DUF26 (CX_8CX_2C) domains, a transmembrane domain (TM), and a serine/threonine kinase-like domain (S-TKc) (Figure 1).

(a)

(b)
A phylogenetic tree for the common conserved amino acid sequences of this protein and its homologs was constructed using MEGA 7.0 and MEME (Figure 2). The tree shows that the protein shares high homology with the CRK proteins of monocotyledonous plants such as Zea mays (Zm), Triticum urartu (Tu), Aegilops tauschii (Ae), and Triticum aestivum (Ta), and it is distantly related to the CRK proteins of dicotyledonous plants such as Populus tomentosa (Ptr), Vitis vinifera (Vv), and Arabidopsis thaliana (At). It is known from the phylogenetic tree that the new member of the CRK family identified in this study is not in the same evolutionary branch as TaCRK1. Bioinformatics analysis revealed that the two DUF26 domains of TaCRK1 contain a CX₈CX₂C motif that differs from the CX₉CX₂C motif contained in the new protein obtained in this study. This suggests that the new protein is a novel cysteine-rich receptor-like protein kinase, so we named it TaCRK2. Vaattovaara reported that DUF26-containing proteins are grouped into three categories: CRRSPs, PDLPs, and CRKs. CRKs contain a signal peptide, two DUF26 domains, and a transmembrane region followed by an intracellular protein kinase domain. CRKs were defined as basal group CRKs (bCRKs) or variable group CRKs (vCRKs) based on their...
phylogenetic positions (Vaattovaara et al., 2019). In order to identify which group TaCRK2 belongs to, we selected some representative sequences of clade 3 in Vaattovaara’s paper. We merged these sequences with TaCRK2 together, then we reconstructed the maximum-likelihood (ML) phylogenetic tree using MEGA 7.0. The result showed that TaCRK2 was located in the vCRK group (Figure S1).

According to others’ research on the kinase domain (Hanks et al., 1988; Stone and Walker, 1995), we found that the catalytic core domain (VIb and VII) was deleted in the 11 subdomain of TaCRK2 kinase domain. For this reason, we carefully checked the correctness of the gene sequence (Figure 1b). The transcript of TaCRK2 was assembled from the RNA sequencing data. The unfiltered reads using integrative genomics viewer (IGV) (Robinson et al., 2011) alignment results showed that the six introns interrupted the transcript. The first four introns were located in the TaCRK2 coding region. The exon sequence completely matched the genome sequence (Figure S2a). The fragment of genome sequence comes from the lineage-specific region of Tc Lr26. HISAT2 (Kim et al., 2019) was used to align the transcriptome data to the draft genome sequence. However, it is worth mentioning that the sequence around the introns has been double checked and all the introns obeyed the GT-AG rule. This type of splicing is termed canonical splicing, and is reported to account for >99% of splicing for randomly selected transcripts (Yang et al., 2004). In addition, we merged all the overlapping exons into the transcripts of TaCRK2. We could only find one long open reading frame (ORF) in the transcript Vector NTI for overexons into the transcripts of TaCRK2. We could only find one long open reading frame (ORF) in the transcript Vector NTI for the analysis (Figure S2b(b)). The last 16 amino acids (48 bp) differed greatly from other CRK proteins as 1 bp was deleted at this position (point of blue arrow) during evolution (Figure S2a). It is possible this was the reason why TaCRK2 had no HRDLK (VIb) and DFG (VII). If we were to add 1 bp or delete 2 bp before the stop codon, it would have the whole kinase domain, with the catalytic core domain VIb and VII. Perhaps it was a longer protein with a whole kinase domain before, but something happened to it and 1 bp was lost. We cloned the coding sequence of TaCRK2, and the sequencing result of cloning was consistent with the ORF prediction. No differences were found during the last 48 bp. Tc Lr26 contains an exogenous chromosome fragment lr26. The fragment lr26 is different from the gene of cultivated wheat, for example hexaploid bread wheat. Now we have sufficient information to suggest that TaCRK2 is not from cultivated wheat, but from the exogenous fragment lr26 carried by Tc Lr26 (Figure S2c).

2.2 TaCRK2 was induced by P. triticina infection in the incompatible combination

The transcriptional levels of TaCRK2 in Tc Lr26 after inoculating with P. triticina races 260 and 165 were evaluated by quantitative reverse transcription PCR (RT-qPCR). The results showed that in the incompatible combination the expression of TaCRK2 increased gradually, and its expression at 48 hr post-inoculation (hpi) was 9.45-fold higher than at 0 hpi, whereas the expression level of TaCRK2 in the compatible combination was almost unchanged with inoculation time compared with 0 hpi (Figure 3). This result indicates that TaCRK2 is induced by P. triticina infection in the incompatible combination.

2.3 Ca2+-chelating agent EGTA inhibits the transcription of TaCRK2

The calcium signalling pathway mediates the interaction between wheat and P. triticina, and extracellular Ca2+ influx is required for the induction of HR during wheat resistance to P. triticina (Hou et al., 2007; Liu et al., 2010, 2015). We tested whether changes of TaCRK2 transcripts in the incompatible combination are affected by 5 mM EGTA, a chelating agent that can chelate extracellular Ca2+ effectively. As shown in Figure 4, the expression of TaCRK2 was strongly inhibited following EGTA treatment compared with the control (preinjection of deionized distilled H2O). This result indicates that the expression of TaCRK2 is regulated by calcium signals in the interaction between Tc Lr26 and P. triticina race 260.

**FIGURE 3** Relative transcript levels of TaCRK2 in different combinations assayed using quantitative reverse transcription PCR. Seven-day-old wheat seedlings (Thatcher Lr26) were inoculated with Puccinia triticina spore suspensions (race 165 or 260) by brushing spores on the surface of the first leaf. The inoculated leaves were collected at 0, 4, 8, 12, 16, 24, and 48 hr post-inoculation (hpi) for RNA extraction. Values are means ± SEM of three independent experiments. Means denoted by the same letter did not significantly differ at according to one-way analysis of variance (ANOVA) least-significant difference (LSD) analysis (p < .01) [Correction added on 6 April 2020, after first online publication: figure caption has been amended in this version.]
2.4 TaCRK2 protein is located in the endoplasmic reticulum membrane

To investigate the subcellular localization of TaCRK2, the ORF lacking the stop codon was ligated upstream of an enhanced green fluorescent protein (GFP) gene under the control of pSuper promoter (pSuper1300). Recombinant pSuper1300:TaCRK2-GFP was infiltrated into the leaf of Nicotiana benthamiana for heterologous overexpression. The empty vector (EV) pSuper1300:GFP was used as the control. As shown in Figure 5a, the TaCRK2-GFP fusion protein was located in the endoplasmic reticulum (ER). GFP alone exhibited characteristic diffuse cytoplasmic fluorescence and...
dense nuclear fluorescence. To further confirm TaCRK2 localization, pSuper1300-TaCRK2-GFP was coexpressed with HDEL-mCherry, which is an ER marker protein. It was clearly observed that the green fluorescence of TaCRK2-GFP overlapped with the red fluorescence of HDEL-mCherry (Figure 5b). These results suggest that the TaCRK2 protein is localized in the ER.

The less hydrophobic transmembrane domain (TMD) is characteristic of the ER location (Bretscher and Munro, 1993). The highly hydrophobic amino acids (LVL) at the C-terminus of the TMD of PDLP1a were replaced by less hydrophobic ones (AAA), and the localization of PDLP1a changed from the plasma membrane to the ER (Thomas et al., 2008). Therefore, the less hydrophobic C-terminus amino acids (YLW) of the TMD might be the key factors that determine the localization of TaCRK2 in the ER. Arginine (R) and lysine (K) behind the TMD have been demonstrated to be an important signal for localization to the ER (Eugster et al., 2004; Michelsen et al., 2007). To test whether TaCRK2 located in ER was correlated with the TMD hydrophobic C-terminus amino acid (YLW) and RKAR motif, we examined the subcellular localization of TaCRK2Y323V, TaCRK2R326A329A, and the triple mutant TaCRK2R326A327A329A by confocal microscopy of C-terminal GFP-tagged proteins transiently expressed in N. benthamiana. However, when the three mutant proteins were coexpressed with HDEL-mCherry, we observed that the fluorescence did not completely overlap (Figure 5c). This suggests that the RKAR motif and hydrophobic amino acids at the C-terminus of the TMD affect TaCRK2 localization in the ER.

2.5 TaCRK2 induced HR-like cell death in N. benthamiana

To investigate its potential role in programmed cell death (PCD), we overexpressed TaCRK2 in N. benthamiana through an Agrobacterium tumefaciens-mediated infiltration assay. ATRP52 is a protein with leucine-rich repeats and a typical nucleotide-binding domain that recognizes the bacterial effector AvrRpt2 and causes strong HR-like cell death (Qi et al., 2011). ATRP52 was used as a positive control, GFP was used as a negative control. When expressed alone, 3 days post-infiltration with A. tumefaciens carrying ATRP52 or TaCRK2, cell death was observed in the sites infiltrated; however, TaCRK2Y323V, TaCRK2R326A329A, and the triple mutant TaCRK2R326A327A329A, and GFP did not cause cell death (Figure 5d). Therefore, accurate positioning of TaCRK2 in ER is necessary for induced strong HR-like cell death.

2.6 Knockdown of TaCRK2 enhances wheat susceptibility to P. triticina

VIGS is a promising approach in plant functional genomics and has been widely used to study gene function in barley and wheat (Holzberg et al., 2002; Hein et al., 2005; Scofield et al., 2005; Hu et al., 2018; Mamun et al., 2018; Singh et al., 2018). We employed barley stripe mosaic virus (BSMV)-mediated VIGS to investigate the function of TaCRK2 in defence against P. triticina infection. One pair of primers was designed to specifically knockdown TaCRK2. The silencing of the wheat phytoene desaturase (PDS) gene was used as the positive control for the gene-silencing system to confirm whether our VIGS conditions were functioning correctly, and this system generated photobleaching symptoms by 12 days post-inoculation (dpi) (Figure 6a). The result showed that the plants treated with the BSMV:TaCRK2 displayed mild chlorotic mosaic symptoms on the third or fourth leaves at 12 dpi but exhibited no obvious defects in further leaf growth (Figure 6a). To determine the efficiency of VIGS, we used RT-qPCR to analyse the expression level of TaCRK2. Compared with the control, the abundance of TaCRK2 transcripts was significantly down-regulated in the TaCRK2-silenced plants (Figure 6b).

P. triticina is an obligate biotrophic pathogen, and the pattern of how it infects its host is very similar to that of Puccinia striiformis f. sp. tritici (Pst). Cantu has described the infection process of Pst in more detail (Cantu et al., 2013). In a compatible combination, P. triticina forms intimate connections with the host through the formation of haustoria. As we know, the haustoria bind the cell membrane tightly but do not penetrate the cell. Nutrients are obtained from the host cells by haustoria, which promotes the growth and development of fungus, and finally forms the urediospore on the leaf surface. However, in an incompatible combination, after a haustorium contacts a mesophyll cell it will induce the host cell to produce an HR. HR is a rapid plant-initiated cell death that is associated with the recognition of avirulence products by the corresponding resistance genes (Panstruga and Dodds, 2009). HR can limit pathogen growth.

To analyse the effect of gene knockdown on plant resistance, the third leaf of a silenced plant was inoculated with P. triticina race 260. Portions of the leaf were sampled at 16, 24, 48, 72, 96, and 120 hpi and subjected to Rohringer fluorescence staining. The results (Figure 6c) showed that silencing of TaCRK2 resulted in a significantly larger area of fluorescence in the HR cells (Figure 6c(d)) compared to that observed in the control group at 120 hpi (Figure 6c(a)). To investigate the HR and haustorial mother cells (HMCs) at each of the infection spots, the leaves at 120 hpi were stained with Rohringer fluorescence and analysed (Figure 6d). The results showed that there were usually two or three HMCs (red arrows) and two or three HR cells (white arrows) at the infection site in the control group (a). However, the area of HR cells and the number of HMCs were greatly increased following TaCRK2 knockdown (d).

To further assess the effects of TaCRK2 gene silencing on HR and P. triticina development, the HR areas and numbers of HMCs at 50 individual infected sites were statistically analysed (Figure 6e). Compared with the control, the HR area and the numbers of HMCs in TaCRK2 knockdown plants gradually increased with inoculation time, reaching maximum values at 120 hpi (Figure 6e). This result indicates that knockdown of TaCRK2 expression enhances the development of P. triticina.

The above results indicate that silencing the TaCRK2 gene reduces wheat resistance to P. triticina and accelerates the development of P. triticina (Figure 6d(e) shows the branching of P. triticina). The reason for this might be that silencing the gene slows down the death process.
FIGURE 6 Effects of silencing the TaCRK2 gene using the virus-induced gene-silencing (VIGS) technique on Thatcher (Tc) Lr26, which is resistant to Puccinia triticina infection. (a) Phenotypic assessment of the barley stripe mosaic virus (BSMV) infection symptoms. All BSMV-inoculated plants exhibited BSMV symptoms at 12 days post-inoculation (dpi). The third leaf of BSMV: PDS-inoculated plants exhibited full leaf bleaching. CK, healthy control. (b) The relative transcription level of TaCRK2 in the third leaves of plants infected with BSMV: γ0 or BSMV: TaCRK2 determined by quantitative reverse transcription PCR. Values are means ± SEM of three independent experiments. Means denoted by the same letter did not significantly differ at according to one-way analysis of variance (ANOVA) least-significant difference (LSD) analysis (p < .01). (c) A portion of the third leaf sampled at 120 hr after P. triticina inoculation was subjected to Rohringer fluorescence staining. Leaves inoculated with BSMV: γ0 were used as controls. Confocal laser scanning microscopy was used for examination. (A)–(C) Plants inoculated with BSMV: γ0 at 120 hr after P. triticina inoculation; (D)–(F) Plants inoculated with BSMV: TaCRK2 at 120 hr after P. triticina inoculation; (A), (D) Superimposed images of (B), (C) and (E), (F), respectively. Bar = 100 µm. (d) Relationship between hypersensitive reaction (HR) and haustorial mother cells (HMCs) at a single infection site. (A)–(C) Plants inoculated with BSMV: γ0 at 120 hr after P. triticina inoculation; (D)–(F) Plants inoculated with BSMV: TaCRK2 at 120 hr after P. triticina inoculation; (A), (D) Superimposed images of (B), (C) and (E), (F), respectively. Bar = 50 µm. Note: The red arrows indicate P. triticina HMCs and the white arrows indicate HR cells. (e) Statistical results for HR areas (A) and the number of HMCs (B) of 50 single infection sites observed by fluorescence microscopy. Values are means ± SEM of three independent experiments. Means denoted by the same letter did not significantly differ at according to one-way analysis of variance (ANOVA) least-significant difference (LSD) analysis (p < .05). (f) Phenotype of TaCRK2 gene-silenced leaves at 15 days after P. triticina inoculation. Note: Plant 1–plant 5 refer to the third leaves of five different BSMV: TaCRK2-silenced plants and the red arrows indicate P. triticina uredosori [Correction added on 6 April 2020, after first online publication: figure caption has been amended in this version.]
of HR cells, allowing *P. triticina* to obtain more nutrients to promote secondary growth. These changes could result in the formation of more HMCs and haustoria (Figure 6d(e),e(b)), leading to more host cells developing HR (Figure 6c(d),d(d),e(a)). To investigate whether *P. triticina* urediniospores form on leaves after gene silencing, portions of the leaves were left on the plants and allowed to continue to grow, and the phenotypic changes were examined. At 15 dpi, sporadic small uredosori of *P. triticina* appeared on the surfaces of the leaves (Figure 6f).

The role of TaCRK2 in the resistance of wheat to *P. triticina* was further assessed based on RNAi analysis. An RNAi-TaCRK2 vector was transferred into *Agrobacterium* and transformed TcLr26 using the wheat spike genetic transduction technique (Patent No. 201,110,418,620.3) (Figure S3). The expression of TaCRK2 in RNAi-1 and RNAi-2 was significantly down-regulated compared with the control. The numbers of HMCs and the HR areas in the RNAi-transduced plants were higher than those in nontransduced ones (Figure S4e). By 12 dpi, *P. triticina* uredosori were observed on the surfaces of the leaves, showing a distinct infection phenotype (Figure S4f) and displaying consistency with the VIGS results. These results further confirm that the TaCRK2 gene positively regulates wheat resistance to *P. triticina*.

### 2.7 TaCRK2 knockdown suppresses transcriptional expression of a series of defence-related genes

HR-associated programmed cell death is frequently accompanied by activation of many pathogenesis-related (PR) genes (Nimchuk *et al.*, 2003). To determine whether the expression of PR genes was affected by TaCRK2 knockdown, RT-qPCR was used to analyse the expression of PR genes. The transcript levels of the PR genes, including TaPR1, TaPR2, and TaPR5, were down-regulated in TaCRK2 knockdown plants (BSMV:TaCRK2 positive plants) infected with *P. triticina* (Figure 7). These results indicate that silencing TaCRK2 reduced the expression of PR genes in the interaction between wheat and *P. triticina*.

### 3 DISCUSSION

Results from previous studies showed that calcium ions, as a secondary intracellular messenger, are involved in the HR induced by *P. triticina* infection in wheat. Calmodulin (CaM) (Guan *et al.*, 2006; Hou *et al.*, 2007), calcium-dependent protein kinase (CDPK) (Cheng *et al.*, 2012; Yan *et al.*, 2016), and calcineurin B-like protein (CBL) (Wang *et al.*, 2016) participate in this process as calcium signal sensors. Ca$^{2+}$ signalling has a regulatory effect on HR induced by *P. triticina* infection (Guan *et al.*, 2006), and an extracellular calcium influx to the cell was shown to be a major source of calcium signalling (Liu *et al.*, 2010). Plants overexpressing CBL-interacting protein kinase 6 negatively regulate immunity and were more susceptible to *Pseudomonas syringae* in *Arabidopsis* (Sardar *et al.*, 2017). Wheat calcium-dependent protein kinase TaCPK7-D overexpression activates defence-related genes, leading to enhanced resistance to sharp eyespot caused by *Rhizoctonia cerealis*, and positively regulates host resistance to sharp eyespot disease (Wei *et al.*, 2016).

To further study the calcium signal transduction mechanism involved in the interaction between wheat and *P. triticina*, it is vital
to identify the component molecules of this signalling pathway. Our group obtained RNA-Seq data of Tc Lr26/Tc infected by P. triticina and data of differentially expressed genes regulated by calcium ions. We also examined differentially expressed sequences between Tc Lr26 and Tc. TaCRK2 was isolated from Tc Lr26 using the RACE technique. RT-qPCR showed that TaCRK2 was gradually up-regulated after inoculation in the incompatible combination (Figure 3), consistent with the transcriptome results. EGTA inhibited transcription of TaCRK2 (Figure 4). Therefore, we speculate that TaCRK2 plays an important role in the defence response induced by P. triticina infection, and the expression of its transcription level is regulated by a calcium signal.

Bioinformatics analysis showed that TaCRK2 is 1,482 bp in length and encodes a protein of 493 amino acids. It is predicted that the protein contains one signal peptide (SP), two DUF26 domains (including two CX_8CX_2C motifs), a transmembrane domain, and a kinase-like domain (Figure 1). The cloning of TaCRK1 was also based on RNA-Seq analysis. TaCRK1 was highly expressed in the disease-resistant wheat variety CI12633 when the plants were infected by R. solani and ABA treatment also enhanced its expression (Yang et al., 2013). Although both TaCRK2 and TaCRK1 belong to the wheat CRK family (Figure 2), the sequence of the former is only 34% homologous to that of the latter. Bioinformatics analysis revealed that there is a CX_8CX_2C motif in the DUF26 domain of the TaCRK1 protein. This motif differs from the CX_2CX_2C motif of the protein encoded by TaCRK2. Therefore, this gene is the second member of the CRK family identified in wheat.

To investigate the functions of TaCRK2, we conducted VIGS and RNAi experiments. The results showed that knockdown of the gene reduced the resistance of wheat to P. triticina. Transcription of TaPR1, TaPR2, and TaPR5 was significantly decreased in the gene-silenced plants compared to those in the controls (Figure 7). P. triticina in the gene-silenced plants formed more secondary hyphae and HMCs (Figures 6d,e and S4d,e), and urediniospores appeared on the leaves at approximately 15 days following P. triticina inoculation (Figures 6f and S4f). Therefore, down-regulation of TaCRK2 in Tc Lr26 did impair wheat resistance to P. triticina. Thus, we deduced that TaCRK2 positively regulates wheat resistance to P. triticina. Although little is known about the mechanism of their actions, it has been reported that the CRK family is involved in regulating plant resistance to pathogens. Current studies of CRKs in Arabidopsis have shown that overexpression of AtCRK28, AtCRK5 or AtCRK13 enhances resistance to P. syringae (Chen et al., 2003; Acharya et al., 2007; Yadeta et al., 2017). Overexpression of AtCRK5 or its homologous genes AtCRK4, AtCRK19, and AtCRK20 has been shown to induce HR-like cell death in plants (Chen et al., 2004; Ederli et al., 2011). In this study, overexpression of TaCRK2 in N. benthamiana induced strong HR-like cell death (Figure 5d). It was reported that silencing OsCRK1 by the RNAi technique increased susceptibility of the plants, indicating that OsCRK1 positively regulates the resistance of rice to X. oryzae (Zhang, 2015). HvCRK1 was shown to be highly expressed in barley plants infected with powdery mildew, and transient silencing of the gene by VIGS enhanced resistance to powdery mildew. These results indicate that HvCRK1 plays a negative regulatory role in resistance to powdery mildew in barley (Rayapuram et al., 2012).

The functions of any protein are closely related to its distribution in the cell. N. benthamiana and its leaves are often used as a model plant for subcellular localization of proteins (Sun et al., 2013; Zhang et al., 2017; Li et al., 2018; Qi et al., 2019). Results from the subcellular localization of TaCRK2 in N. benthamiana indicated that the pSuper1300:TaCRK2-GFP fusion protein was primarily located in the ER (Figure 5). TaCRK1 is reported to localize in the plasma membrane (Yang et al., 2013). However, HvCRK1 is localized to the ER (Rayapuram et al., 2012). An important feature of proteins that are localized in the plasma membrane is the length of the hydrophobic transmembrane domains (Rayapuram et al., 2012). It is speculated that the main difference between proteins located on the ER membrane or the plasma membrane is the length of the hydrophobic TMD; shorter being characteristic of the ER, and longer being characteristic of the plasma membrane (Bretscher and Munro, 1993). Some studies have also noted that the specific amino acid sequences in the transmembrane domain are a key factor in determining the subcellular localization (Thomas et al., 2008). The deletion of the amino acids in the hydrophobic domain of the human lysosomal protein LAMP1, which changed the length of the transmembrane domain from 23 amino acids to 20 or 17 amino acids, resulted in retention of GFP-fused deletion proteins in the ER (Brandizzi et al., 2002). When the highly hydrophobic amino acids (LVL) at the C-terminus of the transmembrane domain of PDLP1a were replaced by less hydrophobic ones (AAA), the localization of PDLP1a changed from the plasma membrane to the ER (Thomas et al., 2008). Based on these studies and our results (Figure 5c), it seems that the less hydrophobic C-terminal amino acids (YLV) in the transmembrane domain of TaCRK2 might be a specific signal for ER localization. Therefore, the short transmembrane domain (19 amino acids) and less hydrophobic C-terminal amino acids (YLV) might be the key factors that determine the localization of TaCRK2 in the ER. It was reported that the arginine (R) and lysine (K) motif behind the TM domain is a localization signal that directs proteins to the ER (Eugster et al., 2004; Michelsen et al., 2007). This study proves that the RKAR motif adjacent to the transmembrane region in the amino acid sequence of TaCRK2 (Figure 1b) is also an important factor for TaCRK2 localization in ER (Figure 5c).

We analysed the phylogenetic relationships and motif composition of CRKs, as shown in Figure 2, and found that some CRKs appear to lack certain modules. TaCRK2 lacks the catalytic core domain among the 11 protein subdomains based on the kinase domain analysis of the sequence (Figures 1 and S2). AcrCRK11 (GenBank OAY85793.1) is similar to TaCRK2, but its function is still unclarified. The STRAD protein is a pseudokinase that lacks the key residues necessary for phosphorylation of proteins (Vib and VII motifs) and is a pseudokinase. LKB1 is activated through its interaction with STRAD; MO25 enhances the formation of the LKB1-STRAD complex in vivo, stimulating the catalytic activity of LKB1 c.10-fold (Boudeau et al., 2003; Zhang et al., 2011). The GHR1 is a pseudokinase and CPK3 is an interaction partner of GHR1. GHR1-mediated activation of SLAC1 occurs via interacting proteins (Sierla et al., 2018). SIBIR3 lacks the conserved RD motif required for kinase activity in the intracellular kinase domain.
Vib subdomain. There is no autophosphorylation and transphosphorylation activity, but SIBIR3 can be phosphorylated by SIBAK1 (Huang, 2018). HopZ1a-triggered immunity requires the nucleotide-binding, leucine-rich repeat domain (NLR) protein ZAR1, and the pseudokinase ZED1. HopZ1a can acetylate members of a family of PBLs (receptor-like cytoplasmic kinases) and promote their interaction with ZED1 and ZAR1 to form a ZAR1-ZED1-PBL ternary complex. Interactions between ZED1 and PBL kinases are determined by the pseudokinase features of ZED1, and mutants designed to restore ZED1 kinase motifs can bind to PBLs, recruit ZAR1, and trigger ZAR1-dependent immunity in planta (Bastedo et al., 2019). TaCRK2 may have kinase activity. It may also lack kinase activity and be activated or promote the activity of other proteins by interacting with other proteins. The mechanism of TaCRK2 is the main direction of future research.

Previous studies have shown that Ca²⁺ acts as an important signalling molecule during the interaction between wheat and _P. triticina_ (Hou et al., 2007; Liu et al., 2010). Ca²⁺ signalling has a regulatory effect on HR induced by _P. triticina_ infection (Guan et al., 2006), and extracellular calcium influx into the cell was shown to be a major source of calcium signalling (Liu et al., 2010). We show in this study that the expression of TaCRK2 at the transcriptional level is inhibited by EGTA (Figure 4), preliminary proof that the gene is regulated by a Ca²⁺ signal. Based on these results and on the results obtained from VIGS and RNAi assessments in this study, we deduced that TaCRK2 may participate in calcium signal transduction and that it plays a positive regulatory role in the HR induced by _P. triticina_ infection in wheat.

In summary, our findings indicate that the TaCRK2 gene is regulated by a Ca²⁺ signal and positively contributes to leaf rust resistance in wheat, which will expand our understanding of the potential function of CRKs proteins and provide valuable insight into the molecular mechanism of plant immunity.

## 4 | MATERIALS AND METHODS

### 4.1 | Plant genotype and fungal race

The wheat genotype Tc Lr26 and the _P. triticina_ physiological races 260 and 165 were used in this study. Tc Lr26 and race 260 comprise the incompatible combination, and Tc Lr26 and race 165 the compatible combination.

Seedlings of Tc Lr26 were raised in a greenhouse at 23–25 °C. Seven-day-old seedlings were inoculated with the _P. triticina_ race 260 or 165 according to procedure described by Qiao et al. (2015). The inoculated seedlings were cultivated in the dark for 12–16 hr in high humidity at 23–25 °C and then transferred to and grown in the greenhouse.

### 4.2 | RNA extraction and RT-qPCR analysis

Leaves were collected at different time points following _P. triticina_ infection. The plant total RNA isolation kit from Sangon Biotech (Shanghai) was used to extract total RNA from the leaves. The PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) was used to purify the total mRNA (TaKaRa). RT-qPCR was performed using SYBR Green I Master Mix in a volume of 10 μl on a LightCycler 96 RT-PCR system (Roche). All RT-qPCRs were repeated three times. _TaGAPDH_ served as the reference gene to normalize the total amount of cDNA in each reaction. The relative expression of the TaCRK2 was calculated with the 2−ΔΔCt method. The primer sequences are listed in Table S1.

### 4.3 | Gene cloning

We identified a unigene (GeneID: CL13444.Contig1_All) that was up-regulated on _P. triticina_ inoculation from an RNA-Seq database. RACE primers GSP1 and GSP2 (Table S1) were designed based on the sequence of CL13444.Contig1_All. A total of 1 mg RNA was reverse transcribed to cDNA using the SMART RACE cDNA Amplification Kit (Clontech). A touchdown amplification procedure was applied following the instructions of the RACE kit. PCR products were purified using agarose gel electrophoresis and recovered. PCR products were then cloned into the pEASY-T1 Simple Cloning Vector (TransGen Biotech) and verified by sequencing.

### 4.4 | Alignment and phylogenetic analysis

For construction of CRK phylogenetic trees using the common conserved amino acid sequences of CRKs in different plants, the full lengths of CRK amino acid sequences were downloaded from NCBI. The phylogenetic trees were generated using the neighbour-joining (NJ) method of MEGA 7.0, with the following parameters: Poisson model, pairwise deletion, and 1,000 bootstrap replications. The MEME online program (http://meme.nbcr.net/meme/intro.html) for protein sequence analysis was used to identify conserved motifs in CRK proteins (Bailey et al., 2009).

### 4.5 | Subcellular localization analysis of TaCRK2-GFP

Using gene-specific primers GFP-CRK2 F and GFP-CRK2 R (Table S1) with XbaI and Kpn1 restriction sites, the coding sequence of TaCRK2 was amplified without the stop codon. The T vector including TaCRK2 and pSuper1300:GFP vector was double digested with XbaI and Kpn1. The DNA fragment was gel-extracted using the SanPrep Column DNA Gel Extraction Kit (Sangon Biotech). Solution I DNA ligase was then applied, generating the TaCRK2-GFP fusion expression vector pSuper1300:TaCRK2-GFP. The recombinant plasmid was transformed into _A. tumefaciens_ GV3101. The agrobacteria were cultivated and infiltrated according to Yang et al. (2018). The GFP signals were then examined and photographed using confocal laser scanning microscopy.
4.6 | Agrobacterium-mediated transient expression of TaCRK2 in N. benthamiana

The TaCRK2 and AtRPS2 overexpression vectors were constructed using the specific primers TaCRK2-F, TaCRK2-R, AtRPS2-F, and AtRPS2-R (Table S1) with Kpn1 and Sac1 restriction sites. The recombinant plasmid was transformed into A. tumefaciens GV3101. The method used for injection was the same as for the subcellular localization above. Disease symptoms were examined and photographed at 72 hr post-transformation.

4.7 | BSMV-mediated silencing of TaCRK2 in wheat-P. triticina interactions

A 250 bp cDNA fragment of TaCRK2 with NotI restriction sites was amplified using primers of V1-CRK2-F and V1-CRK2-R (Table S1). It was then cloned into the vector of pSL038-1 to construct BSMV:TaCRK2 fusion vector (Holzberg et al., 2002; Wang et al., 2019). In vitro transcription was performed using an in vitro RNA transcription kit (m MESSAGE mMACHINE T7; Thermo) (Petty et al., 1990). The tripartite BSMV RNAs BSMV:α, BSMV:β, and BSMV:γ were inoculated on the first fully expanded leaves of 7-day-old Tc Lr26 by rub inoculation with a gloved finger (Scofield et al., 2005; Fu et al., 2014; Niu et al., 2015). When viral symptoms appeared, the third leaves were inoculated with urediospores of P. triticina race 260. The inoculated leaves were collected at various time points for histological examination and RNA extraction. At least 50 infection sites were examined on each of five randomly selected leaf segments for each treatment. HR areas and number of HMCs were observed with an Olympus BX-53 microscope and estimated with DP2-BSW software. Statistical analysis was performed using SPSS software.

4.8 | Rohringer staining (HR staining)

At different time points after inoculation, samples with 1.5 cm segments were collected from the lower, middle, and bottom of each leaf. Rohringer staining was carried out according to the method described previously (Qi et al., 2008).

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AUTHOR CONTRIBUTIONS

J.G., J.W.S., N.L., X.Z.S., S.F.H., W.C.Z., and D.M.W. conceived and designed the experiments. J.G., J.W.S., N.L., and X.Z.S. performed the experiments. J.G., J.W.S., N.L., X.Z.S., C.J.L., L.Z.W., G.L., F.L.Z., C.Y.H., S.F.H., W.C.Z., and D.M.W. analysed the data. J.G., J.W.S., N.L., and X.Z.S. contributed reagents/materials/analysis tools. J.G., D.M.W., and C.J.L. wrote the paper.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the Supporting Information section.

**FIGURE S1** Phylogenetic tree of DUF26-containing proteins. The phylogenetic tree was estimated with the maximum-likelihood method using the protein sequence of TaCRK2 and some representative sequences of three clade DUF26-containing proteins.

**FIGURE S2** The validation of the TaCRK2 gene model. (a) The alignment of TaCRK2 with other CRK protein sequences illustrates that TaCRK2 lacks the catalytic core domain HRDLK (the position of the red arrow) and DFG (the position of the black arrow). (b) The transcript of TaCRK2 was assembled from the RNA sequencing data (a).
The reads alignment showed that the transcript was separated by six introns (b). All the overlapping exons of this gene were merged into the transcripts of TaCRK2 and only one long ORF was found in the transcript. (c) The alignment of the TaCRK2 sequence and the exogenous fragment from the lineage-specific region of Thatcher Lr26.

**FIGURE S3** Young spike transduction process. RNAi-mediated genetic transformation of the TaCRK2 gene in the Thatcher Lr26 was performed using the wheat panicle genetic transformation technique. (a) Wheat young panicle. (b) Pruned young panicle for transformation. (c) Bagging after Agrobacterium infection. (d) Natural maturity. (e) Drying and harvesting.

**FIGURE S4** Effects of TaCRK2 knockdown by RNAi on the resistance of Thatcher Lr26 to Puccinia triticina infection. (a) The knockdown efficiency of RNAi-TaCRK2 in the transduced plants was determined by quantitative reverse transcription PCR. CK refers to nontransduced plants; RNAi-1 and RNAi-2 are RNAi-TaCRK2 positive plants. Error bars represent variations among three independent replicates. Different letters represent significant differences at p < .05. (b) Rohringer fluorescence staining of leaves from RNAi-TaCRK2 positively transduced plants at 120 hr post-inoculation (hpi). Leaves of nontransduced plants were used as controls. All samples were examined using confocal laser scanning microscopy. (a)–(c) CK plants; (d)–(f) RNAi-TaCRK2 plants; (a), (d) superimposed images of (b), (c) and (e), (f), respectively. Bar = 100 µm. (c) Rohringer fluorescence staining of CK and RNAi-TaCRK2 plants at 24 hpi. (a), (b) CK plants, bar = 100 µm; (c), (d) RNAi-TaCRK2 plants, bar = 50 µm. Note: The red arrows indicate haustorial mother cells (HMCs) and the white arrow indicates hypersensitive reaction (HR) cells. (d) Relationship between HR and HMCs at a single infection site. Rohringer fluorescence staining of CK and RNAi-TaCRK2 plants at 120 hpi. (a)–(c) CK plants; (d)–(f) RNAi-TaCRK2 plants, bar = 50 µm; (a), (d) superimposed images of (b), (c) and (e), (f), respectively. Note: The red arrows indicate HMCs and the white arrows indicate HR cells. (e) Statistical results for HR area (a) and number of HMCs (b) from 50 individually infected sites detected using a fluorescence microscopy. Error bars represent variations among three independent replicates. The different letters represent significant differences at p < .05. (f) Phenotype of RNAi-TaCRK2 positive plants at 12 days after P. triticina inoculation. Note: The red arrows indicate P. triticina uredosori.

**TABLE S1** List of primers used for PCR and quantitative reverse transcription PCR analyses.

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