The wheat NB-LRR gene TaRCR1 is required for host defence response to the necrotrophic fungal pathogen Rhizoctonia cerealis

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Summary

The necrotrophic fungus Rhizoctonia cerealis is the major pathogen causing sharp eyespot disease in wheat (Triticum aestivum). Nucleotide-binding leucine-rich repeat (NB-LRR) proteins often mediate plant disease resistance to biotrophic pathogens. Little is known about the role of NB-LRR genes involved in wheat response to R. cerealis. In this study, a wheat NB-LRR gene, named TaRCR1, was identified in response to R. cerealis infection using Artificial Neural Network analysis based on comparative transcriptomics and its defence role was characterized. The transcriptional level of TaRCR1 was enhanced after R. cerealis inoculation and associated with the resistance level of wheat. TaRCR1 was located on wheat chromosome 3BS and encoded an NB-LRR protein that was consisting of a coiled-coil domain, an NB-ARC domain and 13 imperfect leucine-rich repeats. TaRCR1 was localized in both the cytoplasm and the nucleus. Silencing of TaRCR1 impaired wheat resistance to R. cerealis, whereas TaRCR1 overexpression significantly increased the resistance in transgenic wheat. TaRCR1 regulated certain reactive oxygen species (ROS)-scavenging and production, and defence-related genes, and peroxidase activity. Furthermore, H2O2 pretreatment for 12-h elevated expression levels of TaRCR1 and the above defence-related genes, whereas treatment with a peroxidase inhibitor for 12 h reduced the resistance of TaRCR1-overexpressing transgenic plants and expression levels of these defence-related genes. Taken together, TaRCR1 positively contributes to defence response to R. cerealis through maintaining ROS homeoeostasis and regulating the expression of defence-related genes.

Keywords: NB-LRR, Rhizoctonia cerealis, resistance, Triticum aestivum, common wheat, reactive oxygen species (ROS).

Introduction

Bread wheat (Triticum aestivum) is one of the most important staple crops. The wheat sharp eyespot disease, primarily caused by a necrotrophic fungal pathogen Rhizoctonia cerealis, is one of the destructive diseases of wheat in some regions of the world. In terms of wheat acreage affected by sharp eyespot, China is the largest epidemic region in the world, as exemplified by the 8.1 million hectares of wheat infected in 2005 (Chen et al., 2013) and 9.33 million hectares in 2015 (http://www.agri.cn/V20/bchb/201501/20150121_4344729.htm). Breeding to host resistance is an effective and environmentally friendly way to protect wheat from R. cerealis infection. However, traditional resistance breeding is difficult as no wheat lines/cultivars with complete resistance to sharp eyespot have been identified, and the resistance in wheat accessions (CJ12633, Luke and AQ24788-83) is partial and controlled by multiple QTLs (quantitative trait loci, Cai et al., 2006; Chen et al., 2013). To improve wheat resistance to sharp eyespot, it is vital to identify genes that play important roles in the defence response and unravel their underlying functional mechanisms.

To control pathogens, plants activate defence mechanisms followed by pathogen detection through cell surface and intracellular immune receptors. Plants recognize pathogen-associated molecular patterns (PAMPs) through cell-surface pattern-recognition receptors and sense pathogen effectors via intracellular nucleotide-binding leucine-rich repeat (NB-LRR) proteins, resulting in PTI (PAMP-triggered immunity) and ETI (effector-triggered immunity), respectively (Jones and Dangl, 2006). Recently, to explain interactions between fungal pathogens and their host plants, Stotz et al. (2014) summarized another defence mechanism termed effector-triggered defence (ETD). Compared with ETI, ETD responses against pathogens are relatively slow and not associated with a fast hypersensitive cell death response in hosts (Boys et al., 2012; Bozkurt et al., 2012; Jones and Dangl, 2006; Stotz et al., 2014; Thirugnanasambandam et al., 2011; Valent and Chang, 2010). Several dozens of NB-LRR genes, acting as intracellular immune receptors to effectors of bacterial, viral and fungal pathogens, have been cloned from diverse plant species (Anderson et al., 1997; Cloutier et al., 2007; Deslandes et al., 1998; Ellis et al., 1999; Feuillet et al., 2003; Hinsch and Staskawicz, 1996; Huang et al., 2003, 2004; Inoue et al., 2013; Periyannan et al., 2013; Saintenac et al., 2013; Shen et al., 2007; Whitham et al., 1994). Recently, an emerging model is that NB-LRR proteins often function in pairs, with ‘helper’ proteins required for the activity of ‘sensors’ that mediate pathogen recognition (Bonardi et al., 2011; Wu et al., 2016). Certain NB-LRR proteins contribute to signal transduction and/or
amplification (Bonardi et al., 2011; Césari et al., 2014; Gabriëls et al., 2007). The above-mentioned NB-LRRs play a pivotal role in plant resistance responses to biotrophic pathogens. However, in certain plant-necrotrophic fungus pathosystems, the recognition of pathogen-produced effectors by NB-LRR proteins leads to effector-triggered susceptibility (Faris et al., 2010; Lorang et al., 2007; Nagy and Bennetzen, 2008). For example, the wheat Trns1 governs effector-triggered susceptibility to two necrotrophic fungi Stagonospora nodorum and Pyrenophora tritici-repentis (Faris et al., 2010). To our knowledge, no study about NB-LRR genes involved positively in plant resistance responses to necrotrophic fungal pathogens has been reported yet.

Cellular redox status, including generation and scavenging of reactive oxygen species (ROS; including H2O2 and O2⁻), plays an important role in plant defence responses to pathogens. At the early stage of plant-pathogen interaction, oxidative burst is a common phenomenon coupled with the generation of ROS (García-Brugger et al., 2006). Necrotrophic pathogens also induce the generation of ROS (Foley et al., 2016; Heller and Tuzdymski, 2011; Shetty et al., 2008). An appropriate level of ROS not only can promote cell wall reinforcement and phytoalexin production, but also has a signalling role in mounting a defence response (Quan et al., 2008). However, the overproduction of ROS may lead to oxidative stress that can damage some cellular compounds including proteins, lipids, carbohydrates and nucleotides of plant cells (Wu et al., 2008). ROS-generating enzymes like NADPH-dependent oxidase (NOX) complex, and various ROS-scavenging systems, such as peroxidase, ascorbate peroxidase, catalase (CAT) and superoxide dismutase (SOD), are involved in fine-tuning of ROS levels in the plant cells, resulting in the activation of plant defence responses (Mittler, 2002). However, knowledge about the involvement of ROS signalling in NB-LRR-mediated defence responses to fungal pathogens is limited.

An Artificial Neural Network (ANN) analysis on transcriptomic data has been used successfully to identify regulators of developmental processes in plants (Pan et al., 2013). In our laboratory, comparative transcriptomics based on microarray or RNA-seq analysis have been used to identify genes expressed differentially between sharp eyespot-resistant wheat CI12633 and susceptible wheat Wenmai 6 following infection with R. cerealis. In this study, ANN analysis of these transcriptomic data resulted in a regulatory network model of defence-related genes, in which several potentially important genes (including the wheat NB-LRR gene TaRCR1) were predicted several genes with major highly interacting nodes, including one key gene TaRCR1 (NCBI accession no. AK335348 harbouring the probe sequence with TIGR number TC376099) (Fig. 1a). The transcriptional levels of the probe (TIGR number: TC376099), corresponding to the 3’ terminal sequence of TaRCR1, were significantly higher in these two-resistant wheat lines CI12633 (about 341-fold at 4 dpi with R. cerealis R0301 and 409-fold at 21 dpi) and Shanhongmai (about 162-fold at 4 dpi and 319-fold at 21 dpi) than in the susceptible wheat Wenmai 6 (Figure S1). Without or with R. cerealis inoculation for 4 day, the transcriptional levels of TaRCR1 in six wheat lines/cultivars with different resistance degrees, including sharp eyespot-resistant lines CI12633 and Shanhongmai, moderately resistant lines Naivt 14 and Shannong 0431, moderately susceptible wheat cultivar (cv.) Yangmai 158 and susceptible cv. Wenmai 6 were investigated (Figure 1b). Either with or without R. cerealis infection, the expression level of TaRCR1 was the highest in CI12633, slightly decreased in Shanhongmai, gradually declined in Naiv 14 and Shannong 0431, and reached the lowest in Wenmai 6 (Figure 1b). The results suggested that the transcriptional level of TaRCR1 was associated with wheat resistance degrees to R. cerealis. Furthermore, TaRCR1 transcription in CI12633 stems was enhanced by R. cerealis, and the induction reached a peak at 7 dpi with R. cerealis (Figure 1c). Additionally, the expression analyses of TaRCR1 in organs of CI12633 plants showed that, under control treatment with sterile toothpick without R. cerealis, TaRCR1 was expressed in higher levels in the leaves than in the other organs; after 7 dpi with R. cerealis, levels in the roots and stems, the main disease-occurring sites, were more abundant than those in the leaves and spikes (Figure 1d). These results suggested that TaRCR1 may participate in the wheat defence response to R. cerealis.

**Sequence characterization of TaRCR1 in wheat**

The full-length cDNA and genomic sequences of TaRCR1 were cloned from CI12633. The comparison of the cDNA and genomic sequences showed that TaRCR1 genomic sequence with 4602-bp length was comprised of two introns and three exons. The TaRCR1 mRNA (GenBank accession no. KU161103) contains an open reading frame (ORF) with 2838-bp length, the 5’-untranslated region (UTR) with 235 bp and 3’-UTR with 129 bp (Figure 2a). The deduced protein TaRCR1 consisted of 945 amino acid (AA) residues with a molecular weight of 106.28 kD and a theoretical isolectric point (pI) of 8.65. Analysis of the protein sequence showed that TaRCR1 was a typical NB-LRR and contained an N-terminal coiled-coil (CC) domain (AAAs 1-180), an NB-ARC domain (AAAs 181-567) and 13 imperfect LRRs (AAAs 568-945) at the C-terminus. A conserved EDVID motif (EDCID in TaRCR1, AAs 80-84) was identified in the CC domain of TaRCR1 (Figure S2). All of the important motifs, including P-loop, RNBS-A, Walker B, RNBS-B, GLPL, RNBS-D and MHD, present in the NB-ARC domain characteristic of typical NB-LRR proteins (Ellis et al., 2000), were found in TaRCR1 protein (Figure S2). Although the full length of TaRCR1 was not obtained from R. cerealis-susceptible wheat, the 3’-terminal sequence of TaRCR1 was obtained from R. cerealis-susceptible cultivar Wenmai 6. Comparison of the 3’-terminal sequences of TaRCR1 from CI12633 and Wenmai 6 showed that shared 60.44% identity and many single nucleotide polymorphisms (SNPs) existed at their 3’-terminal sequences (Figure S3).

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Sequence alignment of TaRCR1 with the draft sequences of hexaploid bread wheat chromosomes from the International Wheat Genome Sequencing Consortium (IWGSC, http://www.wheatgenome.org) suggested that TaRCR1 should be located on wheat chromosome 3B. TaRCR1 gene-specific PCR amplification on the templates from the genomic DNAs of wheat Chinese Spring (CS) nulli-tetrasomic and di-telosomic lines further indicated that TaRCR1 was located on the short arm of wheat chromosome 3B (Fig. 2b). The ORF sequences of TaRCR1-A (GenBank accession no. KX840356) and TaRCR1-D (GenBank accession no. KX840357) located on chromosome 3AS and 3DS were cloned from CI12633, respectively. Both homoeologous proteins consisted of 955 AAs. TaRCR1, its homologs and some NB-LRR proteins with known resistance function were subjected to identity and phylogenetic tree analyses. The results indicated that the TaRCR1 protein sequence shared 82.45%, 81.38% and 61.28% identities with TaRCR1-A, TaRCR1-D and rice Yr10 (GenBank accession no. BAM28949.1), respectively, and they were clustered on the same branch (Figure 2c). However, the TaRCR1 protein shared quite low identities (7.05%–26.12%) with other plant NB-LRRs (Figure 2c; Table S1). These results suggested that TaRCR1 was a member of the NB-LRR family in wheat.

TaRCR1 localizes in both the cytoplasm and the nucleus

To investigate the subcellular localization of TaRCR1 in the plant cells, the p3SS:GFP-TaRCR1 fusion vector was constructed, and the p3SS:GFP-TaRCR1 and the control p3SS:GFP vectors were separately introduced into and transiently expressed in either onion epidermal cells or wheat mesophyll protoplasts. Confocal microscopic examination showed that in the transformed onion epidermal cells, the GFP-TaRCR1 protein was expressed and localized in both the cytoplasm and nucleus (Figure 3a). After plasmolysis of the onion epidermal cells, TaRCR1 was more clearly observed in both the cytoplasm and nucleus (Figure 3a). Moreover, in the wheat mesophyll protoplasts, the GFP-TaRCR1 protein distributed in both the cytoplasm and nucleus (Figure 3b). The red colour was auto-fluorescence from wheat chloroplast (Figure 3b). The control p3SS:GFP protein diffused in both the nucleus and cytoplasm (Figure 3a, b). These results indicated that TaRCR1 localizes in both the cytoplasm and nucleus in wheat.
Silencing of TaRCR1 impairs host resistance to *R. cerealis*

To explore whether TaRCR1 was required for wheat resistance to *R. cerealis*, barley stripe mosaic virus (BSMV)-based virus-induced gene silencing (VIGS) was used to knockdown TaRCR1 transcript in the partially resistant line CI12633. A 3′-terminal fragment specific to TaRCR1 was inserted in an antisense orientation into Nhe I restriction site of the BSMV RNA gene to generate BSMV:TaRCR1 recombinant construct (Figure S4). Following inoculation with BSMV:TaRCR1 or BSMV:00 (as a control) viruses for 10 day, the mild chlorotic mosaic symptoms of BSMV appeared in the leaves of the infected CI12633 plants (Figure 4a), and the expression of BSMV coat protein (CP) gene was detected (Figure 4b), proving that these inoculated plants were successfully infected by BSMV. The BSMV:00 infection did not significantly affect the expression of TaRCR1 in CI12633 plants (Figure S5), whereas the transcript level of TaRCR1 was markedly reduced in BSMV:TaRCR1-infected (TaRCR1-silencing) plants (Figure 4b).

Figure 2 Gene structure, chromosome location and phylogenetic tree analysis of TaRCR1. (a) Genomic structure of TaRCR1 gene; dark grey portions and dotted lines represent exons and introns, respectively. The dark line is the complete open reading frame. (b) Chromosome location of TaRCR1 using nulli-tetrasomic and double ditelosomic lines derived from wheat cv. Chinese Spring (CS). Marker, DL2, 000 DNA marker; N3A/T3B, N3B/T3D, N3D/T3B, three nulli-tetrasomic lines; 3BL and 3BS, two di-telosomic lines of chromosome 3B. (c) Phylogenetic analysis of the deduced amino acid sequences of TaRCR1 and other plant NB-LRR proteins. Yr10 and Xa1, *Oryza sativa* Yr10 and Xa1; Lr1, Lr10, and Sr33, *Triticum aestivum* Lr1, Lr10, and Sr33; Sr35 and TmMla1, *Triticum monococcum* Sr35 and TmMla1; HvMla1, HvMla7, and HvMla10, *Hordeum vulgare* HvMla1, HvMla7, and HvMla10; NRC1, *Solanum lycopersicum* NRC1; Rp1-D, *Zea mays* Rp1-D; RLM, *Arabidopsis thaliana* RLM. The black diamond indicates the position of TaRCR1.
Subsequently, R. cerealis WK207 was inoculated on the stems of these BSMV-infected plants to evaluate the defence role of TaRCR1. At 14 dpi with R. cerealis, lesions (symptom of sharp eyespot disease) were present on the stems of TaRCR1-silencing plants, but to a lesser extent in BSMV:00-treated control plants (Figure 4c). At 21 dpi with R. cerealis, the lesion areas on the stems of TaRCR1-silencing plants were 1.24–1.96 cm², while the average lesion area of BSMV:00-treated plants was only 0.75 cm² (Figure 4d); the infection types (ITs) of TaRCR1-silencing plants were ranged from 2.7 to 4.0, whereas the average IT of BSMV:00-treated plants was 1.8 (Figure 4c). Relative abundances of R. cerealis actin mRNA, as a measure of the fungal growth in the pathogen-inoculated wheat, were consistent with the symptoms of these plants (Figure 4e). These results indicated that the silence of TaRCR1 in CI12633 plants impaired resistance to R. cerealis and that TaRCR1 is required for the wheat defence response to R. cerealis infection.

**Figure 3** Subcellular localization of the green fluorescent protein (GFP)-TaRCR1 fusion protein in onion epidermal cells and wheat protoplasts. (a) The control GFP and fused GFP-TaRCR1 in onion epidermal cells. After transformed with GFP-TaRCR1 for 20 h, the onion epidermal cells were plasmolysed by 30% sucrose treatment for 10 min, and then images were taken using a confocal microscope with 536 nm wavelengths. Bars = 100 μm. (b) The control GFP and fused GFP-TaRCR1 in wheat protoplasts. The autofluorescence was from wheat chloroplast. Confocal images were taken at 20 h after transformation using 536 nm wavelengths. Bars = 20 μm.

**TaRCR1 overexpression improves resistance to R. cerealis in transgenic wheat**

To further investigate the role of TaRCR1 in the defence against R. cerealis, the overexpression transformation construct pUbi:myc-TaRCR1 (Figure 5a) was generated and transformed into moderately susceptible wheat cultivar Yangmai 16. PCR analysis using the primers specific to the c-myc-TaRCR1-Tnos chimera showed that the introduced transgene could be detected in six TaRCR1-overexpressing lines (R1, R2, R4, R12, R13 and R27) from T0 to T4 generations (Figure 5b). qRT-PCR results showed that the transcript abundances of TaRCR1 in these six transgenic lines were markedly elevated compared with wild-type (WT) Yangmai 16 (Figure 5c). Western blotting indicated that the introduced c-myc-TaRCR1 gene could be expression in the fusion protein in these TaRCR1-overexpressing transgenic lines, but not in WT Yangmai 16 (Figure 5d). Following inoculation with R. cerealis
isolates R0301 (T1–T2 plants) and WK207 (T3–T4 plants), compared with WT Yangmai 16, these TaRCR1-overexpressing lines exhibited significantly enhanced resistance to *R. cerealis* (Figure 5e; Table 1). The average disease indexes of these TaRCR1-overexpressing lines in T1–T4 generations infected with *R. cerealis* were ranged from 24.75 to 45.00, whereas those of WT Yangmai 16 were 50.82–57.11 (Table 1). Furthermore, microscopic observation indicated that the hyphae abundances of *R. cerealis* strain WK207 were less on the inoculated base sheaths of the TaRCR1-overexpressing line R27 than on those of WT Yangmai 16 (Figure 5f), providing supporting evidence that TaRCR1 overexpression increases resistance to hyphae development of *R. cerealis*. These results indicated that TaRCR1 positively regulates wheat resistance response to sharp eyespot caused by *R. cerealis*.

**TaRCR1 modulates expression levels of defence-associated genes**

Seven wheat defence-associated genes, including *TaPIE1* (GenBank accession no. EF583940), *defensin* (GenBank accession no. CA630387), *PR-1.2* (GenBank accession no. AJ007349), *PR2* (GenBank accession no. AF112965), *PR10* (GenBank accession no. CA613496), *chitinase1* (GenBank accession no. CK207575) and *chitinase2* (GenBank accession no. TC426538), have been implicated in resistance responses to *R. cerealis* (Chen et al., 2008; Wei et al., 2016; Zhu et al., 2014, 2015). Additionally, based on the microarray data (GEO accession number GSE69245), *chitinase2* and *TaPIE1* were expressed in higher levels in CI12633 and Shanhongmai than in Wenmai6 (Table S2). To examine whether TaRCR1 regulates defence-associated genes in wheat response to *R. cerealis*, we have analyzed expression patterns of the above-mentioned seven defence-associated genes in TaRCR1-overexpressing and TaRCR1-silencing wheat plants as well as the control plants. The results showed that following inoculation with *R. cerealis* WK207 for 7 day, the transcriptional levels of *PR2* and *chitinase2* were significantly decreased in TaRCR1-silencing plants compared with BSMV:00-infected control plants, whereas they were significantly increased in TaRCR1-overexpressing lines compared with WT Yangmai 16 (Figure 6). The transcriptional levels of *PR-1.2* and *TaPIE1* were higher in TaRCR1-overexpressing lines than in WT Yangmai 16 plants (Figure 6). Additionally, the transcriptional levels of *PR2*, *chitinase2*, *PR-1.2* and *TaPIE1* were significantly induced in WT Yangmai 16 and more markedly induced in TaRCR1-overexpressing wheat lines than the WT after *R. cerealis* WK207 inoculation for 7 day (Figure S6).

**ROS homoeostasis is crucial for TaRCR1-mediated resistance against *R. cerealis***

To explore whether TaRCR1-mediated resistance against *R. cerealis* is closely linked to the homoeostasis between ROS scavenging and production, we performed the following experiments. After
H$_2$O$_2$ treatment, the expression level of TaRCR1 was dramatically induced from 10 min to 24 h, which reached the first peak at 30 min (16.20-fold); and the second peak at 3 h (7.30-fold) (Figure 7a). Interestingly, H$_2$O$_2$ pretreatment elevated the transcriptional induction level of TaRCR1 by R. cerealis WK207 inoculation (Figure 7b). The 3,3’-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) stains were used to detect H$_2$O$_2$ and O$_2^-$ in wheat sheaths. Without R. cerealis inoculation, little H$_2$O$_2$ and O$_2^-$ were detected (Figure 7c). After R. cerealis inoculation for 12 and 36 h, accumulation of H$_2$O$_2$ and O$_2^-$ was more in

Table 1  

| Lines | Infection type | Disease index |
|-------|----------------|---------------|
|       | T$_1$ | T$_2$ | T$_3$ | T$_4$ | T$_1$ | T$_2$ | T$_3$ | T$_4$ |
| R1    | 1.52** | 2.25*  | 1.83*  | 1.51** | 30.33** | 45.00* | 36.67* | 30.33** |
| R2    | 2.10*  | 1.32** | 1.75** | 2.25*  | 42.05*  | 26.43** | 35.00** | 45.00*  |
| R4    | 1.72** | 1.72** | 1.67** | 1.83*  | 34.47** | 34.39** | 33.33** | 36.61*  |
| R12   | 1.28** | 1.36** | 2.17*  | 2.17*  | 25.50** | 27.27** | 43.33*  | 43.33*  |
| R13   | 1.58** | 1.54** | 1.63** | 1.81*  | 31.66** | 30.86** | 32.50** | 36.25*  |
| R27   | 1.80*  | 1.24** | 1.71** | 1.63** | 36.06*  | 24.75** | 34.29** | 33.28** |
| WT    | 2.86   | 2.70   | 2.63   | 2.54   | 57.11   | 53.90   | 52.63   | 50.82   |

* or ** significant difference between each transgenic line and WT wheat at P < 0.05 or 0.01 (t-test).

1R1, R2, R4, R12, R13, R27 indicate TaRCR1-overexpressing wheat lines; WT indicates untransformed wild-type Yangmai 16. R. cerealis isolate R0301 was used to infect plants in T$_1$ and T$_2$ generations, and R. cerealis isolate WK207 was used to infect plants in T$_3$ and T$_4$ generations. At least 10 plants for each line were assessed for disease intensity.
A wheat NB-LRR gene TaRCR1 defends sharp eyespot

Discussion

In this study, a wheat NB-LRR gene, namely TaRCR1, was identified and cloned based on comparative transcriptomics. Microarray data showed that TaRCR1 processed significantly higher transcriptional levels in R. cerealis-resistant wheat lines CI12633 and Shanhongmai than in the susceptible wheat Wenmai 6. Additionally, the RNA-Seq analysis (data not shown) showed that the transcriptional level of TaRCR1 was markedly higher (76.01-fold) in C112633 than in Wenmai 6. Usually, the change in expression of R genes (encoding NB-LRR immune receptors) is small and these R genes could not be picked up by microarray or other transcriptomic analyses. Accumulating evidence indicates that plant NB-LRR proteins not only act as immune receptors, but also contribute to signal transduction and/or amplification in plant–pathogens (Wu et al., 2016). For example, the tomato NRC1, which was identified by combination techniques of cDNA-amplified fragment length polymorphism and VIGS, functioned in cell death signalling pathways and contributed to resistance response to Cladosporium fulvum (Gabriëls et al., 2007). We thus deduced that TaRCR1 might...
participate in defence signalling transduction and/or positive regulator. The qRT-PCR analysis showed that the transcriptional level of TaRCR1 was associated with the resistance degree in six different wheat lines/cultivars. The 3’-terminal sequences of TaRCR1 from the R. cerealis-resistant wheat line CI12633 and the homologous sequence from R. cerealis-susceptible wheat cv. Wenmai 6 shared 60.44% identity and many SNPs existed at their 3’-terminal sequences, resulting in some nonsynonymous mutations and the transcript stability (Spies et al., 2013), which might be one reason for the distinct transcript abundance in the resistant and susceptible wheat genotypes. Our analyses indicated that TaRCR1 was located on chromosome 3BS of wheat. The ORF sequence of TaRCR1 shares 87.35% and 87.07% identities with TaRCR1-A and TaRCR1-D, respectively. Based on the 3’-terminal sequence of TaRCR1, a marker linked to sharp eyespot resistance was developed and could explain 8.00% of phenotypic variance in the recombinant inbred line (RIL) population of Shanhongmai/Wenmai6 (Table S3), which might partially explain the higher expression in the resistance lines even in the absence of infection. Several QTLs have been identified (Cai et al., 2006; Chen et al., 2010).

Figure 7 TaRCR1-mediated resistance to Rhizoctonia cerealis is associated with ROS homeostasis. (a) Expression of TaRCR1 in leaves of Yangmai 16 wheat in response to exogenous applications of H2O2. Wheat plants at four-leaf stage were sprayed with 10 mM H2O2 and 0.1% Tween-20 (as a control). (b) Transcript induction of TaRCR1 by R. cerealis is regulated by H2O2 treatment. Total RNA was extracted from the sheaths of Yangmai 16 plants at four-leaf stage pretreated with H2O or H2O2 for 12 h, followed by R. cerealis treatment for 4 day. (c) Detection of hydrogen peroxide (H2O2) and superoxide anion (O2−) in wheat. Sheaths were harvested from wheat plants at 0, 12 and 36 hpi with R. cerealis and were then stained with 3,3′-diaminobenzidine (DAB) for H2O2 detection and nitroblue tetrazolium (NBT) for O2− detection, respectively. Similar results were obtained from three independent replicates. WT: untransformed wheat Yangmai 16. R1, R12, R27: TaRCR1-overexpressing lines. (d) Transcriptional analysis of genes encoding ROS-scavenging enzymes (CAT1 and POX2) and ROS-producing enzyme NOX in wheat sheaths. Statistically significant differences of TaRCR1-overexpressing (R1, R12, R27) or TaRCR1-silencing wheat plants were compared with the WT or the BSMV:00-infected control based on three technical replications (t-test; *P < 0.05, **P < 0.01). Bars indicate standard error of the mean.
inoculated CI12633 plants, leading to lesions in these sheaths and stems, while the lesions in BSMV:00-inoculated CI12633 were obviously smaller than in BSMV:TaRCR1-inoculated CI12633. These data indicated that TaRCR1, acting as a positive regulator, was required for wheat defence response to *R. cerealis*. The majority of the reported NB-LRR proteins positively contribute to plant immunity to diverse biotrophic pathogens. In more recent documents, three distinct NB-LRR proteins have been implicated in host susceptibility to necrotrophic fungal pathogens (Faris et al., 2010; Lorang et al., 2007; Nagy and Bennetzen, 2008). Here, to our knowledge, this study is the first to uncover the positive regulation of an NB-LRR protein in plant resistance responses to the necrotrophic fungal pathogens. This work undoubtedly broadens understanding of biological functions of NB-LRRs in plant species.

In plants, activation of R proteins leads to extensive transcriptional reprogramming of defence genes (Chang et al., 2013; Inoue et al., 2013; Padmanabhan et al., 2013; Shen et al., 2007; Zhu et al., 2010). Different regulatory proteins may activate different kinds of genes in wheat defence response to *R. cerealis*. In this study, the expression levels of *PR2*, chitinase2, *PR-1.2* and *TaPIE1* were higher in TaRCR1-overexpression lines compared with WT plants, while the expression levels of *PR2* and chitinase2 were the lowest in TaRCR1-silencing plants. These results indicate that TaRCR1 may positively modulate the expression of *PR2* and chitinase2, whereas the expression of *PR1* and TaPIE1 may be regulated not only by TaRCR1, but also by other proteins. The exact reprogramming mechanisms underlying TaRCR1 remain to be elucidated.

ROS signalling plays an important role in plant defence responses to pathogens. For example, ROS signalling is involved in resistance of endochitinase gene transgenic cotton to *R. solani* (Kumar et al., 2009). In this study, TaRCR1 was highly induced at early stage (30 min and 3 h) of H$_2$O$_2$ stimulus. Furthermore, the induced transcriptional abundance of TaRCR1 by *R. cerealis* infection was significantly elevated following by H$_2$O$_2$ pretreatment, suggesting that the response of TaRCR1 to *R. cerealis* infection was associated with ROS signalling. ROS-scavenging enzymes, including CAT, peroxidase and SOD, and ROS-producing enzyme NOX that is also known as respiratory burst oxidase homolog (RBOH), are crucial for maintaining the ROS homeostasis in plant cells (Kuzniak and Sklodowska, 2004; Sharma et al., 2007). RBOH proteins trigger cell death which is favourable for necrotrophic pathogens’ infection, whereas an AtRbohD AtRboHF double mutant displayed reduced cell death after infiltration with an avirulent bacterium strain (Torres et al., 2002; Yoshioka et al., 2003). Here, TaRCR1 regulates the expression of *CAT1*, *POX2* and NOX genes, and peroxidase activity. Consequently, the production of ROS induced after *R. cerealis* inoculation at early stage (12 and 36 h) was less in TaRCR1-overexpressing wheat plants than in WT plants. At 4 dpi with *R. cerealis*, the level of ROS accumulation was reduced. These results suggested that TaRCR1 play an important role in maintaining ROS homeostasis in wheat under *R. cerealis* stress. Further experiments indicated that peroxidase inhibition compromised TaRCR1-mediated resistance to *R. cerealis* and that peroxidase-dependent ROS-scavenging might contribute to the TaRCR1-mediated resistance. Additionally, the expression of defence-associated genes (*PR2*, *PR-1.2*, chitinase2 and *TaPIE1*) positively regulated by TaRCR1 was enhanced upon H$_2$O$_2$ treatment, but markedly reduced after NaN$_3$ treatment except for *PR-1.2*, suggesting that ROS homeostasis also regulated the expression of these genes. NaN$_3$ treatment does not influence *PR-1.2* expression, which is

![Figure 8](image)

Figure 8 Analysis of peroxidase activity and effect of NaN$_3$ treatment on TaRCR1-mediated resistance to *Rhizoctonia cerealis*. (a, b) Analysis of peroxidase activity in TaRCR1-overexpressing lines (R1, R12, R27), WT, TaRCR1-silencing plants (BSMV:TaRCR1-1, BSMV:TaRCR1-2 and BSMV:TaRCR1-3) and BSMV:00-infected controls before and after *R. cerealis* inoculation for 12 h. Statistically significant differences of TaRCR1-overexpressing or TaRCR1-silencing wheat plants were compared with the WT or the control based on three independent replications (t-test; *P < 0.05, **P < 0.01*). mgprot, mg protein. (c) qRT-PCR analysis of *R. cerealis* actin (RcActin) gene represented the biomass of *R. cerealis* in TaRCR1-overexpressing and WT plants after pretreatments with H$_2$O$_2$ and NaN$_3$ (peroxidase biosynthesis inhibitor) for 12 h then *R. cerealis* inoculation for 7 day.
perhaps regulated by SOD or CAT-mediated ROS scavenging. These results suggested that the functional role of TaRCR1 in defence response to R. cerealis was correlated with the expression of these defence-associated genes that were modulated by ROS homeostasis.

In conclusion, the wheat NB-LRR gene TaRCR1 was identified to be required for defence response to R. cerealis. TaRCR1 can regulate the expression of ROS-scavenging and production genes, which maintains ROS homeostasis, subsequently modulating the expression of defence genes, finally leading to enhanced resistance to R. cerealis. This study provides novel insights into biological functions of NB-LRR genes in defence responses to necrotrophic pathogens.

Experimental procedures

Plant and fungal materials and growth conditions

The wheat lines/cultivars used in this study were ShanHongmai, CI12633, Yangmai 158, Yangmai 16 and Wenmai 6. Yangmai 16, an important planting variety in south China, is susceptible to R. cerealis isolates R0301 and WK207 are prevailing in wheat plants of Jiangsu province and Shandong province, respectively.

All wheat plants were grown in the field or in a glasshouse at 22 °C, 14-h light (intensity of 300 μmol/m²/s) and 12 °C, 10-h dark conditions. Seedlings at the four-leaf stage of the WT Yangmai 16 plants were sprayed with 10 mM H₂O₂ and 5 mM NaN₃ (an inhibitor of peroxidase) plus 0.1% (v/v) Tween-20 for the indicated times. Plants sprayed with water containing 0.1% Tween-20 were used as a control for all treatments.

Cloning and sequence analysis of TaRCR1

To clone the full-length sequence of TaRCR1 from resistant wheat line CI12633, based on the sequence of the microarray probe TC376099, primers (TaRCR1-3’-F1 and TaRCR1-3’-F2) were designed, synthesized and used to amplify the 3’-UTR using 3’-RACE kit v.2.0 (TaKaRa, Japan) in CI12633 and Wenmai 6 wheat plant infected by R. cerealis R0301 for 7 day. Then, the ORF sequence of TaRCR1 was amplified from cDNA of CI12633 stems. A phylogenetic tree was constructed using a neighbour-joining method implemented with MEGA 5.0 software.

TaRCR1 chromosomal localization

This sequence of TaRCR1 was aligned with the wheat cv. CS genome using the service provided by IWGSC (http://wheat-urgi.versailles.inra.fr/Seq-Repository/BLAST), and the predicted chromosomal localization was obtained from this website. CS nullisomic and di-telosomic lines were used to verify the chromosomal localization by gene-specific PCR.

Subcellular localization of TaRCR1

The coding sequence of TaRCR1 was amplified by PCR and ligated to the 3’ end of the GFP coding region without the stop codon in p35S:GFP vector, generating the GFP-TaRCR1 fusion construct p35S:GFP-TaRCR1. The resulting p35S:GFP-TaRCR1 and p35S:GFP constructs were separately introduced into white onion epidermal cells and wheat protoplasts following Yoo et al. (2007) and Zhang et al. (2007). After incubation at 25 °C for 20 h, GFP signals were observed and photographed using a confocal laser scanning microscope (Zeiss LSM 700) with a Fluor 10X/0.50 M27 objective lens and SP640 filter.

BSMV-mediated TaRCR1 gene silencing

To generate the BSMV:TaRCR1 recombinant construct, a 315-bp sequence of TaRCR1 (from 2682 to 2996 nucleotides in TaRCR1 cDNA sequence) was subcloned in an antisense orientation into the Nhel restriction site of the RNAi of BSMV (Figure S4). At the three-leaf stage, at least 20 plants of CI12633 were inoculated with BSMV:TaRCR1 or BSMV:00 (as a control) following Holzberg et al. (2002). At 10 day after virus infection, the four leaves were collected to monitor BSMV infection and to evaluate the transcript changes of TaRCR1. At 14 day after BSMV infection, each stem of the BSMV-infected CI12633 plants was inoculated with one sterile toothpick harbouring R. cerealis WK207 mycelia. They were scored at 14 and 21 dpi with R. cerealis, respectively.

TaRCR1 overexpression transformation vector and wheat transformation

The full ORF sequence of the TaRCR1 gene was subcloned into modified pAH25 vector (Christensen and Quail, 1996) with c-myc epitope tag, resulting in the transformation vector pUbi:myc-TaRCR1. The vector contains the Ubi:myc-TaRCR1-Tnos chimera, in which c-myc-TaRCR1 was driven by the maize ubiquitin (Ubi) promoter and terminated by the nopaline synthase gene (Tnos). Plasmid pUbi:myc-TaRCR1 was introduced into 2,000 immature embryos of the wheat cv. Yangmai 16 by biolistic bombardment according to the protocol described by Chen et al. (2008).

PCR and Western blot analyses on TaRCR1-overexpressing transgenic wheat

The presence of the introduced TaRCR1 gene in the transformed wheat plants was monitored by PCR using a primer pair specific to the Ubi:myc-TaRCR1-Tnos chimera, TaRCR1-F located in TaRCR1 gene region and TNOS-R located in the Tnos sequence region of the transformation vector. PCR was performed in a 20-μl volume containing 1 μl genomic DNA (200 ng/μl), 10 μl 2 × PCR Mixture (TaKaRa), 0.5 μl each primer (10 μm) and 8 μl double distilled water.

According to Zhu et al. (2015), Western blots were incubated with 100-fold diluted anti-c-myc antibody and secondary antibody conjugated to horseradish peroxidase (TIANGEN).

RT-PCR and qRT-PCR

The expression patterns of BSMV CP, TaRCR1, ROS- and defence-related genes were analyzed by RT-PCR or qRT-PCR. The qRT-PCR was operated on an ABI 7500 RT-PCR system (Applied Biosystems, USA) following Dong et al. (2010). The relative expression of the target genes was calculated using the 2−ΔΔCT method (Livak and Schmittgen, 2001), where the wheat Actin gene, TaActin, was used as the internal reference. Three independent biological replicates were performed for each RNA sample/primer combination.

All the primers in the study are listed in Table S4.

Assessments of R. cerealis responses in wheat plants

Rhizoctonia cerealis isolate R0301 was used to inoculate plants in T₁ and T₂ generations, and R. cerealis isolate WK207 was used to inoculate plants in T₃ and T₄ generations. In T₁ and T₂ generations in greenhouse, the TaRCR1-overexpressing transgenic and WT wheat plants were grown and inoculated with R. cerealis. In T₃ and T₄ generations, the trials were conducted in nursery field. At the tillering growth stage, the plants were inoculated on each stem base with 8–10 sterile wheat kernels harbouring R. cerealis.
mycelia. To enhance humidity and R. cereals infection and development, the plants were sprinkled with water twice a day during the first 7 days and then with frequency depending on rainfall and soil moisture until final disease recording. Ten to thirty plants for each line were assessed for disease severity. ITS and disease index of wheat plants were scored at ~50 dpi following Cai et al. (2006). At 14 dpi with R. cereals WK207, the base sheaths of plants in T4 generation were sampled and stained by trypsin blue to observe R. cereals hyphae colonization according to Peterhansel et al. (1997).

Assay of ROS level and peroxidase activity

Detection of H$_2$O$_2$ and O$_2^-$ via histochemical staining by DAB and NBT, respectively, was performed as described by Lee et al. (2002). Peroxidase activity was measured using Peroxidase Activity Assay Kit (Nanjing Jiancheng Bioengineering Institute) based on the manufacturer’s instruction.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1** The FoldChange of TaRCR1 transcriptional level derived from microarray analysis (GEO accession number GSE69245) between the *R. cerealis*-resistant wheat line CI12633/Shanhnongai and susceptible wheat cultivar Wenmai 6 at 4 and 21 days postinoculation (dpi) with *R. cerealis*.

**Figure S2** Deduced amino acid sequence of the wheat (*Triticum aestivum* CC-NB-LRR gene TaRCR1. The conserved motifs including EDTID, P-loop, RNBS-A, RNBS-B, GLPL, RNBS-D, and MHD were indicated in yellow.

**Figure S3** Alignment of 3’ terminal sequences of TaRCR1 in resistant wheat line CI12633 and its homolog in susceptible wheat cultivar Wenmai 6. The software DANMAN was used to perform the sequence alignment.

**Figure S4** Scheme of genomic RNAs of the barley stripe mosaic virus (BSMV) construct and the construct of the recombinant virus expressing the wheat (*Triticum aestivum*) NB-LRR gene TaRCR1, BSMV:TaRCR1. The orientation of the TaRCR1 insert is indicated by dark boxes.

**Figure S5** qRT-PCR analysis of TaRCR1 in the mock (buffer-inoculated) and BSMV/00 infected CI12633 plants. Total RNA was extracted from sheaths of mock or plants post-BSMV/00 inoculation for 10 day. The expression level of TaRCR1 in the mock plants was set to 1.

**Figure S6** Transcription analysis of four defence genes in the wild type (WT) wheat (*Triticum aestivum*) Yangmai 16 plants after *Rhizoctonia cerealis* inoculation for 7 day. Total RNA was extracted from sheaths of WT plants after *R. cerealis* inoculation for 7 day. The expression levels of those genes in the WT plants
under normal conditions (mock treated with sterile toothpicks without pathogen) were set to 1. Significant differences between *R. cerealis* inoculation and normal conditions were derived from the results of three independent replications (t-test: **, *P* < 0.01). Error bars indicate SE.

**Figure S7** Detection of hydrogen peroxide (H$_2$O$_2$) and superoxide anion (O$_2^-$) in wheat. Sheaths were harvested from TaRCR1-overexpressing lines (R1, R12, R27), WT and TaRCR1-silencing plants (BSMV:TaRCR1) and BSMV:00-infected controls at 4 day postinfection with *R. cerealis*, and were then stained with 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT), respectively. Similar results were obtained from three independent replicates.

**Figure S8** Transcription analysis of four defence genes in the wheat (*Triticum aestivum*) Yangmai 16 plants after H$_2$O$_2$ and NaN$_3$ treatment. Total RNA was extracted from leaves of wheat plants after H$_2$O, H$_2$O$_2$ and NaN$_3$ treatments for 12 h. The expression levels of those genes in the wheat plants treated with H$_2$O were set to 1. Significant differences between H$_2$O$_2$ or NaN$_3$ and H$_2$O treatments were derived from the results of three independent replications (t-test: **, *P* < 0.01). Error bars indicate SE.

**Table S1** The identities between TaRCR1 and other NLR proteins.

**Table S2** The FoldChange of Chitinase2 and TaPIE1 transcriptional level derived from microarray analysis (GEO accession number GSE69245).

**Table S3** TaRCR1 conferring resistance to sharp eyespot in the Shanhongmai/Wenmai 6 RIL population.

**Table S4** Primers used in this study.

A wheat NB-LRR gene *TaRCR1* defends sharp eyespot.