

Expression and Functional Analysis of a Pathogenesis-Related Protein 1 Gene, *TcLr19PR1*, Involved in Wheat Resistance Against Leaf Rust Fungus

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Published online: 3 September 2014
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Abstract Pathogenesis-related (PR) proteins encoded by plant defense genes play key roles in plant disease-resistance responses, specialized in systemic-acquired resistance. However, their roles in the response of wheat to fungal infection are still not well known. Our earlier studies have reported that a full-length *TcLr19PR1* gene (818 bp) was isolated from wheat infected by leaf rust (*Puccinia triticina*). Here, we showed that *TcLr19PR1* was induced earlier and its expression level was higher in the incompatible interaction between seedling stage of wheat and *P. triticina* than in compatible interactions. *TcLr19PR1* was strongly induced after *P. triticina* inoculation and ABA and SA treatments, in which the expression level of *TcLr19PR1* significantly increased and reached the maximum at 12 and 72 h, respectively. Furthermore, the transgenic T₁-stable *TcLr19PR1* lines generated in the susceptible wheat cultivar Zhengzhou 5389 background exhibited certain extent disease resistance against *P. triticina* infection by preventing disease development. In addition, Western blotting was conducted to confirm that *TcLr19PR1* protein was induced by *P. triticina* infection in positive transgenic plants at the protein expression level. These findings suggest that *TcLr19PR1* gene plays an important role in wheat development and resistance to leaf rust pathogen attack.

Keywords *TcLr19PR1* · Real-time quantitative RT-PCR · Transcripts expression profiles · Gene gun transformation

Electronic supplementary material The online version of this article (doi:10.1007/s11105-014-0790-5) contains supplementary material, which is available to authorized users.

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Abbreviations

PR	Pathogenesis related
HR	Hypersensitive response
ABA	Absciscic acid
SA	Salicylic acid
SAR	Systemic acquired resistance
ET	Ethylene
Hpi	Hours postinoculation
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

Introduction

Leaf rust, caused by *Puccinia triticina*, is one of the most destructive diseases of common wheat all over the world (Komer 1996). Its epidemic commonly causes both yield losses and production with lower quality, and it occurs more frequently and in more worldwide regions as compared with stem rust of wheat (*P. graminis* f. sp. *tritici*) or stripe rust of wheat (*Puccinia striiformis* f. sp. *tritici*) (Bolton et al. 2008). Development of genetic resistance to rust has been approved as the most efficient, cost-effective, and environment-friendly approach to prevent the losses caused by rust epidemics (Singh et al. 2004). However, new wheat cultivars lose their resistance due to the occurrence of new virulent races. Therefore, it is important to elucidate the molecular mechanism of resistance to leaf rust and to use wheat cultivars with durable resistance.

Plants show a series of complex physiological and biochemical changes in response to pathogen attack in co-evolution process. Recognition of a pathogen often triggers a localized resistance reaction, known as the hypersensitive response (HR) which is characterized by

rapid cell death around the site of infection, so that the growth of pathogen is inhibited and a series of defense responses are stimulated causing plant systemic-acquired resistance (SAR) (Hammond-Kosack and Jones 1996; Ryals et al. 1996).

The SAR is a "whole-plant" resistance response that occurs following an earlier localized exposure to a pathogen. The resistance conferred is long-lasting and effective against a broad spectrum of pathogens, including viruses, bacteria, fungi, and oomycetes (Durrant and Dong 2004). SAR can be activated in many plants by pathogens, either as part of the HR or as a symptom of disease. SAR is characterized by the increased expression of large number pathogenesis-related genes (PR genes) (Yoti et al. 2013; Uwe. 2006).

In host-pathogen interactions, various novel proteins are induced which are collectively referred to as "pathogenesis-related proteins" (PRs) (Hamamouch et al. 2011), does not only accumulate locally in the infected leaf but also induced systemically, which defined as proteins coded by the host plant but induced specifically in pathological or related situations (Hamamouch et al. 2011; Van Loon et al. 2006). Induction of PRs has been found in many plant species belonging to various families, PR1 to PR17 (Sels et al. 2008; Liu and Ekramoddoullah 2006), which suggests a general role for these proteins in resistant reaction and in adaptation to biotic stress conditions. Many PRs have direct antibacterial activity (Reddy. 2013). In different families of PRs, PR1 is one of the most important proteins whose function is unclear. PR1 gene induced by pathogenic bacteria, salicylic acid (SA), or ethylene (ET) is often used as a molecular marker gene of SAR (Van Loon and Van 1999), and the research on the proteins encoded by PR1 is a "hot spot" in the area of resistance of plant.

PR1 genes were first discovered in tobacco (*Nicotiana tabacum* L.) (Antoniw et al. 1980), then many PR1 genes were identified from many monocotyledonous and dicotyledonous plants (Mitsuhashi et al. 2008; Li et al. 2011). Most PR1 proteins with molecular weights of 14–17 kD are alkaline. PR1 family is found most abundantly in PRs. PR1 family is highly conserved in structure and shows a high level of similarity in some domains. Their amino acid sequences share a certain degree of homology. PR1 proteins have the same functions in eukaryotic cells (Aglika. 2005) and participate in plant responses to various biotic and abiotic stresses. Some research results showed that PR1 proteins mediated resistance of host plant to fungi. Yang et al. (2013) found that PR1 was upregulated in *Paeonia suffruticosa* ('Luhehong') infected by *Cylindrocladium canadense* at 24 h postinoculation (hpi), which suggested that PR1 was closely associate with *P. suffruticosa* resistance to *C. canadense*. Sujon et al. (2005) found that highly expressed PR1 gene in *Phytophthora nicotianae* can enhance plant resistance to tobacco mosaic virus.

Although many PR1 genes were cloned and reported to be involved in plant disease-resistance responses and SAR, fewer studies targeted to the function of PR gene. In our earlier studies, a PR1 gene designated *TcLr19PR1* with an open reading frame (ORF) of 495 bp encoding 164-amino acids from wheat near-isogenic lines TcLr19 infected by leaf rust (*P. triticina*) was obtained and located on chromosome 7D by nulli-tetrasomic lines of "Chinese Spring," which is in common with the location of wheat-leaf rust-resistance gene *Lr19*. Semiquantitative PCR showed that the expression of *TcLr19PR1* gene was induced by leaf rust pathogen (Wang et al. 2012). In the present study, the expression of *TcLr19PR1* gene induced by pathogen, salicylic acid (SA) and abscisic acid (ABA) was measured temporally and spatially in wheat tissues to explore the relationship between *TcLr19PR1* gene and leaf rust disease resistance. To further understand the gene's function, the constructed vector pAHC-*TcLr19PR1* was transformed into immature embryo-derived calli of a susceptible wheat cultivar ZhengZhou 5389 through particle bombardment, and molecular detection and disease-resistance identification of transgenic wheat with *TcLr19PR1* gene were studied. These results will provide systematic data for further study on the function and the mechanism of *TcLr19PR1* in defending adversity stress.

Materials and Methods

Plant Materials and Inoculation

Wheat (*Triticum aestivum* L.) near isogenic line TcLr19, Thatcher, and leaf rust (*P. triticina*) race 07-10-421-3 (FHJT) were the biological materials used for expression analyses. The susceptible wheat cultivar ZhengZhou 5389 was used for generating transgenic lines. Based on the gene-for-gene model, wheat TcLr19 and leaf rust race 07-10-421-3 showed the incompatible reaction (type). However, Thatcher and leaf rust race 07-10-421-3 showed the compatible reaction (type 4) according to Roelfs standard (1985). The plants were grown in a 15-cm diameter pot in the greenhouse for about 7 days when primary leaves were fully expanded, leaf rust urediospores collected from the susceptible wheat Zhengzhou 5389 were applied with a brush to the surface of the primary leaves of TcLr19; sterile water was inoculated to wheat leaves as control. After inoculation, the plants were kept at 100 % relative humidity in the dark for 24 h at 20 °C, and then moved to the greenhouse. Wheat leaves were harvested at 0, 6, 12, 24, 36, 48, 60, 72, 96, and 120 hpi and immediately frozen in liquid nitrogen and stored at –80 °C prior to extraction of total RNA. Following the same inoculation method, leaves of T₁ generate transgenic plants and ZhengZhou 5389 were harvested at 0, 12, 24, 48, 72, 96, and 120 hpi and stored at –80 °C for extraction of total RNA. Young leaves, young

stems, and young roots collected from noninfected *TcLr19* were used for spatial expression analyses.

SA/ABA Treatments

Fifty micromolars SA or ABA solution was sprayed on the leaf surface of seedlings with one mature leaf and one new leaf until the solution drops down. Wheat leaves were harvested at 0, 12, 24, 48, 72, and 96 h after SA or ABA solution treatment and immediately frozen in liquid nitrogen and stored at -80°C for total RNA extraction.

Temporal and Spatial Expression Profiles of *TcLr19PR1* Gene

Total RNA was extracted from infected wheat leaves at different time points from 0 to 120 hpi and noninoculated wheat leaves, stems, and roots according to the protocol of the Triazol reagent. Subsequently, they were treated with amplification-grade DNase I (TaKaRa, Japan) to remove any residual DNA, and reverse-transcript PCR was performed using SuperScript II reverse transcriptase, oligo(dT)12–18, and RNase OUT (Invitrogen). Synthesized complementary DNA (cDNA) was treated with RNase H and used as the template for quantitative reverse transcription PCR (qRT-PCR). Amplification, detection, and data analyses of qRT-PCR were performed with the iCycler IQ real-time detection system (Bio-Rad, Amsterdam, Netherlands). A pair of *TcLr19PR1*-specific primers (q*TcLr19PR1*-F: 5'-CAATAA CCTCGGCGTCTTCATC-3'; q*TcLr19PR1*-R: 5'-ATTTAC TCGCTCGGTCCCTC-3') was designed and synthesized based on the sequence obtained from RT-PCR. Wheat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as a normalization factor for equal RNA concentrations. A pair of primers for GAPDH gene (qGAPDH-F: 5'-CTGCCCTTGCTCGTCTTGCTAA-3'; qGAPDH-R: 5'-CTTGATGGAAGGACCATCAAC-3') was designed based on the deposited wheat GAPDH sequence (GeneBank ID: AF251217). PCR reaction system was as follows: 12.5 μL 2 \times TransStartTM Green qPCR SuperMix (TransGen Biotech), 2.0 μL cDNA, 0.5 μL of each primer (10 μM) in a total volume of 25 μL . PCR was performed according to the following amplification procedure: an initial denaturation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. Quantification of the target gene was assessed by relative standard curves. The $2^{-\Delta\Delta\text{CT}}$ (Thomas and Kenneth 2008) method was employed to quantify the relative gene expression. Three independent biological replicates were performed for each sample.

Vectors Construction

TcLr19PR1 was amplified from *TcLr19* DNA by PCR using the primer pairs (*TcLr19PR1*-F-*Sma*I: 5'-TCCCCCGGGCGA AACGAGAATG-3'; *TcLr19PR1*-R-*Sac*I: 5'-CGAGCTCG TGTGCCATAGAAAGGCTCAT-3'). The amplified fragment was cloned to the pEGM-T vector following the manufacturer's protocol. Subsequently, the obtained construct pEGM-T:: *TcLr19PR1* was digested with *Sma*I and *Sac*I. Meanwhile, the binary expression pAHC25, driven by the strong *ubi* promoter, was digested with *Sma*I and *Sac*I as well. The digested *TcLr19PR1* fragment was inserted into the digested pAHC25 vector. Consequently, the vector pAHC25:: *TcLr19PR1* was generated.

Particle Bombardment-Mediated Wheat Transformation

The generated vector pAHC:: *TcLr19PR1* was transformed into immature embryo-derived calli of a susceptible wheat cultivar ZhengZhou 5389 using particle bombardment, following the method described by Wang et al. (2006). After several rounds of regeneration, calli were transferred to the differentiation medium for 1–2 weeks when adventitious buds were formed. When bud seedlings reach 3 cm, they were transferred to rooting and strengthening medium. Subsequently, the containers with open lid in which seedling has above 7 cm in height and over 5 cm in root length are moved to greenhouse for adaptation for 3 days. After that, the seedlings were transferred to soil. The advantages of cultivar 'Zheng-Zhou 5389' that was used for transformation are because it does not have leaf rust resistance gene *Lr19* and is efficiently transformed by particle bombardment.

Screening of T_0 and T_1 Transgenic Plants and Histological Observation

To test the transgenic plants, a single leaf from each plant was harvested. Subsequently, DNA and RNA were extracted using a modified CTAB method (Saghai-Marooof et al. 1984) and RNeasy kits, respectively. PCR screening for the presence of the transgenic gene was performed using the right primer of the *bar* gene and the left primer of *TcLr19PR1* gene (*TcLr19PR1*-F: 5'-TCCCCCGGGCGAAACGAGAATG-3'; *Bar*-R: 5'-AAACCCACGTCATGCCAGTTC-3'). Fourteen-day old transformed plantlets (T_0 and T_1) and ZhengZhou 5389 (approximately three- to four-leaf stage) were inoculated with leaf rust race (FHJT) following the method described earlier. Incidence of leaf rust was scored following Roelfs' (1985) standard after inoculation of 12–14 days, and pictures were photographed as well.

T_0 plants were bagged and selfed seeds were harvested. Between 5 and 20 T_1 plants for each T_0 plant were grown, inoculated, and scored as previously described. Tissue

sampling for RNA and DNA extraction, PCR procedures were the same as for the T_0 plants. The fourth leaves were collected at 6, 12, 24, 48, 72, and 120 hpi for histological observation. The infected leaves were examined by a rapid staining method using a protein-specific dye (Coomassie Brilliant Blue R-250). Stained leaf segments were examined with an Olympus BX-51 microscope (Olympus Corp., Tokyo) for infection sites and lengths of infection hyphae. Percentage of germinating spores was calculated by counting about 500 spores measured by ProgResCapturePro2.6 software. Fifty infection sites were examined on each randomly selected leaf segment pretreatment. Procedures and methods including identification of successful infection sites, hyphae staining, and measurement of fungal structures followed the method described by Wang et al. (2007).

Preparations for *TcLr19PR1* Recombinant Protein and Its Antibody

Isolation of total RNA and synthesis of the first-strand cDNA were performed above. The ORF of *TcLr19PR1* gene without signal peptide was amplified by PCR with the special primers: *TcLr19PR1*-F: 5'-CGCGGATCC AACTCGCCTCAGGACT AC-3', and *TcLr19PR1*-R: 5'-CCC AAGCTTTTAGTATGGT TTCTGTCCAATGAT-3'. PCR products were cloned into plasmid expression vector pEASY-E1 according to the instructions of pEASY-E1 expression kit (TransGen). Recombinant plasmid pEASY-*TcLr19PR1* were transferred to *Escherichia coli*-competent cell DH5 α . The positive recombinant plasmid pEASY-*TcLr19PR1* which had the correct connection direction was identified by PCR with the special primers *TcLr19PR1*-F and T7 terminator (5'-TAGTTATTGC TCAGCGGTGG-3'). After sequencing identification, positive recombinant plasmids were transferred into *E. coli* strain BL21 (DE3). Single colony was cultured in liquid Luria-Bertani (LB) medium (with 50 mg/mL Ampicillin) at 37 °C overnight and used to inoculate a new LB liquid medium until the culture reached an optical density of 0.5–0.8 at a wavelength of 600 nm in a spectrophotometer. Different concentrations of isopropyl b-D-thiogalactopyranoside (IPTG: 0, 0.01, 0.1, 0.3, 0.5, 0.8, and 1.0 mmol/L) and induction times (2, 4, 6, 8, 10, and overnight) were used to determine the optimum conditions for producing the target protein. The expression of recombinant protein was induced at the optimal condition and purged with the Ni-Agarose HIS trap column (TransGen). The targeted protein were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 15 % polyacrylamide gels and stained with Coomassie brilliant blue R-250. Preparation and purification of the antibodies were entrusted to the Hebei Academy of Sciences Institute of Biology.

Immunodetection of *TcLr19PR1* Recombinant Protein

The wheat proteins were extracted from the leaves inoculated with leaf rust race 07-10-421-3, respectively, at 0, 24, 48, 72, 96, 120, 144, and 168 hpi. One constitutively present band in the Ponceau S staining was used to normalize the protein concentration in the samples. For Western blotting, fusion protein and wheat proteins were subjected to 15 % SDS-PAGE and transferred to a PVDF membrane. After blocking the membrane using 3 % BSA in Tris-buffered saline (TBS) buffer at room temperature for 1 h, the membrane was incubated with 1:10,000 diluted polyclonal antibodies of *TcLr19PR1* and 1:3,000 diluted goat anti-rabbit IgG antibody conjugated with alkaline phosphatase for 1 h at room temperature. The membrane was washed with 1 \times TBS, 0.1 % Tween-20 for four times at room temperature. Bound antibodies were visualized by color development for NBT formazan. When the color developed to a desirable intensity, the reaction was stopped by washing the membrane in deionized water. Image J software was used to scan the signal intensity and calculated the average and standard deviation among three repeats of WB analysis.

Results

Expression Analysis of Wheat *TcLr19PR1* in Wheat Tissues

Real-time qRT-PCR specific primers were first tested with regular PCR to amplify the *TcLr19PR1* gene using cDNA as a template in order to determine gene-specific amplification. A single 165-bp PCR fragment was amplified and confirmed by sequencing to be the expected fragment of the *TcLr19PR1* gene (data not shown). The primers were then used in qRT-PCR analyses to determine the gene expression profiles of *TcLr19PR1* in different wheat tissues. Gene expression level of *TcLr19PR1* was measured relative to *GAPDH* expression in different tissues of seedling, including roots, stems, and leaves. Gene expression level of *TcLr19PR1* showed significant variations among different plant tissues. More transcripts of *TcLr19PR1* gene accumulated in leaves and stems than that in roots, and the accumulation of *TcLr19PR1* transcripts in leaves was 3.5 times higher than that in roots and 2.0 times than that in stems (Fig. 1).

TcLr19PR1 is Significantly Induced Upon Leaf Rust Pathogen

To investigate the expression profile of *TcLr19PR1* in response to the infection by leaf rust pathogen, a more precise analysis of the expression using qPCR was performed. The transcripts quantity of *TcLr19PR1* in incompatible interactions

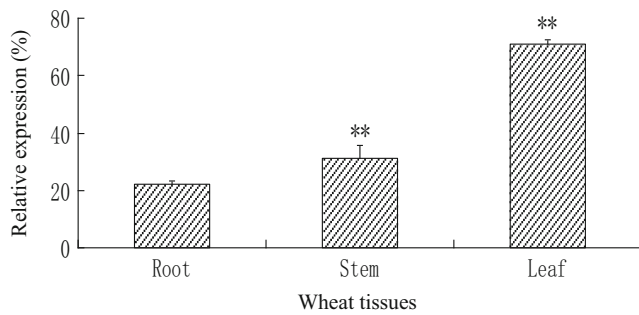


Fig. 1 Expression profiles of the *TcLr19PR1* gene in different wheat tissues. The y-axis indicates the amount of wheat *TcLr19PR1* transcript is normalized to the *GAPDH* gene and express relative to the transcripts of the noninoculated control. The x-axis indicates the different wheat tissues. Error bars represent variation among three biological replicates. Reference gene is *GAPDH*; ** $p < 0.01$; * $p < 0.05$; $n = 3$

was detected at a low level at 0 hpi, then increased at 6 hpi, and peaked at 12 hpi (about 10-fold more expressed than the noninoculated control). The transcript levels then declined sharply to a relatively low expression from 18 to 24 hpi. At 60 hpi, there was a second expression peak that was lower than the first and 6-fold than the noninoculated control (Fig. 2). In compatible interactions, the *TcLr19PR1* transcripts maintained at the lower levels were compared with noninoculated control at most time points. However, there were also expression peaks at 60 and 72 hpi, and then declined (Fig. 2). The accumulation of *TcLr19PR1* transcripts in compatible interaction at 60 hpi was 1.9 times higher than that at 0 hpi. However, the expression levels in incompatible interactions were higher than that in compatible interactions except at 72 hpi, which was consistent with the result of semiquantitative PCR obtained by Wang (Wang et al. 2012).

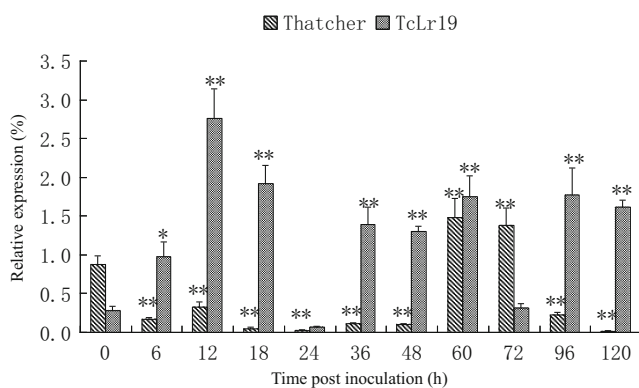


Fig. 2 Expression profiles of the *TcLr19PR1* gene in Thatcher and TcLr19 leaves infected by the avirulent *P. triticina* isolate 07-10-421-3 at different postinoculation times. The y-axis indicates the amount of *TcLr19PR1* transcript normalized to the *GAPDH* gene and express relative to that of noninoculated control, and the x-axis indicates sampling times. Error bars represent variation among three biological replicates. Reference gene is *GAPDH*. ** $p < 0.01$; * $p < 0.05$; $n = 3$

Expression Analysis of *TcLr19PR1* Induced by Signaling Molecules

The expression profile of *TcLr19PR1* transcripts following the treatments of ABA and SA was investigated by qPCR respectively (Fig. 3). The *TcLr19PR1* transcripts reached the peak level at 12 hpi rapidly after ABA treatment, then declined, and reached another peak level at 96 hpi. The accumulation of *TcLr19PR1* transcripts at 12 hpi was 3.1 times higher than that at 0 hpi. For SA treatment, the *TcLr19PR1* transcripts were detected at low level from 0 to 48 hpi, and suddenly peaked at 72 hpi (about 5.4 times higher than control), then declined. There were obvious differences in expression times between ABA and SA induction, but the accumulation of *TcLr19PR1* transcripts were similar. The results showed that *TcLr19PR1* gene could be induced by signaling molecules ABA and SA, thus we hypothesized that the expression of wheat *TcLr19PR1* gene might be regulated by SA and ABA signaling pathway.

Identification of Resistance to Leaf Rust in Transgenic Wheat

To further assess the role of *TcLr19PR1* in responses to the leaf rust infection, transgenic plants were generated using particle bombardment-mediated transformation on susceptible wheat cultivar Zhengzhou 5389. Upon inoculation of T_0 plants with leaf rust *P. triticina* race FHJT, all lines did not display an obvious resistant phenotype, but the severity of the infection was lessened compared with the control group (Table 1). To determine the presence of *TcLr19PR1* gene in T_0 transgenic wheat plants, 20 transgenic plants were subjected to PCR analysis using the forward primer of *TcLr19PR1* gene and the reverse primer of the *bar* gene. A 3,000-bp DNA fragment as expected was amplified from 3 T_0 transgenic plants and positive control (pAHC-*TcLr19PR1*), which was not amplified from the negative control (Zhengzhou 5389 and pAHC25) (Fig. 4a). The ultimate transformation efficiency

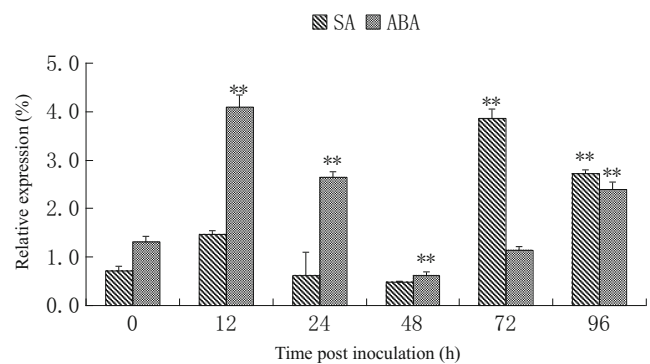


Fig. 3 Expression profiles of the *TcLr19PR1* gene in TcLr19 leaves treated by the SA and ABA at different postinoculation times. The y-axis indicates the amount of *TcLr19PR1* transcript normalized to the *GAPDH* gene and express relative to that of noninoculated control, and the x-axis indicates sampling times. Error bars represent variation among three biological replicates. Reference gene is *GAPDH*; ** $p < 0.01$; $n = 3$

Table 1 Segregation analysis of the transgenic plants produced by transforming *TcLr19PR1* into cultivar Zhengzhou 5389

T ₀ plant	Number of T ₁ plants analyzed	PCR-positive/negative plants	Segregation observed	Segregation expected	χ^2 value	Likelihood (<i>P</i>) according to χ^2 value
T ₀ -1	40	26:14	1.9:1	3:1	1.64	>0.50
T ₀ -2	43	30:13	2.3:1	3:1	0.37	<0.50
T ₀ -3	45	31:14	2.2:1	3:1	0.60	>0.50
Total	128	87:41	2.1:1	3:1	3.00	>0.50

was 0.58 % (the proportion of positive plants of callus from immature embryos). A total of 87 positive plants with *TcLr19PR1* gene were obtained among 128 T₁ transgenic plants derived from the 3 T₀ transgenic plants (Table 1).

To test the T₁ plants resistance to leaf rust, the individual 87 positive plants, wheat-leaf rust resistance NILs TcLr19, and susceptible cultivar Zhengzhou 5389 were used for infectivity analysis after inoculation with *P. triticina*, and different degrees of infection (severity) were visualized on the surface of leaf blades on 10 dpi according to susceptible and resistant behavior of different plants. The results showed that TcLr19 did not show any infection whereas Zhengzhou 5389 started appearing infection courts within 4 dpi and scattered pustules surrounded by pale-halo region of chlorosis on the surface of leaf. Compared with the control group, the occurrence of disease symptoms of positive transgenic plants was postponed and the severity of the infection was lessened, which was reflected by less leaf rust uredinia (Fig. 4b). Furthermore, percentage of germinating spores was measured by microscopic observation and found that the germination of spores

on transgenic plants was inhibited compared with those in Zhengzhou 5389 (Fig. 5), and the percentage of germinating spores in Zhengzhou 5389 was much higher than that in TcLr19 and transgenic plants especially at 24 hpi (Table 2). In addition, specific infection structures of leaf rust fungi were analyzed, including the length of hyphae, the number of hyphal branches, and the formation of haustorial mother cells. The fungal hyphal lengths were shorter ($p < 0.01$) compared with those in the control treatment (Zhengzhou 5389) from 12 to 120 hpi, and the numbers of hyphal branches were lower ($p < 0.01$) compared with those in the control treatment (Zhengzhou 5389) at 12 hpi (Table 2; Fig. 5). The delayed symptoms implicated that *TcLr19PR1* gene has the role against wheat-leaf rust infection to some extent, reflected by reducing the severity of the infection.

Accumulation of *TcLr19PR1* Protein Is Strongly Increased in the Transgenic Lines Upon Leaf Rust Pathogen Infection

To detect the accumulation level of *TcLr19PR1* protein in transgenic lines, polyclonal antibody was raised in rabbits against His-tagged *TcLr19PR1* protein that was heterologously produced in *E. coli*. SDS-PAGE showed that the MW of the recombinant protein was about 16 kDa (Supplementary Fig. 1), which is consistent with the deduced amino acids composition of *TcLr19PR1* and with the related sequence on pEASY plasmid. The ELISA result indicated that the antibody concentration of the antiserum reached 1:768,000, which was sufficient for further experimentation. The generated antibody was then used to detect the accumulation level of *TcLr19PR1* in one of the transgenic lines that showed the significant increase of *TcLr19PR1* upon rust pathogen challenge. As shown in Fig. 6, application of the *TcLr19PR1* antibody resulted in a 16-kDa signal band detected in the positive at the expected height for *TcLr19PR1*, in the transgenic plant and its parental line Zhengzhou 5389 infected by leaf rust from 0 to 168 hpi. It was obvious that the signal intensity of *TcLr19PR1* band in the transgenic line steadily increased from 0 to 120 hpi, then declined at 144 hpi. However, in parental line Zhengzhou 5389, a much weaker signal was detected compared with the transgenic line. Notably, the accumulation of *TcLr19PR1* is consistent and maintained similar levels from 0 to 168 hpi in parental line as well. These results strongly

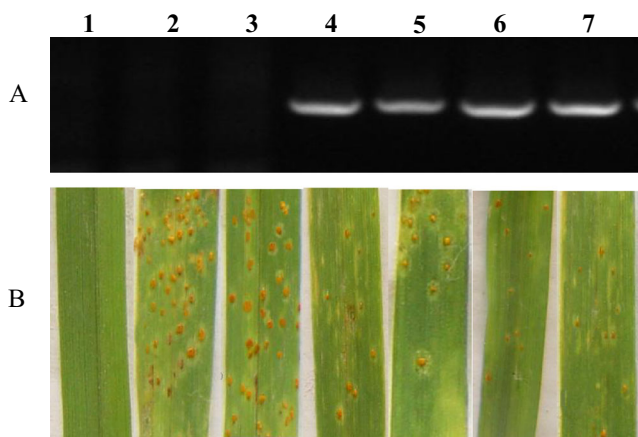
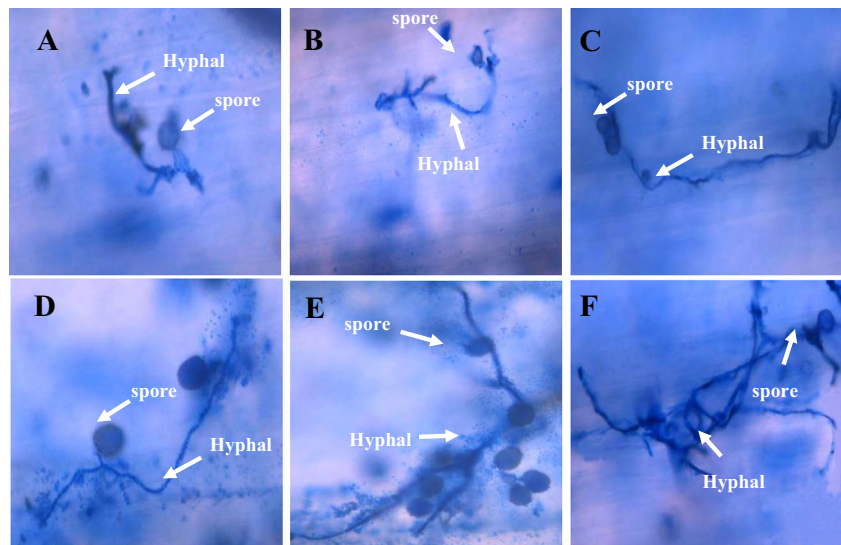


Fig. 4 Transgenic plants showed segregation for resistance to leaf rust. **a** PCR amplification of genomic DNA using the forward primer of *TcLr19PR1* gene and the reverse primer of the *bar* gene. *TcLr19* T₁-negative transgenic plants derived from T₀ lines and susceptible control could not amplify the aim band and all T₁-positive transgenic plants could amplify the aim band. **b** Leaf samples showing typical rust symptoms 14 days after inoculation with *Puccinia triticina* race (FHJT). 1, TcLr19 high resistant control; 2, Zhengzhou 5389 high susceptible control; 3, T₁-negative transgenic plants derived from T₀ lines; 4–7, T₁-positive transgenic plants derived from T₀ lines

Fig. 5 Histological observation of wheat leaves infected with *Puccinia triticina*. Observation of hyphal and germinating spores (a–c at 24 hpi and d–f at 48 hpi; a, d TcLr19; b, e transgenic positive plants; c, f Zhengzhou 5389)



implied that accumulation of *TcLr19PR1* protein was correlated with resistance against leaf rust infection that has been demonstrated in Fig. 4.

Discussion

Previous studies showed that *PR1* expression is activated by various biotic and abiotic stresses, such as pathogen infection, salt, drought, and heavy metals (Mitsuhashi et al. 2008; Thierry et al. 1995). For example, *PsPR1* obtained from leaves of *P. suffruticosa* was significantly induced after *C. canadense* inoculation, and its expression peaked in 24 hpi, which implicated that *PsPR1* might be involved in the disease defense (Yang et al. 2013). Similarly, *VvPR1* isolated from leaves of *Vitis vinifera* was highly induced by *Plasmopara viticola* inoculation and expression level of *PsPR1* reached the highest peak at 24 hpi, suggesting that *VvPR1* might be related to

biotic stresses (Hou et al. 2012). Our present studies showed that the expression of *TcLr19PR1* was highly induced by leaf rust pathogen in incompatible interaction in contrast to compatible interaction at the onset stage of the infection (Fig. 2). However, the expression of *TcLr19PR1* displayed certain variations. It was hardly detected at 24 hpi in both interactions, but accumulation of *TcLr19PR1* transcripts peaked for the second time at 60 hpi. All in all, there was a consistent trend that significantly higher and rapid accumulation of *TcLr19PR1* transcripts in the incompatible combination compared with the compatible combination at most observed time points. The expression profiles of *TcLr19PR1* were similar to the previous studies though the time points of gene expression were slightly different, which may be potentially caused by individual features of independent plant or pathogen that was investigated.

About the tissue-specific expression of *PR1* gene, the real-time PCR analysis indicated that the expression level of *PsPR1* obtained from leaves of *P. suffruticosa* was the highest

Table 2 Histological observation of infection development between transgenic plants and *P. triticina*

Plant materials	Observed spore	Percentage of germinating spores (%) ^a		Hyphal length ^b				Hyphal branch ^c
		6 hpi	24 hpi	12 hpi	24 hpi	48 hpi	120 hpi	72 hpi
TcLr19	535	3	72	12 hpi	24 hpi	48 hpi	120 hpi	2.3
Zhengzhou 5389	485	8	93*	231.9**	244.6**	351.7**	510.5*	5.3**
Positive plants	511	6	86	41.3	50.4	246.1	414.5	3.7

Analysis of significance was calculated according to paired sample *t* test method with SPSS software. *n*=3

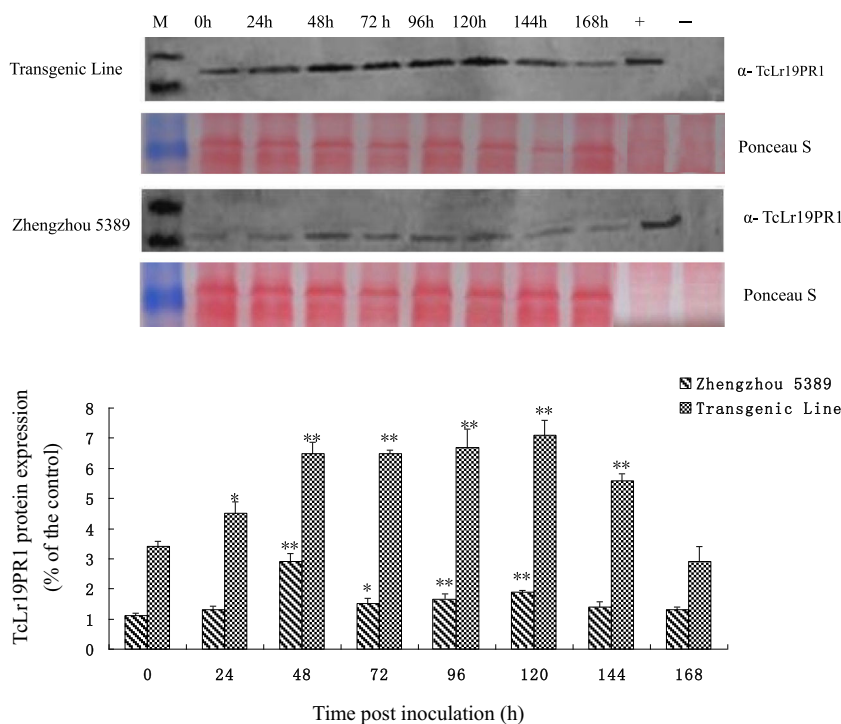
***p*<0.01; **p*<0.05

^a Percentage of germinating spores was calculated by counting about 500 spores measured by ProgResCapturePro2.6 software

^b Average length of hyphal growth measured from the base of substomatal vesicles to hyphal tips from at (unit in μm , measured by ProgResCapturePro2.6 software)

^c Average number of hyphal branches counted from at least 50 infection sites

Fig. 6 Accumulation of *TcLr19PR1* protein in transgenic plant upon leaf rust infection. One of transgenic lines and its parental line Zhengzhou 5389 were inoculated with leaf rust pathogen and the protein was sampled from 0 to 168 hpi. Western blot was probed with the *TcLr19PR1* antibody. The purified *TcLr19PR1* protein was used as a positive control (+). Plasmid pEASY was used as a negative control (-). Lower panel, one constitutively present band in the Ponceau S staining was used to normalize for loading variations. Bar graphs are summary data of normalize densitometric ratio



in sepal but the lowest in leaves (Yang et al. 2013). In this study, the tissue organ-specific expression profiles of the *TcLr19PR1* showed that *TcLr19PR1* is expressed in leaves, stems, and roots, but its transcripts were detected abundantly in leaves and weakly stems in and roots. *TcLr19PR1* was widely distributed in different tissues but differentially expressed in these tissues, which is in accordance with the research conducted by Tornero on *PR1* gene from tomato (Tornero et al. 1997); *TcLr19PR1* was constitutively expressed, which suggested that *TcLr19PR1* directly interacted with pathogen and formed a barriers to tackle the invasion of pathogen together with other PRs.

Extensive studies have shown that phytohormones, such as SA and ABA, played important roles in adaptation to the biotic and abiotic stresses and can induce expression of *PR1* (Sabater et al. 2010; Le et al. 2009). SA has received particular attention to function as a signal molecule in acquired resistance to pathogens in several plant species, resulting in the synthesis of certain PRs and SAR (Yin and Hou 2007). ABA has durable roles not only being involved in abiotic stress responses but also plays an important role in adaptation to various biotic stresses. ABA is known to function in response to biotic stresses through cross-interaction with SA, JA, and ET in signal transduction pathway, thus conferring resistance of plants against adverse environment. Many elicitors enable the induction of *PR1* in plant, but differential elicitors are required for the *PR1* induction in individual plant species (Lorenzo et al. 2003; Nakano et al. 2006). *VvPR1* from *Vitis vinifera* was induced by SA and ABA, and the response to ABA was earlier than SA (Hou et al. 2012). Our studies

showed that *TcLr19PR1* was significantly induced by signaling molecules such as SA and ABA, but differences of induction time were observed in expression of *TcLr19PR1*, which was consistent with the previous studies (Hou et al. 2012). Furthermore, the induction of *TcLr19PR1* by ABA was earlier than SA, implying that ABA might be an upstream signaling molecule to activate the downstream signaling SA, which led to significant difference in expression pattern of *TcLr19PR1*.

Due to the longer time consumption of conventional wheat breeding and of map-based cloning of resistance genes being challenged by huge wheat genome, it is essential to identify and exploit new genetic resources such as resistance-related genes that exhibit effective capability to alleviate disease. Molecular and genetic studies of wheat-leaf rust have been carried out by wheat researchers worldwide. This study was focused on elucidating the behavior of the defense gene, *TcLr19PR1*, in susceptible and resistant plants during leaf rust infection, as well as the role of leaf rust-resistance gene *Lr19* during wheat-leaf rust fungus interaction. In order to get insight into the role of *PR1* in wheat growth and resistance to leaf rust pathogens attack, the expression of *TcLr19PR1* and its function were analyzed. The results indicated that the expression level of *TcLr19PR1* is much higher in the resistant plant than in the susceptible one after leaf rust pathogen challenging, SA treatment, and ABA treatment. Analysis of T₁-positive transgenic plants showed challenged with leaf rust pathogen indicated that *TcLr19PR1* gene was involved in the wheat defense via attenuating the pathogen infection process, and Western blotting results correlated with the phenotype due to the interaction of the gene product. However, the

segregation analysis of the putative transgenic plants could not be reflected by obvious phenotype due to *TcLr19PR1* belonging to the defense gene and not to the resistance gene, so we have to obtain more generation of the transgenic plant used to confirm homozygous in order to further study *TcLr19PR1* gene contributing to the defense against *P. triticina* infection. In future experiments, we should exploit more *PR* genes from wheat to broaden our understanding toward the role of *PR* genes in plant defense against pathogen attack.

Acknowledgments This study was supported financially by the Natural Science Foundation of Hebei Province (no. C2012204005) and National key Basic Research Program of China (no. 2013CB127700).

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