Characteristic expression of wheat *PR5* gene in response to infection by the leaf rust pathogen, *Puccinia triticina*

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RESEARCH ARTICLE

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TaLr35PR5 gene was obtained from the gDNA and cDNA of TcLr35 wheat. It was induced by *Puccinia triticina*, ABA and SA, but *TaLr35PR5* was induced earlier and its expression level was higher in the incompatible interaction than that in the compatible interaction. In addition, the accumulations of *TaLr35PR5* increased stably and showed significant peak challenged by *P. triticina* at different growth and development periods of TcLr35 wheat while it maintained similar level and changed little in mock inoculated. Western blottings were conducted to confirm that TaLr35PR5 be induced by *P. triticina* infection at the protein expression level. Similar to the expression patterns of *TaLr35PR5* at RNA levels, the accumulations of TaLr35PR5 protein were weak in the seedling stage, then increased to the peak and kept constant levels at the mature stage which is consistent with the expression feature of *Lr35* gene as an adult plant resistance gene.

Keywords: pathogenesis-related protein 5; *Puccinia triticina*; adult plant resistance; expression profiles; western blotting

1. Introduction

Wheat (*Triticum aestivum* L.) leaf rust caused by *Puccinia triticina* is one of the most serious diseases worldwide, leading to considerable losses and causing pandemic in the short term in wheat production. Presently, wheat leaf rust disease has attracted worldwide attention, and various researches had been conducted on wheat symptoms, epidemiology and physiological races of the *P. triticina* fungus. 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, single R gene tends to be quickly overcome by changes in the *P. triticina* population. More durable resistance can be achieved by gene pyramiding, the stacking of multiple leaf rust genes, such as the Canadian cultivar Pasqua (Liu and Kolmer 1998; Kolmer 2001). Therefore, the application of transgenic technology to realize directional improvement and molecular assisted breeding technology linked to target trait provide an alternative way to obtain resistant varieties, which can overcome the above-mentioned problems related to conventional breeding.

To survive and adapt to their native surroundings, plants develop the mechanism that detect and rapidly respond to invasions of phytopathogens and arthropods with coordinated and/or integrated gene expression. Resistance genes and defense response gene are two main types of genes mediating defense responses. In host-pathogen interactions, various novel proteins, referred to as pathogenesis-related proteins (PR), are induced systemically and accumulated locally both at the site of infection and in distal parts of the plants, leading to development of the hypersensitive reaction (HR) and systemic acquired resistance (SAR) (Hammond-Kosack and Jones 1996). PR proteins are defined as proteins coded by the host plant but induced specifically in pathological or related situations (Hamamouch et al. 2011). Induction of PRs has been found in many plant species belonging to various families, PR1–PR17 (Jan et al. 2008), playing important roles in the resistance of the host against the pathogen and biotic stress conditions. Meanwhile, an increasing body of research suggests that they exist as constitutive components in different plant organs, irrespective of stress conditions (Borad and Sriram 2008). Analysis of the expression patterns of PR genes would be useful to understand their possible functions and for use as characteristic markers in studies on plant defense reactions.

In different families of PRs, PR5 family is known as thaumatin-like proteins (TLPs) because the amino acid sequences of these proteins exhibit extensive homology with that of thaumatin, a sweet-tasting protein derived from *Thaumatococcus daniellii* (Eden et al. 1982). PR5 proteins of the high molecular weight group exhibit antifungal activities and are found in cell vacuoles. The smaller TLPs located in the extracellular are also involved in plant defense against fungal infection (Chan et al. 1998; Wang et al. 2013), their amino acid sequences share a certain degree of homology, and the research on the proteins encoded by PR5 is a 'hot spot' in the area of resistance of plant. *PR5* genes have been identified from many plants, such as *Prunus domestica* (El-kereamy et al. 2011), *Brassica rapa* (Ahmed et al. 2013), corn (Liu et al. 2011), poplar (Lei et al. 2012), and so on, and proved that PR5 protein could obviously improve plant disease

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resistance. For example, over expression of thaumatin-like protein gene from *Pyrus pyrifolia* and in tobacco increased resistance to *pathogenic fungito* (Liu et al. 2012); rice TLP-D34 enhanced resistance to the sheath blight pathogen *Rhizoctonia solani* (Datta et al. 1999); rice TLP enhanced resistance to the head blight pathogen *Fusarium graminearum* (Mackintosh et al. 2007). Furthermore, expression changes in fungal-related genes between compatible and incompatible fungal strains (rust fungus *Melampsora larici-populina*) in *P. trichocarpa* 9P. deltoides 'Beaupre' indicated that the gene could strongly induced transcript coding PR-5 protein showed a 28.8-fold induction based on cDNA microarray (Rinaldi et al. 2007). Dharmendra Singh et al. (2012) examined 5 target genes encoding for chitinase 3, β-1, 3/1, 4 glucanase, thaumatin-like protein, peroxidase 2 mitogen activated protein kinase 1 to unravel their coordinated action during compatible and incompatible interaction, and attributed the differences to the presence of seedling leaf rust resistance *Lr28* gene, which facilitated prevention of leaf rust infection in resistant wheat plants.

Although many *PR5* genes have been cloned and reported to be involved in plant disease resistance responses and SAR, the role of *PR5* genes in resistance in wheat to leaf rust is not fully understood and so far there isn't any report to prove that PR5 be related to the wheat growth and adult plant resistance (APR). Wheat leaf rust resistance gene, *Lr35*, provides an important source of partial resistance that is expressed in adult plants during the critical grain-filling stage and is the most effective in flag leaf. Understanding the molecular nature of this kind of resistance has important implications for long-term control of rust and other diseases. Previous studies showed that the gene was originally introgressed into chromosome 2B from *Aegilops speltoides*, a diploid relative of wheat (Kerber and Dyck 1990), and localized between the two markers BCD260 and UBC836 by RFLP and ISSR (Seyfarth et al. 1999). As this gene has not yet been used in wheat breeding programs, it is still effective against wheat leaf rust worldwide. Its resistance expression starts from the second leaf stage, fully expressed till six leaf stage, and it is a very effective APR gene, also known as 'slow rusting' confers a broad spectrum of durable resistance. In this study, we isolated a *PR5* gene, designated *TaLr35PR5,* from wheat near-isogenic lines TcLr35 infected by *P. triticina*. To further identify the relationship between *TaLr35PR5* and *Lr35*-mediated resistance response to leaf rust, the expression profiles of *TaLr35PR5* induced by pathogen, SA and ABA were measured temporally and spatially in wheat tissues at the transcript and protein levels. These results will provide systematic dada for further study the function and the mechanism of *TaLr35PR5* in defensing adversity stress.

2. Materials and methods

2.1. Plant material, inoculation system and ABA/SA elicitor pre-treatments

Wheat near-isogenic line TcLr35, Thatcher and leaf rust race 07-10-426-1(FHNT) were the biological materials used for gene cloning and expression analyses. Based on the gene-for-gene model, wheat TcLr35 was resistant to leaf rust race 07-10-426-1 and showed incompatible reaction (type 1). But, Thatcher was susceptible to leaf rust race 07-10-426-1 and showed compatible reaction (type 4) according to Roelfs standard (1984). The plants were grown under ideal conditions of temperature (20° C), relative humidity (80%), and light periods (16 h day with 350 lux light, 8h dark periods) in the greenhouse. Wheat seedlings were grown in the greenhouse about 4 months when up to mature-plant stage. Fresh uredospores collected from the susceptible wheat cv. Zhengzhou5389 were applied to the surface of the primary leaves of TcLr35 and Thatcher with a brush. After inoculation, the plants were kept at 100% relative humidity environment in the dark for 24 hours at 20°C, and then moved to the greenhouse. Inoculated wheat leaves were excised at 0, 6, 24, 36, 48, 72, 96, and 120 hour post inoculation (hpi) for expression analyses in the compatible interaction and incompatible interaction. Inoculated wheat leaves, root and stem were excised at 0, 12, 24, 36, 48, 72, 96, and 120 hpi for temporal and spatial expression analyses when the adult stage. Wheat leaves at different growth periods including seedling stages [one-leaf stage (1 week), two-leaf stage (2 weeks), trefoil stage (3–4 weeks)], tillering stage (4–5 weeks), booting stage (10–11 weeks), flowering stage (13–15 weeks) and mature stage (16–18 weeks) were inoculated at expression peak (96 hpi). Leaves samples collected at 0, 12, 24, 48, 72, 96, 120, 144, and 168 hpi were used to analyze the expression profiles at protein levels while samples at expression peak (144 hpi) were used to analyze the accumulation of TaLr35PR5 at different growing stages of wheat, and noninoculation treatment (inoculation with water) as control.

According to the method of Zambounis et al. (2012) and our pre-experiments, the adult wheat leaves were drenched with 50μmol/L SA and 50μmol/L ABA prior to leaf rust pathogen inoculation. Tween 20 (0.01%) was added as a surfactant (Fisher Scientific, Nepean, ONT, Canada). Afterward, the seedlings were placed on a greenhouse bench at 20°C for three days and then inoculated with *Puccinia triticina*. All samples collected at 0, 12, 24, 48, 72, and 120 hpi were immediately frozen in liquid nitrogen and stored at −80°C prior to the extraction of total RNA.

2.2. Cloning and expression of TaLr35PR5

Genomic DNA (gDNA) was extracted from leaves of wheat TcLr35 using a modified CTAB method (Saghai-Maroof et al. 1984). Total RNA was extracted from wheat leaves at different time points from 0 to 120 hpi according to the protocol of the Triozol reagent. Subsequently, they were treated with amplification grade DNaseI (TaKaRa, Japan) to remove any residual DNA and reverse-transcript PCR was performed using Super-Script II reverse transcriptase, oligo(dT)12–18, and RNAse OUT (Invitrogen), then gDNA and cDNA were used as the templates for PCR using primer pairs

TaLr35PR5-F(5′-CCCAAGCTTTCAGAAGCTGATGG
GGTAGAC-3′) and TaLr35PR5-R (5′-CGGGATCCT
GGGTCAGGAGCAACATCCAG-3′) designed accord-
ing to the conserved domains of *PR5* gene (Gabriela
et al. 1991). PCRs were done on a thermal cycler
(Applied Biosystems 2720, ABI, Foster City CA) with
the following systems: 2.5 μL buffer, 0.5 μL dNTP,
0.3 μL Taq, 1.0 μL cDNA, 1.0 μL of each primer
(10 μmol/L) in a total volume of 25 μL. PCR was
performed according to the following amplification
procedure: an initial denaturation step at 95°C 1 min,
followed by 35 cycles of denaturation at 94°C 1 s,
annealing at 61.6°C 30 s and extension at 72°C 2 min,
sample volume loaded is 5 μL. The PCR products were
detected by an agarose gel electrophoresis and cloned
into pGEM-T easy vector (Sambrook et al. 1998).
Positive clones were sequenced by the Shanghai Gene-
Core BioTechnologies Co., Ltd. Then we use SignalP
(http://www.cbs.dtu.dk/services/ SignalP/) and TMHMM
(http://www.hsls.pitt.edu/obrc/index.) on line tools to
analyze the Schematic structure of *TaLr35PR5* gene.

Total RNA extracted from wheat leaves, steams, and
roots at different time points from 0 to 120 hpi or
different growing periods of wheat was used to analyze
the expression profiles of *TaLr35PR5*. Wheat glyceral-
dehyde 3-phosphate dehydrogenase gene (GAPDH,
GenBank accession No. AF251217) amplified by primer
pairs GAPDH-F (5′-AACTGCCTTGCTCCTCTTGC-3′)
and GAPDH-R (5′-CTGTTGTCACCCTGGAAGTCA-
3′) was selected as an endogenous control to normalize
differences in input RNAs and check efficiencies of
reverse transcription among the various samples. Gene-
tools (SYNGENE, USA) and Statistical Package for the
Social Sciences (SPSS) software (IBM, USA) were used
to calculate the experiment data and analyze the average
and standard deviation respectively in order to get the
expression levels of *TaLr35PR5* relative to noninocu-
lated control. Three independent biological replicates
were performed for each sample.

2.3. Expression of recombinant protein

The ORF of *TaLr35PR5* without signal peptide was
amplified by PCR using the primer pairs YTaLr35PR5-
R (5′-CCCAAGCTTTCATGGACAGAAGGTGATCT
GGTA-3′) and YTaLr35PR5-F (5′-CGCGGATCCATGG
CGACCTCCGCGGTGCTC-3′). PCR products were
cloned into pEASY-E1 vector according to the instruc-
tions of pEASY-E1 expression kit (TransGen). The
positive recombinant plasmid pEASY-TaLr35PR5 was
identified by PCR and transformed 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 inoculated to a new LB liquid medium
until the culture reached an optical density of 0.5–0.8 at a
600 nm wavelength spectrophotometer in order to identify
condition of the optimal protein production, a series of
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 h, and overnight were set.
The expression of recombinant protein was induced at the
optimal condition and pured with the Ni-Agarose HIS trap
column (TransGen, China). The targeted protein were
analyzed by SDS-PAGE with 15% poly-acrylamide gels
and stained with coomassie brilliant blue R-250.

2.4. Antibody preparation and immunodetection

The preparation of antibody was entrusted to the Biology
Institute of Academy of Sciences of Hebei Province.
Purified recombinant protein (2 mg) was injected into
rabbits to produce antibodies. The titer of antibody was
monitored by indirect ELISA. The rabbit antiserum was
collected when the antibody reached a high level. The
proteins of wheat leaves with different treatments were
extracted according to instruction of one step plant active
protein extraction kit (Sangon). For western blotting
analysis, approximately 10μg of protein per lane was
separated using SDS-PAGE and electroblotted to a
PVDF membrane (Sangon) for 45 min at 100 V. The
nonspecific binding sites on the membrane were then
blocked with Blotto. Incubation with the primary anti-
bodies (1:200) was performed in 3% bovine serum
albumin in TBST for 1 h at room temperature followed
by three 10 min washes in TBST. Then the membrane
was incubated with the secondary antibody (1:10,000)
for 1 h at room temperature followed by three 10 min
washes in TBST. The blot was developed using the
SuperSignal Trial kit (Thermo). The heat shock proteins
(HSPs) were used as reference to normalize the protein
concentration in the samples. Gel-pro analyzer software
was used to scan the signal intensity and calculated the
average and standard deviation among three repeats of
WB analysis.

3. Results

3.1. Rust resistance phenotypes

Wheat leaf rust resistance NILs TcLr35 and susceptible
parent cultivar Thatcher 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 at different
growth periods. The mock inoculated plants did not
show any infection whereas Thatcher started appearing
infection courts within 4 dpi and scattered pustules
surrounded by pale-halo region of chlorosis on the
surface of leaf in whole wheat growth stage (Figure 1a,
1b). However, resistant NIL plants TcLr35 showed
necrosis on leaf surface at the adult stage (Figure 1d),
while it showed similar leaf rust symptoms to Thatcher
at the seedling stage (Figure 1c). These results of
infection development and resistance against pathogen
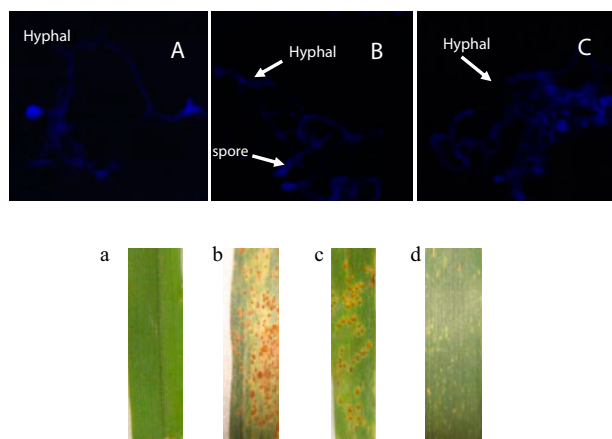at different wheat growth stage respectively warranted
further investigation.

Figure 1. Histological observation and phenotypic expression of wheat leaves infected with *Puccinia triticina*. Higher panels: Observation of hyphal and germinating spores; A: TcLr35 at the adult stage; B: TcLr35 at the seeding stage; C: Thatcher. Lower panels: Phenotypic expression of wheat leaves showing differences in severity of leaf rust symptoms on 10 dpi at different growth period. a: TcLr35 mock inoculated; b: susceptible parent lines Thatcher pathogen inoculated; c: TcLr35 pathogen inoculated at the seeding stage; d: TcLr35 pathogen inoculated at the adult stage.

3.2. Cloning and characterization of TaLr35PR5

A wheat 516-bp homologue of thaumatin-like proteins was identified in wheat TcLr35 cDNA and gDNA and designated as *TaLr35PR5* (GenBank accession number KJ764822, Supplementary Figure 1). The predicated opening reading frame (ORF) of *TaLr35PR5* encoding 171 amino acid protein with a molecular weight of approximately 17.3 kDa. Analysis of the protein domain features indicated that TaLr35PR5 contains thaumatin-like proteins conserved domains. Multisequence alignment showed that TaLr35PR5 is 90%, 92%, 92%, 81%, 70%, 66%, 59%, and 53% identical to *Secale cereale* (AAC83824.1), *Hordeum vulgare* Linn. (P32937.1), *Triticum aestivum* Linn.(P27357.1), *Avena sativa* (P50695.1), *Brachypodium distachyon* (XP_003571622.1), *Oryza* (ACA50508.1), *Setaria italica* (XP_004963267.1) and *Pinus monticola* (S3ADB97933.1). ProtScale software

predict (Kyte and Doolittle 1982) *TaLr35PR5* encoded hydrophobic protein, and Spidey forecast analysis showed that the gene do not contain introns. SignalP 4.1 predict (Nielsen et al. 1997) the *TaLr35PR5* amino acid sequence contain a signal peptide between 1~20 amino acid residues (Supplementary Figure 2).

3.3. Pathogen-induced expression pattern of TaLr35PR5

To investigate the expression profiles of *TaLr35PR5* in response to the infection by leaf rust pathogen, semi-quantitative RT-PCR was performed to analyze the accumulations of *TaLr35PR5* at different treatments. The transcripts quantity of *TaLr35PR5* in incompatible interactions (TcLr35) was detected at 0 hpi, then the transcript levels declined sharply to a relatively low level from 6 to 24 hpi, but increased at 36 hpi (about 1.1–fold more than 0 hpi). At 96 hpi, there was an expression peak that was 1.6-fold higher than 0 hpi. However, in compatible interaction (Thatcher), the transcripts of *TaLr35PR5* maintained similar levels and changed little. Especially at 96 hpi, the accumulations of *TaLr35PR5* transcripts in incompatible interaction was 2.0 times higher than that in compatible interaction (Figure 2 Supplementary Figure 3). All in all, the expression levels of *TaLr35PR5* were higher in incompatible interaction than compatible interaction at most time points.

3.4. Tissue-specific expression of TaLr35PR5 transcripts

The roots, stems, and leaves of the mature TcLr35 wheat infected with leaf rust pathogen were used to detect the tissue-specific expression of *TaLr35PR5*. Gene expression pattern of *TaLr35PR5* showed significant variation among different plant tissues and different time points after inoculation which indicated that the expression of *TaLr35PR5* have tissue-specific and could be induced by leaf rust pathogen. More transcripts of *TaLr35PR5* were accumulated in stems than that in leaves and roots from 0 to 120 hpi, however, the expression trend in different
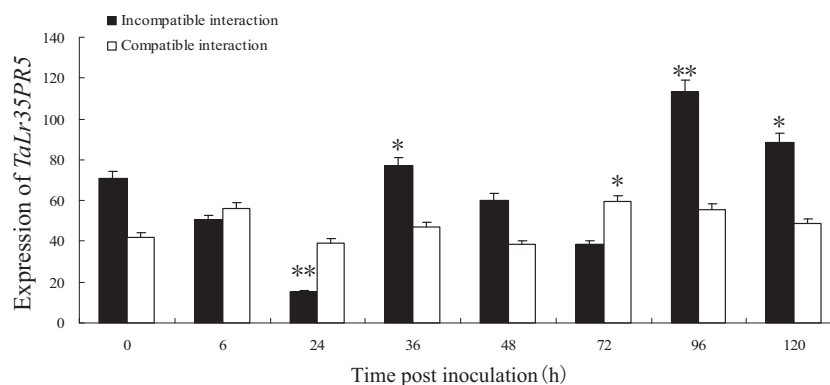

Figure 2. Expression profiles of *TaLr35PR5* in incompatible and compatible interaction leaves infected by the avirulent *P. triticina* isolate 07-10-426-1 at different times post-inoculation. The relative expression is expressed as fold change relative to mock inoculated plants on 0 hpi. The *y*-axis indicates the amounts of *TaLr35PR5* transcript normalized to the *GAPDH* gene. The *x*-axis indicates sampling times. Error bars represent variation among three biological replicates. *$p < 0.05$, $n = 3$.
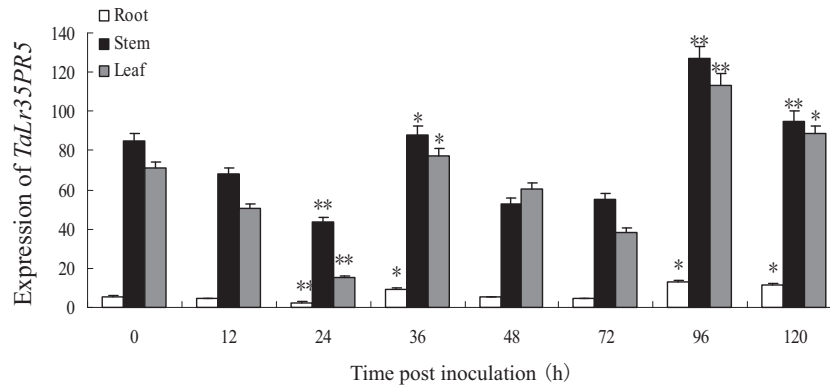
Figure 3. Expression profiles of *TaLr35PR5* in different wheat tissues after inoculation with *P. triticina* at different time points. The relative expression is expressed as fold change relative to mock inoculated plants on 0 hpi. The *y*-axis indicates the amounts of wheat *TaLr35PR5* transcript normalized to the *GAPDH* gene. The *x*-axis indicates different wheat tissues at different hpi. Error bars represent variation among three biological replicates. **$p < 0.01$, *$p < 0.05$, $n = 3$.

tissues is similar which increased or decreased at the same hpi and reached the peaks at 96 hpi in three different tissues (Figure 3 Supplementary Figure 4).

3.5. Responses of **TaLr35PR5** *gene in wheat induced by chemical reagents*

The assessment of gene expression between treated and untreated samples of TcLr35 was investigated by semi-quantitative RT-PCR respectively. The transcripts of *TaLr35PR5* treated with 50 μmol/L SA reached the peak at 48 hpi rapidly which was 1.7 times higher than 0 hpi, then declined. For those using 50 μmol/L SA treatment then inoculating with leaf rust pathogen, the expression trend was consistent with that by SA treatment and reached the peak at 48 hpi, and the accumulation of *TaLr35PR5* transcripts was about 1.9 times higher than the control group (0 hpi). Moreover, the expression levels of *TaLr35PR5* in wheat leaves pre-treated with SA chemical inducers prior to *P. triticina* inoculation were much higher than SA treatment (Figure 4 Supplementary Figure 5). The *TaLr35PR5* transcripts treated by ABA reached peak at 12 hpi

rapidly (about 1.8 times higher than control), then declined. After 50μmol/L ABA stress treatment, and then inoculated with leaf rust pathogen, the expression level of *TaLr35PR5* gene was significantly increased at 24hpi and reached the maximum which was about 2.0 times higher than that in the control group, then a slight downturn, but the expression level was higher than the overall treatment group without leaf rust pathogen (Supplementary Figure 6, Figure 5). In addition, there were obvious differences in expression time between ABA and SA infection reaction. The results showed that *TaLr35PR5* gene could be induced by signaling molecules ABA and SA, and the gene expression levels were significantly increased especially pre-treatment with SA and ABA to infection with leaf rust pathogen.

3.6. Accumulation of TaLr35PR5 is strongly increased at wheat different developmental stages upon leave rust pathogen infection

In order to define the relationship between *TaLr35PR5* gene and wheat adult leaf rust disease resistance gene *Lr35*, we detected the expression levels of *TaLr35PR5* at
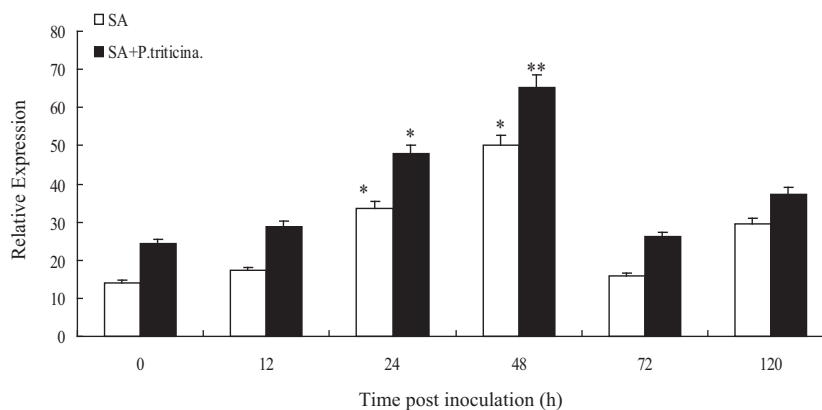

Figure 4. Expression profiles of *TaLr35PR5* in wheat leaves treated by SA and pre-treated with SA chemical inducers prior to *P. triticina* inoculation at different time points. The *y*-axis indicates the amounts of *TaLr35PR5* transcript normalized to the *GAPDH* gene and express relative to that of susceptible cultivar treated with SA. The *x*-axis indicates sampling times. Error bars represent variation among three biological replicates. **$p < 0.01$, *$p < 0.05$, $n = 3$.
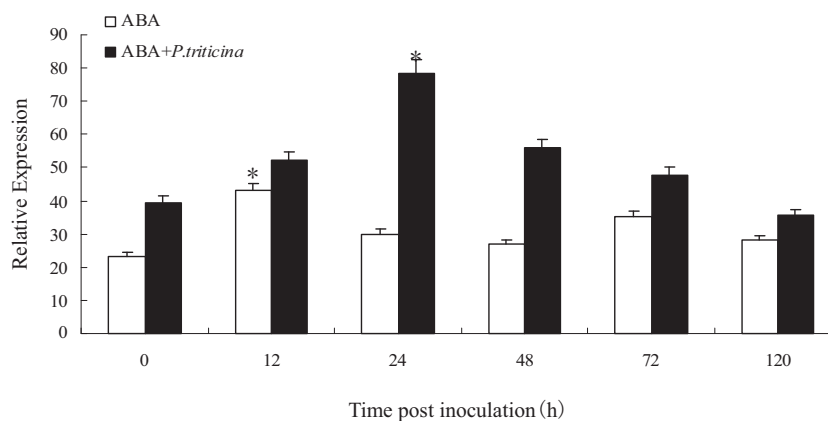
Figure 5. Expression profiles of the *TaLr35PR5* gene in wheat leaves treated by ABA and pre-treated with ABA chemical inducers prior to *P. triticina* inoculation at different time points. The *y*-axis indicates the amounts of *TaLr35PR5* transcript normalized to the *GAPDH* gene and express relative to that of susceptible cultivar treated with ABA. The *x*-axis indicates sampling times. Error bars represent variation among three biological replicates. $**p < 0.01$, $*p < 0.05$, $n = 3$.
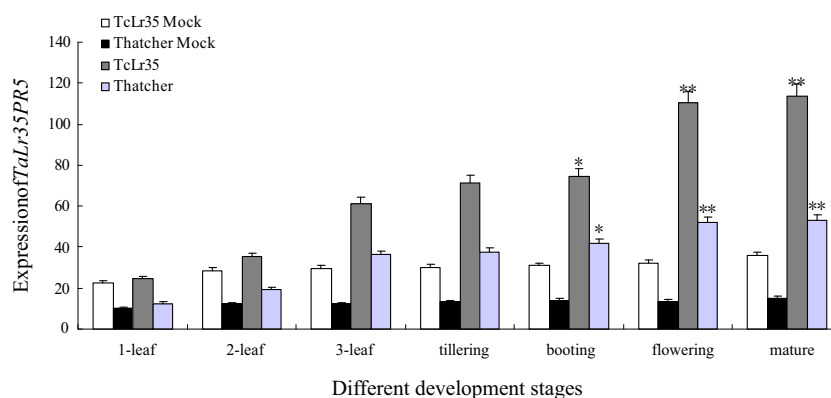

Figure 6. Expression profiles of *TaLr35PR5* at different growth and development of wheat. The *y*-axis indicates the amounts of wheat *TaLr35PR5* normalized to the GAPDH gene and express relative to mock inoculated plants on 0 hpi. The *x*-axis indicates the different development stage. Error bars represent variation among three biological replicates. $**p < 0.01$, $*p < 0.05$, $n = 3$.

different growth periods based on the reported expression features of *Lr35* gene (Seyfarth et al. 1999). As can be seen from Figure 6, at different growth and development periods of wheat TcLr35 and Thatcher, *TaLr35PR5* could be expressed at certain levels without inoculation (Mock) both in compatible and incompatible interaction, and the expression levels remained stable and showed the similar profiles at different periods, but the expression levels were much lower than the treatment with leaf rust pathogen. It showed that the expression level of *TaLr35PR5* was much higher in incompatible interaction than compatible interaction, and then expressed stably at the mature stage. In short, the accumulations of *TaLr35PR5* induced by leaf rust pathogen was significantly increased from the second-leaf stage and reached peak at the mature stage, and the expression levels at booting stage, flowering stage, and mature stage were 3.1 times, 3.9 times and 4.2 times higher than that at the second leaf stage, which is consistent with the expression profile of *Lr35* gene (Figure 6, Supplementary Figure 7).

3.7. Expression profiles of **TaLr35PR5** protein in wheat leaves infected by leaf rust

To detect the accumulation level of TaLr35PR5 protein in wheat leaves, polyclonal antibody was raised in rabbits against his-tagged TaLr35PR5 protein that was heterologously produced in *E. coli*. SDS-PAGE showed that the MW of the recombinant protein was about 28 kDa (Supplementary Figure 8), which is consistent with the deduced amino acids composition of TaLr35PR5 including the related sequence on pEASY plasmid. The ELISA result indicated that the antibody concentration of the antiserum reached 1: 768,000, which is sufficient for further experimentation. The generated antibody was then used to detect the accumulation level of TaLr35PR5 during incompatible (TcLr35) and compatible (Thatcher) interaction. As shown in Figure 7(a), a single or major band was detected in most of the WB results, which demonstrated the specificity of the antibodies and a 28 kDa signal band as expected was detected in TcLr35 and Thatcher infected by *P. triticina* from 0 hpi to 168 hpi. It was obvious that the signal intensity of TaLr35PR5 band
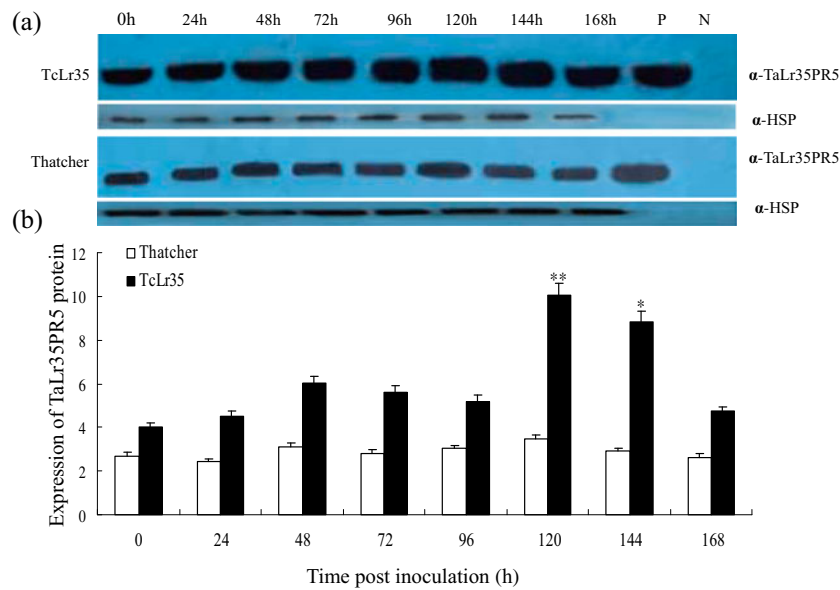
(a)

(b)

Figure 7. Accumulation of TaLr35PR5 protein in compatible (Thatcher) and incompatible (TcLr35) leaves infected by the avirulent *P. triticina* isolate 07-10-426-1 at different times post-inoculation. A: Western blot was probed with the TaLr35PR5 antibody. The purified TaLr35PR5 protein was used as a positive control. Plasmid pEASY was used as a negative control. Heat shock protein (HSP) was taken as the loading control. B: Plot of average and standard deviation among three repeats of WB analysis. The *y*-axis indicates the amount of TaLr35PR5 protein level normalized to the HSP and express relative to that of noninoculated control, and the *x*-axis indicates sampling times. **$p < 0.01$, *$p < 0.05$, $n = 3$.

in TcLr35 steadily increased from 0 to 120 hpi, then declined at 144 hpi. However, a much weaker signal was detected in Thatcher compared with TcLr35, in another words, the accumulation of TaLr35PR5 protein in TcLr35 was much higher than Thatcher (Figure 7b). These results strongly implied that accumulation of TaLr35PR5 protein was correlated with resistance against leaf rust pathogen infection.

3.8. Expression of TaLr35PR5 protein in wheat leaves at different developmental stages

Western blotting was used to detect the expression of TaLr35PR5 in wheat leaves at different developmental stages. As shown in Figure 8, the expression of TaLr35PR5 showed variable patterns in wheat leaves at different developmental stages. TaLr35PR5 could express at certain levels without inoculation (Mock) both in compatible (Thatcher) and incompatible (TcLr35) inter-action and increased slowly from the second leaf stage to mature stage. Compared to Mock, TaLr35PR5 infected by *P. triticina* increased rapidly both in compatible and incompatible interaction. However, TaLr35PR5 expressed weakly at the first leaf stage and began to increase at the second leaf stage and contained constant levels at mature stage in TcLr35. The accumulation of TaLr35PR5 at the mature stage was 2.0 and 1.7 times higher than the first leaf stage and the second leaf stage. In addition, TaLr35PR5 increased more rapidly and its expression level was much higher in incompatible interaction than compatible interaction (Figure 8b).

4. Discussions

A number of reports have demonstrated that PR proteins play roles in the late periods of the resistance response (Datta and Muthukrishnan 1999). In *Arabidopsis thaliana* and tobacco, PR family number such as PR1, PR2, and PR5 have been proved to be involved in the SAR reaction. Datta et al. (1999) found that over expressing of thaumatin protein gene in rice could enhance the resistance of transgenic rice to *Thanatephorus cucul'neri* (Frank) Donk; Chen et al. (1999) translate thaumatin protein gene into wheat, transgenic plants have a certain resistance to *Fusarium graminearum Schw*; Jayasanka (Jayasankar et al. 2003) found that over expression of thaumatin protein gene could enhance the resistance of grape (*Vitis vinifera* Linn.) to *Anthracnose*. This study firstly detected that *TaLr35PR5* was induced by leaf rust pathogen both at RNA and protein levels. It was interesting to find the expression profiles of *TaLr35PR5* gene changes occurred in the incompatible and compatible reaction. The significant differences of *TaLr35PR5* expression between mock inoculated, resistant infected and susceptible infected plants occurred can be attributed to the presence of adult leaf rust resistance *Lr35* gene, which facilitated prevention of leaf rust infection in resistant wheat plants. This is the first example of study on the expression of *TLPs* gene in the interactions between TcLr35 wheat and *P. triticina*.

When plants face adverse natural environment, the accumulation of ABA can increase in plants and then promote the growth of plants. Plants have ABA and non-ABA two regulating system (Von Dahl and Baldwin 2007). In addition, plants in the process of long-term
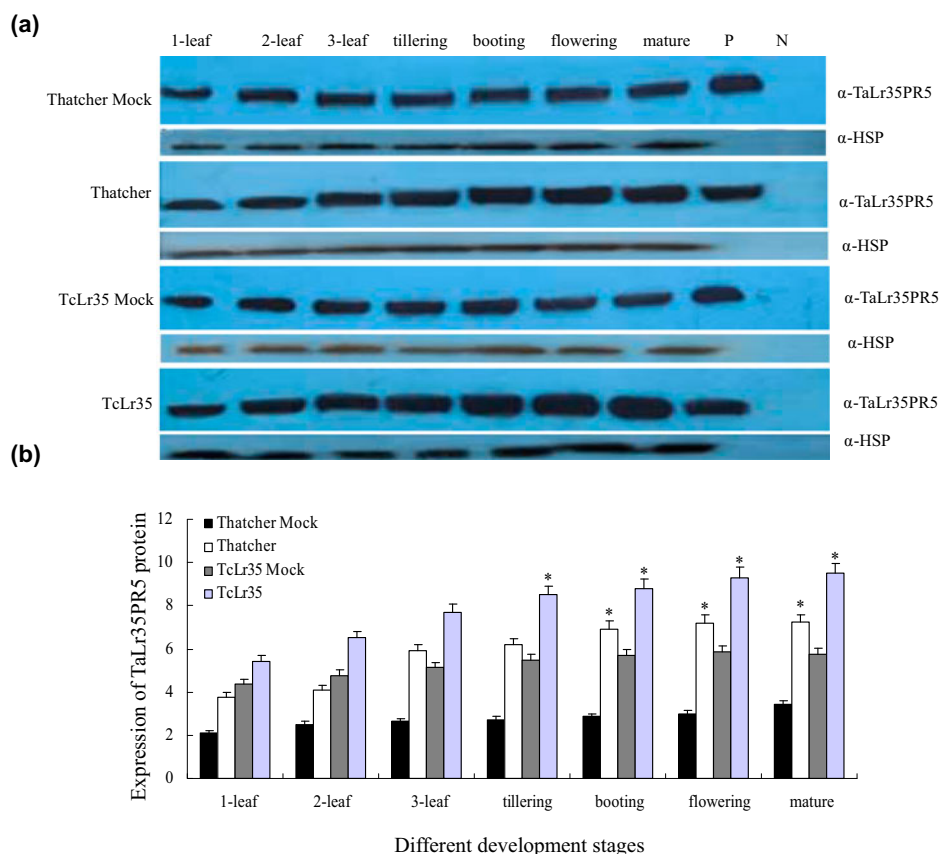
Figure 8. Expression profiles of TaLr35PR5 proteins in wheat leaves at different growth stages A: Western blot was probed with the TaLr35PR5 antibody. Lanes 1–9: Protein samples isolated from normal wheat leaves at seedling stage (1-leaf, 2-leaf, and 3-leaf stage), tillering stage, booting stage, flowering stage, mature stage, positive control, and negative control. The purified TaLr35PR5 protein was used as a positive control. Plasmid pEASY was used as a negative control. Heat shock protein (HSP) was taken as the loading control. B: Plot of average and standard deviation among three repeats of WB analysis. The y-axis indicates the amount of TaLr35PR5 protein level normalized to the HSP and express relative to that of noninoculated control, and the x-axis indicates different development stages. $**p < 0.01$, $*p < 0.05$, $n = 3$.

evolution formed two different signal transduction pathways including SA/JA (Jane 2001; Von Dahl and Baldwin 2007). In this study, we found that *TaLr35PR5* gene was significantly induced by ABA and SA, especially the transcripts of *TaLr35PR5* genes accumulated at higher levels inoculated with *P. triticina* following ABA or SA pre-treatment rather than only with ABA or SA treatment, but the expression trends of *TaLr35PR5* were consistent in both inoculation and noninoculation treatment which are in accord with Zambounis et al. (2012) suggest a correlation between increasing disease resistance and elevated PR transcript accumulation. So, we hypothesized that *TaLr35PR5* may play a role in mediating plant responses to above chemical inducers and pathogen challenge in TcLr35 wheat and the expression of *TaLr35PR5* might be regulated by SA and ABA signaling pathway.

Most of PR proteins expressed in normal leaf tissues, but with different patterns. Some of them expressed constitutively, while others expressed at low levels at the seeding stage and high levels at the adult stage (Hou et al. 2012). Different expression patterns might implicate different functions. These data added to our knowledge of PR proteins expressed in various tissues have

illustrated that they are not only important components of the defense mechanism in wheat but also can sustain the function and regulate normal development. Studies have shown that the differences of different genes in different tissues and organs, such as hubei haitang *MhPR8* gene expression in the root is the largest, followed by the stem, the leaf expression of minimum (Zhang et al. 2012); Zhang found that *PR5* gene expressed differently in the different tissues and organs in strawberry (Zhang and Shih 2007). In our study, we found that *TaLr35PR5* genes expressed in different tissues and organs of wheat including roots, stems, and leaf, but had obvious tissue specificity according to the significant differences in gene expression. The localization analysis of TLPs in poplar indicated that TLPs were present at the tissue level in the phloem, specifically in sieve elements, and were detected in cell walls and within cells associated with starch at the cellular level (Dafoe et al. 2010). So, we speculate that the expression in stem higher than leave and root may be associated with starch.

Moreover, In order to define the relationship between *TaLr35PR5* genes and wheat leaf rust disease resistance gene *Lr35*, we detected the expression levels of *TaLr35PR5* at different growth periods based on the

reported expression features of *Lr35* gene (Seyfarth et al. 1999). It was reported that *Lr35* gene in wheat was not expressed at the first leaf stage, similar to that, a little accumulation of *TaLr35PR5* was detected at the first leaf stage. The expression level of *TaLr35PR5* began to increase in the different treatment since the second leaf stage which is consistent with that *Lr35* gene begins express at certain degree since the second leaf stage. All in all, *TaLr35PR5* gene expressed at lower levels at the seeding stage but higher levels at the adult stage, and the expression at the adult stage was stable. These results indicated that the expression profiles of *TaLr35PR5* at different growth periods be accord with the expression features of *Lr35* gene and showed that the gene might play an important role in wheat resistance during adult stage. In addition, there was a certain amount of background expression in different growth periods in TcLr35 wheat reflected from the expression profiles of *TaLr35PR5* in noninoculated treatments including resistant and susceptible materials, which suggested that *TaLr35PR5* play roles not only in plant disease resistance but also in the growth and development of wheat. On this basis, we should better to understand the expression profiles of *TaLr35PR5* at protein level. Variation in expression level of TaLr35PR5 using western blot showed that the target protein was highly induced by leaf rust pathogen, and the induction peak time of *TaLr35PR5* in the incompatible interaction occurred earlier and higher compared to the compatible one. Western blot showed that the expression trends of target protein were consistent with its expression at nucleic acid level. However, compared with the expression peak (at 96 hpi) at nucleotide level, the maximal expression of TaLr35PR5 protein (144 hpi) was delayed both in incompatible and compatible combinations. On the other hand, *TaLr35PR5* transcript began to increase at 36 hpi, while TaLr35PR5 protein was detected to begin increasing at 48 hpi in incompatible interaction, which might because protein expressed after transcription and post-transcriptional modification.

In conclusion, *TaLr35PR5* was initially cloned and identified the expression profiles infected by *P. triticina* which might take part in SA and ABA signaling pathways and be involved in the interactions between leaf rust pathogen and TcLr35 wheat. In order to get insight into the role of *TaLr35PR5* in wheat growth and resistance to leaf rust pathogens attack, its function should be analyzed. In the future experiment, we have to obtain positive generation of the transgenic plant used to analysis the co-expression between *TaLr35PR5* gene and *Lr35* gene, and contributing to the defense against *P. triticina* infection.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Supplemental data

Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/17429145.2015.1036140

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