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Expression analysis and functional characterization of a pathogen-induced thaumatin-like gene in wheat conferring enhanced resistance to *Puccinia triticina*

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ABSTRACT

Pathogenesis-related (PR) protein-5, is involved in host defense system against both biotic and abiotic stresses as well as the regulation of physiological processes in numerous plant species. Our earlier studies have reported the isolation of a full-length *TaLr19TLP1* gene (516 bp, GenBank accession No. KJ764822) from wheat infected by leaf rust. Quantitative real-time polymerase chain reaction analyses revealed that *TaLr19TLP1* transcript was significantly induced and upregulated during incompatible interaction, while a relatively low level of the transcript was detected during compatible interaction. In the current study, we demonstrate that the accumulation of *TaLr19TLP1* transcript is significantly different in tested wheat organs. *TaLr19TLP1* was induced by salicylic acid (SA), methyl jasmonic (MeJA), ethephon (ETH) and abscisic acid (ABA). The transcripts of *TaLr19TLP1* accumulated at higher levels following pretreatment with SA, MeJA and ABA prior to infection with *P. triticina*. A slight induction was observed in ETH pretreated seedlings compared with the treatment without inoculation. In addition, *TaLr19TLP1* was found to be predominately localized to extracellular spaces of onion epidermal cell. Knocking down the expression of *TaLr19TLP1* through virus-induced gene silencing reduced wheat resistance against leaf rust pathogen. These results suggested that *TaLr19TLP1* mediated disease resistance in wheat exposed to leaf rust pathogen.

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Thaumatin-like protein; qRT-PCR; leaf rust; subcellular localization; gene silencing

1. Introduction

Plants have evolved well-established defense mechanisms to protect themselves against different types of environmental biotic stresses such as plant pathogens and insect predators. Pathogen-related (PR) proteins are encoded by the host plant *PR* genes. They are mostly induced in plants exposed to invasive pathogens or environmental stress (Kim and Hwang 2000). PR proteins provide protection from biotic as well as abiotic stresses (van Loon et al. 2006). Nearly 17 families of PR proteins have been identified in mono- and dicotyledonous plants based on structural and serological relationships as well as biological activities. Their functions range from cell wall rigidity to signal transduction and antimicrobial activity (Christensen et al. 2002).

Proteins of the PR5 family are also referred to thaumatin-like proteins (TLPs) because of their amino acid sequence and structural similarities to sweet tasting proteins from the fruits of West African rain forest shrub *Thaumatococcus daniellii* (Edens et al. 1982). TLPs are involved in plant defense against biotic and abiotic stresses (Petre et al. 2011). They are induced in plants following pathogen attack and exposure to elicitors, stress and developmental signals. Antifungal effects of TLPs involve alterations in fungal cell membrane integrity leading to inhibition of fungal growth, spore lyses, reduced spore number or reduced viability of germinated spores (Abad et al. 1996; Tobias et al. 2007) or degradation of cell walls (Osmond et al 2001; Zareie et al. 2002). Over-expression of TLPs promotes stress resistance in different transgenic plants (Liu et al. 1994; Datta et al. 1999; Rajam et al. 2007; Munis et al. 2010; Wang et al. 2010; Subramanyam et al. 2012; Acharya et al. 2013). The antifungal activities of TLPs can be modified by genetic engineering to produce disease-resistant plants. Cao discovered an evolutionary origin of this gene family. Tandem and segmental duplication plays a dominant role in their expansion (Cao et al. 2015). Furthermore, several proteins of the PR-5 group have been used successfully to enhance plant resistance to fungal pathogens. The transcriptional levels of *ZzPR5* in wild ginger (*Zingiber zerumbet*) were remarkably increased post-infection with soft rot pathogen (*Pythium aphanidermatum*) (Nair et al. 2010). A *PR5* gene family in wild peanut (*Arachis diogoi*) was reported as significantly upregulated in response to infection by *Phaeoisariopsis personata* (Singh et al. 2013). *DcTLP* from carrot (*Daucus carota*) was highly expressed in dehydration stress and its promoter was highly activated following drought (Jung et al. 2005). TaPR5, a TLP homologue from wheat (*Triticum aestivum*) notably increased after treatment of wheat leaves with methyl jasmonic (MeJA), salicylic acid (SA) and abscisic acid (ABA) (Wang et al. 2010). Recently, a *PR5* gene showing a high degree of homology with osmotin-like protein was first found in sweet basil (*Ocinmum basilicum* L.) (Rather et al. 2015). As *PR5* induced in response to *Fusarium oxysporum* f. sp. *cepae* (FOC) infection in garlic (*Allium sativum*) was isolated and characterized (Rout et al. 2016). Furthermore, a few TLPs were also induced in response to developmental signals and fruit ripening (Sassa and Hirano 1998; Kim et al. 2002).

Wheat leaf rust caused by *Puccinia triticina* is one of the most common and widespread diseases, which attacks leaves and leaf sheaths of growing wheat plants at different stages of growth. Leaf rust causes yield losses of 30–70% depending

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upon disease emergence in the adult plant stage (flag leaf infection) or early infection of seedlings (Huerta-Espino et al. 2011). Currently, wheat leaf rust disease has attracted a worldwide attention, and studies have investigated the symptoms, epidemiology and physiological races of *P. triticina* fungus. Development of genetic resistance represents the most effective and environmentally sustainable mechanism of prevention of losses caused by rust epidemics. In a previous study, a *PR5* gene, designated as *TaLr19TLP1*, was identified in TcLr19, a wheat near-isogenic line infected by *P. triticina*, with a high similarity to *PR5* from other plants. The molecular characteristics of *TaLr19TLP1* were analyzed and its transcriptional profiles in response to leaf rust pathogen were determined using quantitative real-time polymerase chain reaction (qRT-PCR) (Li et al. 2014). In this study, the expression profiles of *TaLr19TLP1* in different tissues induced by *P. triticina* fungus were identified. In addition, we analyzed its transcriptional regulation in response to chemical treatments and its subcellular localization by translational fusion with green fluorescent protein (GFP) in onion epidermal cells. Finally, we verified the functional role of *TaLr19TLP1* by silencing it to reduce wheat resistance against leaf rust pathogen. Our results have provided a basis for studies investigating the function and mechanisms underlying the role of *TaLr19TLP1* in biotic stress.

2. Materials and methods

2.1. Plant materials and inoculation system

Wheat near-isogenic line TcLr19, Thatcher, susceptible wheat cv. Zhengzhou5389 and leaf rust race 07-10-421-3 (FHJT) were used for expression and functional analyses. TcLr19, containing the leaf rust resistance gene *Lr19*, expresses a typical HR to the avirulent pathotype FHJT. However, Thatcher is susceptible to the virulent pathotype FHJT and shows compatible reaction (Scale 4) according to Roelfs' standard (Roelfs et al. 1984). Seven-day seedlings at the primary leaf stage were inoculated with fresh urediniospores of FHJT collected from the susceptible wheat cv. Zhengzhou5389 using a paintbrush. Simultaneously, control plants were inoculated with sterile water. Initially, the inoculated and control plants were stored for 24 h at 100% humidity and then transferred to a growth chamber with a 16-h photoperiod (light intensity, 2000 lx) at 15°C. Samples of inoculated and control wheat leaves, stems and roots were obtained at 0, 6, 12, 24, 48, 72, 96, 120, 144 and 168 h after inoculation (hpi) for temporal and spatial expression analyses, quickly frozen in liquid nitrogen and stored at −80°C until extraction of total RNA. Plants were rated for symptom development 15 days during post-inoculation (dpi).

2.2. Treatment of plants with different stimuli

Exogenous hormone treatments were conducted by spraying the leaves of 4-week plants with a solution of 0.5 mM SA, 0.1 mM MeJA, 0.05 mM ethephon (ETH) or 0.5 mM ABA dissolved in 0.1% (v/v) ethanol. Leaf rust pathotype FHJT was used for the inoculation of wheat leaves on various days (0, 1, 3, 5 and 10 d) after the hormone treatment. Control plants were similarly treated with 0.1% (v/v) ethanol. Leaves of wheat seedlings exposed to chemical treatment prior to leaf rust pathogen infection along with control plants were sampled at 0, 6, 12, 24, 48, 72, 96 and 120 h after treatment (hpt). All samples were quickly frozen in liquid nitrogen and stored at −80°C. Each experiment was conducted in triplicate.

2.3. RNA preparation and expression

The total RNA from wheat tissues at different time points was extracted with TRIzol (Invitrogen) according to the manufacturer's protocol, and genomic DNA contamination was removed by DNaseI treatment. First-strand cDNA was synthesized using M-MLV reverse transcriptase (Promega) with an oligo (dT) primer for gene isolation and PCR analysis. Semi-quantitative RT–PCR was performed to analyze the accumulation of *TaLr19TLP1* in different treatments which were prior to qRT-PCR using the iCycler IQ real-time detection system (Bio-Rad, Amsterdam, Netherlands). The gene-specific primers (qTcLr19-F and qTcLr19-R) for real-time PCR are listed in Table 1. Real-time PCR primers were mixed with 12.5 μL 2 × TransStartTM Green qPCR SuperMix (TransGen Biotech), 2.0 μL cDNA and 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 was performed at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s, with annealing at 60°C for 30 s and an extension was done at 72°C for 30 s. Quantification of the target gene was assessed by relative standard curves. The $2^{-\Delta\Delta CT}$ (Thomas and Kenneth 2008) method was employed to quantify the relative gene expression. Three independent biological replicates were maintained for each sample. Wheat glyceraldehyde 3-phosphate dehydrogenase gene *GAPDH* (GenBank accession No. AF251217) amplified by primer pairs qGAPDH-F and qGAPDH-R (Table 1) was selected as an endogenous control to normalize the differences in input RNAs and check efficiencies of reverse transcription among the various samples in triplicate.

2.4. Subcellular localization of TaLr19TLP1–GFP fusion protein

To generate the TaLr19TLP1–GFP fusion construct, the *TaLr19TLP1* coding sequence was amplified with a forward primer designated as Y-GPF-PR5-F containing an *Xba*I site and a reverse primer named Y-GPF-PR5-R containing a *Kpn*I site. The resulting product was cloned into pGEM-T easy vector (Promega) and the positive clones were sequenced. The *TaLr19TLP1* ORF fragment was released from the recombinant following *Xba*I and *Kpn*I digestion and inserted into pCamA: GFP vector. The resulting gene

Table 1. Primers used in this study.

Primer	5′–3′ sequence
qTcLr19-F	5′-CAACGAGAACCAGAAGGACAGC-3′
qTcLr19-R	5′-TACGGACGGACATACGGACACT-3′
qGAPDH-F	5′-CTGCCTTGCTCGTCTTGCTAA-3′
qGAPDH-R	5′-CTTGATGGAAGGACCATCAAC-3′
V-PR5-F1	5′-GTTAATTAAGGTGCTCCTTCAATGGCG-3′
V-PR5-R1	5′ -TTGCGGCCGCTCATGGACAGAAGGTGATCTGGTA-3′
V-PR5-F2	5′-GTTAATTAA ATGGCGACCTCCGCGGTGCTC-3′
V-PR5-R2	5′-TTGCGGCCGCCTACCGCCATTGAAGGAG-3′
Y-GPF-PR5-F	5′- GCTCTAGAATGGCGACCTCCGCGGTGCTC-3′
Y-GPF-PR5-R	5′- GGGGTACCTCATGGACAGAAGGTGATC-3′

constructing pCamA: TaLr19TLP1–GFP contained the *TaLr19TLP1* coding sequence inserted just before *GFP* sequence, which generated an in-frame fusion between *TaLr19TLP1* and *GFP* genes. Onion epidermal cell layers were incubated inside-out centrally on the Murashige and Skoog (MS) medium plates for 4–6 h before bombardment. The fusion expression construct and control plasmid (pCamA: GFP) were transformed into onion epidermal cells by particle bombardment at a helium pressure of 7.6 MPa (1100 psi) using the PDS-1000/He system (Bio-Rad, Hercules, CA). The transformed onion epidermal cells were incubated in a growth chamber at 25°C for 16 h. GFP fluorescence was observed with an Olympus LEXT OLS4100 confocal laser microscope using a 480-nm filter (Olympus, America).

2.5. BSMV construction, virus inoculation and histological observation

Virus-induced gene silencing (VIGS) experiments were conducted with two fragments of 307 and 230 bp of TaLr19TLP1 amplified with *Not*I and *Pac*I restriction sites using the specific primer combination of V-PR5-F1: V-PR5-R1 and V-PR5-F2: V-PR5-R2, respectively (Table 1). The plasmids utilized for barley stripe mosaic virus (BSMV)-VIGS are based on the constructs described by Holzberg et al. (2002). The RNA γ vector BSMV:PDS was constructed by digesting p γ. bPDS4-as with *Not*I + *Pac*I, and replacing the PDS4 insert with 307-bp or 230-bp fragment of *TaLr19TLP1*, respectively. To produce in vitro transcripts of viral RNAs, plasmids containing the tripartite BSMV genome were linearized. Capped in vitro transcripts from the linearized plasmids were prepared using the mMESSAGE mMACHINE® Kit High Yield Capped RNA Transcription Kit (Ambion), according to the manufacturer's instructions. BSMV transcript mixtures were used for wheat as described by Scofield et al. (2005). The second leaf of a two-leaf wheat seedling was inoculated by gently rubbing the surface with gloved fingers. The seedlings were lightly misted with (diethyl pyrocarbonate) DEPC-treated water and maintained in the growth chamber at 23 ± 2°C. After approximately 10 days, the viral disease symptoms were observed in plants inoculated with virus or viral constructs. The recombinant virus BSMV: PDS was used as a positive control. In the presence of the photo-bleaching phenotype obtained by silencing the phytoene desaturase gene (*PDS*) gene, the fourth leaf was inoculated with the leaf rust pathotype FHJT, and the RNA was extracted at 96 hpi followed by real-time PCR analysis. The infected phenotypes of leaf rust were observed at 14 dpi.

3. Results

3.1. Tissue-specific expression of TaLr19TLP1 transcripts

The roots, stems and leaves of the TcLr19 wheat infected with leaf rust pathogen were used to determine the tissue-specific expression of *TaLr19TLP1*. The gene expression pattern of *TaLr19TLP1* showed significant variation in different plant tissues and at different time points after inoculation, indicating that the expression of *TaLr19TLP1* was tissue-specific and induced by leaf rust pathogen. The *TaLr19TLP1* accumulation in roots was altered little and maintained at similar levels under different hpi levels, while the transcript levels of *TaLr19TLP1* in stems peaked at 120 hpi, and were slightly downregulated at 144 and 168 hpi. The higher transcription levels persisted during later rather than earlier stages. The expression trend in leaves was similar to that in stems, which was increased or decreased at most hpi and peaked at 96 hpi. However, additional transcripts of *TaLr19TLP1* accumulated in stems than in leaves from 0 to 168 hpi (Figure 1, Supplementary Figure 1).

3.2. Response of TaLr19TLP1 in wheat induced by chemical reagents

The qRT-PCR was used to test the expression of *TaLr19TLP1* induced by exogenous phytohormone application of SA, ETH, MeJA and ABA (Figure 2, Supplementary Figure 2). The expression of *TaLr19TLP1* mRNA was slightly decreased at 6 h post-SA treatment. It was markedly accumulated at 48 hpt, which was nearly 5.4 times higher than that in control group (0 hpt), followed by a slight decrease and slight upregulation at 96 and 120 hpt. During ETH treatment, the expression of *TaLr19TLP1* peaked at 12 hpt followed by a sharp decrease at 12–48 hpt, increase at 48 hpt and another

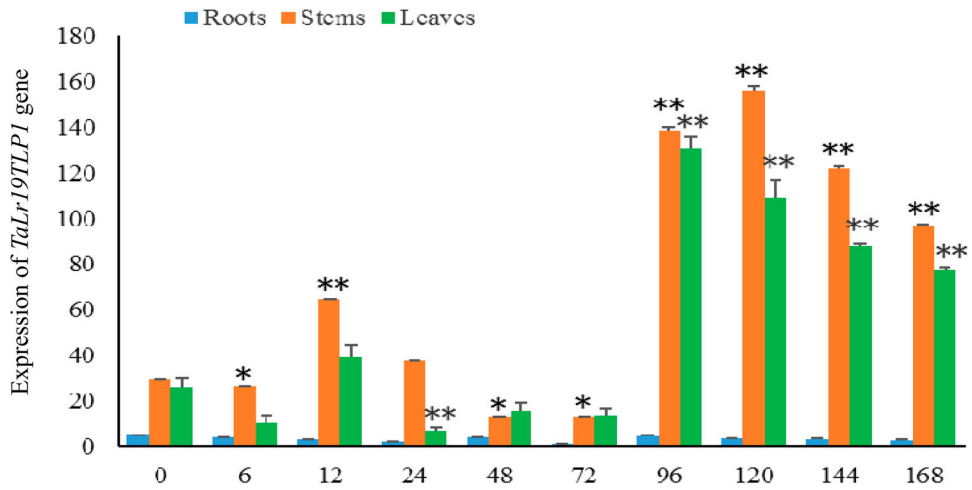

Figure 1. Differential expression of *TaLr19TLP1* mRNA in TcLr19 tissues inoculated with leaf rust pathotype FHJT. The *y*-axis indicates the levels of wheat *TaLr19TLP1* mRNA normalized to *GAPDH* as the reference gene, and the transcriptional expression relative to the non-inoculated control. The *x*-axis shows wheat tissues at different hpi. Error bars represent standard deviations of three independent experiments. $^{**}p < .01$; $^{*}p < .05$, $n = 3$.

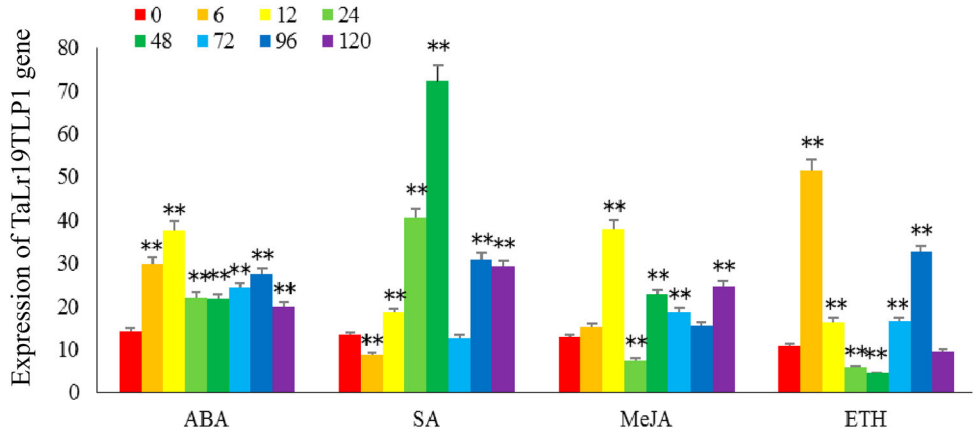

Figure 2. qRT-PCR analysis of *TaLr19TLP1* transcription levels following treatment with different chemical inducers. The y-axis indicates the amounts of *TaLr19TLP1* transcript normalized to the *GAPDH* gene and express relative to that of Mock control plants treated with 0.1% (v/v) ethanol solution. The *x*-axis indicates different chemical inducers. Different coloring means different sampling times. Error bars represent standard deviations of three independent experiments. $^{**}p < .01$; $^{*}p < .05$, $n = 3$.

peak at 96 hpt. The transcriptional expression of *TaLr19TLP1* only slightly increased at 6 h post-MeJA treatment, and peaked at 12 hpt followed by a sharp decrease at 24 hpt and a slight increase from 48 to 120 hpt. However, ABA treatment had no obvious effect on *TaLr19TLP1* expression.

Wheat seedlings were pretreated with chemical inducers including SA, ETH, MeJA and ABA, respectively, and were challenged with pathotype FHJT on various days. The qRT-PCR assays were performed on RNA samples extracted and mixed after inoculation from 0 to 168. The *TaLr19TLP1* transcripts treated with ABA following FHJT inoculation exhibited significant differences compared with uninfected control on all dpi, which was nearly 10 times higher than in uninfected control at 5 dpi. SA and MeJA pretreatment prior to infection resulted in a steady increase in *TaLr19TLP1* expression from 0 to 3 dpi, and persisted at similar levels on 5 and 10 dpi. *TaLr19TLP1* expression showed the highest level at 0 dpi after SA pretreatment, which indicated earlier induction by SA than other chemical reagents. However, pretreating wheat leaves with ETH did not influence *TaLr19TLP1* gene expression. A slight induction was observed at 3, 5 and 10 dpi compared with inoculated but untreated counterparts (Figure 3, Supplementary Figure 3). Since SA was the most effective phytohormone inducing the expression of TaLr19TLP1, leaf rust pathotype FHJT was used for the inoculation of wheat leaves on 0d after 0.5 mM SA treatment. The results showed that TcLr19 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. Compared with Thatcher, percentage of germinating spores was measured by microscopic observation and found that the germination of spores

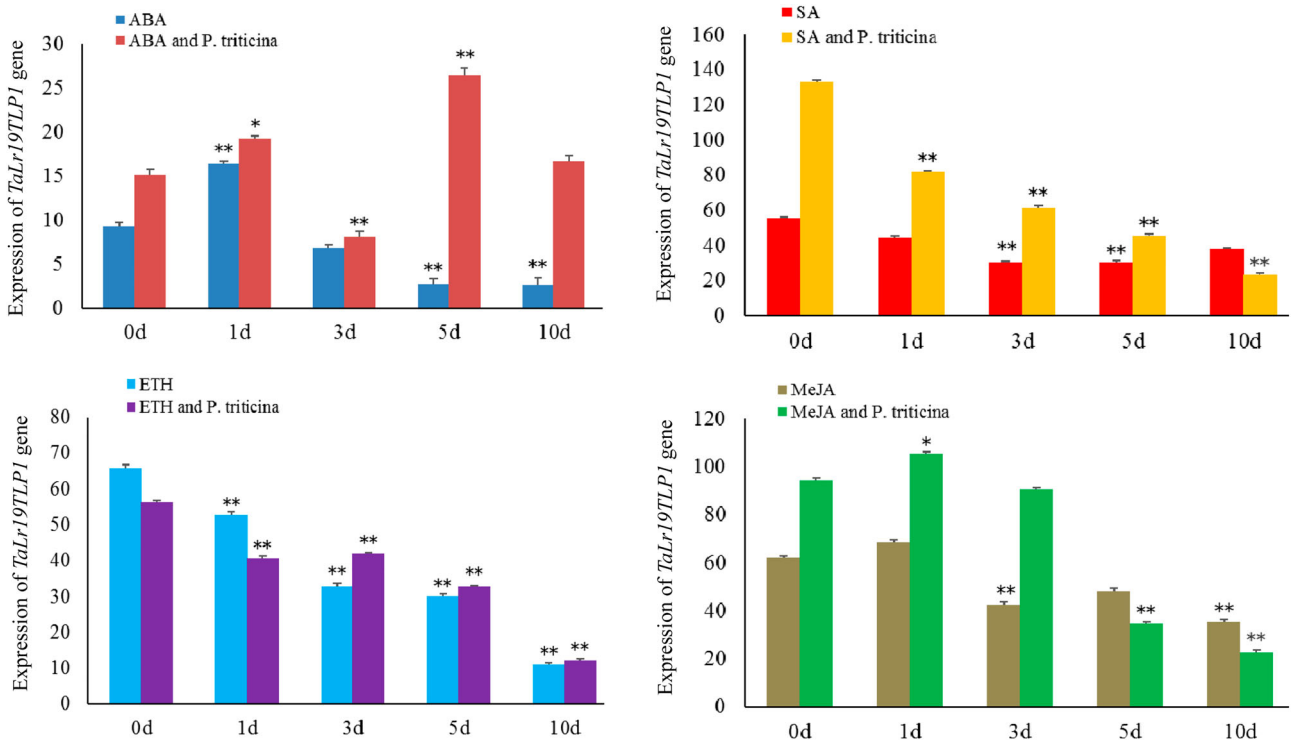

Figure 3. Expression levels of *TaLr19TLP1* induced by different chemical agents prior to wheat leaf rust infection. The *y*-axis indicates the amounts of *TaLr19TLP1* transcript normalized to the *GAPDH* gene and express relative to that of Mock control plants treated with 0.1% (v/v) ethanol solution. The *x*-axis indicates different sampling times. Error bars represent standard deviations of three independent experiments. $^{**}p < .01$, $^{*}p < .05$, $n = 3$.

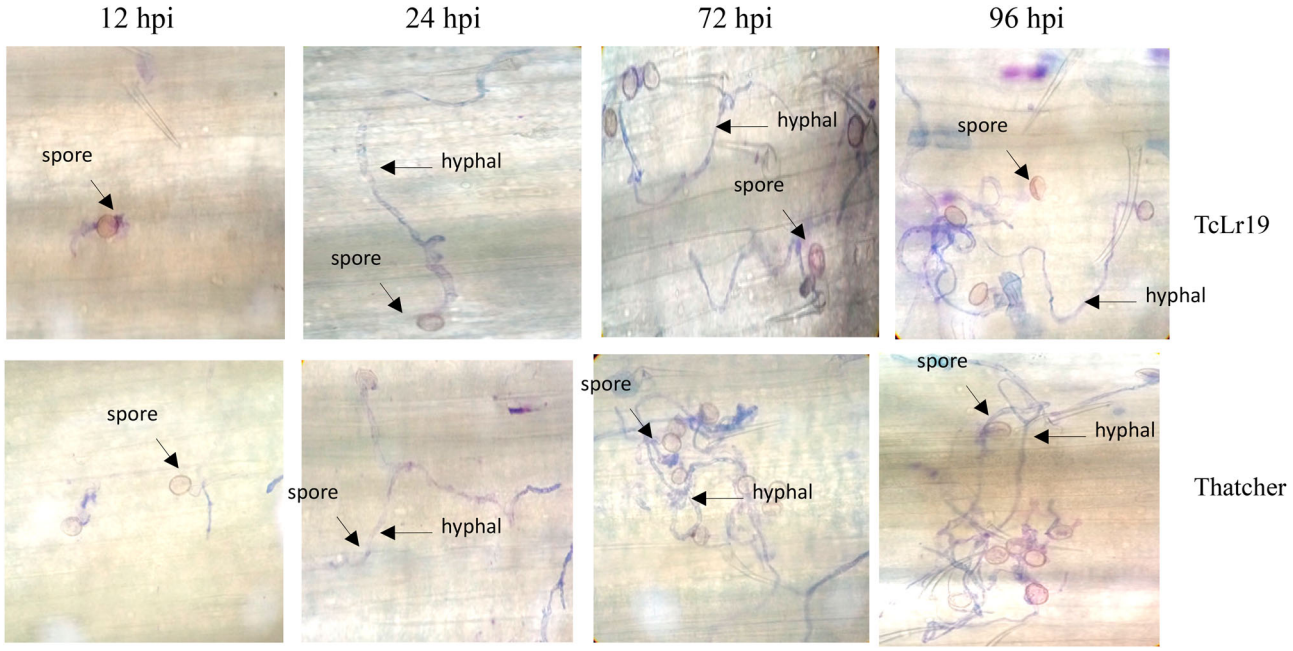

Figure 4. Histological observation of hyphal and germinating spores at 12, 24, 72 and 96 hpi of wheat leaves induced by SA prior to wheat leaf rust infection. Leaf rust pathotype FHJT was used for the inoculation of wheat leaves on 0 d after 0.5 mM SA treatment.

on TcLr19 was inhibited compared with those in Thatcher. In addition, the fungal hyphal lengths were shorter compared with those in Thatcher from 12 to 96 hpi, and the numbers of hyphal branches were lower compared with those in Thatcher at different hpi (Figure 4).

3.3. Extracellular localization of TaLr19TLP1–GFP fusion protein

To investigate the subcellular localization of *TaLr19TLP1*, a transient expression system using onion epidermis cell layers was carried out. A fragment with *Xba*I and *Kpn*I was obtained (Supplementary Figure 4), *GFP* gene was fused to *TaLr19TLP1*, and then bombarded into the epidermal cells of onion. Under the control of a pCamA promoter, the TaLr19TLP1–GFP fusion proteins predominantly accumulated in the cell wall of transformed cells and were randomly scattered in the extracellular region (Figure 5(B)), whereas GFP proteins in the control were uniformly distributed throughout the cell (Figure 5 (A)). These results indicated that the *TaLr19TLP1* gene encodes an extracellular protein, which was consistent with the pSORT prediction of 95% probability (Figure 4).

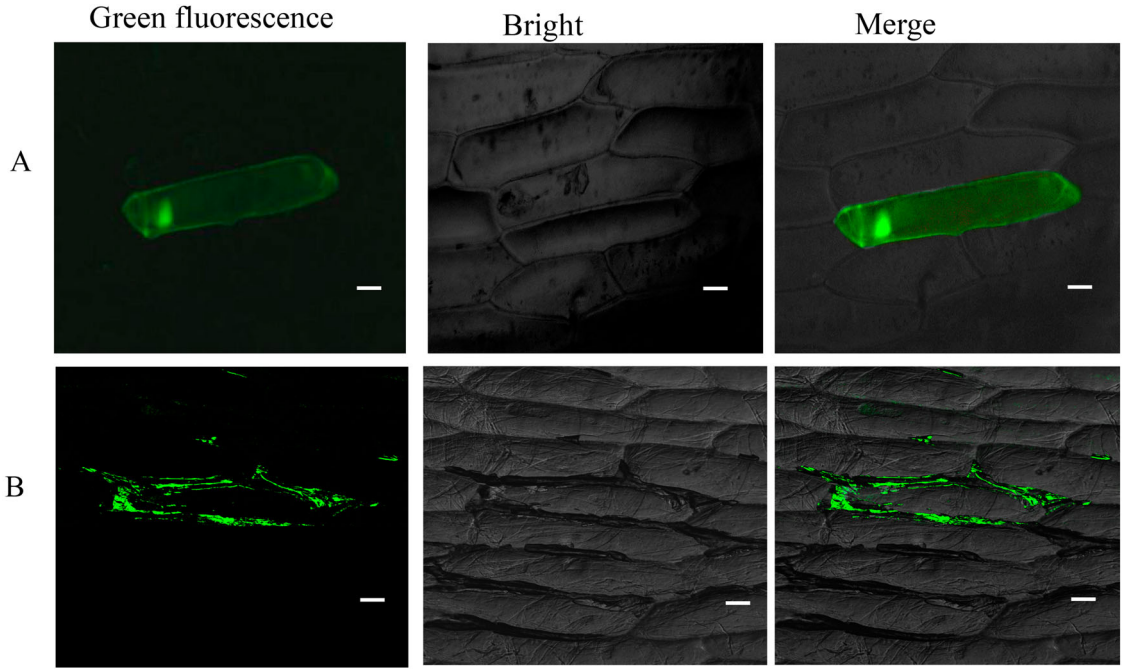

Figure 5. Subcellular localization of *TaLr19TLP1*. Onion epidermal cells were transformed with plasmids expressing the fusion protein (TaLr19TLP1–GFP) and GFP by bombardment. All images were visualized with a laser scanning confocal microscope. (A) Onion epidermal cells expressing the GFP alone. (B) Onion epidermal cells expressing the TaLr19TLP1–GFP fusion protein. Left panel: image of the fusion protein under green fluorescence; middle panel: image of the fusion protein under bright-field; right panel: merged images. Scale bars are 50μM.

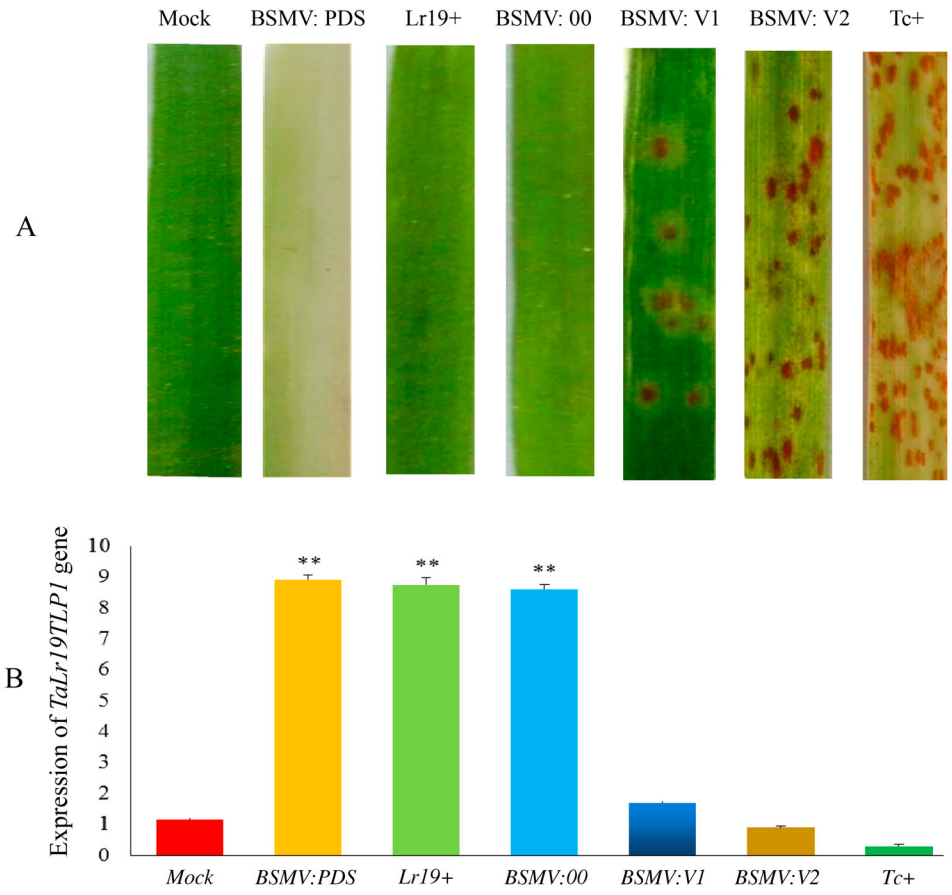

Figure 6. Functional analysis of *TaLr19TLP1* in response to leaf rust infection using BSMV-VIGS. (A) Disease symptoms of gene-knockdown wheat leaves 14 days after inoculation with leaf rust pathotype FHJT. (B) Relative expression of wheat *TaLr19TLP1* in gene-knockdown wheat leaves after inoculation with FHJT. Mock, TcLr19 wheat leaves without BSMV and FHJT inoculation; Lr19+, TcLr19 after FHJT inoculation; Tc+, Thatcher after FHJT inoculation. *GAPDH* was used as the reference. The mean value and standard deviation of the expression were calculated from three independent biological replicates.

3.4. Knockdown of TaLr19TLP1 reduced the resistance of wheat against P. triticina

The expression of *TaLr19TLP1* was knocked down using the BSMV-based VIGS system to further characterize its function in the interaction between wheat and leaf rust pathogen. Silencing of the wheat PDS was used as a positive control because of the visible photo-bleaching phenotype. To ensure the specificity of target gene silencing, two fragments of *TaLr19TLP1* measuring 307 and 230 bp in length were cloned and inserted into the plasmid, respectively (Supplementary Figure 5). Among all the BSMV-inoculated plants, both treatment and control displayed mild chlorotic mosaic symptoms 9 dpi but showed no obvious defects in leaf growth. Photo-bleaching phenotypes were observed in BSMV-PDS knock-down plants 10 days after virus inoculation (Figure 6(A)). The seedlings inoculated with water represented the controls. After inoculating seedlings of wheat cultivar with FHJT, different degrees of infection (severity) were visualized on the surface of leaf blades 14 dpi. An immune phenotype (Scale 0) was observed on wheat leaves preinfected with Mock (buffer inoculated without BSMV), BSMV-PDS, TcLr19, and BSMV-00 (empty vector) compared with Thatcher (Scale 4), whereas fewer leaf rust uredinias (Scale 1) were observed on leaves preinfected with BSMV: V1 and higher leaf rust uredinias (Scale 2) with BSMV: V2 (Figure 6(A)). Thus, knocking down the expression of *TaLr19TLP1* reduced the resistance of wheat to leaf rust fungus.

To determine the efficiency of VIGS, qRT-PCR assays were performed on RNA samples extracted from the fourth leaves of wheat seedlings preinfected with BSMV: 00, BSMV: V1 and BSMV: V2 at 96 hpi with FHJT according to our previous study which showed that the *TaLr19TLP1* expression inoculated with leaf rust pathogen peaked at 96 hpi. Compared with BSMV:00 control, the abundance of the two *TaLr19TLP1* transcripts was significantly suppressed to vary degree in the silenced plants (Figure 6(B)).

4. Discussion

Thaumatin-like proteins have been isolated and characterized from different plants and tissues. They are classified as PR5 proteins and shown to play a key role in alleviating both biotic and abiotic stress tolerance (Goel et al. 2010; Das et al. 2011; Singh et al. 2013). PR5 is a developmentally controlled gene expressed in multiple organs (Jayasankar et al. 2003) suggesting a constitutive functional role and activation in different parts of the plant system. Semi-quantitative RT–PCR showed that the transcriptional levels of *AsPR5* were higher in stem tissues, the primary site of FOC infection, followed by leaves, roots and flowers in garlic (Rout et al. 2016). Earlier, El-Kereamy et al. (2011) reported that *PdPR5-1* was significantly expressed in the fruits of *Prunus persica*, which acts as the primary site of infection by brown rot pathogen *Monilinia fruiticola*. Here, we show that *TaLr19TLP1* is expressed in roots, stems as well as leaves of wheat. However, its expression in roots was relatively lower compared with leaves and stems (Figure 1), which was consistent with the expression pattern of *LePR-5* in

tomato and *AsPR5* in garlic (Ren et al. 2011; Rather et al. 2015).

PR5 proteins may localize to cytoplasm, vacuoles or may be secreted outside the cell depending on their specific function (Melchers et al. 1993). Plants depend on secretory pathways to respond to abiotic or biotic environmental challenges (Wang and Dong, 2011). The recombinant TLP proteins including AdTLP-GFP (Singh et al. 2013), TaPR5-GFP (Wang et al. 2010) and CkTLP-GFP (Wang et al. 2011) were mainly identified as extracellular proteins during transient expression. Other so-called extracellular TLPs such as RlemTLP and CsTL1 were found to predominantly localize to both periphery of plasma membrane and cytoplasm and mediate antifungal activities (Kim et al. 2009). Due to the acidic nature and the presence of 23-amino acid signal peptide at the N-terminus, it suggested that *TaLr19TLP1* may be an extracellularly secreted protein. The extracellular localization was demonstrated by the transient expression assay in the onion epidermal cells. The TaLr19TLP1–GFP fusion protein was detected in the apoplast of the transformed cells.

Synergistic or antagonistic interaction between the pathways regulating these signal molecules mediates the response to specific fungal phytopathogen (Antico et al. 2012). SA triggers the pathway of local acquired resistance (LAR) and systemic acquired resistance (SAR), which are associated with the accumulation of PR proteins (Ryals et al. 1996, Shirasu et al. 1997). Our studies revealed that SA was the most effective phytohormone inducing the expression of *TaLr19TLP1*, which was activated at 12 hpt, peaked at 48 hpt followed by a significant downregulation at 72 hpt. The gaseous hormone ETH often worked in concert with MeJA-mediated signaling leading to constitutive activation of plant genes involved in fungal defense response (Guo and Ecker 2004). Temporal analyses of *TaLr19TLP1* following ET application revealed that the transcript levels of *TaLr19TLP1* peaked at 6 hpt. On the other hand, JA induced a moderate expression at 12 hpt, while ABA was proved to be inactive. Thus, *TaLr19TLP1* expression was most effectively activated by SA signaling resulting in LAR and SAR and accumulation of PR proteins.

We also investigated whether pretreatment with chemical inducers such as SA, ETH, MeJA and ABA, prior to leaf rust pathogen infection, resulted in differential gene expression. Alteration in relative gene expression of the defense-related genes was also assessed after treatment with chemical inducers on various days prior to inoculation with *P. triticina*. Our analyses indicate that pretreatment with SA, MeJA and ABA prior to infection with *P. triticina* enhanced the transcription of *TaLr19TLP1*. A slight induction was observed in ETH pretreated seedlings compared with inoculated but untreated counterparts. The results are consistent with other reports suggesting a correlation between increased disease resistance and elevated PR transcript accumulation (Zambounis et al. 2012). Based on our results, we speculated that pretreatment with SA and MeJA triggers early response to *P. triticina* infection as indicated by the upregulation of *TaLr19TLP1* genes in wheat seedlings, without any noticeable induction during ETH pretreatment.

VIGS has been documented as a rapid and effective reverse genetic approach in studies investigating gene function in barley and wheat (Senthil-kumar and Mysore 2011; Scofield and Brandt 2012). To assess the role of *TaLr19TLP1* in response to *P. triticina* infection, two fragments of *TaLr19TLP1* were knocked down. Compared with the control group, the disease symptoms of silenced *TaLr19TLP* plants were postponed and the severity of infection was attenuated, which was reflected by fewer leaf rust uredinia. The results indicated that silencing of *TaLr19TLP1* did not inhibit or eliminate R gene-mediated resistance to the leaf rust fungus, but suppressed fungal growth and development, suggesting that *TaLr19TLP1* silencing reduced resistance in wheat against *P. triticina*. Real-time PCR was performed to confirm the efficiency of *TaLr19TLP1* silencing. The expression of *TaLr19TLP1* transcripts was suppressed in the knocked down leaves relative to their expression in BSMV:00-inoculated leaves.

In conclusion, the results suggest that *TaLr19TLP1* plays a definitive role in wheat resistance to leaf rust fungus via multiple defense signaling pathways.

Disclosure statement

No potential conflict of interest was reported by the authors.

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