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RESEARCH ARTICLE

Pathogenesis-related protein genes involved in race-specific all-stage resistance and non-race specific high-temperature adult-plant resistance to *Puccinia striiformis* f. sp. *tritici* in wheat

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Abstract

Interactions of the stripe rust pathogen (*Puccinia striiformis* f. sp. *tritici*) with wheat plants activate a wide range of host responses. Among various genes involved in the plant-pathogen interactions, the expressions of particular pathogenesis-related (PR) protein genes determine different defense responses. Different types of resistance have been recognized and utilized for developing wheat cultivars for resistance to stripe rust. All-stage resistance can be detected in seedling stage and remains at high levels throughout the plant growth stages. This type of resistance is race-specific and not durable. In contrast, plants with only high-temperature adult-plant (HTAP) resistance are susceptible in seedling stage, but become resistant when plants grow older and the weather becomes warmer. HTAP resistance controlled by a single gene is partial, but usually non-race specific and durable. The objective of this study was to analyze the expression of PR protein genes involved in different types of wheat resistance to stripe rust. The expression levels of 8 PR protein genes (*PR1*, *PR1.2*, *PR2*, *PR3*, *PR4*, *PR5*, *PR9* and *PR10*) were quantitatively evaluated at 0, 1, 2, 7 and 14 days after inoculation in single resistance gene lines of wheat with all-stage resistance genes *YrTr1*, *Yr76*, *YrSP* and *YrExp2* and lines carrying HTAP resistance genes *Yr52*, *Yr59*, *Yr62* and *Yr7B*. Races PSTv-4 and PSTv-37 for compatible and incompatible interactions were used in evaluation of PR protein gene expression in wheat lines carrying all-stage resistance genes in the seedling-stage experiment while PSTv-37 was used in the HTAP experiment. Analysis of quantitative real-time polymerase chain reaction (qRT-PCR) revealed that all of the PR protein genes were involved in the different types of resistance controlled by different *Yr* genes. However, these genes were upregulated at different time points and at different levels during the infection process among the wheat lines with different *Yr* genes for either all-stage resistance or HTAP resistance. Some of the genes were also induced in compatible interactions, but the levels were almost always higher in the incompatible interaction than in the compatible interaction at the same time point for each *Yr* gene. These results indicate that both

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salicylic acid and jasmonate signaling pathways are involved in both race-specific all-stage resistance and non-race specific HTAP resistance. Although expressing at different stages of infection and at different levels, these PR protein genes work in concert for contribution to different types of resistance controlled by different *Yr* genes.

Keywords: wheat, stripe rust, plant resistance, pathogen-related protein, qRT-PCR

1. Introduction

Stripe rust (yellow rust), caused by *Puccinia striiformis* Westend. f. sp. *tritici* Erikss. (*Pst*), is one of the most damaging diseases of wheat worldwide (Chen 2005; Wellings 2011). Depending upon the severity of epidemic and resistance or susceptibility of wheat cultivars, yield losses caused by stripe rust range from 0 to more than 90% (Chen 2005, 2014). The disease can be controlled by growing resistant cultivars or by timely applying effective fungicides. These different control strategies have their advantages and disadvantages. Use of the fungicides adds extra cost to wheat production; is harmful to humans, animals and the environment; and may be prohibited in the areas that are protected for special habitats or for organic farming. Growing resistant cultivars is the most effective, economic, easy-to-use and environmentally friendly approach for control of the disease (Chen 2005, 2013; Dodds and Rathjen 2010).

When a *Pst* urediniospore adheres to a leaf surface and starts to infect the plant under the conditions of dew formation and temperature range for germination and penetration, the plant exhibits diverse physiological and biochemical changes. Because of these changes, the plant will either show a resistant (incompatible) or susceptible (compatible) response. The onset of these responses involves the recognition of host plants to the attacking pathogen. Wheat plants respond to the infection of *Pst* through various defense compounds induced by the pathogen. The plant response can be completely resistant, completely susceptible, or any level in between. Resistance to stripe rust can be classified into different types based on various ways of categorization, but commonly into all-stage resistance *vs*. high-temperature adult-plant (HTAP) resistance (Line 2002; Chen 2013). All-stage resistance, also called seedling resistance, can be detected at seedling stage, but expresses during all stages of plant growth. This type of resistance usually provides a high level of protection against *Pst* infection but is easily overcome by virulent races of the pathogen, and therefore, not durable. In contrast, HTAP resistance, which starts expressing when plants reach late jointing stage (Zadoks 32) and the weather becomes warm, is non-race specific and durable, but often does not provide complete protection, especially when the disease starts early in the growth season and the weather is favorable for the disease development (Chen 2005, 2013). Although both types of resistance can be controlled by a master gene, referred as a *Yr* gene for yellow rust resistance, numerous defense-related genes that are regulated by master genes are involved in either type of resistance (Coram *et al*. 2008a, b, 2010; Chen *et al*. 2013). Through transcriptomics analysis, different genes were found to be involved in all-stage and/or HTAP resistance (Coram *et al*. 2008a, b, 2010). However, molecular mechanisms underlying the different types of resistance are still not well understood.

In a plant-pathogen interaction, a complex signaling cascade is involved, including the production of reactive oxygen species (ROS), referred as oxidative burst. ROS are not only antimicrobial compounds, but also inducers of hypersensitive response (HR) that is related to the host cell necrosis in the infected cells (Tenhaken *et al*. 1995; Lamb and Dixon 1997). Host cell death, or programmed cell death, is beneficial for plants as a defense response against biotrophic pathogens, because it is detrimental for biotrophic pathogens as they need living host cells to grow and reproduce (Williams and Dickman 2008).

Pathogen-related (PR) proteins occur widely in the plant kingdom and comprise a large group of 17 protein families. These proteins include PR-1 (unknown function), β-1,3-glucanase (PR-2), endochitinases (PR-3, PR-4, PR-8 and PR-11), thaumatin-like proteins (PR-5), proteinase inhibitors (PR-6), proteinase (PR-7), peroxidase (PR-9), ribonuclease-like proteins (PR-10), defensins (PR-12), thionins (PR-13), lipid transfer proteins (PR-14), oxalate oxidase (PR-15), oxalate oxidase-like proteins (PR-16) and PR-17 (unknown function). These PR proteins have been reported to play important roles in plant defense against various biotic and abiotic stresses (Van Loon and Van Strien 1999; Van Loon *et al*. 2006a, b), but the roles of most of them in wheat-*Pst* interactions have not been studied.

At the molecular level, little is known about how different types of stripe rust resistance affect the development of the pathogen, or the response of the plant to pathogen invasion. One approach for understanding the mechanisms of disease resistance is to profile the expression of different genes. Studying the *Yr5* resistance, a typical race-specific all-

stage resistance, and the *Yr39* resistance, a typical non-race specific HTAP resistance, in comparison with a susceptible reaction using the commercial Affymetrix wheat microarray genechip or a custom genechip revealed expression of few PR protein genes (Coram *et al*. 2008a, b). The attempts to expend gene expression to include some other *Yr* genes for either all-stage or HTAP resistance were also limited by the numbers of genes in the custom genechip used in the previous studies (Coram *et al*. 2010; Chen *et al*. 2013). It is not known whether other PR protein genes are involved in the different types of resistance, and if involved, whether PR protein genes express differently in the different types of resistance controlled by different *Yr* genes. Therefore, the objective of this study was to elucidate the roles of different PR protein genes in different types of resistance and their involvement in resistance controlled by different *Yr* genes. The results may be helpful for improving the understanding of race specificity and durability of stripe rust resistance.

2. Materials and methods

2.1. Plant materials

Spring wheat (*Triticum aestivum* L.) near-isogenic lines (NILs) AvSYrTr1NIL (*YrTr1*), AvSYrSPNIL (*YrSP*) and AvSYr76NIL (*Yr76*) and recombinant inbred line (RIL) AvS/Exp 1/1-1 line 7 (*YrExp2*), representing race-specific all-stage resistance, and RILs *Yr52*, *Yr59*, *Yr62* and *YrPI182103-7BL* (*Yr7B*), representing non-race specific HTAP resistance, were used in this study. The NILs for *YrTr1* and *YrSP* were developed by Wellings *et al*. (2004), and the NIL for *Yr76* and the RIL for *YrExp2* were developed by Xiang *et al*. (2016) and Lin and Chen (2008), respectively in our program. The RILs for *Yr52*, *Yr59*, *Yr62* and *Yr7B* were also developed in our program (Ren *et al*. 2012; Feng *et al*. 2013; Lu *et al*. 2014; Zhou *et al*. 2014). All of these lines have the spring wheat 'Avocet Susceptible' (AvS) background.

2.2. Pathogen races

Races PSTv-4 (virulent to *Yr1*, *Yr6*, *Yr9*, *Yr17*, *Yr27*, ***YrSP*** and ***Yr76*** but avirulent to *Yr5*, *Yr7*, *Yr8*, *Yr10*, *Yr15*, *Yr24*, *Yr32*, *Yr43*, *Yr44*, ***YrTr1*** and ***YrExp2***) and PSTv-37 (virulent to *Yr6*, *Yr7*, *Yr8*, *Yr9*, *Yr17*, *Yr27*, *Yr43*, *Yr44*, ***YrTr1*** and ***YrExp2***, but avirulent to *Yr1*, *Yr5*, *Yr10*, *Yr15*, *Yr24*, *Yr32*, ***YrSP*** and ***Yr76***) (Wan and Chen 2014) were used to study the expression of PR protein genes in compatible and incompatible interactions (bold highlighted *Yr* genes are the ones studied in this paper). PSTv-37 was used to study PR protein gene expression in the HTAP resistance experiments, in which RILs *Yr52*, *Yr59*, *Yr62* and *Yr7B* were resistant, but they were susceptible to this race and other races in seedlings (Ren *et al*. 2012; Feng *et al*. 2013; Lu *et al*. 2014; Zhou *et al*. 2014).

2.3. Plant inoculation

The experiments for studying race-specific all-stage resistance and non-race specific HTAP resistance were conducted separately. All experiments were conducted using a randomized complete block design with three biological replications. For each biological replication in the race-specific all-stage experiment, seeds of individual genotypes were planted in separate 25 cm×42.5 cm flats using a potting mix (6 peat moss:4 vermiculite with lime:3 sand:3 commercial potting mix:2 perlite:1.7 g L^{-1} lime:3.3 g L^{-1} Osmocote:2.2 g L^{-1} ammonium nitrate). Each flat contained 5 rows of 10 seedlings each, with rows randomly assigned one of the five time points for sampling, 0, 1, 2, 7 and 14 days after inoculation (dai). Seedlings from one of the rows were used to monitor the expected disease responses to *Pst* inoculation. One row of AvS was also planted in each treatment to check inoculation conditions and efficiency. The seedlings were grown to the two-leaf stage (Zadoks 10, ~12 days after planting) in a greenhouse with a diurnal temperature cycle of 10 to 25°C and a 16-h light/8-h dark cycle. Inoculation was performed by misting the plants with sterile water and applying a 1:20 urediniospore/talcum mixture to leaves with a sterile brush. Talcum was used to aid in the spread and adhesion of spores over leaf surfaces. Control flats were treated in the same way except for the absence of urediniospores in talcum. After inoculation, plants were misted lightly with distilled water. Plastic sleeves were placed around the inoculated pots to prevent contamination. Three biological replications (individual plants) were inoculated in each treatment group. Plants were placed in a dew chamber for 24 h in dark at 10°C with 100% relative humidity and then were removed from the dew chamber and placed in a growth chamber (16 h light at 16°C; 8 h dark at 8°C) (Wan and Chen 2014).

For the HTAP experiment, six seeds were planted in a pot of 15-cm in diameter and 15-cm in height filled with potting soil mixture as described above. Plants were grown in a greenhouse at 18–25°C with a 16-h photoperiod. When the plants reach the boot stage (Zadoks 45), inoculation of flag leaves was performed by misting the plants with sterile water and then applies with a 1:20 urediniospore/talcum mixture with a sterile brush. Mock-inoculated plants were treated in the same way with talcum only. Susceptible check AvS was also included in the experiment. Inoculated plants were placed in a dew chamber for 24 h in dark at 10°C with 100% relative humidity, and then placed in a growth chamber set

at a diurnal cycle of 12°C for 12 h, rising to 30°C at the rate of 4.5°C h^{-1}, staying at 30°C for 4 h and then decreasing to 12°C with rate 4.5°C h^{-1} with 16 h light covering the high temperature range and 8 h dark in the low temperature range (Chen 2013). *Pst*- and mock-inoculated seedlings and adult plants for phenotypic observations were grown under the respective temperature conditions as described above, and infection types were recorded 20 dai using the 0–9 scales described by Line and Qayoum (1992).

2.4. RNA extraction

The inoculated leaves were harvested with sterilized scissors at 0, 1, 2, 7 and 14 dai. These time points were selected based on the major points of the infection process: (1) contact between *Pst* and plants (0 dai, just after inoculation), (2) fungus entered plant tissue (1 dai), (3) formation of haustoria and starting of the parasitic life cycle (2 dai) (Zhang *et al*. 2012), (4) appearance of chlorosis disease symptom (7 dai) and (5) sporulation (14 dai). The leaves were placed in a labeled aluminum foil wiped with 95% ethanol and then immediately frozen in liquid nitrogen.

Total 200 mg of leaf tissue for each sample was ground in liquid nitrogen in a sterilized mortar with a sterilized pestle to fine powder. Total 1 mL Trizol (Invitrogen, Carlsbad, CA, USA) was added to each sample. The suspension was homogenized by shaking moderately for 7 s, incubated for 2–3 min at 20–22°C, and added with 0.2 mL chloroform. The samples were shaken for 15 s and incubated for additional 2–3 min at 20–22°C. Samples were centrifuged for 15 min at 13 000 r min^{-1} at 4°C. Approximately 550 µL of the aqueous phase was transferred to an Eppendorf tube, added with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and mixed well by vortexing and centrifuged for 10 min at 13 000 r min^{-1} at 4°C. The aqueous phase was transferred to a new Eppendorf tube, added with an equal volume of chloroform and centrifuged at the same conditions. The aqueous phase was transferred again to a new tube, added with 0.25 mL isopropanol, mixed by inverting, kept at 20–22°C for 2–3 min and centrifuged for 10 min at 13 000 r min^{-1} at 4°C. After decanting the supernatant, the pellet was washed with 1 mL filter sterilized 70% ethanol and dried in a chemical hood. The RNA pellet was dissolved in 50 µL of sterile water and kept at –80°C until use.

RNA was analyzed using 2% agarose gel electrophoresis to ascertain the quality of RNA and the absence of DNA. The concentration of each RNA sample was measured with a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Only the RNA samples with OD_{260}/OD_{280} ratio of 1.9–2.0, indicating protein free, and OD_{260}/OD_{230} ratio greater than 2.0, indicating reagent free, were used for further analysis.

2.5. qRT-PCR assay

As listed in Appendix A, eight PR protein genes of wheat were selected based on their potential roles in plant defense reported in wheat or other plants. The primer sequences for *PR1*, *PR2*, *PR3*, *PR5* and *PR10* were from Desmond *et al*. (2006), *PR1.2* from Molina *et al*. (1999), *PR4* and *PR9* were from Casassola *et al*. (2015) and *β-actin* from Yin *et al*. (2009). All primers were synthesized by Promega (Madison, WI, USA). The iTaq™ Universal One-Step RT-qPCR Kit (Life Science Research, Hercules, California, USA) was used in quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR amplification was performed in a total volume of 10 µL containing 60 ng $µL^{-1}$ RNA, 1×*iTaq* universal SYBER Green Reaction Mix, 1×iScript reverse transcriptase, 300 nmol L^{-1} of gene-specific primers and nuclease-free water. PCR was performed in a Bio-Rad iQ5 Real-Time PCR Detection System Instrument (Bio-Rad, Hercules, California, USA) with the following cycling program: reverse transcription of RNA at 50°C for 10 min and qRT-PCR performed at 95°C for 1 min, 35 cycles of 95°C for 10 s, 60°C for 30 s and 75°C for 30 s. All products were subjected to the melting curve analysis between 55 and 95°C to determine the specificity of the PCR reaction. Experiments included a non-template control and non-RT control. Transcript levels of all eight PR protein genes in *Pst* inoculated wheat leaf tissue were calculated in relative to their mean expression levels in the mock-inoculated (control) plants, at each time point. The expression value of *β-actin* gene, which is constitutively expressed and not affected by pathogen infection, was used to calibrate the expression values of PR protein genes in each sample. Three biological replicates were analyzed for each time point. Relative fold changes were calculated using the comparative $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001).

2.6. Statistical analysis

Relative gene transcript levels were analyzed using analysis of variance (ANOVA) to determine significant differences between the expression levels using the Statistical Analysis System (SAS) Program (SAS Institute Inc., Cary, NC, USA). The Tukey's test was used to determine significant differences among the genes at each time point.

3. Results

3.1. Phenotypic observations

In all experiments, resistance to stripe rust on all *Pst*-inoculated and mock-inoculated plants were monitored until 20 dai. For all incompatible interactions, leaves of

all genotypes began to show chlorosis at ~7 dai, which developed into necrotic flecks by 14 dai. For the compatible interactions, sporulation was observed starting at ~14 dpi. Infection types (ITs) were recorded at 20 dai. All mock-inoculated plants were free of any disease symptoms over the 20-day observation period. Expected reactions, IT 1 or 2 (necrotic blotches on seedling leaves and necrotic stripes on flag leaves without uredinia) for incompatible (resistant) reaction and IT 8 or 9 (abundant uredinia with or without chlorosis) for compatible (susceptible) reaction, were confirmed for all wheat line — *Pst* race treatments of the wheat *Yr* single-gene lines with all-stage resistance genes *YrTr1*, *YrExp2*, *YrSP* or *Yr76*. Similarly, expected IT 2 (necrotic stripes without uredinia) was observed on flag leaves of wheat lines with HTAP resistance genes *Yr52*, *Yr59*, *Yr62* or *Yr7B* whereas the susceptible check variety AvS had IT 8. These results indicated successful inoculation and right conditions for the plant samples that were used to determine expression of PR protein genes.

3.2. Expression of PR protein genes in race-specific all-stage resistance

YrTr1 The *YrTr1* line was resistant to PSTv-4 (incompatible interaction) but susceptible to PSTv-37 (compatible interaction). The mean relative expression values of the PR protein genes at different time points are present in Appendix B and their patterns are shown in Fig. 1. Genes *PR2* (1.14), *PR3* (1.34) and *PR9* (1.61) were induced in the incompatible interaction at 0 dai, but their expression was not significant compared with other genes and also not significantly different from those in the compatible interaction. At 1 dai, all genes (4.58–9.53 in the incompatible interaction and 2.79–6.90 for the compatible interaction except for *PR2*) were highly induced compared to the mock inoculation. In comparison between the incompatible and compatible interactions, all of these genes, except *PR1* and *PR5*, were significantly higher in the incompatible interaction. *PR1.2* had the highest expression level (9.53) in the incompatible interaction. At 2 dai, all genes, except *PR1.2* and *PR9*, were induced by *Pst* inoculation, and the expression levels of these induced genes (*PR1*, *PR2*, *PR3*, *PR4*, *PR5* and *PR10*) were significantly higher in the incompatible interaction than in the compatible interaction. *PR1* had the highest expression level (8.22) in the incompatible interaction. At 7 dai, *PR1*, *PR2* and *PR10* were induced in the compatible interaction, whereas all genes, except *PR9*, were significantly induced in the incompatible interaction compared to the mock inoculation, and their expression levels were significantly higher than those in the compatible interaction. *PR4* had the highest expression level (6.58) in the incompatible interaction. At 14 dai, none of the PR protein genes were induced in the compatible interaction and only *PR1* (3.21) was significantly induced in the incompatible interaction.

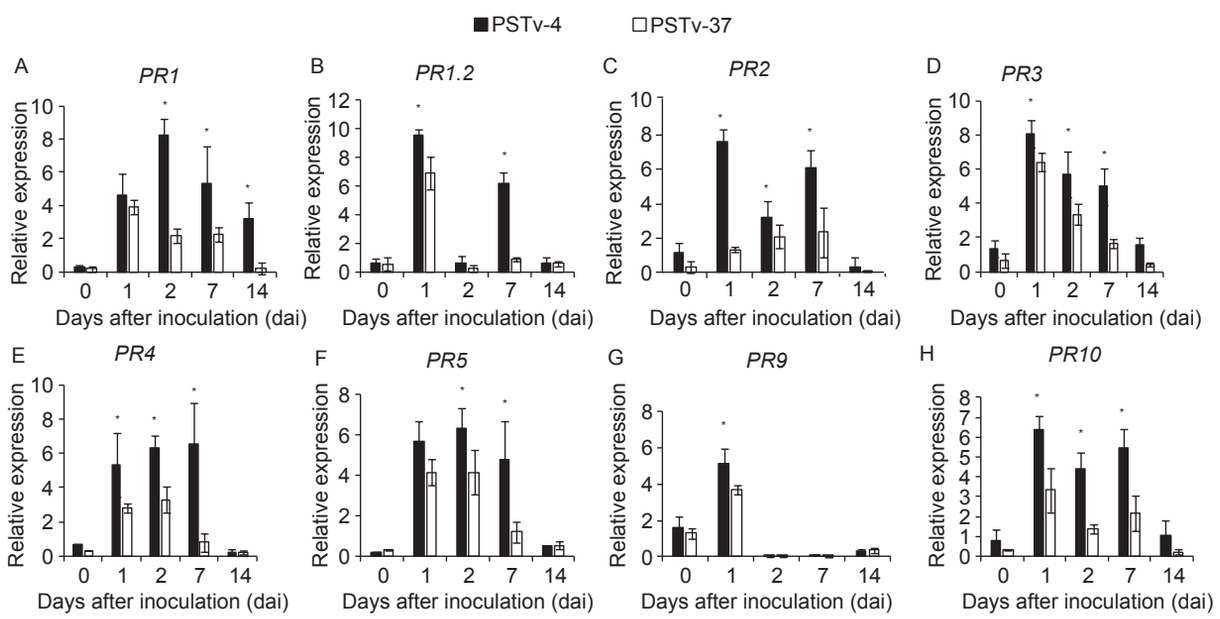

Fig. 1 Quantitative real-time PCR (qRT-PCR) analyses of expression of eight PR protein genes in wheat line of *YrTr1* inoculated with races PSTv-4 (incompatible) and PSTv-37 (compatible) of *Puccinia striiformis* f. sp. *tritici*. Three independent biological replications were performed. All data were normalized to the *β-actin* expression level. The relative expression was expressed as fold change relative to the mock inoculated plants. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta C_T}$ method. The * symbol represents significant difference ($P \leq 0.05$) according to analysis of variance. Bars represent standard deviation among the biological replicates.

YrExp2 Similar to *YrTr1*, the *YrExp2* line was also resistant to PSTv-4 (incompatible interaction) but susceptible to PSTv-37 (compatible interaction). The mean relative expression values of the PR protein genes at different time points are present in Appendix B and their patterns are shown in Fig. 2. At 0 dai, the expression levels of *PR4* and *PR9* in the incompatible interaction were significantly higher than those in the compatible interaction, but none of the eight PR protein genes had the relative expression values greater than 2.00. At 1 dai, all eight PR protein genes were significantly induced in the incompatible, but only *PR5* was significantly induced in the compatible interaction compared to the mock inoculation. All of these genes had higher expression levels in the incompatible interaction than the compatible interaction. *PR10* had the highest expression level (8.03) in the incompatible interaction. At 2 dai, the expression levels of all *PR* genes were significantly higher in the incompatible interactions than those in the compatible interactions. The similar results were observed at 7 dai, however, the expression levels were relatively low compared to 2 dai and *PR2* had the highest expression level (6.87). At 14 dai, *PR1.2*, *PR2*, *PR3*, *PR5* and *PR9* were significantly induced in the incompatible interaction but only *PR1.2* and *PR2* were significantly induced in the compatible interaction in comparison with the mock inoculation. The expression levels of *PR1*, *PR1.2*, *PR2*, *PR3*, *PR5* and *PR9* were significantly higher in the incompatible interaction than the compatible interaction, and *PR2* also had the highest expression level (6.12).

YrSP The *YrSP* line was susceptible to PSTv-4 (compatible interaction) but resistant to PSTv-37 (incompatible interaction). The mean relative expression values of the PR protein genes at different time points are present in Appendix B and their patterns are shown in Fig. 3. At 0 dai, the expression levels of *PR4*, *PR9* and *PR10* were significantly higher in the incompatible interaction than the compatible interaction. At 1 dai, none of the genes, except *PR3*, were significantly induced in the compatible interactions, whereas all of the genes were significantly induced in the incompatible interaction compared to the mock inoculation. All of the genes had significantly higher expression levels in the incompatible interaction than the compatible interaction, with *PR3* had the highest expression level (24.24) in the incompatible interaction. At 2 dai, the expression levels of all of the genes in the incompatible interaction were significantly higher than those in the compatible interaction with *PR2* had the highest expression level (25.88). At 7 dai, the expression levels of *PR1*, *PR1.2*, *PR3*, *PR4* and *PR9* were significantly higher in the incompatible interaction than the compatible interaction with *PR9* the highest (10.05). At 14 dai, only *PR9* was significantly induced in the incompatible interaction.

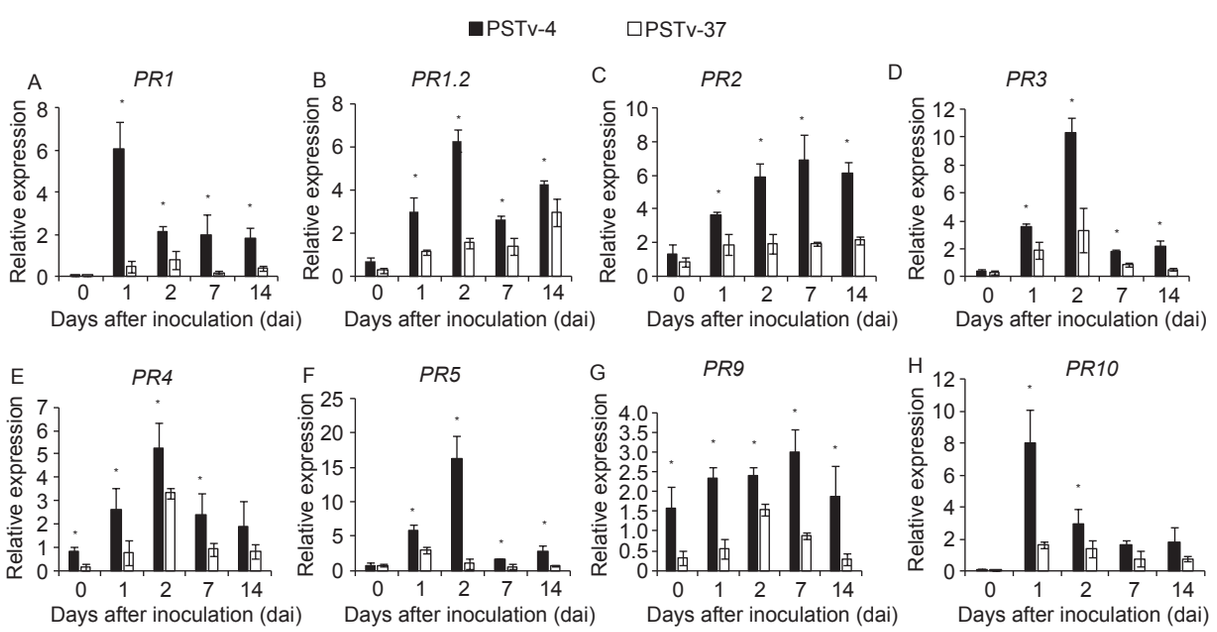

Fig. 2 Quantitative real-time PCR (qRT-PCR) analyses of expression of eight PR protein genes in wheat line of *YrExp2* inoculated with races PSTv-4 (incompatible) and PSTv-37 (compatible) of *Puccinia striiformis* f. sp. *tritici*. Three independent biological replications were performed. All data were normalized to the *β-actin* expression level. The relative expression was expressed as fold change relative to the mock inoculated plants. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta C_T}$ method. The * symbol represents significant difference ($P \leq 0.05$) according to analysis of variance. Bars represent standard deviation among the biological replicates.

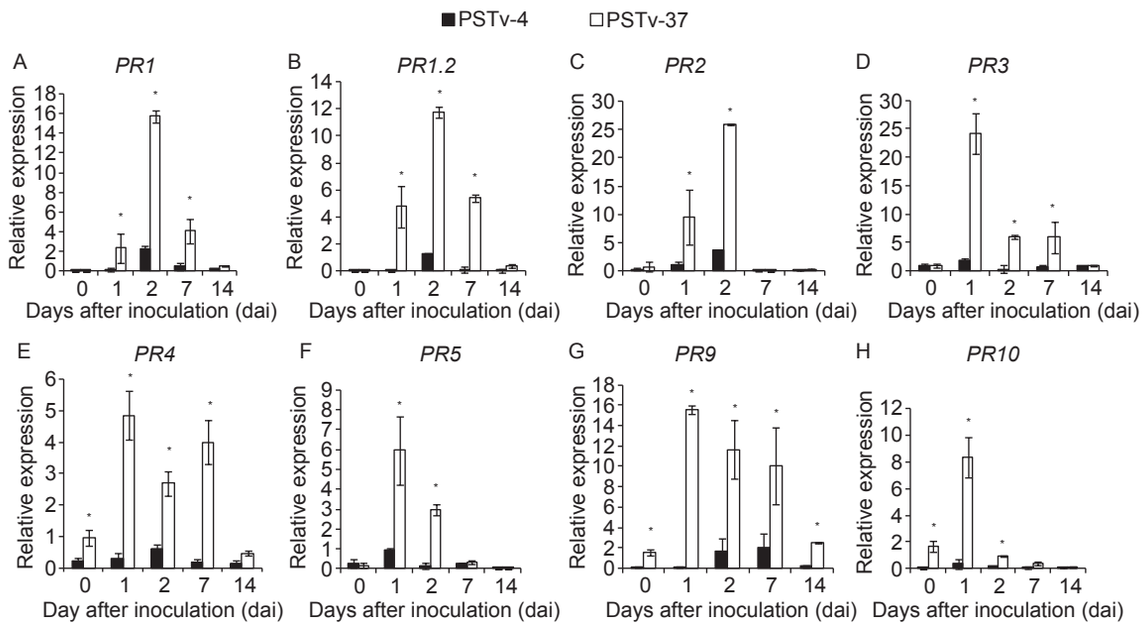

Fig. 3 Quantitative real-time PCR (qRT-PCR) analyses of expression of eight PR protein genes in wheat line of *YrSP* inoculated with races PSTv-4 (compatible) and PSTv-37 (incompatible) of *Puccinia striiformis* f. sp. *tritici*. Three independent biological replications were performed. All data were normalized to the *β-actin* expression level. The relative expression was expressed as fold change relative to the mock inoculated plants. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta C_T}$ method. The * symbol represents significant difference ($P \leq 0.05$) according to analysis of variance. Bars represent standard deviation among the biological replicates.

Yr76 Similar to *YrSP*, the *Yr76* line was susceptible to PSTv-4 (compatible interaction) but resistant to PSTv-37 (incompatible interaction). The mean relative expression values of the PR protein genes at different time points are present in Appendix B and their patterns are shown in Fig. 4. At 0 dai, *PR2*, *PR3*, *PR4*, *PR5* and *PR9* had significantly higher expression levels in the incompatible than the compatible interaction. *PR4* had the highest expression level (13.93) in the incompatible interaction. At 1 dai, the expression levels of *PR3*, *PR4*, *PR5* and *PR10* were significantly higher in the incompatible interaction than the compatible interaction. *PR10* had the highest expression level (18.33) in the incompatible interaction. At 2 dai, all of the genes, except *PR9*, were significantly induced in the incompatible interaction, and *PR1*, *PR1.2* and *PR2* were also induced in the compatible interaction. All genes, except *PR1.2* and *PR9*, had significantly higher expression levels in the incompatible interaction than the compatible interaction. *PR1* had the highest expression level (7.81). At 7 dai, all genes, except *PR2* and *PR5*, were significantly induced in the incompatible interaction, while only *PR3* was significantly induced in the compatible interaction compared to the mock inoculation. *PR1*, *PR1.2*, *PR3* and *PR10* had significantly higher expression levels in the incompatible interaction than in the compatible interaction. *PR1.2* had the highest expression level (10.48) in the incompatible interaction. At 14 dai, only *PR9* was significantly induced in the compatible interaction, but *PR3*, *PR4* and *PR9* were significantly induced in the incompatible interaction and the expression levels of the three genes were significantly higher in the incompatible interaction than in the compatible interaction.

3.3. Expression of PR protein genes in non-race specific HTAP resistance

As there was no a compatible interaction for each of the non-race specific HTAP resistance genes, the expression levels of the PR protein genes were compared with those in the mock inoculation and compared to each other for their relative levels of induction by *Pst* inoculation. The expression levels are presented in Table 1.

Yr52 At 0 dai, *PR1.2*, *PR3*, *PR5* and *PR9* were significantly induced by the *Pst* inoculation compared to the mock inoculation (Table 1). At 1 dai, all genes, except *PR9* and *PR10*, were significantly induced, and *PR5* had the highest expression level (14.03). At 2 dai, all genes, except *PR9*, were significantly induced, and *PR1* had the highest expression level (11.65). At 7 dai, only *PR1.2* was significantly induced. In contrast, all genes, except *PR1*, were significantly induced with *PR2* the highest (14.30) at 14 dai.

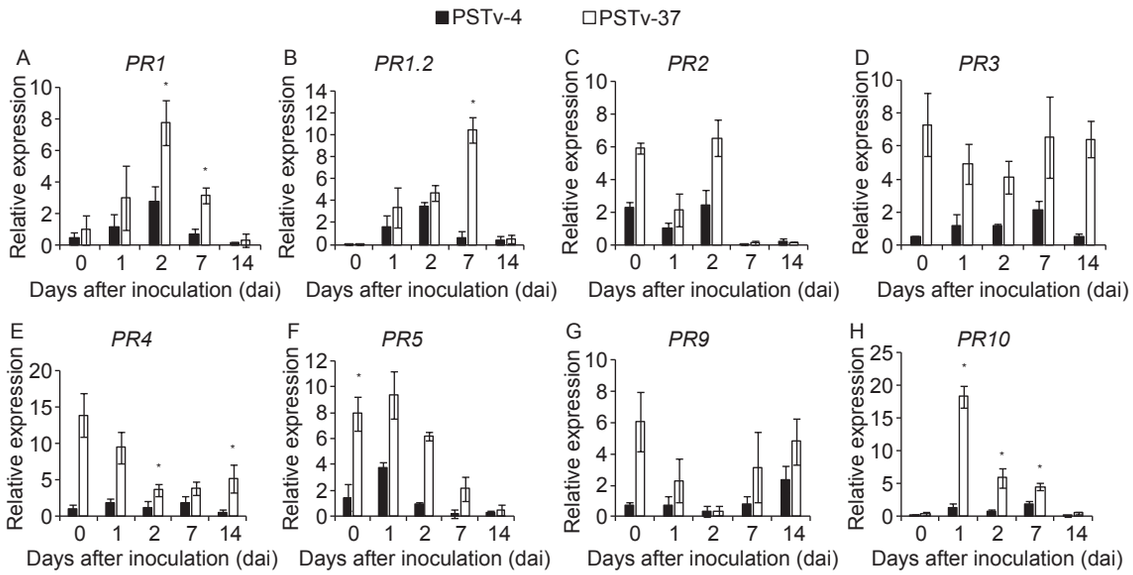

Fig. 4 Quantitative real-time PCR (qRT-PCR) analyses of expression of eight PR protein genes in wheat line of *Yr76* inoculated with races PSTv-4 (compatible) and PSTv-37 (incompatible) of *Puccinia striiformis* f. sp. *tritici*. Three independent biological replications were performed. All data were normalized to the *β-actin* expression level. The relative expression was expressed as fold change relative to the mock inoculated plants. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta C_T}$ method. The * symbol represents significant difference ($P \leq 0.05$) according to analysis of variance. Bars represent standard deviation among the biological replicates.

Yr59 None of the PR protein genes were significantly induced at 0 and 1 dai (Table 1). At 2 dai, *PR2*, *PR5* and *PR9* were significantly induced, and *PR5* had the highest expression level (4.93). At 7 dai, five genes, *PR1.2*, *PR3*, *PR4*, *PR5* and *PR10*, were significantly induced, and *PR1.2* had the highest expression level (5.00). At 14 dai, *PR1*, *PR2*, *PR3*, *PR4* and *PR9* were significantly induced, and *PR9* had the highest expression level (21.04).

Yr62 *PR4*, *PR5* and *PR10* were significantly induced (3.79–5.73) at 0 dai (Table 1). At 1 dai, *PR3*, *PR4*, *PR5*, *PR9* and *PR10* were significantly induced with *PR4* having the highest expression (10.38). At 2 dai, all genes were significantly induced at the levels ranging from 2.91 (*PR10*) to 17.97 (*PR5*). At 7 dai, *PR1*, *PR5* and *PR9* were significantly induced with *PR9* the highest (8.55). At 14 dai, *PR5*, *PR9* and *PR10* were significantly induced, and *PR9* had the highest expression level (3.80).

Yr7B At 0 dai, *PR1*, *PR3* and *PR9* were significantly induced with *PR9* the highest (4.82) (Table 1). At 1 dai, all of the genes were significantly induced at the levels ranging from 5.95 (*PR10*) to 40.30 (*PR9*). Similarly, all of the genes were significantly induced at 2 dai, with the highest expression level (25.72) observed for *PR5*. At 7 dai, all genes, except *PR1.2*, were significantly induced, and *PR4* had the highest expression level (7.90). At 14 dai, five genes, *PR1*, *PR1.2*, *PR2*, *PR3* and *PR4* were significantly induced, and among these *PR1.2* had the highest expression level (3.69).

The expression patterns of the PR protein genes were quite different among the four HTAP resistance gene lines (Table 1). In the *Yr52* lines, highly expressed (>10.0 fold) PR protein genes occurred at 1 dai (*PR1.2*, *PR2*, *PR3* and *PR5*), 2 dai (*PR1* and *PR4*) and 14 dai (*PR2* and *PR10*). In the *Yr59* line, the highest expression levels were mainly observed at 14 dai (*PR1* and *PR9*). In contrast, in the *Yr62* line, the highest expression occurred at 2 dai for all PR protein genes except *PR10*. Similar to the *Yr52* line, in the *Yr7B* line, the majority of the PR protein genes highly expressed (>10.0 fold) at 1 and 2 dai, but none of the genes had expression levels greater than 10 folds at both 7 and 14 dai.

4. Discussion

Pathogen-related proteins play important roles in plant defense against pathogen infection and also elicit resistance. The accumulation of PR protein gene transcripts is one of the best-characterized plant defense responses in many other plant pathosystems. However, PR proteins had not been well characterized for wheat-*Pst* interactions. In the present study, we determined the involvement of eight PR protein genes in race-specific all-stage resistance controlled by four individual genes (*YrTr1*, *YrExp2*, *YrSP* and *Yr76*) and in non-race specific HTAP resistance controlled by four genes independently (*Yr52*, *Yr59*, *Yr62* and *Yr7B*). We

Table 1 Expression levels of pathogen-related (PR) protein genes in wheat near-isogenic lines with *Yr52*, *Yr59*, *Yr62* and *YrPI182103-7BL* (*Yr7B*) for non-race specific high-temperature adult-plant resistance at different days after inoculation (dai) with race PSTv-37 of *Puccinia striiformis* f. sp. *tritici* in adult-plant stage

Yr & PR protein genes	Mean relative expression				
	0 dai	1 dai	2 dai	7 dai	14 dai
Yr52					
PR1	0.77 a	2.98 b*	11.65 e*	0.22 a	1.67 ab
PR1.2	2.10 b*	11.17 e*	7.60 d*	6.13 cd*	3.06 b*
PR2	1.12 a	11.17 e*	7.46 d*	0.10 a	14.30 f*
PR3	2.53 b*	13.86 f*	7.73 d*	1.09 a	5.39 c*
PR4	0.92 a	4.02 c*	10.09 e*	0.33 a	4.20 c*
PR5	2.09 b*	14.03 f*	4.06 c*	1.35 a	5.72 cd*
PR9	2.03 b*	0.46 a	1.75 ab	0.11 a	3.64 b*
PR10	0.32 a	1.69 a	5.60 cd*	0.19 a	10.69 e*
Yr59					
PR1	0.04 a	0.15 a	0.25 a	1.40 ab	13.84 f*
PR1.2	0.08 a	0.12 a	0.09 a	5.00 c*	0.54 a
PR2	0.01 a	0.07 a	2.35 b*	1.43 ab	7.89 d*
PR3	0.56 a	0.20 a	0.36 a	2.94 b*	9.19 e*
PR4	0.06 a	0.16 a	0.20 a	2.59 b*	5.53 c*
PR5	0.02 a	0.15 a	4.93 c*	2.18 b*	1.80 ab
PR9	0.07 a	1.20 ab	3.16 b*	1.67 ab	21.04 g*
PR10	0.01 a	0.10 a	0.54 a	3.82 bc*	0.29 a
Yr62					
PR1	1.80 ab	1.39 ab	16.12 f*	6.14 c*	0.38 a
PR1.2	1.26 ab	0.02 a	13.24 f*	0.27 a	0.23 a
PR2	1.97 ab	1.01 a	14.27 f*	1.86 ab	0.37 a
PR3	1.77 ab	6.17 d*	15.90 f*	1.35 ab	0.46 a
PR4	4.27 c*	10.38 e*	12.39 d*	1.52 ab	0.53 a
PR5	3.79 bc*	2.04 b*	17.97 f*	2.47 b*	2.54 b*
PR9	0.59 a	3.87 bc*	14.97 f*	8.55 d*	3.80 bc*
PR10	5.73 c*	2.40 b*	2.91 b*	1.58 ab	2.37 b*
YrPI182103-7BL (*Yr7B*)					
PR1	3.10 b*	18.40 f*	1.99 ab*	2.63 b*	2.99 b*
PR1.2	0.42 a	11.75 e*	4.17 c*	0.06 a	3.69 bc*
PR2	1.02 a	31.51 g*	11.10 e*	6.10 cd*	2.85 b*
PR3	3.97 bc*	7.17 d*	12.45 e*	2.90 b*	2.53 b*
PR4	1.00 a	13.02 e*	12.29 e*	7.90 d*	2.28 b*
PR5	0.49 a	14.93 e*	25.72 g*	3.55 bc*	1.37 a
PR9	4.82 c*	40.30 h*	4.12 c*	5.30 c*	1.33 a
PR10	0.98 a	5.95 cd*	16.14 f*	5.57 c*	1.33 a

Different letters after the values in a column or a row indicate significant different expression values according to Tukey's test ($P<0.05$), and * indicates the mean expression value is significantly different from that of the mock inoculation ($P<0.05$).

found that all of the tested PR protein genes were involved in resistance controlled by different resistance genes, but different PR protein genes were induced at different stages of infection.

4.1. Expression of PR proteins in plant-pathogen interactions

PR1 proteins have been used as markers for enhanced defense conferred by pathogen-induced systemic acquired resistance (SAR) in various plants (Van Loon *et al*. 2006b; Hou *et al*. 2012; Gkizi *et al*. 2016). As expression of this gene is salicylic acid (SA)-responsive, it is used as an indicator for the SA pathway (Gkizi *et al*. 2016). Li *et al*. (2016) reported the involvement of the wheat PR1 protein in the *Lr35*-mediated adult-plant resistance to leaf rust caused by *Puccinia triticina*. High accumulation of *PR1* transcripts was also reported in wheat lines resistant to *Fusarium graminerum* and *Mycosphaerella graminicola* (Pritsch *et al*. 2000; Ray *et al*. 2003). In the present study, the highest expression of *PR1* was observed in the incompatible interaction for *YrExp2* at 1 dai, *YrTr1* and *Yr76* at 2 dai, and *YrSP* at 7 dai, showing that *PR1* was involved in race-specific all-stage resistance controlled by different *Yr* genes. The highest expression of *PR1* was occurred for *Yr7B* at 1 dai, *Yr52* and *Yr62* at 2 dai and *Yr59* at 14 dai. The results showed that *PR1* is involved in both race-specific all-stage resistance and non-race specific HTAP resistance.

The PR1.2 protein is homologous to PR1 proteins from other plant species. It represents the first described monocot *PR1* gene with constitutive and pathogen-inducible expression in wheat leaves, but does not respond to activators of systemic acquired resistance (Molina *et al*. 1999). Soltanloo *et al*. (2010) reported that the transcript of *PR1.2* was induced significantly at 7 dai in wheat variety Sumai 3 resistant to *F. graminearum*. In the present study, the highest expression of *PR1.2* was detected in the incompatible interaction for races-specific all-stage resistance genes *YrTr1* at 1 dai, *YrExp2* and *YrSP* at 2 dai, and *Yr76* at 7 dai. For HTAP resistance, the highest expression of *PR1.2* was observed for *Yr52* and *Yr7B* at 1 dai, *Yr62* at 2 dai and *Yr59* at 7 dai. The results showed that *PR1.2* was involved in both all-stage resistance and HTAP resistance controlled by all tested *Yr* genes, but its expression pattern differed greatly from those of *PR1* and other genes.

PR2 is an β-1,3-glucanase, a group of enzymes playing a major role in plant defense and general stress responses through the regulation of callose deposition (Levy *et al*. 2007). β-1,3-Glucanases are also capable of hydrolyzing β-1,3-glucans found in the cell wall of fungi. They can degrade the cell wall of the pathogen or disrupt its deposition, which lead to pathogen death (Mauch *et al*. 1988). The deposition of cell wall releases cell wall fragments that act as elicitors of active host defense response (Yoshikawa *et al*. 1993). β-1,3-Glucanase gene transcripts were detected on tobacco leaves within 24 to 48 h and increased up to 21-fold by bacterium *Pseudomonas syringae* (Alonso *et al*. 1995). High expression of *PR2* was reported in wheat adult-plant resistance against *P. triticina* (Casassola *et al*. 2015) and in resistant wheat plants infected with *F. graminearum* (Li *et al*. 2001) or *M. graminicola* (Ray *et al*. 2003). In the present study, the highest expression of *PR2* was detected in the incompatible interaction for race-specific all-stage resistance controlled by *YrTr1* at 1 dai, *YrSP* and *Yr76* at 2 dai, and *YrExp2* at 7 dai. The highest expression of *PR2* in HTAP resistance was detected for *Yr52* and *Yr7B* at 1 dai, *Yr62* at 2 dai and *Yr59* at 14 dai. The results are generally in consistent with the previous reports that β-1,3-glucanase genes express as early as 6 h after inoculation and the highest expression occurred at 1 and 2 dai in all-stage resistance controlled by *Yr5* (Coram *et al*. 2008b) and other genes (Coram *et al*. 2010; Chen *et al*. 2013). They also reported the induction of *PR2* expression in HTAP resistance conferred by *Yr39* and other genes at 2 dai (Coram *et al*. 2008a; Chen *et al*. 2013). The present study and previous studies clearly show that the PR2 protein contributes to both race-specific all-stage resistance and non-race specific HTAP resistance to stripe rust.

PR3 and PR4 are endochitinases that break bonds between the C1 and C4 of two consecutive N-acetylglucosamines of chitin, a main component of the cell wall in fungi (Collinge *et al*. 1993). PR-4 proteins from wheat were shown to inhibit spore germination and hyphal growth in *in vitro* assays (Caruso *et al*. 2001). Chitinase was also reported to be involved in wheat resistance to stripe rust (Coram *et al*. 2008a, b; Chen *et al*. 2013). In the present study, the highest expression of *PR3* was observed in race-specific all-stage resistance controlled by *Yr76* at 0 dai, *YrTr1* and *YrSP* at 1 dai and *YrExp2* at 2 dai; in the HTAP resistance controlled by *Yr52* at 1 dai, *Yr62* and *Yr7B* at 2 dai and *Yr59* at 14 dai. Similarly, the highest expression of *PR4* was observed in race-specific resistance controlled by *Yr76* at 0 dai, *YrSP* at 1 dai, *YrExp2* at 2 dai and *YrTr1* at 2 and 7 dai. The highest expression of *PR4* in HTAP resistance was detected for *Yr7B* at 1 and 2 dai, *Yr52* and *Yr62* at 2 dai and *Yr59* at 7 dai. The results show the involvement of both *PR3* and *PR4* proteins in both race-specific all-stage resistance and non-race specific HTAP resistance.

PR5, encoding a thaumatin-like protein (TLP), plays an active role in resistance biotic and abiotic stresses in cereals (Rebman *et al*. 1991; Pritsch *et al*. 2000). TLP proteins have been reported to be produced in plants under either biotic or abiotic stresses (Walter *et al*. 2010). Several studies revealed that PR5 proteins interact specifically with (1,3)-β-D-glucans of the fungal cell wall (Osmond *et al*. 2001). The binding of PR5 proteins to (1,3)-β-D-glucans result in poor fungal cell wall assembly during hyphal extension, resulting in increased plasma membrane permeability which reduces the growth and development of the fungus in the host (Edreva 2005). Overexpression of *PR5* was shown to enhance rice resistance to *Rhizoctonia solani* (Datta *et al*. 1999). Barley *TLP* gene was induced by *F. graminearum* infection (Geddes *et al*. 2008). Wheat *PR5* induced by the leaf rust pathogen was significantly increased from the second-leaf stage through mature stage with a peak at the mature stage (Li *et al*. 2016). In the present study, the highest expression of *PR5* was detected in race-specific all-stage resistance controlled by *YrSP* at 1 dai and *YrTr1*, *YrExp2* and *Yr76* at 2 dai; and in the HTAP resistance controlled by *Yr52* at 1 dai and *Yr59*, *Yr62* and *Yr7B* at 2 dai. The results generally agreed with Wang *et al*. (2010) that reported high expression of *PR5* at 1–2 dai in the incompatible interaction at seedling stage. In our experiments of non-race specific HTAP resistance, this gene was significantly induced at 0 or 1 dai, and remained at significant levels until 14 dai, except for the *Yr59* line in which the gene was not significantly induced until 2 dai. The highest expression level in HTAP resistance (4.93–25.72) was generally higher than in all-stage resistance (5.94–16.18). This is in contrast to the previous report of

Chen *et al*. (2013). In the previous study, the significant transcription of *PR5* was reported only in all-stage resistance controlled by *Yr5*, but not in the same type of resistance controlled by other *Yr* genes tested, and also not in HTAP resistance conferred by *Yr18*, *Yr29* or *Yr39*. The difference may be due to the different *Yr* genes tested, and also the limited time points (1 and 2 dai) and microarray technique in the previous studies (Coram *et al*. 2008a, b, 2010; Chen *et al*. 2013). Even in the present study, we did not detected significant induction of *PR5* in the *Yr59* line until 2 dai at a relatively low level (4.93). However, the present results are in agreement with a previous report that the expression level of *PR5* was high in adult rice leaves infected by *Xanthomonas oryzae* pv. *oryzae* (Hou *et al*. 2012).

PR9 is a specific type of peroxidase that acts in cell wall reinforcement by catalyzing lignification and enhance resistance against pathogens by producing reactive oxygen species (ROS) (Odjakova and Hadjiivanova 2001). The production of ROS is central to the defense mounted by plants in reaction to challenges from pathogens (Apel and Hirt 2004). Genes participating in oxidative burst are highly induced at 6–8 hours after inoculation, which forms the first step in triggering plant defense (Wang *et al*. 2007). The first burst occurs within minutes in both susceptible and resistant interactions, whereas the second, sustained burst occurs within a few hours of infection and only in a resistant interaction (Baker and Orlandi 1995). Casassola *et al*. (2015) reported high expression of *PR9* in wheat plants resistant to leaf rust at 2 dai. Dmochowska-Boguta *et al*. (2015) identified 12 clones representing transcripts involved in oxidation reduction processes in wheat plants inoculated with *P. triticina*. Through cytological and histological studies of adult-plant resistance of wheat to stripe rust, Zhang *et al*. (2012) reported that the first phase of ROS occurred at 1 and 2 dai, which is associated with the beginning of haustorium formation and subsequent hyposensitive response (HR) in infected host cells. The second phase of ROS burst occurring at 5 dai was associated with an increasing number of necrotic host cells and the formation of secondary hyphae surrounding the infected plant cells. Microarray and cDNA-AFLP studies showed that the transcription levels of wheat peroxidase genes were high at 0.5 and 1 dai in both all-stage resistance and HTAP resistance with *Pst* (Coram *et al*. 2008a, b, 2010; Wang *et al*. 2010; Chen *et al*. 2013). High transcript accumulation of a peroxidase gene was reported for adult plant resistance to stripe rust in wheat cultivar Xingzi 9104 through suppressive subtractive hybridization (Huang *et al*. 2013) and transcriptome sequencing (Hao *et al*. 2016). Transcripts of peroxidases significantly increased from 1 to 2 dpi in adult plants of Xingzi 9104. In the present study, *PR9* was significantly induced at 0 dai for all-stage resistance gene *Yr76* and HTAP resistance genes *Yr52* and *Yr7B*; and at 1 and/or 2 dai for all resistance genes. The highest expression of *PR9* was detected for *Yr76* at 0 dai, *YrTr1* at 1 dai, *YrSP* at 2 dai and *YrExp2* at 7 dai. In HTAP resistance, the highest expression was observed for *Yr7B* at 1 dai, *Yr62* at 2 dai and *Yr52* and *Yr59* at 14 dai. The highest expression level varied greatly among resistance genes, ranging from 3.02 (*YrExp2*) to 11.66 (*YrSP*) for all-stage resistance and from 3.64 (*Yr52*) to 40.30 (*Yr7B*) for HTAP resistance.

PR10 encodes a ribonuclease (RNase)-like protein (Park *et al*. 2004). PR-10 proteins have a wide distribution throughout the plant kingdom, but its functions are not clear although some subclasses are allergic to humans (Fernandes *et al*. 2013). A recent study reported that overexpression of *PR10* in rice plants reduced susceptibility to rice blast and enhanced tolerance to salt and drought (Wu *et al*. 2016). Expression of *PR10* was reported to be high in race-specific all-stage resistance controlled by several genes, such as *Yr1*, *Yr5*, *Yr8* and *Yr10* (Coram *et al*. 2008b, 2010; Chen *et al*. 2013). In the present study, the highest expression of *PR10* was consistently observed in race-specific all-stage resistance controlled by all *YrTr1*, *YrExp2*, *YrSP* and *Yr76* at 1 dai. In contrast, the occurrence of the highest expression of *PR10* in HTAP resistance varied greatly, at 0 dai for *Yr62*, 2 dai for *Yr7B*, 7 dai for *Yr59* and 14 dai for *Yr52*.

The present study showed that all of the tested PR protein genes were involved in both race-specific all-stage resistance and non-race specific HTAP resistance. However, the expression patterns and the levels of expression varied greatly among different resistance genes. The results indicate that the different PR protein genes work in concert leading to the different types of resistance controlled by different *Yr* genes.

4.2. PR protein transcripts with SA and JA pathways

PR1, *PR1.2*, *PR2* and *PR5* can be activated by salicylic acid (SA) application in wheat (Niu *et al*. 2007), whereas jasmonate (JA) activates *PR3*, *PR4*, *PR9* and *PR10* (Duan *et al*. 2014; Wu *et al*. 2016). Thus, these PR proteins can serve as markers for the two different pathways for responding to pathogen interactions. In our study, all of these genes expressed, but at different time points of both all-stage resistance and HTAP resistance after inoculation, indicating that both SA and JA pathways are involved in the different types of resistance. This finding was in agreement with previous reports for resistance to other diseases. Both SA and JA are known to induce wide-spectrum disease resistance in plants, and co-induction of JA and SA signaling pathways has been reported in various plant pathosystems (Pozo *et al*. 2004; Takahashi *et al*. 2004). Specifically,

Duan *et al*. (2014) reported that both SA and JA mediated resistance signaling molecules are involved in wheat resistance to powdery mildew.

4.3. Differences of PR protein gene expression between compatible and incompatible interactions

In the present study, expression of PR protein genes was found in both compatible and incompatible interactions, indicating that incompatible and compatible interactions share signaling pathways. However, when the same gene was induced in both compatible and incompatible interactions, the expression of the gene was most likely higher in the incompatible interaction than the compatible interactions. Also, more PR protein genes were induced in the incompatible interactions than compatible interactions. These results agreed with previous studies of wheat response to stripe rust (Coram *et al*. 2008a, b). Thus, a resistant plant is capable of rapidly deploying a wide variety of defense responses at high levels that prevent or reduce pathogen colonization and growth. In contrast, a susceptible plant exhibits much weaker and slower responses that fail to restrict pathogen colonization and growth.

5. Conclusion

In the present study, we determined expression levels of eight PR protein genes in eight *Yr* genes controlling either race-specific all-stage resistance or non-race specific HTAP resistance at five major time points during the *Pst* infection process. We found that all eight PR protein genes of wheat are involved in resistance to the stripe rust pathogen, no matter which type of resistance or which *Yr* resistance gene controls resistance. The results indicate the involvement of both SA and JA signaling pathways in both race-specific resistance and non-race specific resistance to stripe rust. Thus, both durable resistance and non-durable resistance utilize some common defense mechanisms against the pathogen infection. However, different PR protein genes express at different stages and at different levels during the pathogen infection process. The different signaling pathways may regulate different defense genes, which work in concert to produce final results of different levels of resistance. The expression level of PR protein genes varied significantly at different time points and among different *Yr* gene lines. This finding indicates that not a single PR protein gene is involved in resistance, however, any type of the resistance or any *Yr* gene-controlled resistance is contributed by different defense-related genes that may regulated by different signaling pathways. The results of the present study greatly improve the understanding of the molecular mechanisms underlying wheat resistance to stripe rust.

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Appendices associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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