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Research Article

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Fine-Tuning of *PR* Genes in Wheat Responding to Different *Puccinia* Rust Species

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Abstract

Upregulation of pathogenesis-related genes (*PRs*) has been found to be associated with plant defense response. To better understand the role of *PR* genes in wheat defense response against rust pathogens, we studied the expression of five *PR* genes in six wheat lines during their interactions with three *Puccinia* species. The research revealed three *PR* gene expression patterns associated with resistance to the three rusts, implying different strategies from the host in response to different rusts. In addition, different *PR* gene expression patterns were found in the same genetic background when interacting with different races of the same *Puccinia* species, suggesting different counteractions from the pathogen during infection. Overall, our study revealed fine-tuning of *PR* genes in wheat responding to different *Puccinia* rust species, implicating the limitation of defense when only overexpressing a single *PR* gene in the host.

Keywords

PR genes; *Triticum aestivum*; defense response; qRT-PCR; *Puccinia* species

Introduction

The term "pathogenesis-related proteins (*PRs*)" was used to describe these proteins because they were first identified as additional proteins induced in the host plants under pathogen-inoculated conditions [1]. PR-1a, -1b and -1c were first purified in tobacco plants infected with tobacco mosaic virus (TMV) [2,3]. In TMV infected tobacco, the highest PR1 concentration was found at the hypersensitive lesion margins as well as non-infected leaves from resistant tobacco plants that previously inoculated with TMV, suggesting the role of PR1 in limiting the multiplication or spreading of the virus [4]. Later, PR1 proteins were also found in TMV inoculated susceptible tobacco [5]; healthy plants during flowering [6]; natural senescing plants [7,8] and plants treated by salicylic acid [9]. In 1980, PR proteins were defined as "proteins encoded by the host plant but induced only in pathological or related situations" [1].

Soon after the discovery of PR1, PR2 (β-1, 3-glucanases) [10] and PR3 (chitinase) [11], they were identified to have antifungal activity. So far, sixteen PR proteins have been described [12]. Most of them have antimicrobial functions including antifungal, antibacterial, and antiviral actions. Some have insecticidal or nematicidal activities [12]. These studies suggest that the function of PR proteins is positively associated with host plant defenses. During the past decades, many research efforts have been devoted to understanding of the regulation of PR proteins and their inducers. In *Arabidopsis*, *PR1*, *PR2* and *PR5* are associated with salicylic acid (SA)-regulated defense response [13,14]. Mutations that impaired SA biosynthesis strongly reduced *PR1* expression and systemic acquired resistance (SAR) [15], suggesting that upregulation of *PR1* was SA-dependent and a positive correlation between *PR1* and SAR. However, the same mutations did not affect the expressions of *PR2* and *PR5*, indicating their SA-independency [15]. In tobacco, both *PR-1b* and *PR5* were found induced by the combination of ethylene and methyl jasmonate (MeJA) [16]. However, a protein that blocked ethylene induction of *PR-1b* was unable to block *PR5* induction, suggesting a fine regulation of the two *PR* genes [16]. Desmond et al. [17] reported that either exogenous JA or benzo-(1, 2, 3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH, a SA analog) treatment could result in upregulation of *PR1*, *PR2*, *PR3*, *PR4*, *PR5* and *PR10* in wheat cultivar Kennedy. In contrast, in a different wheat cultivar Sunco, only JA induced the expressions of those six *PR* genes, BTH suppressed the expressions of the same *PR* genes [17]. These reports revealed the complexity of *PR* gene regulation in different species, in different backgrounds of the same species or in response to different pathogens.

In our previous research, we noted that knocking out genes *TaCSN5-2A* or *TaCSN5-2D* in spring wheat cultivar Alpowa increased *PR1* transcription and enhanced resistance to leaf rust [18]. However, enhanced *PR1* level in the *TaCSN5* mutants did not render resistance to stem rust. These findings draw our attention to the expression patterns of several antifungal *PR* genes of wheat, including *PR1*, *PR2*, *PR3*, *PR5* and *PR10*, during the interactions with three rust pathogens, which are *Puccinia triticina (Pt)* causing leaf rust, *P. graminis* f. sp. *tritici* (*Pgt*) causing stem rust and *P. striiformis* f. sp. *tritici* (*Pst*) causing stripe rust. They are fungal pathogens having a similarl biotrophic life style when infecting wheat. In this study, we found different expression patterns of the five *PR* genes associated with wheat defense responses against different rust pathogens.

Materials and Methods

Plant materials

Six wheat lines were used in this study, including two mutants MNR180 and MNR220 and four cultivars containing known *Lr*, *Sr* or *Yr* genes for resistance to leaf rust, stem rust or stripe (yellow) rust, respectively. Alpowa (PI 566596), a soft white spring wheat cultivar, was obtained from the USDA National Plant Germplasm System (NPGS). MNR180 and MNR220 are EMS induced mutants of Alpowa generated by Dr. Michael Giroux at Montana State University, referred as *MNR180* (Alpowa) and *MNR220* (Alpowa) thereafter. Wheat cultivar Scholar with *Lr47*, was provided by Dr. Luther Talbert at Montana State University. The *Sr33* line in the Chinese Spring background, *Sr33* (CS), was provided by Dr. Evans Lagudah at CSIRO, Australia. The *Yr5* line in the Avocet background,

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Yr5 (Avocet), was maintained in Dr. Xianming Chen's lab at USDA-ARS, Pullman, Washington.

Pathogen

The *Pt* race PBJJG used for leaf rust assays was kindly provided by Dr. Robert Bowden, USDA-ARS Manhattan, KS. The *Pgt* races TMLKC and QFCSC were maintained at the Cereal Disease Laboratory, St. Paul, Minnesota. The *Pst* races PSTv-11 and PSTv-37 were maintained at the USDA-ARS Pullman, Washington.

Plant growth conditions and pathogen inoculation

Plant growth conditions: Before inoculation, all wheat seedlings were grown under the following conditions: 22°C/14°C day/night temperatures and a 16 h photoperiod. Plants were watered and fertilized with Peters General Purpose Plant Food (Scotts-Miracle-Gro Company, Marysville, OH) at a concentration of 150 ppm N-P-K every day.

Rust inoculations and hormone treatments: Leaf rust inoculations were performed as described in Campbell et al. [19]. Stem rust inoculations were conducted in a similar manner to leaf rust with the following exceptions: the dew chamber was pre-conditioned to an air temperature of 19–22°C. Inoculated plants were incubated for 24 h followed by at least 3 h under high humidity and light intensity conditions before being transferred to the greenhouse. Stripe rust inoculations were conducted following the methods described by Wan and Chen et al. [20]. Plants were uniformly inoculated with urediniospores mixed with talc in a ratio of 1:20, kept in a dew chamber without light for 24 h at 10°C, and then grown in a growth chamber with diurnal temperature cycles gradually changing from 20°C at 2:00 pm to 4°C at 2:00 am and 16 h light/8 h dark. Mock treatments were done the same as the corresponding rust inoculation except without urediniospores.

For hormone treatments, wheat seedlings were sprayed with 20 mM SA or 2 mM MeJA in 0.1% (v/v) ethanol, respectively at four different time points: 24 hours before rust inoculation (hbri), 0 hours post rust inoculation (hpri) in which, SA or MeJA were sprayed right before rust inoculation, 12 hpri and 24 hpri in which SA or MeJA were sprayed 12 hr or 24 hr post rust inoculation. The check (CK) was the plants without any chemical treatment and mock was the plants sprayed with 0.1% (v/v) ethanol prior to rust inoculation.

Gene expression analysis by qRT-PCR

Leaf tissues were collected at 0, 1, 2, 3, 5, 8 and 10 dpi, snap frozen in liquid nitrogen and stored at –80°C until RNA isolations were performed. Total RNA was isolated and treated with DNase I on a column using a Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The quality and concentration of total RNA were assessed via agarose gel electrophoresis and 260/280ABS measurements on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE).

Basal level of the *PR* genes and time course expression study post inoculation with different rust pathogens on different lines were quantified by quantitative real-time-PCR (qRT- PCR). The primers used to measure the *PR* gene transcripts were according to Desmond [17]. qRT-PCRs were performed using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA) on a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA) following the manufacturer's protocol. Transcript abundance was normalized to the reference gene ACTB (β-actin) [21]. Expression measurement of each gene was conducted in triplets with three biological replicates. Data were used only if the Ct standard deviation among the triplets was ≤ 0.2, and the mean of the triplet's Ct was used for the calculation. Relative expression was calculated using the ΔΔCt method as described in the CFX96 manual (Bio-Rad, Hercules, CA), where fold change = $2^{-\Delta\Delta Ct}$. Relative expression of the *PR* genes at different time points are presented as the relative expression to the 0 hr time point. Standard deviations were calculated among the three biological replicates. Student's t-tests were performed to test whether the expression level at different time points are different. The *p*-values were calculated based on an unpaired two-tailed distribution.

Results

Basal expression levels of the *PR* genes

As the term *PR* gene implies, their expressions should be pathogenesis-related or stress- related. However, when the expression of five *PR* genes were investigated in the six wheat lines without pathogen inoculation or stresses via qRT-PCR, the basal levels of the *PR* genes were different in Alpowa, *MNR180* (Alpowa), *MNR220* (Alpowa), *Lr47* (Scholar), *Sr33* (CS) and *Yr5* (Avocet) (Figure 1). Alpowa was susceptible to all the races of three rust species selected; therefore the level of each *PR* gene in Alpowa was used as a reference and normalized to 1 for calculating the relative expressions of *PR* genes in other lines. The expression levels of the others were calculated as fold changes relative to that in Alpowa. Among the six lines, *Sr33* (CS) had the lowest basal levels of all five *PR* genes. The levels were almost undetectable, consistent with the term of "pathogenesis-related". The remaining five lines, including Alpowa, *PR* genes have been elevated to some extent compared to the levels of *Sr33* (CS). Mutant *MNR180* (Alpowa) was resistant to all three rust species, and had the highest basal levels of *PR1*, *PR2*, *PR3* and *PR5* among all. Although the basal *PR10* level of *MNR180* was not the highest, it was still significantly

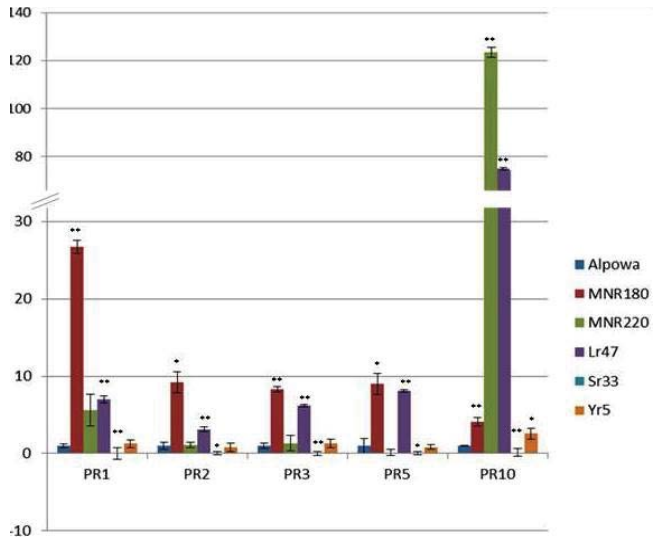

Figure 1: Basal expression of the PR genes in different wheat lines. [Gene expression of five PR genes each in five wheat lines is presented as fold changes relative to the level in Alpowa. Error bars represent standard deviation among three biological replicates, and * and ** denote statistical significance at the P ≤ 0.05 and 0.01 levels, respectively].

higher than the level of Alpowa. *Lr47* (Scholar) was highly resistant to leaf rust and had second highest basal levels of all five *PR* genes. *MNR220* (Alpowa), which is resistant to leaf and stem but not to stripe rust races at the seedling stage, had the highest level of *PR10*. *Yr5* (Avocet), resistant to stripe rust, had the second lowest expression levels of *PR* genes except *PR10*.

Contrast expression profiles of *PR* genes in *MNR220* responding to leaf and stem rust pathogens

MNR220 (Alpowa) is a mutant that acquired new resistance to more than a dozen races of the leaf and stem rust pathogens. The new resistance to the two rusts is conferred by the same locus of *MNR220* [19]. The two rust pathogens belong to the same genus and have a similar life style. To understand if the same strategy reflected by *PR* gene expression patterns was deployed to defend against the two rust species, the five *PR* genes were investigated in *MNR220* (Alpowa) at seven time points after inoculation with *Pt* PBJJG and *Pgt* QFCSC, respectively. Unexpectedly, expression patterns of all five *PR* genes were quite different in *MNR220* in response to leaf and stem rust pathogens (Figure 2). When challenged with *Pt*, *PR1* expression was elevated and reached to the highest at 2 dpi with a 6-fold increase compared to the 0-dpi level. In contrast, there was little change in *PR1* expression in *MNR220* until 8 dpi when infected with *Pgt*. The level of *PR2* was increased for 35 fold at 2 dpi and level of *PR5* was up for 280 fold at 1 dpi after inoculated with *Pt*. Similarly, both *PR2* and *PR5* were upregulated at 1 dpi during *Pgt* infection, but the magnitude was insignificant when compared to the increased levels detected during *Pt* infection. Interestingly, and quite opposite, highly elevated *PR3* and *PR10* expressions were detected in the mutant at 1 dpi and 2 dpi after inoculated with *Pgt*, but not after *Pt* inoculation (Figure 2). These results suggested a different strategy was deployed in *MNR220* (Alpowa) to defend against the two different rust species. *PR1*, *PR2*

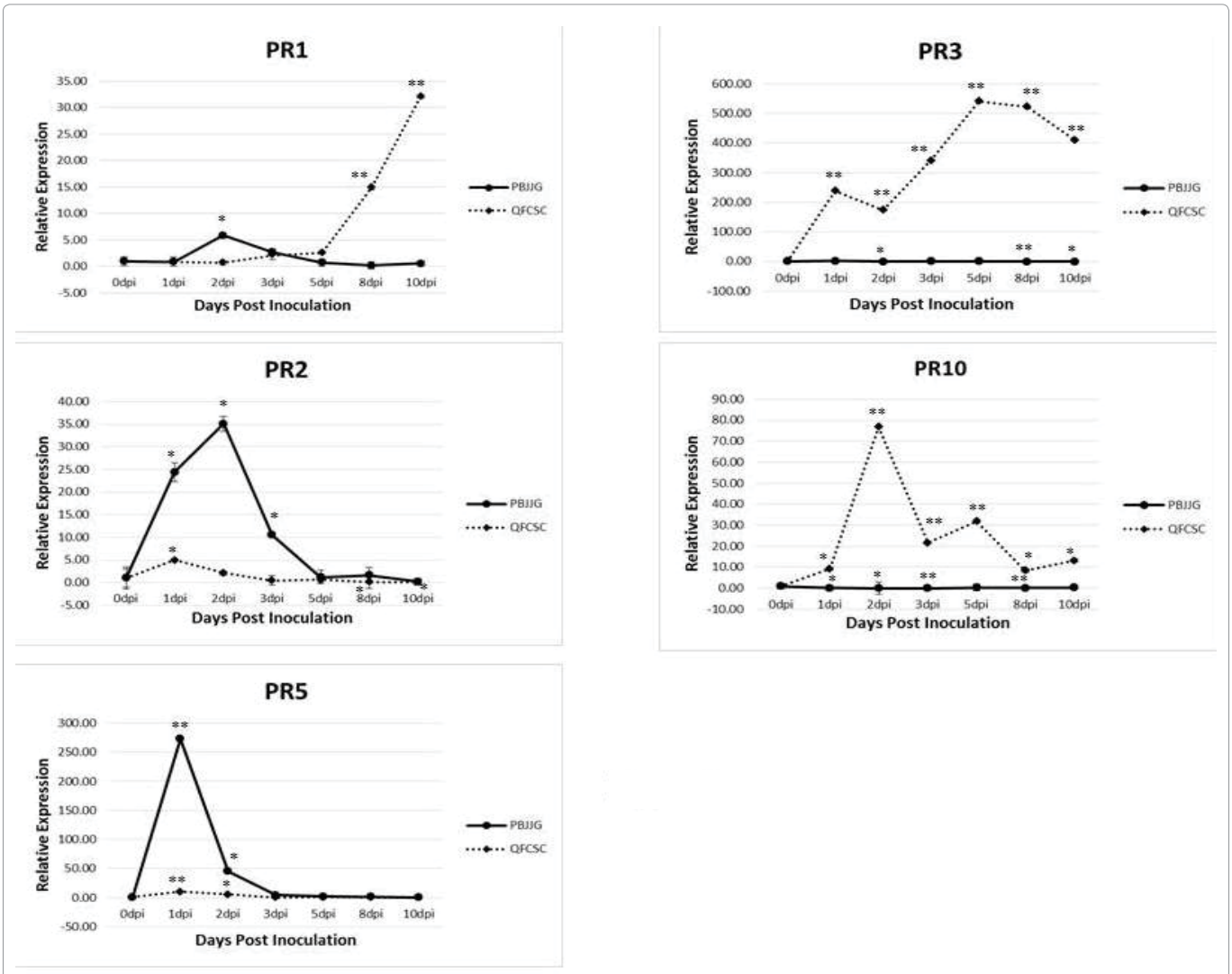

Figure 2: Relative expression of the PR genes in MNR220 (Alpowa) responding to leaf and stem rusts.
[Mutant MNR220 (Alpowa) was inoculated with Pt race PBJJG and Pgt race QFCSC at 2-leaf stage, respectively. RNA samples were extracted from the leaf samples collected at seven time points. Transcript abundances of the five PR genes were measured via real-time PCR in each sample. Relative expression of PR gene at each time point was relative to the level of 0-dpi. Error bars represent standard deviation among three biological replicates, and ˚ and ¨ denote statistical significance at the P ≤ 0.05 and 0.01 levels compared to the level of 0-dpi, respectively].

and *PR5* were more important in *MNR220* (Alpowa) during defense against leaf rust pathogen, and *PR3* and *PR10* were more crucial during defense against stem rust pathogen.

PR gene expression patterns in leaf rust resistant lines during infection

After seeing different *PR* gene expression patterns in the same genetic background *MNR220* (Alpowa) against two different rust species, it was interesting to investigate the *PR* gene expression patterns among different lines with resistance to the same rust species. *Lr47* (Scholar) and *MNR220* (Alpowa) had a similar level of resistance to *Pt* race PBJJG. Expression patterns of the five *PR* genes were compared between the two lines at seven time points from 0~10 dpi (Figure 3). As shown in Figure1, the two lines had different *PR* basal expression levels. *MNR220* (Alpowa) had a lower *PR* basal expression level compared to *Lr47* (Scholar) except *PR10,* therefore, expression levels of *PR1*, *PR2*, *PR3* and *PR5* in *MNR220* (Alpowa) at 0 dpi were normalized to 1 for calculating the relative expressions of other time points for the two lines. In Figure 3, expression pattern of each *PR* gene was presented by the curve plotted by using relative expressions to the level of 0-dpi *MNR220* at seven time points. When the patterns were compared, *PR1*, *PR2* and *PR5* genes in *MNR220* (Alpowa) were upregulated much earlier and higher than that in *Lr47* (Scholar) (Figure 3). However, there was something in common between the two lines, both had significantly elevated *PR1*, *PR2* and *PR5* at early time points, and not much change in *PR3* or *PR10* expression was detected over time. It seemed that the significantly increased *PR1*, *PR2* and *PR5* expression levels at early time points were corresponding to resistance to leaf rust, suggesting that these three *PR* genes in wheat had a more critical role than *PR3* and *PR10* when defending against leaf rust pathogen.

Different *PR* gene expression patterns in the same genetic background to different races of stem rust pathogen

The stem rust resistance gene *Sr33* encodes a protein containing a coiled-coil, nucleotide-binding site and leucine-rich repeat (CC-NBS-LRR) domains [22]. The gene was discovered from *Aegilops tauschii* and introgressed into cultivar Chinese Spring (CS) through a single

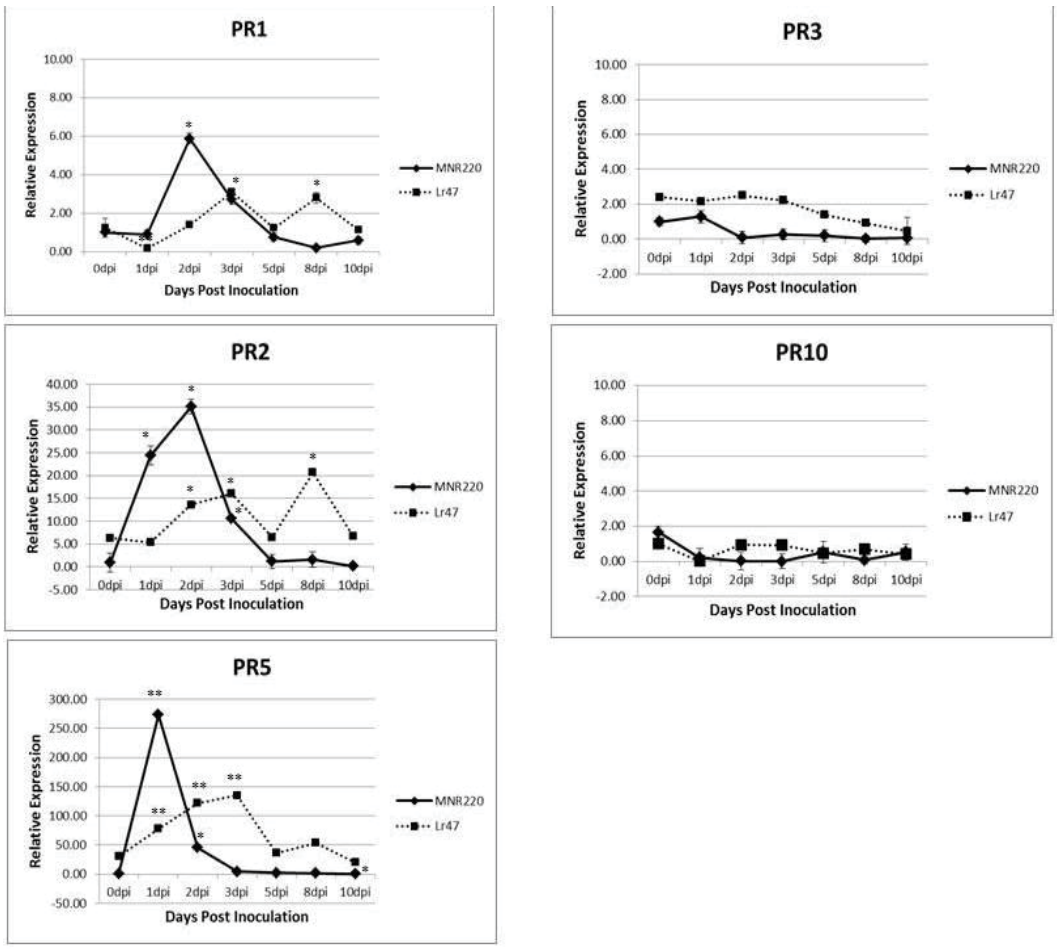

Figure 3: Relative expressions of the PR genes in two different leaf rust resistant lines in response to the same *Puccinia tritici* (Pt) race. [Mutant MNR220 (Alpowa) and Lr47 (Scholar) were inoculated with Pt race PBJJG at 2-leaf stage. RNA samples were extracted from the leaf samples collected at seven time points. Transcript abundances of the five PR genes were measured via real-time PCR in each sample. The lowest expression of a PR gene at 0-dpi between the two lines was used as a reference for calculating the relative expression of the other time points of the PR gene. Error bars represent standard deviation among three biological replicates, and * and ** denote statistical significance at the P ≤ 0.05 and 0.01 levels compared to the corresponding reference, respectively].

chromosome substitution [23]. *Sr33* (CS) is resistant to diverse *Pgt* races including QFCSC and TLMKC. The infection types of *Sr33* (CS) to the two *Pgt* races were similar. Five *PR* genes were monitored in *Sr33* (CS) line after being inoculated with QFCSC and TLMKC, respectively. As shown in Figure 4, the five selected PR genes had very similar patterns at early time points in the host between the two interactions. The genes were all upregulated at 1 or 2 dpi although different patterns were seen after 3 dpi. Interestingly, all five *PR* genes showed a two-peak pattern in *Sr33* (CS) when infected with TLMKC, but a one-peak pattern when infected with QFCSC (Figure 4).

Notably, *PR1* had the highest increased level, upregulated 200 folds compared to the 0-dpi level. The second highest was *PR10*, more than 120-fold increase responding to TLMKC. The rest of the three *PR* genes had similar levels of enhancement, around 20~40 folds increase compared to that at 0-dpi. *PR2* and *PR3* had a very similar expression pattern in *Sr33* (CS) in response to the two races of stem rust pathogen, respectively.

Similar *PR* gene expression patterns in *Yr5* (Avocet) in response to two different races of stripe rust pathogen

The two *Pst* races PSTv-11 and PSTv-37 were collected in the state of Washington. *Yr5* in the Avocet background had similar infection types, 1-2 in a 0-9 scale, when tested with the two races. All five *PR* genes had very similar expression patterns in the host when interacting with the two *Pst* races (Figure 5). *PR5* was the only one that did not show a significant change at the selected time points during the infection process. *PR1* expression showed two peaks. The first peak appeared at a slightly different time point between the two interactions, at 1 dpi in *Yr5*- PSTv-37 interaction and at 2 dpi in *Yr5*-PSTv-11 interaction. The second peak at 5 dpi was the same for both interactions. Again, *PR2* and *PR3* had very similar expression patterns, both were upregulated and reached the highest level at 1 dpi, declined rapidly at 3 dpi, and then a second weak upregulation at 5 dpi was detected in *Yr5*-PSTv-37 interaction. *PR10* expression has a clear two-peak pattern in *Yr5*-PSTv-37 interaction. Although it seemed like only one peak in *Yr5*- PSTv-11 interaction, one common

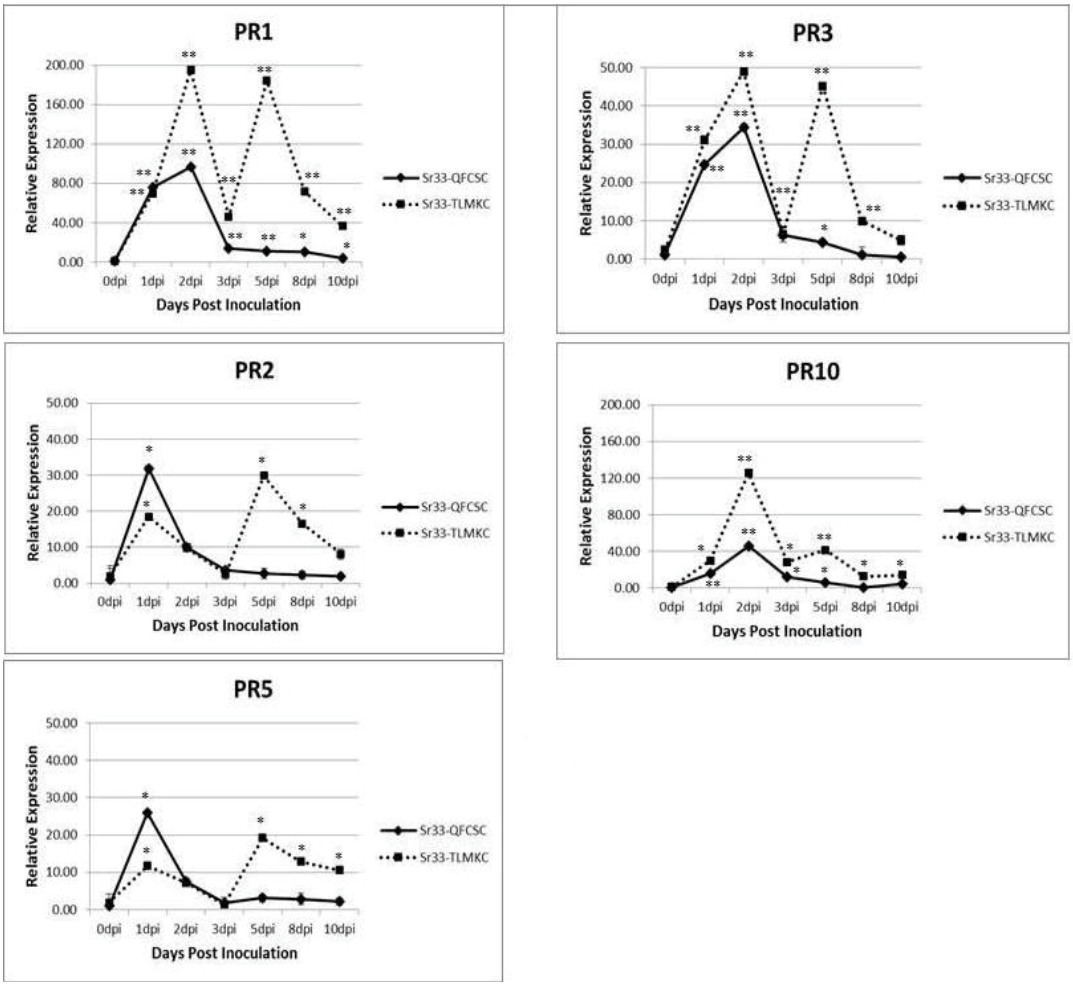

Figure 4: Relative expressions of the PR genes in the same stem rust resistant line in response to different *Puccinia graminis* f. sp. tritici (Pgt) races. [Sr33 (CS) was inoculated with two races of Pgt QFCSC and TMLKC at 2-leaf stage. RNA samples were extracted from the leaf samples collected at seven time points. Transcript abundances of the five PR genes were measured via real-time PCR in each sample. Relative expression of PR gene at each time point was relative to the level of 0-dpi. Error bars represent standard deviation among three biological replicates, and * and ** denote statistical significance at the P ≤ 0.05 and 0.01 levels compared to the level of 0-dpi, respectively].

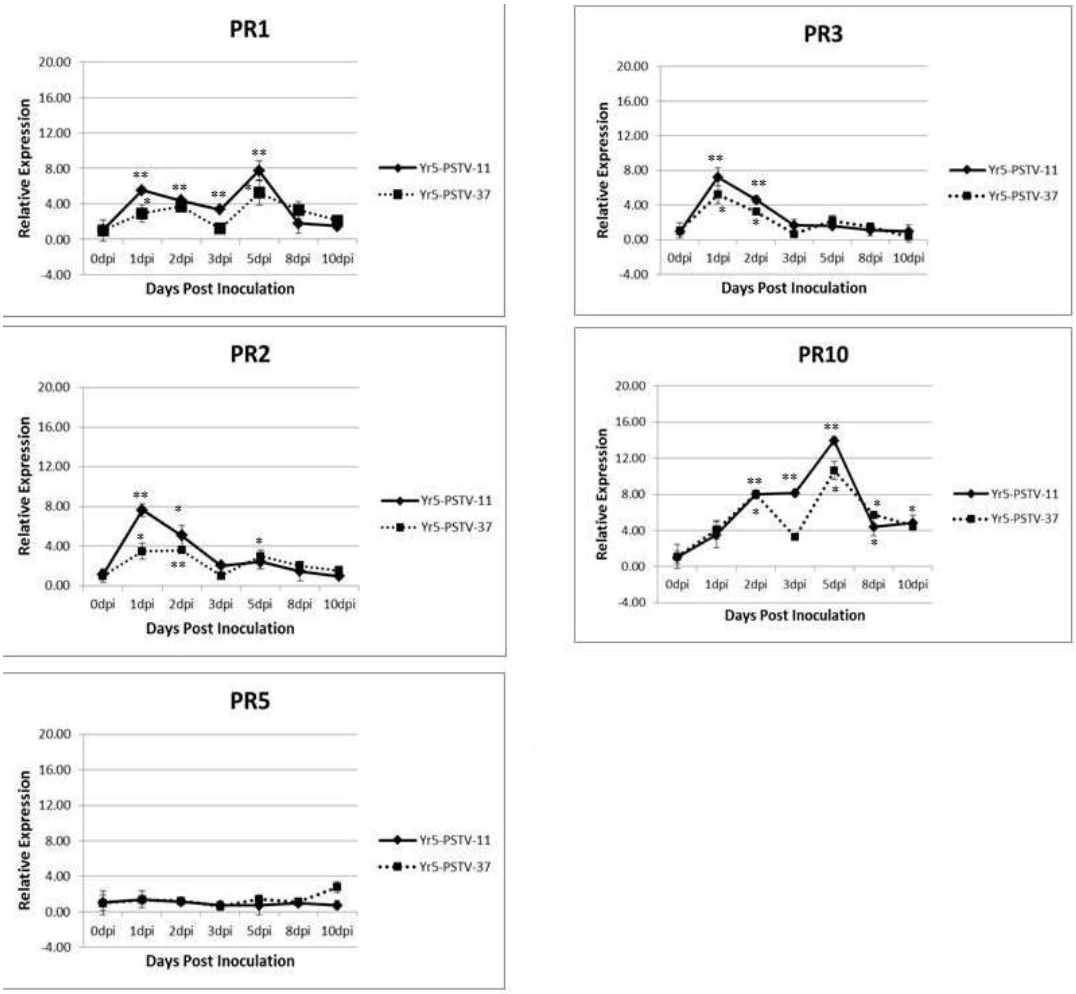

Figure 5: Relative expressions of the PR genes in the same stripe rust resistant line in response to different Puccinia striiformis f. sp. tritici (Pst) races. [Yr5 (Avocet) was inoculated with two races of Pst PSTv-11 and PSTv-37 at 2-leaf stage. RNA samples were extracted from the leaf samples collected at seven time points. Transcript abundances of the five PR genes were measured via real-time PCR in each sample. Relative expression of PR gene at each time point was relative to the level of 0-dpi. Error bars represent standard deviation among three biological replicates, and * and ** denote statistical significance at the P ≤ 0.05 and 0.01 levels compared to the level of 0-dpi, respectively].

observation in the two interactions was that the level of *PR10* at 3 dpi has either stopped rising or declined, and then a further increase at 5 dpi.

Exogenous hormone treatments enhanced resistance to rusts

The observations of more *PR* genes upregulated in resistance lines against *Pgt* than those against *Pst* or *Pt* invited a hypothesis that when a wheat plant is in the resistance status to the *Pgt* races tested then it would also be resistant to the *Pt* race tested. To test this hypothesis, we monitored the infection types of two susceptible wheat lines of Alpowa and CS to *Pt* and *Pgt* after exogenous SA and JA treatments. As shown in Figure 6, post SA treatment, both Alpowa and CS had enhanced resistance to *Pt* PBJJG (Figure 6A and 6C) and *Pgt* QFCSC (Figure 6B and 6D) compared with the CK and the mock treatment. MeJA treatment on two cultivars also enhanced their resistance to leaf rust (Figure 6A and 6C) and a little to stem rust (Figure 6B and 6D). Analysis of qRT-PCR results revealed elevated *PR1*, *PR2*, *PR3* and *PR5* in Alpowa (Figure 6E) and *PR1*, *PR3* and *PR10* in CS (Figure 6F) 24 hr post SA treatment. The results did support the aforementioned hypothesis.

Discussion

Breeding constraints in favor of lines with a high *PR* expression

Plant SAR plays an important role in defense against pathogens. Effective SAR is normally associated with higher expression levels of some *PR* genes, those genes were used as SAR marker genes [24]. However, inductions of *PRs* by endogenous and exogenous signaling compounds in the absence of pathogens suggest their functions besides defense against pathogens. High induction of *PR* genes in tobacco under drought and high salt condition [25], after exposure to UV light or wounding [26] indicated their important roles in maintaining cellular structure under various stresses. From our study, we found that all wheat lines except *Sr33* (CS) had a high basal level expression of the five *PR* genes studied (Figure 1).

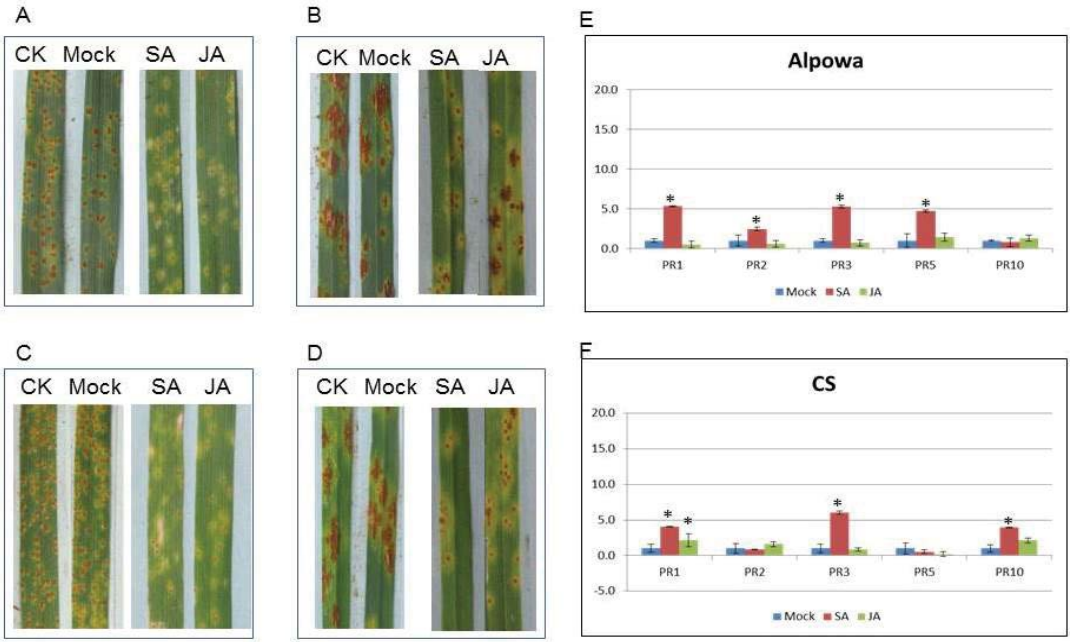

Figure 6: Infection types and PR gene expression of Alpowa and CS after treated with SA and MeJA responding to *Puccinia tritici* (Pt) race PBJJG and P. graminis f. sp. tritici (Pgt) race QFCSC, respectively.

Six plants each from Alpowa and CS were treated with 20 mM SA or 2 mM MeJA in 0.1% (v/v) ethanol at 2-leaf stage. Leaf samples were collected from three plants 24 hr post treatment, and the remaining three plants were inoculated with Pt race PBJJG and Pgt race QFCSC, respectively. Infection types of leaf rust and stem rust were documented and photographed 8 dpi and 14 dpi, respectively. Relative expression of each PR gene was relative to the level of mock inoculation which was sprayed with only 0.1% (v/v) ethanol. CK is the plants without any chemical treatment. Error bars represent standard deviation among three biological replicates, and * denotes statistical significance at the P ≤ 0.05 compared to the mock].

The six selected lines were derived from Alpowa, Scholar and CS three genetic backgrounds. Among them, Alpowa and Scholar were once popular cultivars in Washington and Montana states, respectively. The only low basal *PR* expression line was in the CS background, and CS is a landrace from China with little breeding efforts. This observation may imply that breeding practice in selecting good performance under various stresses resulted in the lines in favor of higher basal *PR* gene expressions.

Expression patterns of *PR* gene combinations associated with resistances to the three rusts

It has been noticed that different *PR* genes associated with the onset of SAR in different species, for example, *PR1*, *PR2* and *PR5* were upregulated when effective SAR was detected in *Arabidopsis* [14]. At least nine *PR* gene families were found associated with SAR in tobacco [3,27,5]. In our study, significantly upregulated *PR1* and *PR2* expressions were detected in each resistant line infected with the corresponding rust at early time points (Figure 2- 5). PR1 protein has been reported to inhibit the broad bean rust hyphae differentiation [28] although the function of the protein has not been clearly elucidated. In wheat, there are 23 *PR1* genes identified [29], and some have been shown to play distinct roles in host-pathogen interactions, such as *PR1.1* and *PR1.3* [29]. In this study, the primers of *PR1* used for qRT-PCR are specific to *PR1.1* and *PR1.3* transcript abundances. PR2 is β-1,3-endoglucanase, an enzyme that catalyzes endo-type β-1,3-glucans which is a basic cell wall compound of almost all higher plants and fungi. β-1,3-endoglucanase may directly degrade fungal cell walls or release cell-wall debris as elicitors of defense response [30] . The primers of *PR2* will detect the three β-1,3-endoglucanase orthologs on the wheat chromosomes 3A, 3B and 3D. Inductions of *PR1* and *PR2* by exogenous SA or JA have been reported in some but not all wheat cultivars tested [17,31-33]. Elevated *PR1* and *PR2* were associated with enhanced resistance to Fusarium head blight caused by a necrotrophic fungal pathogen [32] or to leaf rust caused by a biotrophic fungal pathogen [33].

From our study, we found three expression patterns of the five *PR* gene combinations associated with resistance to different wheat rust. Pattern 1 represents the combination of greater than 2 fold upregulation of *PR1*, *PR2* and *PR5* before 3 dpi and less than 2 fold changes of *PR3* and *PR10* over the time course (Figure 3). This pattern is associated with resistance to leaf rust.

Pattern 2 represents the significant upregulation of all five *PR* genes at 1~2 dpi (Figure 4), this pattern is associated with resistance to stem rust. Pattern 3 represents the significant upregulation of four but *PR5* genes at 1 dpi (Figure 5), and this pattern was associated with resistance to stripe rust. This observation implied the enhancement of only *PR1* gene expression was not sufficient to render resistance to all three rust pathogens, and also explained why a mutant line with increased *PR1* could be resistant to leaf rust but not to stem rust.

Different defense strategies of the hosts and the counteractions of the pathogens

Among the five rust resistance lines, host defense response in *Lr47* (Scholar), *Sr33* (CS) and *Yr5* (Avocet) were mediated by a resistance (*R*) gene. Different *PR* gene expression patterns mediated by the three *R* genes in different genetic backgrounds suggested different defense strategies of the hosts to different rust pathogens. *Sr33* and *Yr5* are

race-specific *R* genes that recognize the corresponding avirulence (*Avr*) gene in the pathogens although each confirms resistance to more than one race of the corresponding rust species. *Sr33* (CS) is resistant to both *Pgt* QFCSC and TMLKV, it is believed that *Sr33* recognizes the same *Avr* gene in the two races and mediates the same defense response in the host. Similarly, *Yr5* should recognize the same *Avr* gene in *Pst* PSTv-11 and PSTv-37. It is reasonable to believe that the same defense response in the same genetic background should result in the same *PR* gene expression pattern. However, when *Sr33* (CS) and *Yr5* (Avocet) each was tested with two races of the corresponding *Puccinia* species, different *PR* gene expression patterns were found in response to different races of the same rust pathogen (Figure 4,5), suggesting different counteractions of the pathogen races in response to the same defense response.

Fine-tuning of *PR* gene regulation

The regulatory regions of the *Arabidopsis PR1* [34-39] and *PR2* genes [40,41] have been well studied. A region mainly comprised of binding sites for WRKY and TGA transcription factors relaying SA-dependent signals has been identified and characterized for *PR1* [38]. This gene is regulated by both positive and negative *cis*-acting factors through *NPR1*. In contrast, the *PR2* regulatory region showed no sequence similarity to *PR1* [40] although the two genes were upregulated in response to the same pathogen [32,33] or negatively regulated by TGA2 in *Arabidopsis* [39]. A 125-bp fragment in the *PR2* regulatory region is sufficient for the activation of the gene by a 61 amino acid-homeodomain protein [41]. To the best of our knowledge, there is no report on the regulatory regions of the five wheat *PR* genes we selected. Based on our observations, it seemed that the expression pattern of each *PR* gene was independent of the expression of the other *PR* genes. More often than not, *PR2* expression was increased when *PR1* gene was upregulated (Figures 1-5). *PR1* increased its expression in CS after treated with MeJA, but *PR2* remained unchanged (Figure 6F). In summary, the expression patterns of the five *PR* genes were the result of host-pathogen interactions, reflecting the host defense strategies and the pathogen counteractions to the defense responses.

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