PROGRESS IN BIOLOGICAL SCIENCES

Quantitative expression analysis of candidate genes for *Septoria tritici* blotch resistance in wheat (*Triticum aestivum* L.)

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Abstract

Septoria tritici blotch (STB), caused by the ascomycete fungus *Mycosphaerella graminicola* (asexual stage: *Septoria tritici*), is one of the most important foliar diseases of wheat. In this research, quantitative expression analysis of five candidate genes for the induction of resistance to STB (*PR-1*, *Bsi*, *Msr, Per*, and *Ppi*) was conducted in the wheat cultivars 'Seri 82' (susceptible) and 'Frontana' (resistant). The study used a randomized complete block design with three replications and five time-points (0, 3, 6, 12, and 24 hours after inoculation). Evaluation of the expression of five candidate genes by real-time PCR showed that expression of *Per, pr-1, Bsi, Msr* genes was higher than 5, 3, 3 and 2 fold in treatment *vs.* control samples in 'Frontana' at 12 hours after inoculation respectively. The results indicated the effectiveness of these genes in the conferral of resistance to STB in wheat.

Keywords: *Septoria tritici,* Wheat, Real-time PCR analysis, Resistance gene

Introduction

Septoria tritici blotch (STB) is caused by *Zymoseptoria tritici* (teleomorph *'Mycosph-aerella' graminicola*). STB is the most important foliar disease of the wheat due to its impact on production in most wheat-growing areas of the world, specifically in areas with high rainfall (1, 2). Asexual spore structure of *M. graminicola* causes chloresis and necrotic lesions which called pycnidia. Incompatible interactions are character-ized by either very limited fungal colonization of the mesophyll or inability of the fungus to form mature pycnidia on leaves (3). Fungicides were used previously to control STB but the effectiveness of disease control decreased after populations of *M. graminicola* developed resistance to the fungicides (4). Use of resistant cultivars is the most effective and economical means of achieving protection against the disease (5).

Similar to most successful pathogens, *M. graminicola* can suppress host defense responses or react to these responses by expressing genes that counteract the mechanisms of stress tolerance (6). Plants have evolved complex mechanisms to recognize pathogen attack and activate an effective natural immune response. An important perception mechanism is based on the expression of R genes in plants whose products enable the identification of related virulence (Avr) proteins in the pathogen. Subsequently, the affected plants are able to activate a wide array of defense mechanisms. To understand these mechanisms, it is necessary to study how plants recognize pathogen attack and transmit this information to activate the transcription of cellular-response mediators such as signal transduction genes. These genes are regulated by resistance genes and defense-related genes in the host. Many genes are involved in the gene cascade that is induced by pathogens (7). Increased levels of the products that are encoded by these genes are required for host defense responses. However, limited evidence indicates that the over-expression of resistance genes, defense-related genes, and

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some signal-transduction genes might not represent a complete schema for the regulation of pathogen-induced defense responses.

The first gene expression analysis of the host response to STB was undertaken by Ray et al., (2003), who studied defense-related genes that are expressed in incompatible interactions. These authors compared gene expression profiles of the wheat line Tadinia (containing the *Stb4* gene for resistance) and the susceptible line Yecora Rojo, non-inoculated or inoculated with *M. graminicola*, by differential-display polymerase chain reaction (DD-PCR), which enabled detection of rapid responses to *M. graminicola* signals within 3 hours of inoculation, and reported that four genes were induced differentially. These early responses began before penetration of the host by the pathogen and were indicated to determine the outcome of the interaction (3). Numerous studies have analyzed the differential expression of defense-related genes in wheat in response to pathogen attack. These include the use of differential-display reverse transcriptase-PCR (DDRT-PCR) to analyze yellow rust (8), cDNA-AFLP screening for yellow rust (9), cDNA library construction for leaf rust (10), cDNA-AFLP analysis for leaf rust (11), and microarray analysis for deoxynivalenol produced by *Fusarium* species (12). The main objective of this research was to estimate the expression level of five resistant candidate genes that are associated with disease resistance by Real time PCR.

Materials and methods

Plant material and greenhouse conditions

Two wheat cultivars were used in this study. 'Frontana' is a Brazilian spring wheat cultivar that is resistant to *Fusarium* head blight (7, 13) and possesses remarkable resistance to STB. 'Seri 82' is a Mexican spring cultivar that is susceptible to STB and lacks any known genes for resistance to *M. graminicola* (14). All experiments were conducted in greenhouse in the Department of Genomics at the Agricultural Biotechnology Research Institute of Iran (ABRII). Sixty 150-mm-diameter plastic pots filled with peat moss were used and ten wheat seeds planted in each pot. Plants were grown in the greenhouse under a 20/18°C and 16/8 h photoperiod (light intensity 250 $\mu E/s/m^2$).

Inoculums preparation and plant inoculation

Inoculums were prepared from four isolates of *M. graminicola* (BL1S1, SA852-2, Kermanshah 19, and MJA) that were collected respectively from Bookan, Safiabad, Kermanshah Province, and Majidabad, Iran, during 2005. Pure cultures of each isolate were obtained by culturing a pieces isolate stock on Potato Dextrose Agar (PDA) medium (39 g of PDA dissolved in 1 liter water and autoclaves 121°C for 15 min) and keeping in incubator 18°C for 4 days The spores were transferred to Yeast Glucose Broth (YGB; 1% yeast extract, 3% glucose) and incubated at 18°C on an orbital shaker at 120 rpm for 5 days to produce spores, which were collected by centrifugation. Inoculums were prepared by adjusting the spore suspension to 1×10^7 spores/ml sterile water using a neobar lam. One drop of Tween 20 (polyoxyethylene-sorbitan monolaurate, Sigma-Aldrich, St. Louis, USA) was added to 100 ml of spore suspension as a surfactant for inoculation. In two-leaf stage, wheat seedlings were inoculated with the spore suspension using a hand-held sprayer. Control seedlings were treated with suspension solution that lacked spores. Plants were cultivated in the greenhouse at 70% RH with a 23/18°C mean temperature and 16/8 h (light/dark) photoperiod (light intensity 166 $\mu E/s/m^2$).

Disease scoring

An experiment to determine isolate aggressiveness was conducted on the susceptible (Seri 82) and resistant (Frontana) cultivars. The aggressiveness of the four isolates (BL1S1, SA852-2, Kermanshah 19, and MJA) was tested in a randomized complete block experimental design with three replications. After 21 days from inoculation, symptoms were examined macroscopically and the percentage area of necrosis was scored (3). Analysis of variance and comparison of means for investigation of isolate's pathogenicity were performed with SAS software.

Sampling procedure

Each treatment was sampled at five time-points following inoculation (0, 3, 6, 12, and 24 Hours Post-Inoculation; HPI), which resulted in the

collection of 60 samples in total (two cultivars, five time-points, two treatments, and three replications). 5 g leaf tissues of 10 plants in each pot were harvested, frozen immediately in liquid nitrogen, and stored at −80°C until use.

RNA extraction and cDNA synthesis

Sampled leaf tissues were ground to a fine powder in liquid nitrogen with a sterile mortar and pestle. Approximately 100 mg of powder were transferred to a 1.5 ml microcentrifuge tube. Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. The purified RNA was quantified with a NanoDrop spectrophotometer (NanoDrop 1000-Spectrophotometer, Thermo Scientific, USA) and visualized on a 1.2% agarose/formaldehyde gel. First-strand cDNA was synthesized from total RNA that had been treated with 1 µg of DNase (Qiagen, Hilden, Germany) using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA).

Table 1. Putative defense-related genes, the sequence (5′-3′), melting temperature (Tm) and the product size of primers used for real-time PCR.

Gene	Primer sequence	T_m (°C)	Product size (bp)
Peptidyl prolyl *cis-trans* isomerase	PPi F:GAGATCGACCCGGACAACAG	81°C	126
	PPi R:CTCCGTCTTCCTCCATTTGG		
Putative protease inhibitor Bsi1	Bsi F:GGGCCCTGCAAGAAGTACTG	80°C	106
	Bsi R:ACACGCATAGGCACGATGAC		
Peroxidase	Per F:CCAGCACGACACGTGAATG	77°C	101
	Per R:CATGATTTGCTGCTGCTCGTA		
Pathogenesis-related protein	PR-1 F:CATGCGATTAGGGACGAAAGA	80°C	120
	PR-1 R:CCGCGGGAATATCATTGG		
Putative methionine sulfoxide reductase	Msr F:CATGCAGATGTTTCGGACAAA	77°C	139
	Msr R:ACCATCGCGTCCCATGTAAA		
Wheat 18S ribosomal RNA	18S F:GTGACGGGTGACGGAGAATT	81°C	151
	18S R:GACACTAATGCGCCCGGTAT		

Real-time PCR analysis

Real-time PCR was performed with five primer pairs and on three independent biological replicates, each of which contained three technical replicates. The reaction volume of 25 µl contained 12.5 µl of Power SYBR Green PCR Master Mix, 1 µl of 10 mM primer, 3 µl of cDNA sample (1/50 dilution), and 5.5 µl of dH_2O. Also, 96-well plates with a MyiQ™ Single-Color iCycler Real-Time PCR Detection System (Bio-Rad, USA) were used. To normalize gene expression, 18S ribosomal RNA was applied. Primers published by (15) were employed to amplify the five genes (*PR-1*, *PPi*, *BSi*, *Per*, and *Msr*) and 18S rRNA. The sequence (*5′-3′*), melting temperature (T_m) and the length of PCR products of six primers that used for real-time PCR were shown in table 1. The following thermal cycling profile was used: 95°C for 5 min; 45 cycles of 95°C for 30 s, 59-62°C for 30 s, and 72°C for 45 s; 72°C for 5 min. Subsequently, for the melting-curve analysis, the temperature was maintained at 50°C for 30 s and then increased slowly (at a 2% ramp rate) to 95°C over 20 min. To estimate fold changes in gene expression, a Microsoft Excel 2007 spreadsheet was compiled and a relative standard curve prepared with the $2^{-\Delta\Delta CT}$ method (16). Reactions were performed twice to test for repeatability. Gene expression in the incompatible (resistant) or compatible (susceptible) interactions was expressed as fold changes relative to the control. Standard deviations and ranking with Duncan's multiple range tests were used to test for significant differences between tran script abundance in resistant and susceptible cultivars at $P<0.05$.

Results

Evaluation of isolates pathogenicity on resistant and susceptible cultivars in the greenhouse

According to analysis of variance, significant difference ($P<0.01$) was observed between

virulence of *M. graminicola* isolates (Table 2). The average percentage area of leaf necrosis in the susceptible cultivar was significantly higher with SA852 isolate than with the other isolates and significantly lower in the resistant cultivar (Fig. 1). For the susceptible cultivarSeri82 that is inoculated with SA852, symptoms were first visible at 15 days post-inoculation (DPI) and the percentage area of leaf necrosis increased to approximately 80% of the leaf area at 24 DPI. In contrast, no significant necrosis or discoloration was observed in Frontana at 27 DPI (Fig. 2).

Table 2. Analysis of variance of effects of *Mycosphaerella graminicola* isolates on necrotic area.

Source of variation	df	Necrosis Area
Block	2	18.09
Cultivar	1	1896.06**
Isolate	3	176.36**
Cultivar*Isolate	3	272.80**
Error	14	7.84
Total	23	
C.V.		24.77

**Significant at $P<0.01$

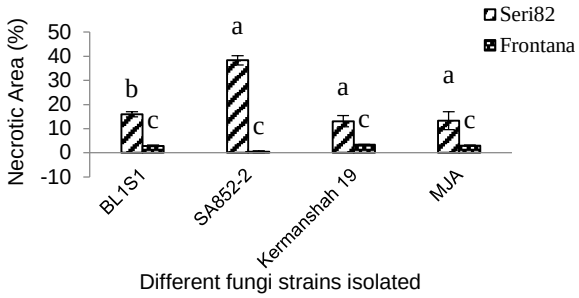

Figure 1. Effects of Mycospha *erella graminicola* isolates on the necrotic area of leaves of inoculated wheat seedlings. Real-Time Quantitative PCR.

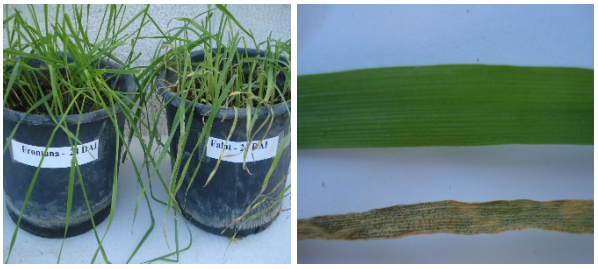

Figure 2. Symptoms of *Septoria tritici* on leaves of two Frontana (resistant) and seri82 (susceptible) cultivars 24 days after infection (left) and leaf necrosis area in susceptible and resistant cultivars 24 days after infection (right).

The expression of five defense-response genes was analyzed by real-time PCR. The specificity of the five primer pairs was tested by PCR amplification and visualization on a 2% agarose gel (Fig. 3). All five primer pairs amplified the target fragment specifically. Expression of 18S rRNA was used as an internal control to normalize all data. The expression profile of these five genes in the resistant (Frontana) and susceptible (Seri82) cultivar in response to pathogen attack are as follows: *PR-1* gene was up regulated significantly at 12 HPI in resistant (Frontana) cultivar, *Per* gene expression was significantly higher at 24 HPI in the resistant cultivar than in the susceptible cultivar, *Msr* and *Bsi* genes were induced significantly at 12 and 24 HPI in the resistant cultivar compared with the susceptible cultivar. *Ppi* gene was induced after inoculation but it was not significant at any of cultivars (Fig. 4). In Frontana, for four genes, a significant difference ($p<0.05$) with control inoculation was observed at 12 and 24 HPI. Significantly, higher expression of these four genes in the resistant cultivar, compared to that in the susceptible cultivar, indicates that these genes play vital roles in the resistance mechanisms of wheat. Briefly, these data indicate that a number of different cellular processes are activated during the incompatible interaction between *M. graminicola* and the resistant wheat cultivar.

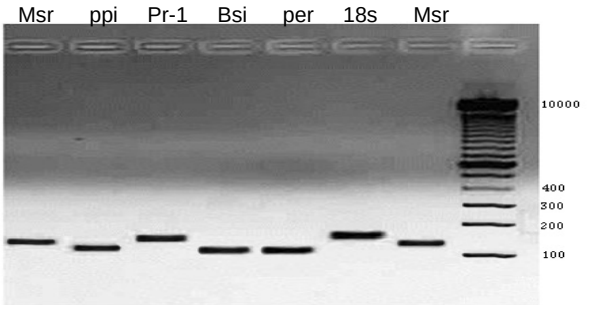

Figure 3. Specificity of five primer pairs for defense-related genes amplified by PCR and visualized with 2% agarose gel electrophoresis.

Discussion

In plant pathology, real-time PCR is an extremely useful tool with which to study various causal agents of plant diseases (17, 18). Defense-response genes in wheat were activated rapidly after inoculation with *M. graminicola*. Transcripts of the

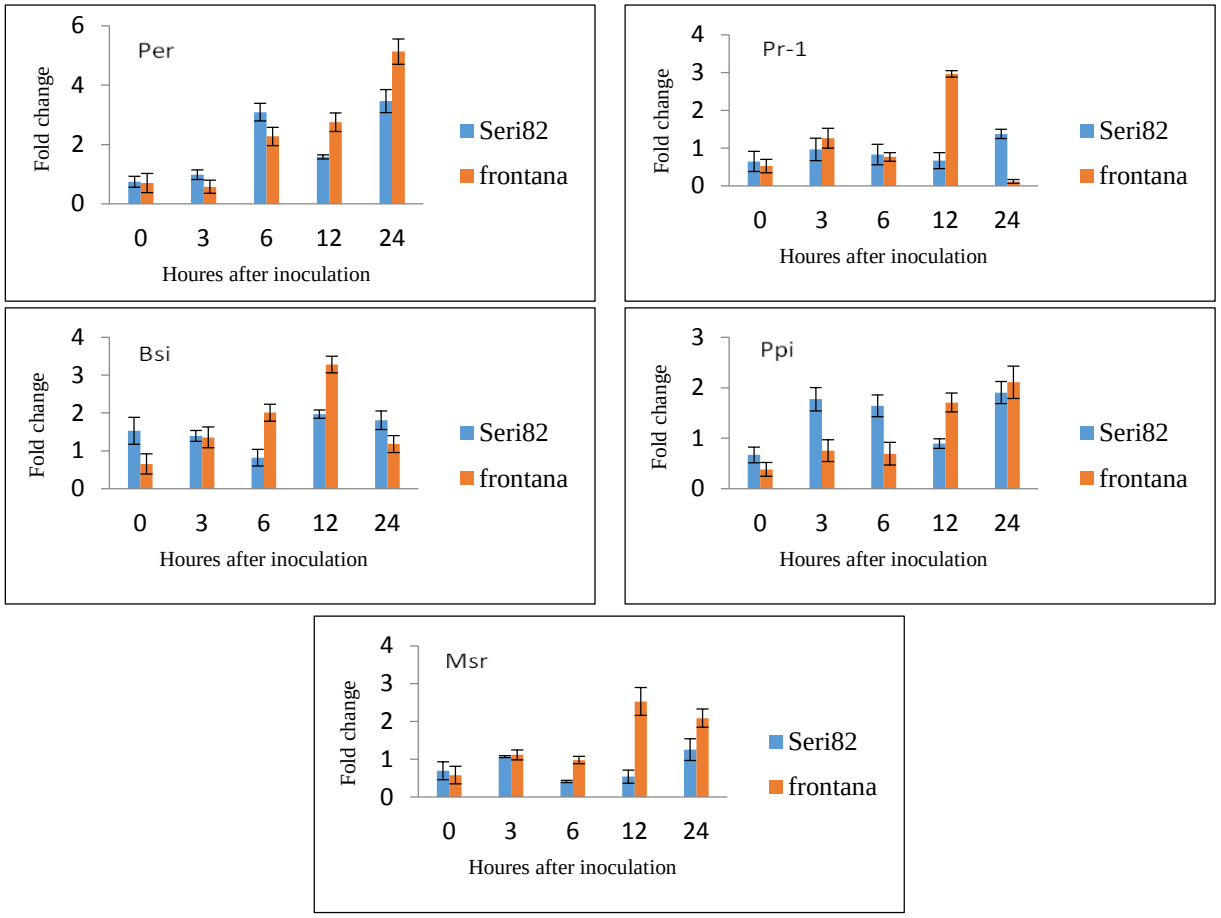

Figure 4. Real-time PCR analysis of five putative defense-related genes in a resistant cultivar (Frontana) and susceptible cultivar (Seri 82) during the 24 hours after inoculation of wheat seedlings with the *Septoria tritici* blotch pathogen, *Mycosphaerella graminicola*. Error bars indicate standard errors of the measurements at each time-point.

defense-response genes begin to accumulate within 3 HPI and reach maximal levels by 12 HPI (3). On the basis of the results of Ray et al., (2003) and Adhikari et al., (2007), we hypothesized that these changes in transcript levels 3-24 HPI determine the outcome of the host-pathogen interaction. All five genes under study were induced in both pathogen-inoculated and water-treated samples. This result was not unexpected because recent studies have indicated that, physical stimuli induce defense responses and the same pathways and key signaling components are induced as in pathogen-infected samples (19, 20).

According to Adhikari researches, the *PR-1* gene is induced during early stages of the defense response (15). In addition, Ray showed that the pathogenesis-related proteins *PR-1*, *PR-2*, and *PR-5* are induced in inoculated wheat leaves within 3-12 HPI (3). In current study Pathogenesis-related (PR) genes are induced in response to *M. graminicola* inoculation. Defense-related genes could have two roles in the resistance response. Pathogen-induced PR proteins that are secreted into intracellular spaces might be involved in controlling infection by the pathogen and the construction of physical barriers that curtail the pathogen's ability to penetrate the plant cell wall (15). In the present study, the expression of *PR-1* gene was higher in a resistant wheat cultivar (Frontana) than in a susceptible cultivar (Seri 82) at 12 HPI, which indicated that this gene might have an important role in the defense response against *M. graminicola* infection. Exactly how PR-1 gene participate in the defense response in wheat is not clear but a variety of roles have been suggested (21). The present results suggest that PR proteins that are induced rapidly in response to pathogen invasion might help the resistant wheat

cultivar to limit infection by *M. graminicola*. Expression of PR genes in transgenic plants results in significantly increased resistance to disease (22). *Per* gene was induced in both resistant (Frontana) and susceptible (Seri 82) cultivars in response to pathogen attack, but the expression of this gene was significantly higher at 24 HPI in the resistant cultivar than in the susceptible cultivar. This result confirms the findings of Adhikari (15). One of the earliest events in the plant defense response against pathogen attack is the production of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) and superoxide (23-25). The early induction of *Per* gene that was observed in the present study suggests that this gene might play a significant role in the generation of H_2O_2 during the wheat defense response and in the conferral of resistance to a wide range of pathogens (26). The roles of peroxidase in the defense reaction in resistant plants have been studied previously (27-29).

Activation of ROS during the interaction of resistant plants and virulent pathogen (30) means that peptidyl methionine is oxidized readily by ROS to form methionine sulfoxide. The enzyme PMSR catalyzes the reduction of methionine sulfoxides to methionine. Consequently, PMSR is indicated to act as a last-chance antioxidant that repairs proteins damaged by oxidative stress (31). Up regulation of the *Msr* gene in the resistant wheat cultivar suggests that the product of this gene might repair proteins that have been damaged following *M. graminicola* inoculation. The *Msr* gene expression up regulating, confirmed the Adhikari results (15), and in *Arabidopsis*, expression of PMSR increases in response to oxidative stress (31).

The other gene that was up regulated 12 hours after *M. graminicola* inoculation was *Bsi* gene, which is reported to be associated specifically with the resistance response. Stevens reported that the sequence of *Bsil* cDNA showed 86% identity with *wali5a,* an aluminium-induced gene of unknown function that is expressed in wheat roots (32). This high level of homology was also observed in the 3′ non-coding regions of them, which indicated that the two genes were closely related. A number of plant proteinase inhibitors have been shown to possess antifungal activity (33), which raises the possibility that *Bsil* might function in the prevention of pathogen infection by inhibiting growth of the penetrating hyphae within the cell wall (34). Although the function of this gene remains unknown, it might be part of the plant's general response to stress and might not play a specific role in defense against *M. graminicola* (15). The *Ppi* gene expression did not show significant changes in response to *M. graminicola* inoculation. The obtained data explain a section of the molecular basis of the infection process and some genes that can help to inhibit the pathogen.

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