Mycopath (2017) 15(1): 1-5 **Research Article**

Differential gene behavior in barley plants challenged with biotrophic and necrotrophic pathogens

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Abstract

The biotrophic [*Blumeria graminis* (DC.) Speer] and necrotrophic [*Cochliobolus sativus* (S. Ito & Kurib.) Drechsler ex Dastur] are economically important pathogens of barley found worldwide. To better understand the defense responses initiated by resistant barley challenged with *B. graminis* and *C. sativus*, important known defense-related genes at early points of infection were analyzed using PCR (qPCR) approach. Time-course experiments revealed a remarkable discrepancy in the expression patterns of the defense-related genes between barley *B. graminis* or *C. sativus* interactions, and all of them exhibited significant differential accumulations compared to the non-inoculated controls. The most outstanding differences were observed in *PR1* and *PR5* expression which were 6 and 9 folds higher respectively at the 72-hpi of barley *B. graminis* interaction as compared with *C. sativus*. Data showed that barley plants triggered different defense mechanisms to strengthen their *B. graminis* and *C. sativus* resistance, and the expression patterns of the same defense-associated genes were altered in adaptation to each pathogen. Taken together, our study might provide useful information for a deeper molecular research on barley defense responses against pathogens with different lifestyles.

Keywords: Barley - Defense response - PCR (qPCR) - Powdery mildew - Spot blotch.

Introduction

Plants posses innate immune systems that recognize the presence of potential pathogens involving biotrophic and necrotrophic fungal species and initiate effective defense responses. Biotrophic lifestyle involves feeding on living plant tissue, whereas a necrotrophic lifestyle is associated with killing plant tissue and feeding on dead or dying cells (Glazebrook, 2005; Spanu and Panstruga, 2017). To help deal with this issue, plants have evolved tightly regulated inducible defence systems that are less costly to maintain and include mechanisms to restrict defence deployment unless completely necessary. Therefore, the biochemical mechanisms of defense against these pathogens are wide-ranging, highly dynamic, and are mediated both by direct and indirect defenses. As a consequent, our understanding of these defensive mechanisms is still limited.

Powdery mildew caused by the biotrophic fungus *Blumeria graminis* f. sp. *hordei*, and spot blotch caused by the necrotrophic fungus *Cochliobolus sativus*. have been the major yield-reducing factors for barley production during the last decade (Ghazvini, 2012). Changes in the expression level of thousands of genes can be detected in barley plants at early stages of *B. graminis* and *C. sativus* infection, which cause an induction of key resistance genes (Panstruga, 2003; Jawhar *et al*., 2017). However, infecting barley plants with these pathogens triggers the constitutive and induced defence responses which are regulated by a concerted expression of different plant pathways (Kumar *et al*., 2002).

A large number of defense-related genes are up or down-regulated during plant-pathogen interactions (Jing *et al*., 2015; Nayanakantha *et al*., 2016). The expression levels of pathogenesis related (*PR*) proteins and other genes like SGT1 and PAL are low or absent in mature healthy plants but become elevated after pathogen attack (Thomma *et al*., 2001). However, the molecular events involved in barley defence response against *B. graminis* and *C. sativus* are not yet fully understood, although an increasing number of potentially involved components were determined (Ge *et al*., 2016; Spanu and Panstruga, 2017). Therefore, quantitative PCR (qPCR) would be an effective method to be utilized, since it allows the measuring of the relative expression level of a particular transcript and determines its expression after being exposed to a specific alteration, such as an infection by a pathogen (Nolan *et al*., 2006; Derveaux *et al*., 2010).

The present study aimed to evaluate the changes in the induction of some well known defense-related genes *viz. PR-1, PR-2, PR-5, SGT1* and *PAL* during barley interactions with *B. graminis* and *C. sativus* deploying qPCR approach.

Materials and Methods

Experimental design

After an extensive screening of barley genotypes for several years in the greenhouse and laboratory experiments, the German cv. Banteng was proved to be the most resistant genotype to all *B.*

graminis and *C. sativus* isolates available so far (Arabi and Jawhar, 2004, 2012), therefore, it was used as a plant material in this study. Plants were grown in plastic flats (60 × 40 × 8 cm) filled with sterilized peatmoss and arranged in a randomized complete block design with three replicates for each pathogen. Each experimental unit consisted of 10 seedlings. Flats were placed in a growth chamber at temperatures 22 ºC (day) and 18 ºC (night) with a day length of 12 h and 85-90% relative humidity.

Inoculation with *C. sativus*

The most virulent pathotype of *C. sativus* (pt4) described by Arabi and Jawhar (2004) was used in this study. Infections were performed by spraying plants with conidial suspension of 2×10^4 conidia mL^{-1} in pure water. Tween 20 (polyoxyethylene-sorbitan monolaurate) was added as a surfactant (100 µL L^{-1}) to the conidial suspension to facilitate dispersion of the inoculum over the leaf surface. Leaves were covered for one night with plastic bags to increase humidity and plants were kept in the same greenhouse at 20 °C with a 16 h photoperiod. Non-inoculated control plants were sprayed with distilled water and surfactant.

Inoculation with *B. graminis*

Seedlings were inoculated with virulent *B. graminis* conidiospores of the mildew culture isolate (Pt1m) by shaking susceptible spreader sporulating plants above them. During the period of inoculation a temperature of 18-20 ºC was maintained. The experiment was conducted in a randomized complete block design with three replicates and was repeated twice.

RNA isolation and cDNA synthesis

Primary leaves were collected at 24, 48, 72 and 96 hours post inoculation (hpi) and were immediately frozen in liquid nitrogen. At the same time points, samples from mock inoculated plants were collected as controls. Mock inoculation was done by spraying plants with pathogen-free water on the plants. mRNA was extracted from samples (100–200 mg) with the Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany) following the manufacturer's protocol. RNA was used for cDNA synthesis with the Quanti Tect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions and the obtained cDNA was stored at −20 °C.

Quantitative real-time PCR (qPCR)

Five known defense-related genes *viz. PR-1, PR-2, PR-5, SGT1* and *PAL* were analyzed in the current study. PCR primers were designed based on the cDNA sequences of barley available at NCBI (http://www.ncbi.nlm.nih.gov) database using Primer 3 software (Table 1). Gene expression was assayed in Step One Plus, 96-well rotor, using SYBR Green Master kit (Qiagen), 3 µL 35x diluted cDNA samples and thermal profile (40 cycles). This was followed by a melt curve analysis and gel electrophoresis of the qPCR products. For each target gene, all of the cDNA samples, standards and no template controls (that was tested not to contain genomic DNA) were assayed in triplicate in a single run. The standard curve calculation and data analysis was performed with Rotor-Gene Q software (Qiagen). The threshold cycle (Ct) value was automatically determined for each reaction by the real time PCR system with default parameters. Raw data (not baseline corrected) of fluorescence levels and the specificity of the amplicons were checked by qRT-PCR dissociation curve analysis using StepOne™ Software v2.3.

Data analysis

Raw data of fluorescence levels and the specificity of the amplicons were checked by qRT-PCR dissociation curve analysis using StepOne™ Software (v2.3). The fluorescence readings of three replicated samples were averaged, and blank value (from no-DNA control) was subtracted. The final Ct values were the mean of three replicates and the coefficient of variance was calculated to evaluate the variation of Ct values for each gene. Each qRT-PCR reaction set included water as a negative no-template control instead of cDNA. The fold change in putative target gene expression levels was determined using the Ct method (Livak and Schmittgen, 2001), with *EF1α* as a reference (housekeeping control) gene. Standard deviation was calculated from the replicated experimental data. The statistical analysis was conducted through the Tukey's test at the 0.05 level.

Results and Discussion

In order to determine the defense responses exhibited by barley plants to overcome biotrophic *B. graminis* and necrotrophic *C. sativus* pathogens infection, the induction of well known defense-related genes *viz. PR-1, PR-2, PR-5, SGT1* and *PAL* was assayed in barley leaves (Fig. 1). Oligonucleotides were designed from the sequence information available on NCBI database and used for RT-PCR on inoculated and non-inoculated samples revealed consistently reproducible and differential amplification profiles. The sequence information and amplified fragments for all qRT-PCR primers are given in Table 1.

Our analysis showed that *PR-1, PR-2, PR-5, SGT1* and *PAL* genes in the resistant barley exhibited a differential expression by $P = 0.05$, and were inversely regulated during different times point post inoculation. However, 72 hpi, *PR1* and *PR5* were significantly expressed with 6 and 9 fold increases, respectively for barley infected with *B. graminis* as compared with *C. sativus*. In contrast, *PR2* was up-regulated 24 hpi and down-regulated 72 hpi in *C.*

sativus inoculated barley plants as compared to its expression in *B. graminis* infected plants, suggesting its role in the first stages of defense against a biotrophic attack (Fig. 1). In addition, increased and consistent transcripts abundance of *SGT1* and *PAL1* were recorded 24 hpi for *C. sativus* as compared with *B. graminis* (Fig. 1).

Conversely, *SGT1* expression was markedly down regulated 96 hpi during *B. graminis* infection. Unexpectedly, genes whose expression patterns were similar under *B. graminis* attack showed different temporal patterns of expression under *C. sativus* infection (Fig. 1). This finding indicated that these genes have multiple or different functional roles in response to different biotic stresses.

This study sheds some light on expression changes of important defense response genes that occur during the biotrophic and necrotrophic– barley interactions. Our results demonstrated that transcripts of the selected genes accumulated earlier and with a higher level in the resistant barley cv. Banteng leaf tissues upon challenge with either *B. graminis* or *C. sativus*. Considering that this cultivar had high levels of resistance to both pathogens (Arabi and Jawhar, 2004, 2012). Importantly, some genes were associated with a multi-gene resistance which dispels the current belief that similar mechanisms are activated in response to *B. graminis* and *C. sativus* infection.

A repertoire of genes is likely to be involved in stress and defence responses, signal transduction and phytohormone regulation in plant tissues in response to fungal pathogen infection (Takahashi *et al.,* 2003; Yan *et al.,* 2014). In agreement with those reports, genes induced in those categories were differentially expressed in the present investigation. However, in contrast to necrotrophs, the biotrophs pathogens secrete limited amounts of lytic enzymes, generally lack toxin production and evade detection or suppress immune responses through manipulation of host defenses (Oliver and Ipcho, 2004).

Results demonstrated that *PR2* was up-regulated 24 hpi in *C. sativus* inoculated barley plants as compared to *B. graminis* inoculated plants, which might indicate that *PRs* are related to the severity of *C. sativus* symptom rather than to resistance (Kumar *et al*., 2002). However, it is well known that *PR2* encodes for 1, 3-ß-glucanase throughout the plant kingdom and belong to the glycoside hydrolases family. 1, 3-ß-glucanase hydrolyses the ß-*O*-glycosidic bond of ß-glucan in plant cell walls, with cell wall loosening and expansion (Akiyama *et al.,* 2009). This phenomenon may be the cause of barley cell wall leakage during *C. sativus* infestations.

It is of a particular interest to highlight the induction of defense-related genes at early stages of infection. It has been well established that biotrophic *Uromyces vignae* and hemibiotrophs *Mycosphaerella graminicola* have suppressed the host defenses for parasitize that invaded host cells during the biotrophic phase (Panstruga, 2003; Doehlemann *et al.,* 2008). These results might support our finding when *SGT1* and *PAL1* suppressed 24 hpi for *B. graminis* infection.

Conclusion

Our study demonstrated that resistant barley revealed a remarkable discrepancy in the expression patterns of the defense-related genes against biotrophs and necrotrophs attacks. This consistency in the response of the defense mechanisms could be in agreement with the well-accepted concept that defense responses are very intense in resistant plants.

Table1: Properties and nucleotide sequences of primers used in this study.

Gene	Gene description	Accession No.	Sequence	Amplified fragment (bp)
EF1α	Elongation foctor-1 Alapha	AT1G07920	TGGATTTGAGGGTGACAACA CCGTTCCAATACCACCAATC	167
SGT1	_	AT4G11260	GGCTGTTGCTCCTGCTACATCTTC CGAGGCTGGAAATGGTATGGTTC	177
PAL	Phenyl alanine amino lyase	AT2G14610	CCATTGATGAAGCCAAAGCAAG ATGAGTGGGTTATCGTTGACGG	123
PR1	Pathogen-related protein	AY005474	ACTACCTTTCACCCCACAACGC TTTCTGTCCAACAACATTCCCG	182
PR2	Beta1,3-glucanase2	AT3G57260	TGGTGTCAGATTCCGGTACA TCATCCCTGAACCTTCCTTG	193
PR5	Pathogen-related protein S	AT1G75040	GGAGACTGTGGCGGTCTAAG GCGTTGAGGTCAGAGACACA	197

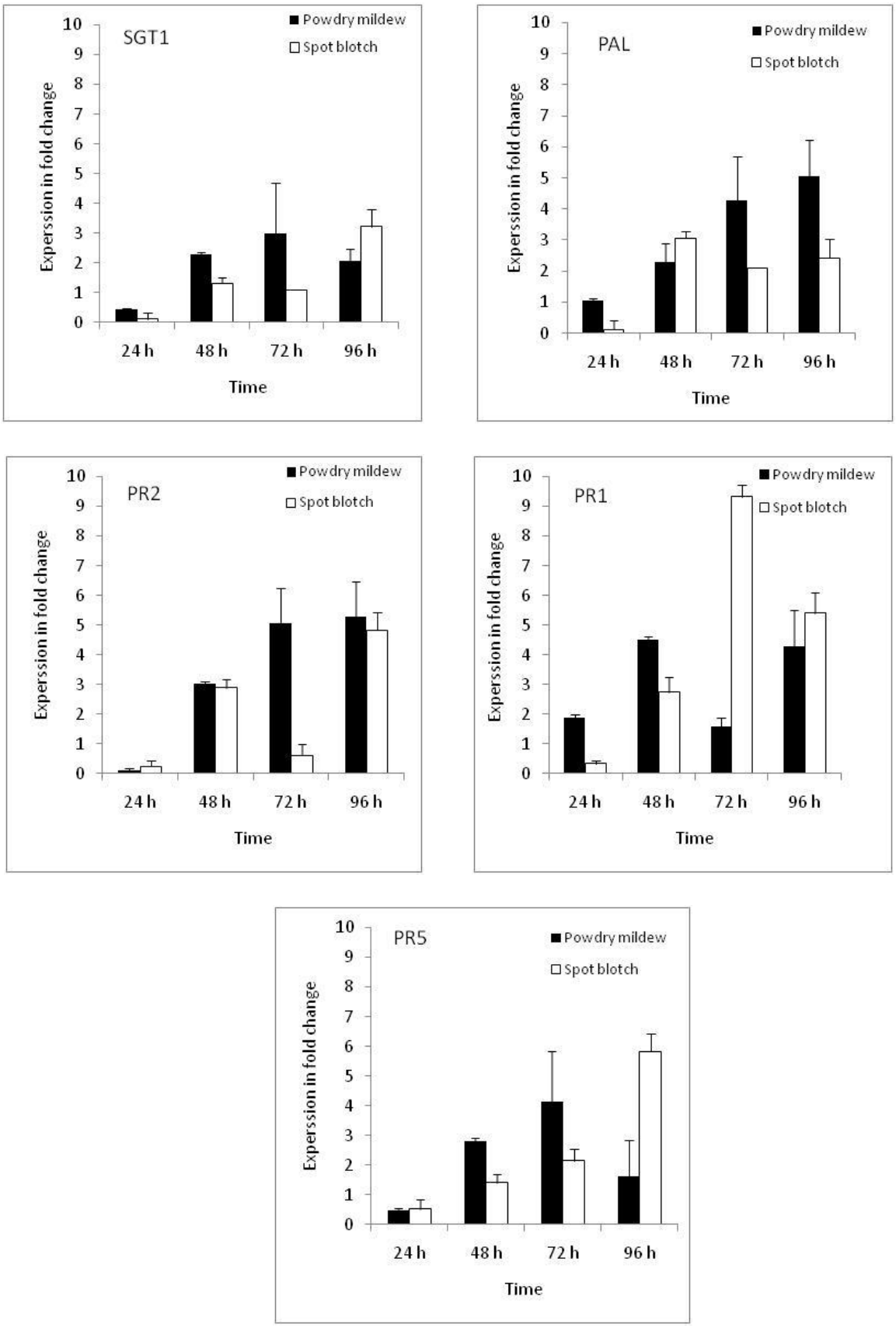

Fig 1. Relative expression profiles of marker in Barley cv. Banteng during the time course of powdery mildew and spot blotch infections. Error bars are representative of the standard error of the mean of three replicates.

The data suggested that resistant barley triggered different defense mechanisms to strengthen its *B. graminis* and *C. sativus* resistance, and the expression patterns of the same defense-associated some genes were altered in adaptation to the pathogens. We were able to identify this role for defense-related genes, because the sensitivity of the qRT-PCR gene expression assay allows detection of low-abundance transcripts that are below the threshold of widely used techniques such as RNA gel blots (Derveaux *et al*., 2010).

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