*Mycopath* (2017) 15(2): 61-66 **Research Article**

# Differential expression of defense-related genes in susceptible versus resistant barley genotypes challenged with *Pyrenophora teres*

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## Abstract

The fungus *Pyrenophora teres* f. sp. *teres* is the causal agent of barley net blotch, a disease responsible for significant yield loss in major production areas throughout the world. In this study, the differential responses of susceptible and resistant barley cultivars upon *P. teres* infection were investigated in infected seedling leaves at 24 and 96 hours post inoculation using RT-PCR method. Our results showed a notable discrepancy in the expression pattern of the studied genes between susceptible and resistant barley-*P. teres* interaction. All tested defense-related genes were up-regulated in both resistant and susceptible genotypes as compared with non-inoculated ones. However, expression was often higher in resistant plants, suggesting a possible role for these genes in the defense response. *PR2* expression level increased by 6.4 folds after 96 h inoculation in the resistant cultivar as revealed by qRT-PCR. Our *preliminary results* will strengthen our basic understanding of the genetic mechanisms governing barley *susceptibility* and *resistance* to net blotch disease.

**Keywords:** Barley - Defense response - *Pyrenophora teres* f. *teres* - Semi-quantitative RT-PCR.

## Introduction

Net blotch (NB) caused by the necrotrophic pathogen *Pyrenophora teres* f. sp. *teres,* is an economically serious disease of barley (*Hordeum vulgare*) worldwide. The net and spot types of NB (Smedegard-Peterson, 1971; McLean *et al.,* 2009) cause significant reductions in the grain yield and quality (Mathre, 1997; Murray and Brennan, 2010). This complex pathosystem requires further and deeper analysis of the involved genetic mechanisms existed during *P. teres*-barley interaction (Liu *et al.,* 2011).

Studies on the inheritance of *P. teres* f. sp. *teres* resistance in barley have indicated the presence of both quantitative (Arabi *et al.,* 1990; Robinson, 1999) and qualitative forms (Schaller, 1955; Ste-Venson *et al.,* 1996; Friesen *et al.,* 2006). This suggests a gene for gene interaction in NB pathosystem. Different researches have indicated to the presence of QTL/genes for resistance on barley chromosome 6H. However, in spite of some of these works may be reporting the same loci for NB resistance, it appears that 6H carries multiple independent genes for resistance (Abu Qamar *et al.,* 2008). In addition, single resistance genes and/or QTL have been reported on other barley chromosomes (Richards *et al.,* 2016). Furthermore, hypersensitive reactions were found in resistant and susceptible barley genotypes during penetration stage of fungal infection of epidermis tissue (Jørgensen *et al.,* 1998). However, mechanisms underlying the genetic variation in the cultivated resistant and susceptible barley- *P. teres* interaction are still obscure.

Different number of defense-related genes has been reported to be up or down-regulated during plant-fungal pathogen interactions (Glazebrook, 2005; Jing *et al.,* 2015). However, the pathogenesis related (PR) proteins expression levels were depressed or missing in uninfected plants and be activated after fungal infection (Thomma *et al.,* 2001). Moreover, PR genes might be expressed also in susceptible species but their expression may be seen at the late stage of disease progression or in low concentration; not sufficient to arrest the lesion development (Nayanakantha *et al.,* 2016). Although, *Rar1, SGT1*and *HSP90* are well-known to have an active function in the plant immune regulation reactions, and their role in barley -*P. teres* interaction is largely indefinite.

However, significant molecular data from barley is available in response to inoculation with *P. teres* (Cakir *et al.,* 2003; Richards *et al.,* 2016), information on defense gene induction in barley after challenge inoculation with this fungus is scarce. In this context, barley susceptible and resistant plants infected with *P. teres* were analyzed for the induction of some important defense-related genes *viz., PR-2, PR-3, PR-5, Rar1, SGT1*and *HSP90* by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR).

# Materials and Methods

**Plant material**

The German resistant genotype 'Banteng', and the Australian universal susceptible genotype 'WI2291'(Arabi *et al.*, 2003) were used in this study. Plants were grown in plastic boxes (60 × 40 × 8 cm) contained sterilized peatmoss in three replicates experimental units (10 plants) and installed in a randomized complete block design. Boxes were placed in a greenhouse at 22 ºC (day) and 18 ºC (night) with a day length of 12h and 85% relative humidity.

***P. teres* inoculation**

The major Syrian virulent pathotype *P. teres* (Pt4) to all barley genotypes available so far was used in this study (Arabi *et al.*, 2003). The fungus was incubated on Petri dishes containing PDA (potato dextrose agar) in the darkness for 8 days at 20-22 °C. Suspension was adjusted to a content of 2 × $10^4$ spore $mL^{-1}$ by hemacytometer. A surfactant (polyoxyethylene-20-sorbitan monolaurate) was added with a rate 100 µL $L^{-1}$ to the spore suspension for dispersion of the inoculum over plants. Inoculation were performed by spraying barley seedlings with the spore suspension, and then covered all the night with plastic bags to keep humidity, and then placed at 20 °C with a 16 h photoperiod. The control plants were sprayed with distilled water and surfactant. NB infection was measured using a scale described by Tekauz (1985).

**mRNA isolation**

mRNA was extracted from primary barley leaves at 24, 48, 72 and 96 (hpi) using liquid nitrogen. Controls were collected at the same periods from un-inoculated plants. RNA isolation was achieved as reported previously by Arabi et al. (2015). mRNA was isolated from 100–200 mg of barley samples using Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany). The QuantiTect Reverse Transcription Kit (Qiagen) was used for cDNA synthesis according to the manufacturer's instructions.

**Semi quantitative RT-PCR**

Six known genes *viz. PR-2, PR-3, PR-5, Rar1, SGT1*and *HSP90* were used in the present study. Primers were designed due to the cDNA sequences of barley available at NCBI (http://www.ncbi.nlm.nih.gov) database using Primer 3 software (Table 1). All the designed primers were blasted to NCBI database for BLASTn search and confirmed to specifically anneal with their template genes. PCR was performed using a thermocycler (Biometra) in a 50-µL including 1µL of ten times diluted cDNA template, 5 µL of 10X amplification buffer (Thermo Scientific, USA), 1 µL of 200 µM deoxynucleotide triphosphates (Thermo Scientific, USA), 1 µL of 10 pico-molar of each primer, and 0.2 µL (1 U) of Taq DNA polymerase (MBI Fermentas, York, UK) and 40.8 µL of PCR grade water. After preliminary experiments, PCR conditions included an initial denaturing step at 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 55-57 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 10 min. PCR products were separated using 1% agarose gels, stained with ethidium bromide and observed on a UV transilluminator. PCR was performed three times for each primer using the same cDNA sample for confirming the reproducibility of the data. PCR (qPCR) was performed for *PR-2* to confirm RT-PCR data using the method reported by Derveaux *et al.* (2010).

# Results and Discussion

Development of NB symptoms was observed in inoculated leaves of both the resistant and susceptible genotypes while un-inoculated controls remained free of symptoms. Yellowing of the tissues surrounding the inoculation droplet appeared as early as 24 hpi in the susceptible cv. WI2291 while it appeared in the resistant cv. Banteng 48 hpi. However, the lesion diameter in WI2291 was smaller compared to that in Banteng 96 hpi and lesions did not progress much beyond the boundaries of the inoculum drop even 10 days post inoculation. The infection responses are presented in Fig. 1.

For deeper understanding of the molecular machinery underpinning the defence response during barley - NB interaction, the differential expression of three PR genes (*PR2*, *PR3* and *PR5*) was evaluated. Moreover, the expression of *Rar1, SGT1*and *HSP90*, was also assisted. RT-PCR changes in the transcript levels of these genes relative to the reference gene EF1*α* were determined at different time intervals as described earlier. The strong up regulation of *PR3* and *Rar1* in both resistant and susceptible varieties at 24 and 96 hpi confirms the defence-inducing capabilities of these genes in barley leaves in response to the *P. teres* attack (Fig. 2). However, the strongest up regulation of these genes was noticed in the resistant cv. Banteng 24 hpi. Conversely, *SGT1* and *HSP90* expression was markedly down regulated 48 and 72 hpi, respectively in the resistant cultivar (Fig. 2 and 3). However, *PR2* increased by 6.4-fold after 96 hpi in the resistant cultivar as revealed by qRT-PCR (Fig. 4).

The data demonstrated strong up regulation of some genes encoding pathogenesis-related (PR) proteins in resistant plants and down regulation in susceptible ones, suggesting their roles in the barley-*P. teres* interaction. RT-PCR expression patterns of most studied genes in the resistant genotype demonstrated their peak 24 hpi and decreased towards 72 hpi, as compared to their patterns of *in planta* discovered fungal proteins and coinciding with the highest fungal biomass in plants after 1.5 days of inoculation. This might to the fact that the defence response of the resistant genotype is strong

enough at 24 hpi to restrict further *P. teres* development.

This work focused on expression changes of some genes involved in plant innate immunity that occurs during *P. teres*-barley interaction. Two barley genotypes were used for this purpose, including *P. teres*, a highly susceptible barley phenotype (S), and a highly resistant barley phenotype (T). The infection responses are presented in Fig. 4. The results are in agreement with our observations under natural field conditions during the past several years (Arabi *et al.*, 1990).

In order to study the genetic changes during the defense responses to *P. teres* infection of susceptible and resistant barley genotypes, the induction of some important defense-related genes *viz., PR-2, PR-3, PR-5, Rar1, SGT1*and *HSP90* were assayed in barley leaves (Fig. 2 and 3). Results indicated that these genes were accumulated at a greater level and earlier in the resistant cv. Banteng than in the susceptible WI2291 leaf tissues upon challenge inoculation with *P. teres*. It has been reported that a large number of genes is implicated in signal transduction, defence responses and phytohormone regulation in plants attacked with fungal diseases (Takahashi *et al.*, 2003; Yan *et al.*, 2014). However, in comparing the response of barley susceptible and resistant genotypes after infection with *P. teres*, several arising discrepancies may be attributed due to the fungus effects (life cycle from biotrophic to necrotrophic) and systemic hormone signalling effects (Glazebrook, 2005; Jing *et al.*, 2015).

**Table1**: Properties and nucleotide sequences of primers.

| Gene | Gene description | Accession No. | Sequence | Amplified fragment (bp) |
|---|---|---|---|---|
| *EF1α* | Elongation foctor-1 Alapha | AT1G07920 | TGGATTTGAGGGTGACAACA<br>CCGTTCCAATACCACCAATC | 167 |
| *PR2* | Beta1,3-glucanase2 | AT3G57260 | TGGTGTCAGATTCCGGTACA<br>TCATCCCTGAACCTTCCTTG | 193 |
| *PR3* | Basic Chitinase | AT3G12500 | GGGGCTACTGTTTCAAGCAA<br>GCAACAAGGTCAGGGTTGTT | 187 |
| *PR5* | Pathogen-related protein S | AT1G75040 | GGAGACTGTGGCGGTCTAAG<br>GCGTTGAGGTCAGAGACACA | 197 |
| *Rar1* | Zinc binding protein | AT5G1700 | TTGCGGCTCCTACTTCATCT<br>AGGCGCTAAGGGTTCAATTT | 173 |
| *SGT1b* | _ | AT4G11260 | GGCTGTTGCTCCTGCTACATCTTC<br>CGAGGCTGGAAATGGTATGGTTC | 177 |
| *HSP90* | Heat shock protein | AT5G56030 | GTCATCCGCAAGAACCTTGT | 162 |

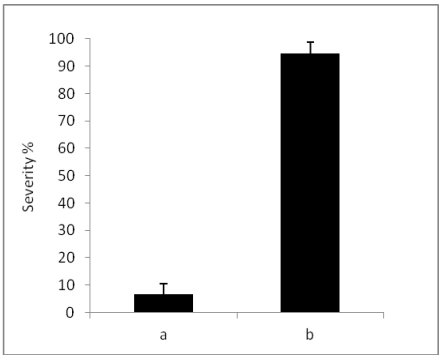


**Fig. 1.** Frequency of disease reactions incited on the barley (a) resistant cv. Banteng and (b) susceptible cv. WI2291 by *P. teres*. Disease infections were scored according to a scale developed by Tekauz (1985).

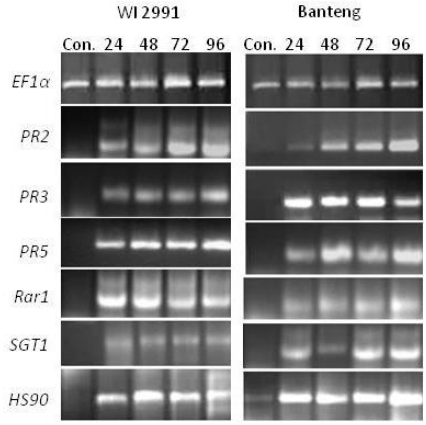


**Fig. 2.** Time course of *PR-2, PR-3, PR-5, Rar1, SGT1*and *HS90* transcript accumulation in barley inoculated leaves from susceptible (WI2991 ) and resistant (Banteng) genotypes. Total RNA was isolated from leaves at 0 to 96 h post infection. Semi quantitative RT-PCR was performed from cDNA made from each RNA sample. *EF1α* transcripts were used to normalize the sample.

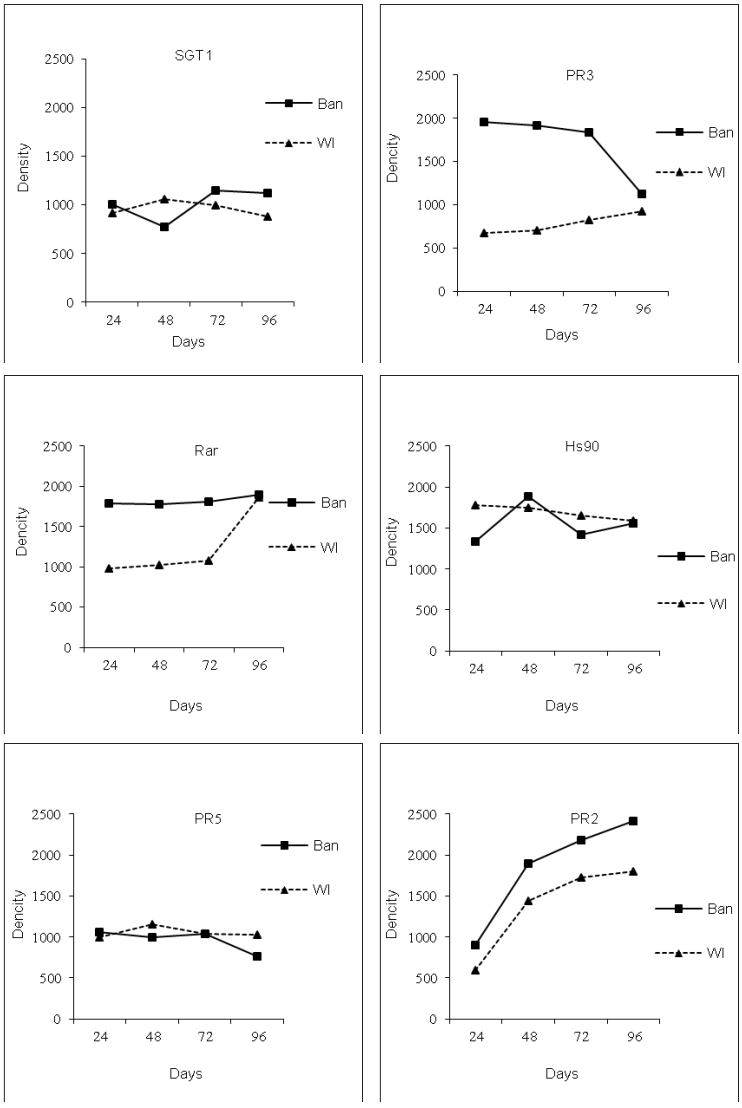


**Fig. 3.** Relative expression profiles of marker genes in resistance cv. Banteng and in susceptible cv. WI2291 during the time course after *P. teres infection*

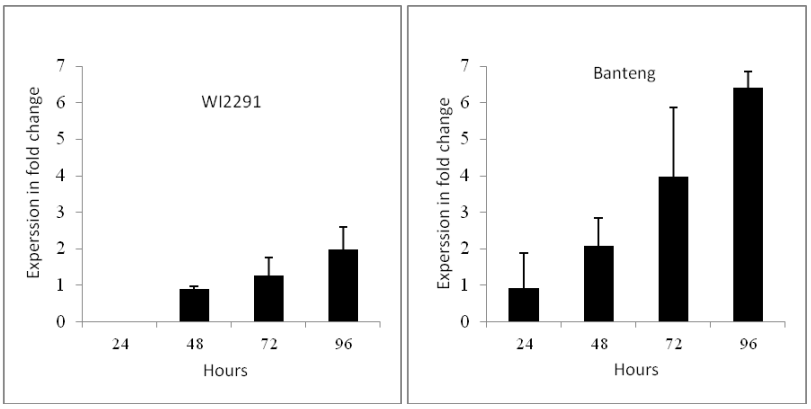


**Fig. 4.** Relative expression profiles of PR2 gene in resistance cv. Banteng and in susceptible cv. cv. WI2291 during the time course after *P. teres infection.* Error bars are representative of the standard errors.

In addition, our results demonstrate that *PR2* and *PR5* were consistently up regulated in both susceptible and resistant genotypes during *P. teres* infection. In contrast, *PR3* was significantly attenuated in the resistant genotype at 96 hpi. Howver, it has been reported that several *PR* genes were suppressed in tomato leaves tissues during their compatible interaction with Verticillium *dahliae* (*Tan et al., 2015*). Similarly, the *PR* gene accumulation was recorded in barley leaf tissues infected with *Cochliobolus sativus* (Arabi *et al.*, 2015).

Our work explains the changes in differential expression of a battery of important genes in susceptible and resistant barley-*P. teres* interaction, that might illustrated the molecular logic in determining agents effects on plant susceptibility to fungal pathogen. Our study shows that early defenses were activated in both susceptible and resistant barley plants upon infection with NB, whereas it is ostensibly suppressed in the susceptible plants at late stage of infection. However, Lightfoot *et al.* (2016) found that cytosolic superoxide may play an important function in several barley-pathogen interactions.

## Conclusion

This study demonstrated that *PR-2, PR-3, PR-5, Rar1, SGT1*and *HSP90* had in general higher constitutive expression and faster induction in infected plants, and this expression was often higher in resistant plants. Further experiments concerning plant intracellular proteins related to the SGT1, Rar1 and Hsp90 regulation genes will be needed to illustrate the molecular mechanisms of *P. teres*-barley interaction. However, in spite of further quantitative validation using Real-Time PCR (qPCR) is required, our work will hopefully serve as a basis to reply new questions and design new experiments to explain further the biology of plant- necrotrophic interactions.

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