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Changes in salicylic acid content and pathogenesis - related (*PR2*) gene expression during barley - *Pyrenophora teres* interaction

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Summary Net blotch (NB), caused by the necrotrophic fungal pathogen *Pyrenophora teres f. teres*, substantially reduces barley grain yield and quality worldwide. The role of salicylic acid (SA) signaling in NB resistance has been poorly documented. In this study, SA levels as well as the expression of the SA-responsive gene *PR2* were monitored in infected leaves of two barley genotypes, Banteng (resistant) and WI2291 (susceptible), at different time points of infection. SA signaling was activated in bothgenotypes 24 hours post infection (hpi) as compared with non-inoculated plants. However, with or without pathogen pretreatment, SA significantly increased (P=0.001) in Banteng comparing with WI2291. RT-PCR analysis revealed that *PR2* expression increases in the resistant and susceptible genotypes over the inoculation time points, with maximum expression (6.4 and 1.99-fold, respectively) observed 6 dpi. *PR2* expression was paralleled by an increase in leaf SA content as shown by the test coincidence ($F_{3, 32} = 4.74$, $P = 0.001$). Based on barley genotype resistance levels, our data strengthen the idea that SA signaling and *PR2* play a role in barley NB reduction.

Additional keywords: barley, *Pyrenophora teres*, *PR2* gene expression, RT-PCR, salicylic acid

Introduction

Net blotch, caused by the fungal pathogen *Pyrenophora Drechs. teres* Smedeg. (anamorph: *Drechslera teres* [Sacc.] Shoem. f. *Teres* Smedeg.), is a common foliar disease of barley (*Hordeum vulgare* L.), a disease responsible for heavy crop losses (Liu *et al.,* 2011; Wang *et al.,* 2015). Various mechanisms for NB resistance and susceptibility appear to operate in barley. *Pyrenophora teres* activates different defense responses which are regulated through different plant signaling pathways, including plant hormones such as SA and pathogenesis-related (PR) proteins (Wang *et al.,* 2011; Bogacki *et al.,* 2008).

A number of studies have demonstrated that SA signaling pathways play important roles in resistance against fungal pathogens in plants (Trusov *et al.,*2009; Zwart *et al.,* 2017). Therefore, discovery of SA targets and the understanding of their molecular modes of action in physiological processes could help in the dissection of the SA signaling network, confirming its important role in plant responses to fungal diseases (Vásquez *et al.,* 2015). After a pathogen attack SA levels often increase and lead to the induction of PR expression and the development of systemic acquired resistance and hypersensitive response. Furthermore, SA appears to regulate the delicate balance between pro- and after- cell death functions during hypersensitive response (Dorey *et al.,* 1997; Alvarez, 2000).

Barley plants produce enzymes that digest fungal cell walls to stop fungal penetration. However, since all true fungi contain chitin as a primary structural component of their cell walls, the chitinase family of PR proteins is of particular importance (Wessels, 1994). Chitin in fungal cell walls can be hydrolyzed by chitinases into smaller oligomers or monomers (Bishop *et al.,* 2002), so PR proteins such as *PR2* are known to play a major role during plant–pathogenic fungus interactions (Collinge *et al.,* 1993; Dangl and Jones, 2001).

Quantitative PCR (qPCR) is now a well-

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established method forquantifying the relative expression level of a particular transcript and determines its expression after being exposed to a specific alteration, such as pathogen infection (Kralik and Ricchi 2017). In the present work, we studied the defense responses of two barley genotypes Banteng and WI 2291, which are integrated in international breeding programs aimed at developing NB resistant barley genotypes. Banteng was described as a highly resistant to *P. teres* (Arabi *et al.,* 2003), i.e. exhibited a lower level (compared with WI2291) of NB symptom development. We thus hypothesized that SA-triggered defenses could drive contrasted levels of resistance in Banteng and WI2291, inoculated by the same pathogen isolate. Thus, the aim of the current study was to evaluate the changes in SA content and induction of *PR2* gene expression in two barley cultivars with different resistance to *P. teres*.

Materials and Methods

Plant materials and pathogen inoculation

The German genotype Banteng has proved to be the most resistant genotype to all NB isolates available so far under field and greenhouse conditions for over fifteen years (Arabi *et al*., 2003). For this reason, it was chosen and used in this study. A universal susceptible control genotype (cv. WI2291) from Australia was also included in the experiments. The *P. teres* single conidium isolate (NB4) tested was the most Syrian virulent pathotype to all barley genotypes available up to now (Arabi *et al*., 2003). The fungus was incubated in Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) for 8 days at 20-22°C in the dark. Conidia were collected in 10 mL sterile distilled water and the suspension was adjusted to 2 x 10^4 conidia/mL using hemacytometer. A surfactant (polyoxyethylene-20-sorbitan monolaurate) was added (100 µL/L) to the conidial suspension to facilitate dispersion of the inoculum over leaf surfaces. *Pyrenophora teres* inoculum preparation, inoculation, and post-inoculation were similar to those described by Abu Qamar *et al.* (2008). Barley plants were grown in the greenhouse and inoculated at the two- to three-leaf stage with the second leaf fully expanded.

SA quantification

Pooled samples containing the fourth leaf of 20 independent plants/genotype where used for SA quantification. Pooled samples were prepared from leaves taken 24, 48 and 72 hpi, respectively. For each time case studied, six pooled sample replicates were used for quantification. SA was extracted from approximately 200 mg of freshly ground leaves in 1.5 ml tubes following the method described by Trapp *et al.* (2014), with minor modifications. Briefly, 100 mg of plant material were dried overnight in a freeze drier at −42°C. The extraction was achieved by adding 1.0 mL of ethyl acetate, dichloromethane, isopropanol, MeOH:H_2O into each tube containing dry plant material. Samples were shaken for 30 min and centrifuged at 16,000 g and 4°C for 5 min. The supernatant was transferred into a new 1.5 micro-centrifuge tube and dried in a speed vac. After drying, 100 µL of MeOH was added to each sample, homogenized under vortex and centrifuged at 16,000 g and 4°C for 10 min. The supernatant was analyzed by a high-performance liquid chromatography coupled mass spectrophotometer (HPLC-MS/MS) system (Agilent Technologies, Böblingen, Germany). Changes in SA content were compared to the control for each time point. Six independent repetitions were performed for each time point. Data were analyzed using the standard deviation and t-test methods.

RNA isolation and cDNA synthesis

Primary leaves from three individual biological replicates were collected at 24, 48 and 72 hpi, and homogenized with a tube pestle in liquid nitrogen. mRNA was extracted with the Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany) following the

manufacturer's instructions. RNA was used for cDNA synthesis with the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions and the resulting cDNA was stored at –20°C. At the same time points, samples from mock inoculated plants were collected as controls.

Semi quantitative RT-PCR

PCR primers for *PR2* were designed based on the cDNA sequences of barley available at NCBI (http://www.ncbi.nlm.nih.gov) database (Id:M23548.1) using Primer 3 software (5′ CAGCGAATGCTCCAATGAAGA 3′ and 5′ TACCCTGCCGTGAACATCAAG 3′). PCR reactions were performed in a 50-µL final volume 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, 0.2 µL (1 U) of Taq DNA polymerase (MBI Fermentas, York, UK) and 40.8 µL of PCR grade water. PCR reactions were performed on a thermocycler (Biometra) with the following program: an initial denaturing step at 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 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 in order to confirm the reproducibility of the results.

qRT-PCR assay

Quantitative real-time PCR (qPCR) was performed using the method described by Derveaux *et al.* (2010). Data was checked by qRT-PCR dissociation curve analysis using stepone software (v2.3). The fluorescence readings of six replicated samples were averaged and the blank value (without DNA control) was subtracted. *PR2* relative expression levels were determined using the average cycle threshold (CT). Average CT values were calculated from the triplicate experiment conducted for each gene, with the ΔCT value determined by subtracting the average CT value of genes from the CT value of *EF1α* gene. Finally, the equation $2^{-\Delta\Delta CT}$ was used to estimate *PR2* relative expression level (Livak and Schmittgen, 2001). Standard deviation was calculated from the replicated experimental data. The statistical analysis was conducted through the Tukey's test at the 0.05 level. The assumption of coincidence was tested using the ANOVA procedure implemented in the software package Statistica 6.1.

Results and Discussion

In this study, we used two barley genotypes with different resistance to *P. teres* infection. As shown in Figure 1, *P. teres* produced net-like striated lesions surrounded by chlorosis or necrosis, and these symptoms were more severe on the susceptible genotype 'WI2291' after 10 days of infection. These results are in agreement with our previous observations under natural field conditions (Arabi *et al.,* 2003).

Further studies of barley-*P. teres* interactions by measuring changes in the leaf SA content and *PR2* gene expression at four early time points after pathogen challenge, showed that SA levels of infected barley leaves increased 24hpi in comparison with non-inoculated plants (Fig. 2). With or without pathogen pretreatment, the tolerant genotype Banteng contained three-fold or greater total SA than the susceptible genotype WI2291 (24hpi). It was found that Banteng contained significantly (P=0.001) higher levels of total SA thanWI2291 a teach time point investigated (Fig. 2), which might reflect the expected role of SA in signaling events during *P. teres* infection. This result could support the findings published by Häffner *et al.* (2014), stating that the endogenous SA level in a plant is the main cause of susceptibility *versus* resistance in barley, since pathogen infection may induce plant responses regulated by SA. In addition, SA accumulation has been widely used as a reliable marker of elevated defense responses

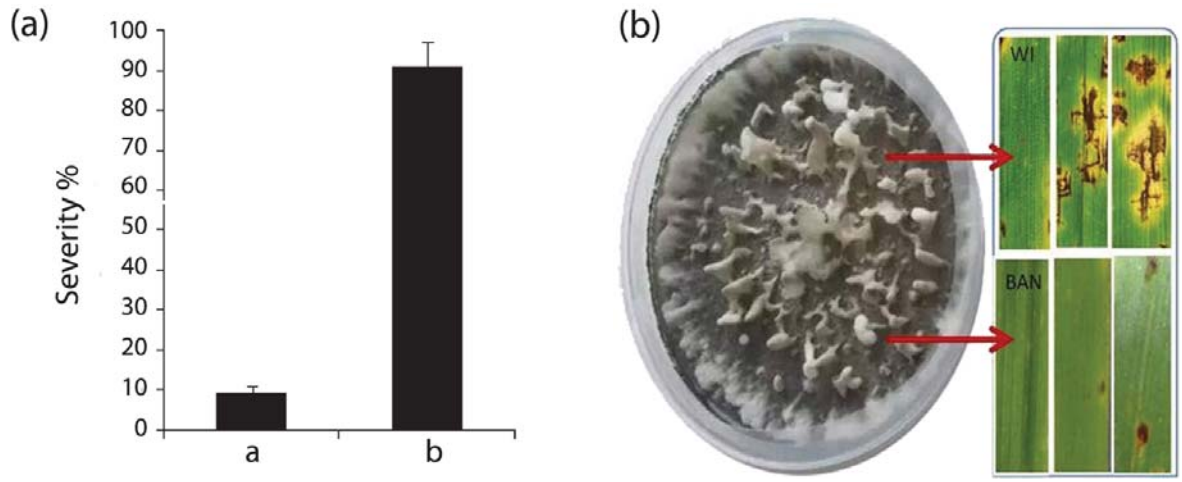

Figure 1. a) Frequency of disease reactions incited on barley (a) resistant cv. Banteng and (b) susceptible cv. WI2291, 10 days after *Pyrenophora teres* infection. b) Disease symptoms on the resistant (BAN) and susceptible (WI) barley genotypes, which were measured using the scale described by Abu Qamar *et al.* (2008).

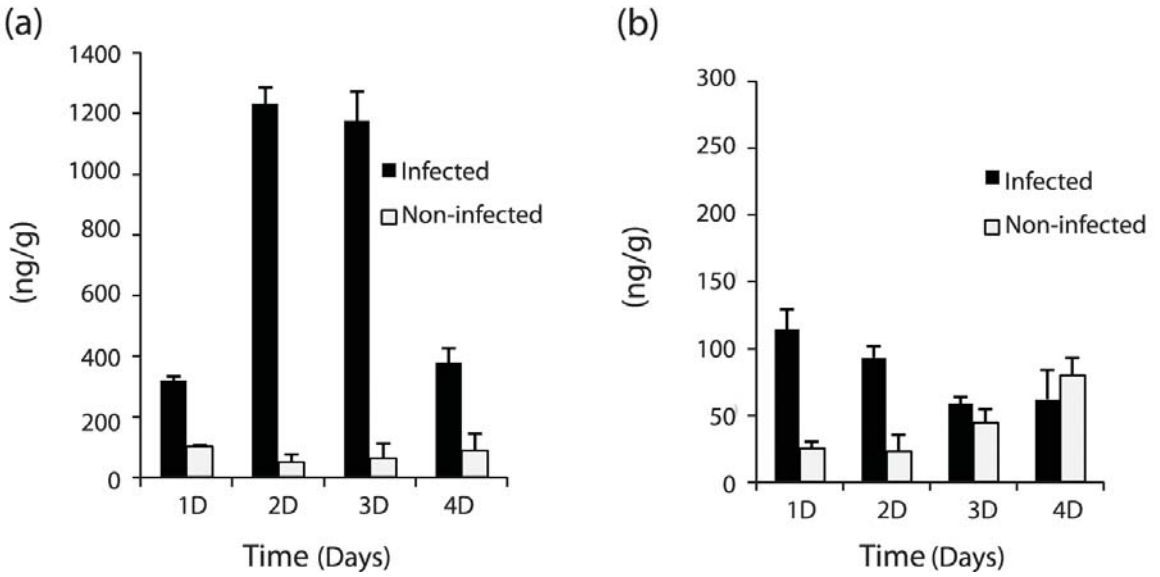

Figure 2. Quantification of total salycilic acid in barley leaves 1, 2, 3 and 4 days post inoculation with *Pyrenophora teres* in (a) the resistant cv. Banteng and (b) the suscepitible cv. WI2291. Error bars represent the stantard error of the means ($n = 3$).

and is closely associated with redox homeostasis, hypersensitive response, or systemic acquired resistance (Alvarez, 2000; Dong, 2004).

Semi quantitative RT-PCR analysis demonstrated that attack of barley by *P. teres* induced *PR2* accumulation in infected plants as compared with the un-infected controls and it was inversely regulated 24h post inoculation i.e, it was repressed in the susceptible cultivar WI2291 while being induced in the tolerant genotype Banteng (Fig. 3). Moreover, RT-PCR expression analysis revealed that the *PR2* expression increased in the resistant and susceptible genotypes over the inoculation time points, with the highest expression (6.4 and 1.99–fold for Banteng and WI2291, respectively) observed at 6 dpi. *PR2*

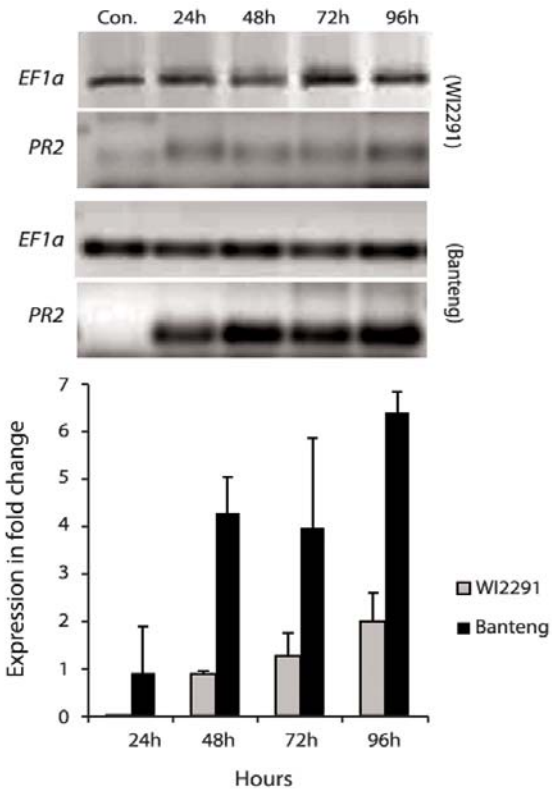

Figure 3. Relative expression profiles of *PR2* gene in the resistant cv. Banteng and in the susceptible cv. WI2291, 24, 48, 72 and 96 hours after infection by *Pyrenophora teres*.

encodes for a 1,3-ß-glucanase (Simmons, 1994), belonging to the glycoside hydrolases family (Opassiri *et al*., 2010). 1, 3-ß-glucanase hydrolyses the ß-*O*-glycosidic bond of ß-glucan in plant cell walls, resulting in cell wall loosening and expansion (Akiyama *et al*., 2009). This phenomenon may be the cause of barley cell wall leakage during *NB* infestations.

Our data showed that *PR2* gene exhibited a differential expression (P=0.01) in the tolerant and susceptible barley genotypes and was closely related to the increase of the SA level. The SA marker *PR2* was upregulated 3-fold in infected leaves of the tolerant Banteng than in the susceptible WI2291 (Fig. 2). *PR2* expression was paralleled by an increase in leaf salicylic acid (SA) content as shown by the coincidence test ($F_{3, 32} = 4.74$, $P = 0.001$). This is supported by previous works indicating that SA is involved in the regulation of induced immunity in barley through the induction of PR proteins with chitinase, β-1, 3-glucanase and peroxidase enzyme activity, both locally and systemically (Bindschedler *et al.,* 1998).

Our data show that the contribution of the SA pathway to the resistance response appears to depend on the plant genotype. The NB tolerant genotype Banteng used for this study was proved to be the most resistant genotype to all *P. teres* isolates available so far. The higher activities of the selected defense genes such as *PR2* and higher level of SA in infected Banteng leaf tissues compared with the susceptible genotype WI 2291 may explain its high level of resistance.

This study provides information about the role of SA in resistance of barley against the necrotrophic foliar pathogen *P. teres*. In addition, it highlights that SA may increase in response to *P. teres* infection in different barley genotypes. It is also noteworthy that *PR2* has a higher constitutive expression and faster induction in the tolerant genotype as compared with the susceptible one. Our results suggested that not only SA is important for the induction of defense-like responses but, in the absence of pathogen attack, SA may sustain basal expression levels of genes associated with resistance responses and may keep the defense system primed.

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Μεταβολές στην περιεκτικότητα του σαλικυλικού οξέος και στην έκφραση του σχετιζόμενου με την παθογένεια γονιδίου *PR2*, στο κριθάρι κατά την αλληλεπίδραση με το μύκητα *Pyrenophora teres*

A. Al-Daoude, M. Jawhar, E. Al-Shehadah, A. Shoaib, M. Orfi and M.I.E Arabi

Περίληψη Η ασθένεια του κριθαριού που είναι γνωστή διεθνώς ως «net blotch» και προκαλείται από το μύκητα *Pyrenophora teres f. teres*, μειώνει σημαντικά την απόδοση και την ποιότητα των σπόρων κριθαριού παγκοσμίως. Ο ρόλος της σηματοδότησης του σαλικυλικού οξέος (SA) ως προς την αντοχή στην ασθένεια δεν έχει τεκμηριωθεί επαρκώς. Σε αυτή τη μελέτη, καταγράφηκαν τα επίπεδα του SA καθώς και η έκφραση του σχετιζόμενου με την σηματοδότηση του SA, *PR2* γονιδίου σε μολυσμένα φύλλα δύο γονότυπων κριθαριού, του Banteng (ανθεκτικό) και WI2291 (ευαίσθητο), σε διαφορετικά χρονικά σημεία της μόλυνσης. Η σηματοδότηση του SA ενεργοποιήθηκε και στους δύο γονότυπους, 24 ώρες μετά τη μόλυνση (hpi) σε σχέση με το μάρτυρα. Ωστόσο, με ή χωρίς την εφαρμογή παθογόνου, το SA αυξήθηκε σημαντικά (P = 0,001) στον ανθεκτικό γονότυπο Banteng συγκριτικά με τον ευαίσθητο WI2291. Η ανάλυση με RT-PCR αποκάλυψε ότι η έκφραση του *PR2* αυξάνει στους ανθεκτικούς και ευαίσθητους γονότυπους μετά την εφαρμογή του παθογόνου, με τη μέγιστη έκφραση (6,4 και 1,99 φορές, αντίστοιχα) να παρατηρείται στις έξι ημέρες μετά την εφαρμογή του παθογόνου. Η έκφραση του *PR2* συνοδεύτηκε με παράλληλη αύξηση της περιεκτικότητας του SA στα φύλλα του κριθαριού (coincidence test , $F_{3, 32} = 4.74$, P = 0,001). Τα αποτελέσματα ενισχύουν την άποψη ότι η σηματοδότηση του SA και το γονίδιο *PR2* σχετίζονται με τη μείωση της ασθένειας «net blotch» στο κριθάρι.

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