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Transcriptional Changes of Salicylic Acid Dependent Signaling Pathways in Barley-*Cochliobolus sativus* Interaction

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Abstract

Spot blotch (SB), caused by the necrotrophic fungal pathogen *Cochliobolus sativus*, is an important disease of barley globally. Following transcriptional changes of salicylic acid (SA)-interacting/binding proteins during C. sativus infection may greatly advance understanding the defense crucial signaling pathways. In this study, changes of four known categories of defense; phosphorylation, ROS, PR proteins and nucleotide-binding sites encoded by genes involved in SA–mediated defense signaling networks were studied in compatible/incompatible barley-SB interactions. The functional categories showed significant differential accumulations when compared to the non-inoculated controls, and they were primarily upregulated during fungal infection in the resistant cultivar compared with the susceptible one. However, SA profiling of resistant and susceptible cultivars indicated a reduction in its levels 72 hours post inoculation; therefore, we hypothesized that this signaling pathways may facilitate SB resistance. Furthermore, the expression of selected categories was induced earlier in resistant barley plants as in susceptible ones, supporting the hypothesis that a delayed defense response may occur in the *C. sativus* susceptible interaction.

Keywords: Barley- cDNA-AFLP; Gene expression; *Cochliobolus sativus*; Salicylic acid

Introduction

Cochliobolus sativus (Ito and Kurib) Drechs. ex Dastur (anamorph: *Bipolaris sorokiniana* (Sacc.) Shoem.), is the causal agent of spot blotch (SB) of barley (*Hordeum vulgare* L.), a disease responsible for high crop losses [1,2]. Comprehensive studies to characterize and compare barley–*C. sativus* interaction micro-phenotypes exhibited by diverse resistant and susceptible barley genotypes were conducted; however, different mechanisms for SB resistance and susceptibility have appeared to operate in barley including plant hormones such as salicylic acid [3,4].

It is widely known that SA-modulated regulation of a fungal disease resistance is primarily achieved through direct effects on gene transcription [5,6] which is carried out by a physical interaction between trans-acting proteins, such as transcription factors, and cis-acting DNA elements. Transcription factors and co-regulators can themselves be monitoring at the transcriptional level, but they are also subject to a post-translational modification through reduction or oxidation, sequestration, phosphorylation, degradation, or interaction with other transcription factors or co-factors [7]. Several transcription factors have been shown to be important for SA activity [8]. However, ways by which these transcriptional regulators regulate SA signaling during barley-*C. sativus* interaction are largely unknown.

On the other hand, transcript profiling plays a basic role in defined gene functions; cDNA-amplified fragment length polymorphism (cDNA-AFLP) is an active and economical tool to present whole transcript profiles of single tissues [9]. In addition, comprehensive sequencing of transcript derived fragments (TDFs) was successfully used to study barley genes expressed during interaction with *C. sativus* [10,11].

In a previous work we found that SA dependent genes were increased in barley following challenge with *Blumeria graminis* [12]. However, the exact role of SA in inducing barley resistance against necrotrophic pathogens has been unclear until recently. The work aimed to evaluate early changes in four major TDFs involved in SA – mediated defense signaling networks believed to encode enzymes of oxidative phosphorylation, reactive oxygen species (ROS), and pathogenesis related proteins during early time points of barley infection with *C. sativus*.

Materials and Methods

Plant materials and inoculation

The highly resistant (Banteng) and highly susceptible (WI 2291) barley cultivars to SB [13] were used as plant material. Plants were grown in 20 cm pots filled with sterilized peatmoss and placed in a greenhouse and arranged in three replicates for each cultivar (each replicate is one pot containing 10 plants) at 22 ± 1°C (day) and 17 ± 1°C (night) with a day length of 12 h and a relative humidity of 80-90%. The most virulent *C. sativus* single conidium isolate (Pt4) to barley genotypes [13] was used in this study. The fungus was incubated on Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) with 13 mg/L kanamycin sulphate and incubated for 10 days at 21 ± 1°C in the dark. Then, conidia were collected and adjusted to 2 × 10^4 conidia/mL using hemacytometer. Inoculation and post-inoculation were similar to those described [13]. Non-inoculated control plants were sprayed with distilled water and surfactant.

Quantification of SA in plant samples

SA measurements were performed at 24, 48 and 72 hours post inoculation (hpi) using a protocol described [14] Trapp et al., 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 either ethyl acetate, dichloromethane, isopropanol, MeOH or MeOH: water (8:2) into each tube containing dry or fresh plant material and 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 speed vac, and 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 samples were analyzed by a high-performance liquid chromatography (HPLC) system (Agilent Technologies, Germany). The capacity of this method to differentiate the analyte from the other sample components was tested using the protocol described [15] Green. Where no additional MS/MS spectrum peaks for the band correspondent to the analyte in the matrix compared with the MS/MS spectrum of original standards were obtained, the method was deemed selective. Changes in SA were compared with the control for the same day. Five independent repetitions were performed for each time point. Data was statistically evaluated using the standard deviation and t-test methods.

RNA extraction and cDNA synthesis

mRNA was extracted from barley primary leaves at 24, 48 and 72 hpi using a Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany) following the manufacturer's guidelines. The RNA was used for cDNA synthesis using the Quanti-Tect Reverse Transcription Kit (Qiagen). Quality and yield of purified doubled strand cDNA were assessed by agarose gel electrophoresis as described [16] Sambrook et al.

cDNA-AFLP analysis

cDNA-AFLP analysis was achieved with minor modifications [11]. PCR products were purified with MultiScreen PCR µ96 plates (Millipore) and sequenced directly (BMR Genomics). PCR products were purified with a QIAgen gel extraction kit due to the manufacturer ´s recommendations. Sequencing was performed on a Genetic Analyzer (ABI 310, Perklin-elmer, Applied Biosystems, USA). Database searches were performed using the BLAST Network Service (NCBI, National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST).

Results and Discussion

SB severity was always more obvious in the highly susceptible cultivar WI9921 compared with the resistant one, Banteng. Infected leaves of susceptible WI 22091 plants showed the typical small, solid dark brown necrotic lesions of SB 72 hpi compared with the control (non-inoculated) cultivar (data not shown).

Measurements taken 72 hpi showed an obvious variation in the development of spot lesions between Banteng and WI2291 (Figure 1). The decreased lesion development in Banteng was therefore harmonious with the ranking of this genotype as resistant due to Arabi et al. [13]. Based on the symptoms observed on leaves of both cultivars, 24, 48 and 72h were chosen as inspection points for this study. No lesion development was seen in non-inoculated controls, while there was an indication that SB had an effect on barley plants defense responses as observed in *C. sativus* infection system [17].

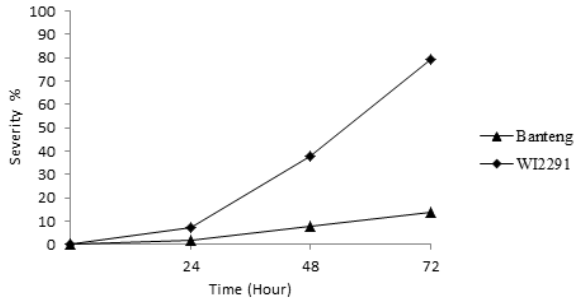

Figure 1: Progression of SB lesion lengths over a 72 h period in barley cultivars, Banteng and WI 2291.

Our experiments were designed to use a SB non- inoculated control which closely reflects a natural style of the penetration of fungus into barley tissues. While non-infected controls would provide insight into barley responses due to the inoculation procedure. The non-inoculated control was considered as a more biologically likeness to study the effects of *C. sativus* directly.

Data showed that SA levels were found to be significantly higher in the resistant cv. Banteng than the susceptible WI 2291 (Figure 2).

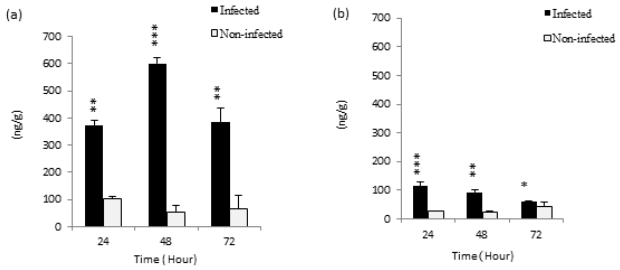

Figure 2: Quantification of salicylic acid contents in barley leaves at three harvest times post inoculation with *C. sativus*. (a) Banteng and (b) WI2291. Error bars are representative of the standard error (Mean ± SD, n=3). Significance at $^{*}p<0.05$; $^{**}p<0.01$ and $^{***}p<0.001$ within each cultivar during different periods comparing with the non-infected control.

It is possible that higher SA levels may create a systemic acquired resistance (SAR) response in Banteng earlier than in the susceptible cultivar. At earlier time points (24 hpi), significant changes were observed in both cultivars with hormones profiled. However, SA profiling of susceptible and resistant barley cultivars indicated a reduction in its level at 72 hpi (Figure 2).

Diseases caused by fungal pathogens affect most plants in their natural environment. Plants combat the majority of these intruders by activating elaborate immune responses, which typically result in a disease resistance response [18,19]. Nevertheless, pathogens have typically evolved ways to bypass plant defenses, and susceptibility to pathogens re-appears. In addition to this occasional immune failure of the host, other immune-response independent processes allow further

ingress of the invading pathogen and contribute to plant pathogen susceptibility. In this work, we focused on four major TDFs functional categories involved in SA –mediated defense signaling networks that are required for effective resistance to *Cochliobolus sativus* necrotrophs. These TDFs regulate critical aspects of disease resistance/susceptibility to necrotrophs without interfering with immune signaling [11]. Using a cDNA-AFLP approach [10], 456 TDFs were visualized and grouped in four functional categories; phosphorylation, pathogenesis related protein (PR), ROS, and nucleotide binding protein (Table 1 and Figure 3).

Cultivar	Gene down regulated			Gene up regulated		
	24 h	48h	72h	24h	48h	72h
Banteng (Ban.)	A, B	A, B	A, B, C	A, B	A, B, C, D	A, B, C, D
WI 2291 (WI.)	B	B	A, B, C	A	A, C	A, C
Common between Ban. And WI	B	B	B	A	A, C	A
Unique Ban.	_	_	C	_	B, C	B, C
Unique WI.	C, D	C, D	_	A	A	B, C
A: Phosphorylation, B: Pathogenesis related protein (PR), C: ROS and D: Nucleotide binding protein						

Table 1: Significant differentially accumulated functional categories in barley resistant (Banteng) and susceptible (WI2291) cultivars by *C. saivus* and hours after inoculation, detected at $p<0.0001$.

The analysis provided an indication of the overall changes occurring within each category and allowed for a broad comparison of the processes occurring in Banteng and WI 2291 at different stages of the barley defense response. Mitogen-activated protein kinases (MPKs) was unique to Banteng during infection time as compared with the susceptible one (Figure 3), which is involved in the phosphorylation of several transcriptional regulators.

SA pathways have been widely shown to be implicated in barley resistance against biotrophs [20] and thus it was assumed that these pathways would be induced towards necrotrophs (Table 1 and Figure 3). In harmony, genes connected with SA that were found to be up-regulated in both barley cultivars, in the nucleotide binding protein category a gene known as NBS-LRR, involved in hormone signaling regulation was up-regulated in WI 2291 but down-regulated in the resistant Banteng (Table 1 and Figure 3). This highlights a time in the defense series at which SA may increase resistance in Banteng which is supported by a decrease of the hormone at the metabolite level 72 hpi. NBS-LRR are thought to facilitate rapid R-gene evolution [21]. On the other hand, pathogenesis related proteins PR-5 also found to have a function in the resistant plants via delayed or halted the onset of disease symptoms of fungal pathogens [22,23].

Moreover, the ROS category highlighted candidates in cv. Banteng (Figure 3) that were GTP-binding proteins, is well-known to play a basic role in barley resistance to fungal diseases [24-26]. Wrzaczek et al., reported that activation of SA signaling in infected plants was preceded by oxidative bursts originating in various cellular compartments.

Barley susceptibility to SB could either result from that susceptible cv. WI 2291 lacking the required defense genes i.e., uncertainty in activating the correct response or it could be attributed to a later activation in defense genes as compared to a resistant cv. Banteng [27]. The pattern of a possible delayed defense response was observed in WI 2291 compared to Banteng. It is evident from this work that selected functional categories were activated a quicker and more robust compared in cv. Banteng as compared to cv. WI 2291 which may contribute to SB resistance.

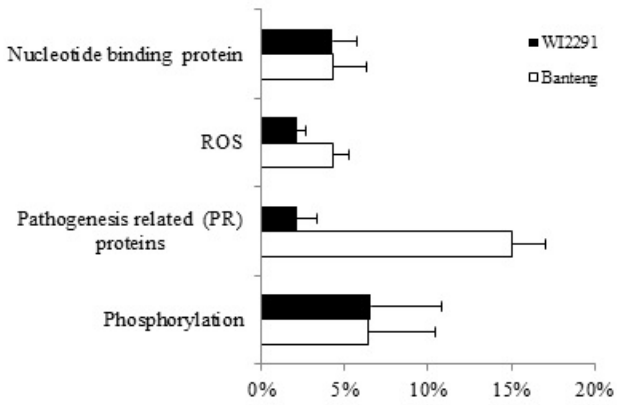

Figure 3: Functional overview of selected categories in barley resistant. Banteng and susceptible WI 2291 cultivars following *C. sativus* attack at the three harvest times. The X-axis indicates the percentage of differentially-expressed genes in each category.

Conclusion

In this study, the four selected TDF-annotation categories were found to be crucial for the fine-tune gene expression regulation mediated by SA during barley-*C. sativus* interaction. It is also noteworthy that TDF-annotation categories had higher and faster expression in the resistant cultivar as compared with the susceptible one, which suggest that the resistant cv. Banteng was able to regulate its defense responses at different points of infection. This supports the hypothesis that a delayed defense response may occur in the *C. sativus* susceptible interaction. However, future functional and protein interaction studies would further enable identification of essential elements in SA signaling in defense resistance of *C. sativus*.

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