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Functional cooperation between jasmonic acid and *PR3* signaling pathways during barley-*Cochliobolus sativus* interaction

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Abstract

Spot blotch (SB) caused by the fungus *Cochliobolus sativus* is a destructive disease of barley worldwide. Functional cooperation between Jasmonic acid (JA) and *pathogenesis-related 3* (*PR3*) gene signaling pathways in barley with SB have been poorly documented. Keeping in view this objective, *SB*–resistant 'Banteng' and susceptible 'WI2291' genotypes were evaluated using qRT-PCR across four-time points after pathogen challenge. Data showed significant variance in the expression patterns of JA and *PR3* between inoculated and non-inoculated plants in both genotypes, and their expressions were higher and faster in the resistant cultivar 'Banteng' as compared with the susceptible one 'WI2291' at each infection time point. However, qRT-PCR showed that 24 h constitute a significant starting time-point for *PR3* and JA in demonstrating the differential response of both genotypes towards *C. sativus*. The maximum expressions were recorded for *PR3* (5.43 and 2.81-fold) at 48 hours post inoculation (hpi) and for JA (152 and 142 ng/g) in the resistant and susceptible genotypes respectively, at 72 hpi. Taken together, our results confirm the importance of *PR3* and JA - dependent signaling during barley- *C. sativus* interactions.

Keywords: Barley, defense signaling, jasmonic acid, pathogenesis-related 3 (PR3), Cochliobolus sativus, RT-PCR

1. Introduction

Cochliobolus sativus (Ito & Kurib.) Drechsl. ex Dast. [anamorph: *Bipolaris sorokiniana* (Sacc. in Sorok.) Shoem.], the causal agent of spot blotch disease, is an important pathogen responsible for significant losses in barley yield worldwide [7]. The use of resistant genotypes is considered the most helpful and eco-friendly for managing SB [15]. However, genetic interaction between barley and *C. sativus* complicated the studies of resistance breeding [25], thus, more detailed works are needed to better understand of the defensive mechanisms.

Barley plants have developed various defense mechanisms against *C. sativus* attack and trigger an effective natural immune response.

Therefore, understanding of signaling pathways that play a key role in barley resistance mechanisms is important for the development of novel SB management strategies related with barley resistance. Barley–*C. sativus* interaction is known to stimulate various defense responses including formation of reactive oxygen species (ROS), generation of *phytohormones such as* jasmonic acid (JA) signaling and activation of pathogenesis-related (*PR*) genes [1, 14]. However, many of their specific functions are still unknown.

The synthesis of *PR* proteins is one of the inducible mechanisms that plants possess to resist pathogen attack. JA pathway, mainly activated by necrotrophic pathogens, provides local acquired resistance (LAR) through the upregulation of a number of *PR* genes including *PR3* [2].

In barley, *PR-3* showed a high constitutive presence mainly in the epidermis of leaves and to some extent in the phloem [25].

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Proteins of the *PR-3* family are endochitinases, which hydrolyze. β -1,4-linkages between N-acetylglucosamines of chitin, releasing oligosaccharides from the cell walls of many fungi [32]. The high chitinase activity of *PR-3* class I is also reflected in the antifungal activity demonstrated in *in vitro* studies of *Rhizoctonia solani* [27], and has been shown to inhibit growth of most fungi [6].

Different relationships between the JA synthesis and other metabolic pathways have been reported [16]. JA pathways are linked with enhanced transcription of PRs that are particularly stimulated both around infection sites and systemically [2, 31]. However, these molecular events involved during barley-*C. sativus* are not yet fully understood [16].

Quantitative PCR (qPCR) is an effective method to analyze modulations in gene expression due to its efficiency to detect and precisely quantify the target genes after plant infection by pathogens [9].

In our previous study, we have shown that *PR3* are involved in the barley immune response against *C. sativus* infection [1]. However, there is little information regarding the functional cooperation between JA and *PR3* gene signaling pathways. The present study aimed to evaluate the changes in JA, and induction of *PR3* gene in two barley genotypes with different levels of resistance towards *C. sativus* using quantitative PCR (qPCR).

2. Materials and Method

Barley genotypes. After several years of extensive screening in the greenhouse and field experiments, the German cv. Banteng was proved to be highly resistant to all *C. sativus* isolates available so far [4, 5], therefore, it was used in this study. The universal susceptible cv. WI2291 from Australia was also included in the experiments. Plants were grown in plastic pots (30 *cm* in diameter cm) filled with sterilized peatmoss with three replicates. Each experimental unit consisted of 10 seedlings. Pots were kept at temperatures 22 ºC (day) and 16ºC (night) with a day length of 12 h and 90% relative humidity.

***Inoculation with C. sativus*.** The virulent isolate of *C. sativus* (pt4) described by Arabi and Jawhar [5] was used in this study. Inoculation was performed by spraying plants with conidial suspension of 2 $\times 10^4$ conidia mL^{-1} contained Tween 20 (polyoxyethylene-sorbitan monolaurate) as a surfactant (100 µL L^{-1}) to facilitate dispersion of the inoculum over barley leaves. Pots were kept in greenhouse at 20 °C with a 16 h photoperiod. The control plants were sprayed with distilled water and surfactant.

Quantification of JA in plant samples. JA quantification was performed at four time points 24, 48, 72 and 96 hours post inoculation (hpi) as described by Trapp et al. [29]. Briefly, leaf samples were ground in liquid nitrogen , and freeze dried. JA extraction was done by adding 1.0 mL of ethyl acetate, dichloromethane, isopropanol, MeOH:H_2O into each tube containing dry leaves. Tubes were centrifuged at 16,000 g for 5 min, and the supernatant phase was moved into a new 1.5 micro-centrifuge tube and was passed through carbon-packed solid phase extraction tubes (Supelclean ENVI-Carb SPE tubes), dried in speed vac. Then, 1000µL of MeOH were added to each tube, homogenized under vortex and centrifuged at 16,000 g for 10 min. The supernatant was applied on a thin layer chromatography plate (Silica gel on TLC Al foil with fluorescence indicator 254 nm<Supelco) using automatic TLC sampler 4 (Camag, Switezerland). TLC plate was developed in a glass chamber using isopropanol: ammonia: water (9:1:1, v/v) as development solvent. The plate was dried and read using TLC Scanner 3 (Camag, Switezerland). Five replicates were performed for each time point.

RNA isolation and cDNA synthesis. Barley seedling leaves were collected at 24, 47, 72 and 96 hpi and ground to a fine powder in liquid nitrogen, and total RNA were extracted using Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany). cDNA synthesis was performed by the Quanti Tect Reverse Transcription Kit (Qiagen) following the manufacturer's protocol. Samples from non-inoculated plants at each time point were collected as controls.

Quantitative real-time PCR (qPCR). *PR3* was assayed using SYBR Green Master kit (Roche). The sequence information for all RT-PCR primers is given in Table 1. The expression level of *PR3* was determined according to the $2^{-\Delta\Delta CT}$ method of Livak and Schmittgen [17] using Ct-values of EF1α gene for normalization. Standard deviation was calculated from the replicated experimental data Vandesompele et al. [30]. The statistical analysis was conducted using ANOVA software and Tukey's test at the 0.05 significance level.

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

Gene	Gene description	Accession No.	Sequence	Amplified fragment (bp)
EF1α	Elongation factor-1 Alpha	AT1G07920	TGGATTTGAGGGTGACAACA CCGTTCCAATACCACCAATC	167
PR3	Basic Chitinase	AT3G12500	GGGGCTACTGTTTCAAGCAA GCAACAAGGTCAGGGTTGTT	187

3. Results and Discussion

In this work, two barley genotypes with different resistance levels towards the pathogen *C. sativus* were used. As shown in Fig. 1A, B, the fungus caused more severe infection on the susceptible cv. 'WI2291' as compared with the resistant one 'Banteng'. *C. sativus* produced spots surrounded by chlorosis or necrosis, and these symptoms were more severe on the susceptible cv.'WI2291' after 14 days of infection. These results accord with our earlier observations under natural field experiments [5].

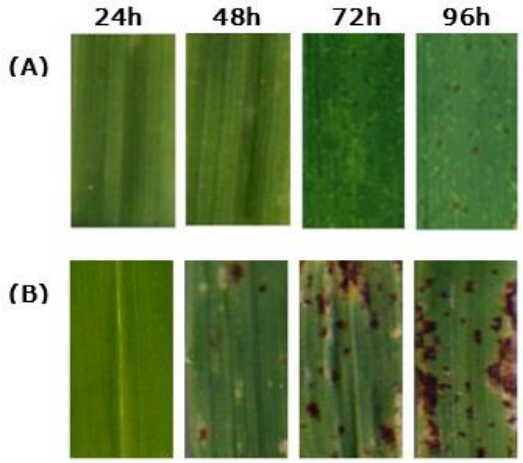

Figure 1. SB symptoms in barley resistant (A) and susceptible (B) at different points post inoculation with *C. sativus.*

To better understand these interactions, changes in *PR3* and JA were investigated at four early time points after pathogen challenge. Seedlings inoculated with distilled water were also analyzed at 0h. *PR3* and JA levels were observed post inoculation of seedlings in both compatible and incompatible interactions, and it was noteworthy that these signaling pathways were higher and faster in the resistant cultivar as compared with the susceptible one (Fig. 2a,b).

Data showed that 24 and 72 hpi constitutes a significant starting time-point for *PR3* and JA in demonstrating the differential response of resistant and susceptible barley plants towards *C. sativus* (Fig. 2a,b). However, qRT-PCR analysis revealed that maximum expression for *PR3* (5.43 and 2.81-fold) at 48 hpi and JA (152 and 142 ng/g) in resistant and susceptible genotypes, respectively, mainly at 72hpi, and their expressions decreased at 96hpi (Fig. 2a,b).

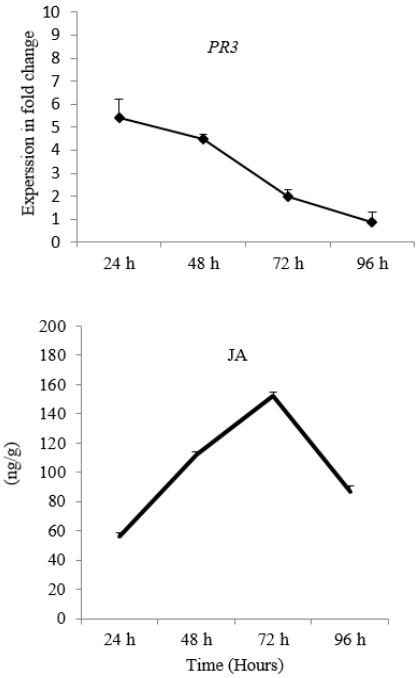

Figure 2a. Relative *PR3* and JA expression profiles in the resistant barley genotype Banteng during the time course following *Cochliobolus sativus infection.* Error bars are representative of the standard error (Mean ± SD, $n = 3$). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 1.00).

Our analysis showed that *PR3* and JA in the resistant cv. 'Banteng' and in the susceptible cv. WI2291 exhibited differential expressions by $P = 0.05$, and were inversely regulated during different time points post inoculation.

Considering that these genotypes had high different levels of resistance to *C. sativus* [3, 4]. The *PR3* and JA were up-regulated 24 hpi in inoculated barley plants as compared to non- inoculated plants, which might indicate that their roles are related to the severity of SB symptoms rather than to resistance. These upregulations were higher in the resistant cultivar than in the susceptible cultivar (Fig. 2a,b).

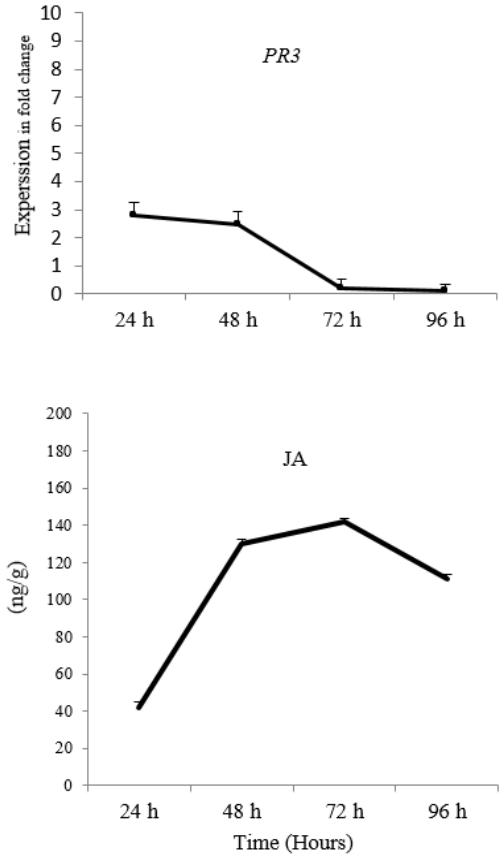

Figure 2b. Relative *PR3* and JA expression profiles in the susceptible barley genotype WI2291 during the time course following *Cochliobolus sativus infection.* Error bars are representative of the standard error (Mean ± SD, $n = 3$). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 1.00).

It is well known that, PR proteins, individually or in combination, have been widely reported to impair or uplift the level of defense response in plants to a wide range of pathogens [3]. It has been reported that JA stimulates antimicrobial compounds production such as phytoalexins and PR proteins [31], and also found that JA is produced in reaction to pathogen infection, most possibly due to an increase in lipoxygenase and 1-amino-cyclopropane-1-carboxylic acid (ACC) oxidase activities, respectively [13].

These events might support their roles in barley plants during *C. sativus* infestation. However, the fact that *PR3* and JA expressions were higher in the resistant cv. 'Banteng' than in the susceptible barley cv 'WI2291' indicates that these signaling pathways might have specific roles in activating barley resistance.

Our results are in agreement with previous studies which reported that higher levels of *PR3* were observed in cucumber resistant cultivars than that of susceptible ones upon infection with *Fusarium oxysporum (*22*) and in wheat after infection with Bipolaris sorokiniana* [2]. In addition, our data could be supported by the results of JA activation in *Arabidopsis after infection with Alternaria brassicicola, Botrytis cinerea and Pseudomonas syringae* [10, 12, 28].

In conclusion, our data demonstrated that, significant increases in *PR3* and JA expression were found upon barley challenged with the *C. sativus*, and can contribute to SB resistance, since these signaling responses induced together in each genotype. It is also noteworthy that *PR3* and JA have higher expressions and faster induction in the resistant cultivar as compared with the susceptible one. In addition, we highlighted the fact that two different signaling pathways may be induced in response to the same isolate of *C. sativus* in different barley cultivars.

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Compliance with Ethics Requirements: Authors declare that they respect the journal's ethics requirements. Authors declare that they have no conflict of interest and all procedures involving human or animal subjects (if exist) respect the specific regulation and standards.

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