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Systemic phenylalanine ammonia-lyase gene *expression* during barley- *Pyrenophora graminea* interaction

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Abstract

Leaf stripe, caused by the fungus *Pyrenophora graminea* (*Pg*), is an important seed-borne disease of barley causing significant yield and quality losses worldwide. In this work, expression of phenylalanine ammonia-lyase *(PAL)* was monitored in infected leaves of two barley genotypes, Banteng (resistant) and Furat1 (susceptible) across four-time points of *Pg* systemic movement using qRT-PCR approach. Data showed that the systemic movement of the fungus was slower in the resistant cultivar than in the susceptible one, as confirmed by both microscopic examination and culturing on PDA media. qRT-PCR revealed that the expression patterns of *PAL* gene significantly increased in infected plants in comparison with non-infected controls. Its expression correlated with *Pg* systemic movement in the root, stem and leaf fractions of the resistant and susceptible barley cultivars. Taken together, based on the *Pg* systemic movement within barley plants with different resistance levels, our data strengthen the idea that *PAL* plays a role in barley leaf stripe reduction.

Keywords: Barley (*Hordeum vulgare* L.), *Pyrenophora graminea*, RT-PCR,*PAL* expression

1. Introduction

Barley leaf stripe caused by *Pyrenophora graminea* Ito & Kuribayashi [anamorph *Drechslera graminea* (Rabenh. ex. Schlech. Shoem)] is a seed-borne disease of barley (*Hordeum vulgare* L.), a disease responsible for heavy crop losses [11]. It can infect barley plants during seed germination, and hyphae accelerate its intercellular growth within the coleorhizae, the embryo, the roots and scutellar node, in order to establish a full-scale infection in the seedling [14]. Movement of *Pg* mycelium within barley plants was suggested to be a useful criterion in studying susceptibility to this pathogen [3].

Various mechanisms for leaf stripe resistance and susceptibility appear to operate in barley [15]. However, several works have suggested that phenylalanine ammonia-lyase (*PAL*) expression increased in plants attacked by fungal pathogens [4, 12].Their expression level is low or absent in mature healthy barley plants but becomes activated after pathogen attack [9]. Therefore, focusing on this essential gene, considered as hallmark of typical defense plant responses is needed.

A number of works have demonstrated that *PAL* plays important role in resistance against fungal pathogens in plants, and its expression may be detected at different stages of disease progression [7, 13]. In plant-pathogen interactions, quantitative PCR (qPCR) provides a comprehensive understanding of the molecular responses by measuring the relative expression levels of gene products after infection by pathogens [1, 5].

So far, little is known about the defense response mechanisms occurring during the barley-*Pg* interactions. In the current work, we studied the changes in *PAL* expression during *Pg* systemic movement in two barley cultivars Banteng and Furat1, which are integrated in international breeding programs aimed at developing leaf stripe resistant barley genotypes. Banteng was described as a highly resistant cultivar to *P.graminea* [2], i.e. exhibited a lower level (compared with Furat1) of leaf stripe symptom development.

We thus hypothesized that *PAL*-triggered defenses could drive contrasted levels of resistance during *Pg* movement in Banteng and Furat1, inoculated by the same pathogen isolate

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2. Material and Methods

2.1 Plant materials and pathogen inoculation

This study was carried out by comparing two barley cultivars Banteng and Furat-1.The German cultivar Banteng was previously proved to be resistant to all *Pg* isolates originated and isolated from Syrian barley fields [2], and thus it was chosen and used in this study. The susceptible cultivar Furat-1 from Syria was also included in the experiments. The *Pg* single conidium isolate (*Sy3*) was the most virulent and prevalent in the barley-growing areas of Syria [2], and therefore it was used in the present study.The fungus was grown in Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) supplemented with 13 mg/l kanamycin sulfate and incubated for 10 days at 20± 1 °C in the dark. Barley seeds were inoculated using the protocol of Hammouda (1986) [6]. Briefly, fifty seeds of each cultivar were surface-sterilized in 2% sodium hypochlorite for 5 min, dried for 3-4 h, then incubated at 6 °C in the dark in Petri dishes containing an actively growing mycelium of *Pg* cultured on PDA medium. As a control, seeds were incubated on PDA medium alone. After 14 days of incubation, inoculated and control seeds of each cultivar were planted in plastic pots (d=20 cm) filled with sterilized peat moss with five replicates. Each replicate comprised five pots each of ten seeds. All plants were maintained in a growth chamber at 16 ± 2 °C (for day light)and 12 ± 2°C (for night) at 60–70 % relative humidity with a photoperiod of 10 h.

2.2 In vitro PDA tests

Ten plants per cultivar were harvested 6, 10, 14 and 18dayspost inoculation (dpi). Each plant was spilt into roots, monocotyled on, second leaf and stem. Each part was surface sterilized using 2% sodium hypochlorite (NaOCl) for 5 min, rinsed three times (5 min each) in sterile distilled water and dried using sterilized filter paper. Each part was separately plated on PDA medium in Petri dishes, and incubated for 72 h at 21 ± 1ºC in the dark.

In order to prove that the colonies identified on each part of a plant were of *Pg* origin, Petri dishes were incubated for additional 4 days in a cycle of 12 h darkness/12 h UV light.

The presence of conidia was confirmed under a light microscope. In addition, the presence of mycelia in each plant part was also examined microscopically. Disease rating was estimated based on the scale of 0-3 as described by Arabi and Jawhar (2005) where: 0= resistant; 1-25% of the plant part infected with fungus, moderately resistant; 26 - 50% of the plant part infected with fungus, and susceptible; 3 > 50% of the plant part infected with fungus. Leaf stripe severity (%) value for each cultivar was estimated according to the following formula: Disease severity (relative *in vitro* value) for one cultivar = Total *in vitro* scores for all plant parts of the cultivar /100.

2.3 RNA isolation and cDNA synthesis

Infected and non-infected seedling parts were collected at the time course 6, 10, 14 and 18 dpi, and immediately frozen in liquid nitrogen. At the same time points, samples from mock inoculated plants were used as controls. Mock inoculation was done by spraying plants with pathogen-free water. mRNA was extracted from collected samples 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.

2.4 qRT-PCR assay

*PAL*expression was verified by Quantitative real-time PCR (qPCR) according to the protocol described by Derveaux et al. (2010) [5]. The sequence information for all RT-PCR primers is given in Table 1. The fluorescence readings of five replicated samples were averaged, and blank value (without DNA control) was subtracted.*PAL* relative expression levels were measured using the average cycle threshold (CT) which was automatically determined for each reaction by the real time PCR system with default parameters. The ΔCT value determined by subtracting the average CT value of gene from the CT value of *EF1α* gene. Finally, the equation $2^{-\Delta\Delta CT}$ was used to estimate *PAL* relative expression level [10]. The statistical analysis was conducted through the Tukey's test at the 0.05 significance level. Data obtained was the mean of the five replicates for each plant part.

Table 1. List of the oligonucleotides used in this study

Gene	Gene description	Accession No.	Sequence	Amplified fragment (bp)
EF1α	Elongation factor-1 Alapha	CV066174	GGCTGATTGTGCTGTGCTTA	153
			TGGTGGCATCCATCTTGTTA	
PAL	Phenyl alanine amino lyase	AT2G14610	CCATTGATGAAGCCAAAGCAAG	123
			ATGAGTGGGTTATCGTTGACGG	

3. Results and Discussion

In this study, we used two barley cultivars with different resistance to *Pg*. Leaf stripe symptoms on susceptible 'Furat1'plants appeared a spale green lines at *Pg*6 dpi and several days later, these stripes became thicken and turning brown darker as the fungus sporulates on the leaf surface. In contrast, the resistant cultivar 'Banteng', showed normal growth in comparison with 'Furat1 even at 18 dpi (data not shown).

Host responses of the two selected barley cultivars to *Pg* are presented in Figure 1.

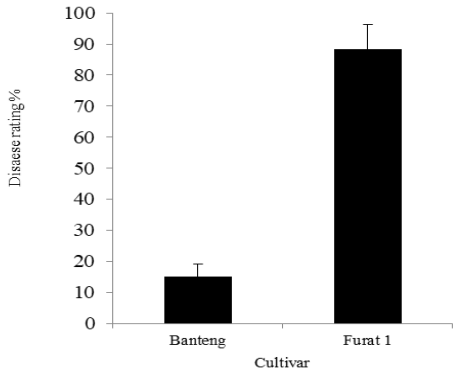

Figure 1. Frequency of leaf stripe reactions incited on the barley resistant cv. 'Banteng' and susceptible cv. 'Furat-1'. *Pg* infections were scored according to the scale 0-3 described by Arabi and Jawhar (2005) [3].

Table 2. *Pg* detection in different plant parts of resistant `Banteng` and susceptible `Furat – 1` barley cultivars at different times after inoculation

Cultivar	Plant part	Days after inoculation			
		6	10	14	18
Furat-1	Root	+	++	++	+++
	Stem	+	++	++	+++
	Leaf	-	+	++	++
Banteng	Root	+	+	+	-
	Stem	+	+	+	-
	Leaf	-	+	-	-

Disease score based on the percentage of parts that produced hyphae when cultured on PDA media (Arabi and Jawhar, 2005), Where; - = 0; (No reaction) + =1; (1-25%) ++ = 2; (26-50%) +++ = 3; (50%<) of plant part produced mycelia on PDA.

The data from PDA cultures of plant parts and the microscope showed that the movement of *Pg* was slower in the resistant cultivar 'Banteng' than in the susceptible one. *Pg* was detected from root, stem and leaf at 6 dpiin the highly susceptible cultivar 'Furat-1',by contrast, the fungus was detected in the resistant cultivar 'Banteng' at 10 dpi, and *Pg* undetectable at 18 dpi in the new root and stem growth (Table 2).

However, symptoms started to appear with a very low percentage of leaf stripe (less than 2 %) in the resistant cultivar 'Banteng' which was accompanied with *Pg* mycelium presence within the plant parts.

In contrast, noticeable necrotic symptoms and fungal mycelium were observed in the parts of the susceptible cultivar'Furat-1'inoculated with the same *Pg* isolateSy3 10 dpi in the new root and stem growth. This result confirmed previous findings which demonstrated that *Pg* mycelia penetrate rapidly through the coleorhiza during seed germination of susceptible barley plants [2,14]. Additionally, plants of the resistant cultivar inoculated with isolate Sy3showed no leaf stripe symptoms at 18 dpi, which was correlated with undetectable fungal mycelium within the tested parts.

The results demonstrated that the values obtained from *in vitro* PDAfor plant parts could clearly differentiate between resistant and susceptible barley cultivars based on the *Pg* movement (Table 2).

As interactions between the fungal pathogen and the host plant are critical for disease development, signaling pathways that mediate these interactions are also important. Here, in order to understand the *PAL* expression changes in *Pg*-resistant and *Pg*-susceptible barley during mycelial movement in plant parts, we have inspected the differential movement of *Pg* by comparing the time-window of the infection within two barley cultivars having different resistant levels towards this fungus.

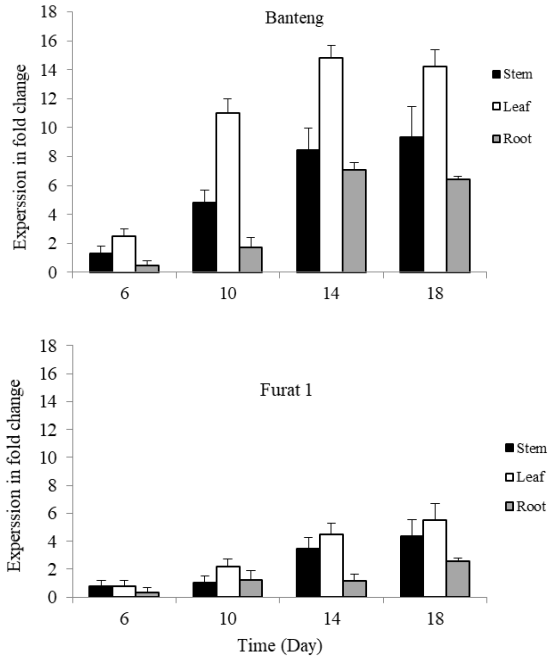

Figure 2. Relative expression profiles of *PAL* gene in barley plant parts of the resistant cv. Banteng and in susceptible cv. Furat1 during the time course following *Pyrenophora graminea infection.* Error bars are representative of the standard error (Mean ± SD, *n* = 3). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 1.00).

Our data showed that *PAL* signaling was activated in the two barley cultivars as compared with the non-inoculated plants (Fig. 2), however at 14 days, *PAL* expression was significantly expressed with 14.8, 8.45 and 7.1 fold increases in leaf, stem and root, respectively in the resistant cultivar 'Banteng', whereas in the susceptible cultivar 'Furat1' this expression was 3.48, 4.5 and 1.15fold increases in these parts, respectively (Fig.2), this result indicates that *PAL* might have specific roles in triggering barley resistance. Our findings correlate with earlier work where *PAL* expression was higher in the resistant than in the susceptible barley seedlings after *Pg* inoculation showing that *PAL* was involved in the development of resistance [9].

Our results showed that the *Pg* isolate *Sy3* was able to grow within all examined barely susceptible seedling tissues comparing with the resistant plants. It has been reported that during colonization process the *Pg* might produce enzymes that function in the fungal growth through interactions with the barley seedling [9]. In our study, *PAL* expression increased both in the resistant and susceptible barley cultivars over the inoculation time points, with the highest expression at 16 dpi. It has been reported that *PAL* catalyses the non-oxidative deamination of phenylalanine to *trans*-cinnamate as a first step in the phenylpropanoid pathway, which is a crucial regulation point between primary and secondary metabolism [4, 7]. Geetha *et. al.* (2005) [8] reported that *PAL* expression was increased in pearl millet after *Sclerospora graminicola* infection.

This work illustrated that the relative *in vitro* growth on PDA values for plant parts could clearly differentiate between resistant and susceptible barley cultivars based on the *Pg* movement. It is also noticeable that the *PAL* expression patterns were well correlated with the *Pg* movement in the root, stem and leaf fractions as confirmed by *in vitro* PDA. In addition, a remarkable conflict in *PAL* expression was observed in the resistant cultivar accompanied with a slow movement of the *Pg* mycelium within plant parts as compared with the susceptible one. This uniformity in response could be in convention with the well-accepted concept that barley defense mechanisms against *Pg* pathogen are very intense in resistant plants during infection time points.

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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