Changes in PR2 and PAL Patterns in Barley Challenged with Leaf Stripe (Pyrenophora graminea) and Powdery Mildew (Blumeria graminis) Diseases

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The seed-borne (Pyrenophora graminea; Pg) and foliar (Blumeria graminis; Bg) are two economically important fungal pathogens of barley worldwide. Barley plant resistance genes, as the pathogenesis related proteins play an important role in defense mechanisms. This study aimed to monitor the expression of PR2 and PAL pathogenesis related genes during compatible/incompatible barley interaction with Pg and Bg at different time points of disease development using the Quantitative Real-time PCR technique (qRT-PCR).

Comparison of data showed that PR2 and PAL were significantly over expressed in infected resistant and susceptible plants as against their lower expression in controls.. Upregulation of these defense-related genes during Pg and Bg infections was companied with a slow development of disease symptoms at the time course in the resistant genotype. qRT-PCR analysis revealed higher gene expression in resistant barley plants inoculated with Pg as compared with Bg, with a maximum expression for PR2 (13.8 and 5.06-fold) and PAL (14.8 and 4.51-fold) respectively, at the latest stage of each disease development. It was also noteworthy that PR2 and PAL genes, had higher constitutive expression and faster induction for the both pathogens in the resistant genotype as compared with the susceptible one.

Obtained results suggest that both genes, PR2 and PAL, positively regulate Pg- and Bg-resistance in barley plants during disease progress. These expression patterns can provide useful insights to better understanding of the barley–fungus interactions with different fungal lifestyles.

Keywords: barley, Blumeria graminis, Pyrenophora graminea, defense response, PR2, PAL gene expression.

Leaf stripe caused by the seed-borne pathogen, Pyrenophora graminea Ito and Kuribayashi [anamorph Drechslera graminea (Rabenh.) Shoemaker], and powdery mildew caused by the foliar pathogen, Blumeria graminis f. sp. hordei (Bg) are among the most devastating fungal diseases of barley (Hordeum vulgare L.) causing significant crop yield losses worldwide (Arabi et al., 2004; Dean et al., 2012). During infection, barley plants operate different resistance mechanisms in response to these both fungi that are regulated through various signaling pathways, including pathogenesis-related (PR) proteins (Häffner et al., 2014; Vásquez et al., 2015). Therefore, understanding the molecular
basis of more plant–pathogen interactions would significantly assist the development of new control strategies through the identification of host-plant factors required for the \( P_g \) and \( B_g \) infection establishment (Boyd et al., 2013; Peyraud et al., 2017).

A notable number of defense-related genes is known to be involved in plant–pathogen interactions (Nayanakantha et al., 2016; Samsatly et al., 2018). However, understanding the expression changes of genes involved in signaling pathways in resistant barley plants during \( P_g \) and \( B_g \) infection requires exploring information about the infection kinetic. Therefore, focusing on essential genes that considered as hallmarks of typical defense plant responses such as that encode for PR proteins like \( PR2 \) or phytoalexin biosynthesis-related proteins like phenylalanine ammonia lyase (\( PAL \)) are needed. These genes protect plants against pathogens by accumulating in infected areas (Ebrahim et al., 2011), and they could be depressed or missing in healthy plants in different species and their expression may be seen at the various stages of disease progression (Pagán and García-Arenal, 2018).

Although, \( PR2 \) and \( PAL \) genes are well known to have an active function in the plant immune regulation reactions, however their role in barley – seed-borne and foliar fungi interactions is not entirely defined. qRT-PCR is now a relatively simple method for measurement of gene expression levels after being exposed to a specific alteration, such as pathogen infection (Kralik and Ricchi, 2017).

To complete the picture of barley biochemical responses drawn by Ghannam et al. (2016), the current work was aimed to study the possible changes of \( PR2 \) and \( PAL \) pathogenesis related genes in barley inoculated with seed-borne (\( P_g \)) and foliar (\( B_g \)) pathogens at different time points of disease development by deploying a qRT-PCR approach.

**Materials and Methods**

*Plant material*

In our barley seed bank the German cv. Banteng has proved to be the most resistant genotype to all \( P_g \) and \( B_g \) isolates available so far under field and greenhouse experiments for over fifteen years (Arabi et al., 2004; Arabi and Jawhar, 2012). Therefore, it was chosen and used in this study. The susceptible control genotype (cv. Furat1) from Syria was also included in the experiments.

*Inoculation with \( P_g \)*

The most Syrian virulent isolate Sy3 (Arabi et al., 2004) was used in this study. The fungus was grown on potato dextrose agar (PDA, DIFCO, Detroit, MI, USA) and incubated for 7 days at 20 ± 1 °C in the darkness. Inoculation was carried out using the modified technique of Hammouda (1986). Seeds were surface-sterilized in 2% sodium hypochlorite for 5 min, dried for 3–4 h, then placed on 8-day-old mycelial culture growing on PDA medium in Petri dishes and incubated at 6 °C for 14 days in the dark. As a control, seeds were incubated on PDA medium alone. Inoculated and control seeds were sown in plastic 20-cm pots filled with sterilized peat moss with five replicates. Each replicate comprised five pots each of ten seeds. Plants were grown in a growth chamber at about
12 °C with a daylength of 12 h. They were watered every 3-4 days and fertilized once a week using water soluble 20-20-20, NPK. Leaf stripe infection was recorded according to the Delogu et al. (1989).

**Inoculation with Bg**

Seeds were sown in 20-cm pots filled with sterilized peat moss, and each replicate comprised five pots of ten seeds. Pots were placed in greenhouse at 17 °C, with a 16 h-light/8 h-dark cycle. Leaves of 12-day-old seedlings were inoculated at the middle part of their abaxial surface with conidiospores of a virulent Bg isolate by employing a soft hair brush to give about 10–20 conidia per one microscope field at ×150 magnification (Chaure et al., 2000). Disease ratings were scored according to the scale described by Moseman and Baenziger (1981).

**RNA isolation and cDNA synthesis**

Samples from barley leaves of each genotype were collected at different periods due to the infection stages for each pathogen and they were 0, 6, 10, 14 and 18 days for 

**Quantitative real-time PCR (qPCR) assay**

Differential expression of PR2 and PAL genes was verified by PCR (qPCR) according to the method described by Derveaux et al. (2010). The fluorescence readings of five replicated samples were averaged and the blank value (without DNA control) was subtracted. PR2 and PAL relative expression levels were determined using the average cycle threshold (CT) which was 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−ΔΔCT was used to estimate PR2 and PAL 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 significance level.

**Data analysis**

Data generated was the average of the five replicates for each disease. Comparison of means between Pg and Bg was performed using analysis of variance (ANOVA) at the 5% level using the software package Statistica 6.1. When the main effect was significant, differences between means were evaluated for significance by the Scheffe F-test. The standard deviation was calculated.
Results

In this study, we used two barley genotypes with different resistance to \( P_g \) and \( B_g \) infections. As shown in Figs. 1 and 2, both pathogens caused more severe infection on the susceptible genotype ‘Furat1’ as compared with the resistant one Banteng. These results

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene description</th>
<th>Accession No.</th>
<th>Sequence</th>
<th>Amplified fragment (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAL</td>
<td>Phenylalanine ammonialyase</td>
<td>AT2G14610</td>
<td>5’ CCATTGATGAAGCCAAAGCAAG 3’</td>
<td>123</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’ ATGAGTGGTGTTATCGTGACGG 3’</td>
<td></td>
</tr>
<tr>
<td>PR2</td>
<td>Beta1.3-glucanase2</td>
<td>AT3G57260</td>
<td>5’ TGGTGTCAGATTCCGGTACA 3’</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’ TCATTTCCTGAAACCTTCTTG 3’</td>
<td></td>
</tr>
<tr>
<td>EF1α</td>
<td>Elongation factor-1 Alapha</td>
<td>AT1G07920</td>
<td>5’ TGGATTTGAGGGTGACAAACA 3’</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’ CGTCCAATACCACCAATC3’</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Disease severity and gene expression by RT-PCR in barley resistant (cv. Banteng) and susceptible (cv. Furat1) plants inoculated with \( P. graminea \) at 8–18 days.
are in agreement with our previous observations under natural field conditions (Arabi et al., 2004; Arabi and Jawhar, 2012).

To better understand the expression changes of genes involved in signaling pathways in the compatible/incompatible barley plants during \( P_g \) and \( B_g \) infection exploring information about the infection kinetic is required. Therefore, we have first inspected the differential dynamics of leaf stripe and powdery mildew development during the time-window of symptoms for the two pathogens, before \( PR2 \) and \( PAL \) expression patterns were compared in infected plants (Table 1, Figs 1 and 2).

Results demonstrated that 1 and 6 dpi have permitted the display of different plant responses to \( B_g \) and \( P_g \) pathogens, respectively (Figs 1 and 2), which are in agreement with our previous observations under natural field conditions (Arabi et al., 2004; Arabi and Jawhar, 2012). It was also noteworthy that \( PR2 \) and \( PAL \) genes, had higher constitutive expression and faster induction for the both pathogens in the resistant genotype as compared with the susceptible one.

Data showed that 1 and 14 days constitutes a significant starting time-point (\( PR2 \)) in demonstrating the differential response of resistant and susceptible barley plants towards \( B_g \) and \( P_g \) and that the optimal time-point was 4 and 18 days, respectively (Fig. 3).
Whereas, for PAL expression, this response was at 1 and 14 days towards Bg and Pg, respectively, with optimal ones at 6 and 14 days post inoculation (Fig 4).

qRT-PCR analysis revealed higher expression in the resistant barley plants inoculated with Pg compared with Bg, with a maximum expression (13.8- and 5.06-fold) for PR2 and (14.8- and 4.51-fold) for PAL, respectively, mainly at the latest stage of each disease development. However, expression of PAL gene in the resistant barley plants found to be significantly upregulated at 14 and 6 days, and for PR2, were 14 and 4 days for Pg and Bg, respectively (Figs. 3, 4).

**Discussion**

In this work, for a deeper understanding of molecular machinery underpinning the defense response during barley – seed-borne and foliar diseases interactions, expression levels of PR2 and PAL pathogenesis related genes in compatible/incompatible barley inoculated with Pg and Bg were evaluated at different time points of infection. No lesion development was seen in non-inoculated controls for both diseases, while there was an indication that Pg and Bg had an effect on barley plants defense responses as observed in their infection systems (Chaure et al., 2000; Ghannam et al., 2016). Our design was to use
Pg and Bg non-inoculated controls which closely reflects a natural mode of entry of the fungus in barley tissues, which was considered as a more biologically relevant comparison to study the effects of both pathogens directly.

Data showed that both PR2 and PAL were significantly overexpressed in the infected resistant and susceptible genotypes as against their lower expression in controls, and higher expression in resistant barley plants inoculated with Pg compared with Bg was observed as compared with the susceptible one. This might be attributed to the nature of Bg and Pg infection, since the foliar biotrophic pathogen Bg needs around 24 hours to establish the infection through penetration of spore germinating tube into the leaf epidermis tissue (Carver and Thomas, 1990). Whereas, in Pg when a barley seed germinates the hyphae accelerates its intercellular growth within tissue, in order to establish a full-scale infection and therefore plants need to increase their defense expression in order to resist Pg attack (Platenkamp, 1976; Haegi et al., 2008).

It is well known that PR2 encodes 1,3-β-glucanase which hydrolyses the β-O-glycosidic bond of β-glucan in plant cell walls that led to cell wall loosening and expansion (Akiyama et al., 2009). In addition, PAL plays an important function in the biosynthesis of salicylic acid which is an essential signal involved in plant systemic resistance (Cha-
man et al., 2003). However, in this study, \textit{PR2} presented difference in expression levels between the \textit{Pg} and \textit{Bg} infected plants and the control (\( P = 0.05 \)), with values being higher in plants infected with the seed-borne pathogen \textit{Pg}. Similarly, the \textit{PR} gene accumulation was recorded in barley leaf tissues infected with \textit{Cochliobolus sativus} (Arabi et al., 2015). Furthermore, previous works indicate that \textit{PR} genes are associated with MLO powdery mildew resistance (Opalski et al., 2005; Tayeh et al., 2015) that should be further investigated to support the current results.

### Conclusion

Results of the present work demonstrate that, significant increases in \textit{PR2} and \textit{PAL} expression were found upon barley challenged with seed-borne (\textit{Pg}) and foliar (\textit{Bg}) pathogens, with values being consistently higher in plants inoculated with \textit{Pg}. It was also noteworthy that \textit{PR2} and \textit{PAL} genes, had higher constitutive expression and faster induction for the both pathogens in the resistant genotype as compared with the susceptible one, and their patterns accumulation clearly differentiated during the time course of infection and corresponded to the pathogen lifestyle, which could indicate that these changes might have roles in barley – \textit{Pg} – \textit{Bg} interactions. This may be an effective complementary option for leaf stripe and powdery mildew diseases management in barley.

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### Literature


