

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

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(Received: 5 November 2018; accepted: 10 December 2018)

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 accompanied 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

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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 *Pg* and *Bg* 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 *Pg* and *Bg* 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 (*Pg*) and foliar (*Bg*) 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 *Pg* and *Bg* 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 Pg*

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  $20 \pm 1$  °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  $\times 150$  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 *Pg*, 1, 2, 4 and 6 days for *Bg*. mRNA was extracted and 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. Samples from non-inoculated plants at each time point were collected as controls.

### *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  $\Delta CT$  value determined by subtracting the average CT value of genes from the CT value of *EF1 $\alpha$*  gene. Finally, the equation  $2^{-\Delta\Delta CT}$  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 *Pg* and *Bg* 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 1**

Properties and nucleotide sequence of primers used in qRT-PCR

Gene	Gene description	Accession No.	Sequence	Amplified fragment (bp)
<i>PAL</i>	Phenylalanine ammonia-lyase	AT2G14610	5' CCATTGATGAAGCCAAAGCAAG 3' 5' ATGAGTGGGTTATCGTTGACGG 3'	123
<i>PR2</i>	Beta1,3-glucanase2	AT3G57260	5' TGGTGTCAAGATCCGGTACA 3' 5' TCATTCTGAACCTTCCTTG 3'	193
<i>EF1α</i>	Elongation factor-1 Alpha	AT1G07920	5' TGGATTGAGGGTGACAACA 3' 5' CCGTTCCAATACCACCAATC 3'	167

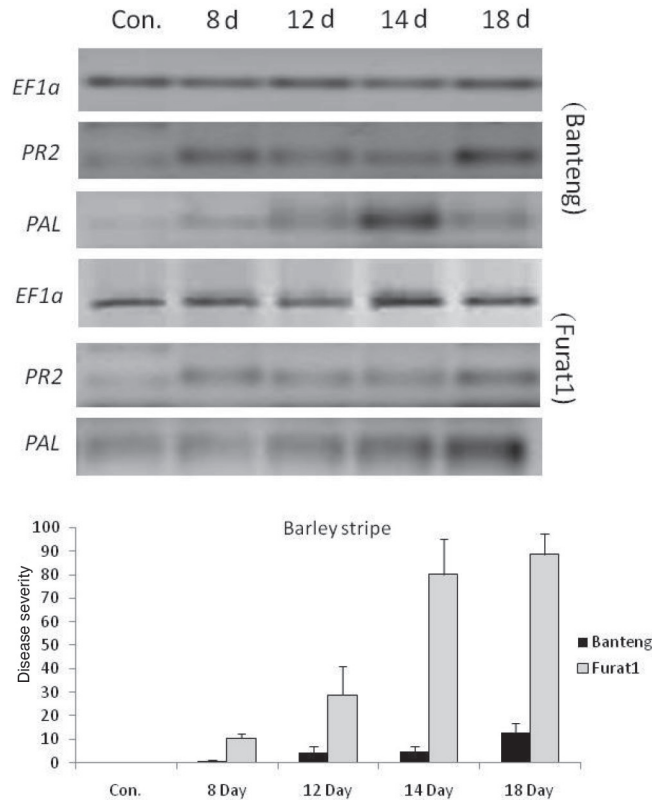


Fig. 1. Disease severity and gene expression by RT-PCR in barley resistant (cv. Banteng) and susceptible (cv. Furat1) plants inoculated with *P. graminea* at 8–18 days

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 *Pg* and *Bg* 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 *Bg* and *Pg* 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 *Bg* and *Pg* and that the optimal time-point was 4 and 18 days, respectively (Fig. 3).

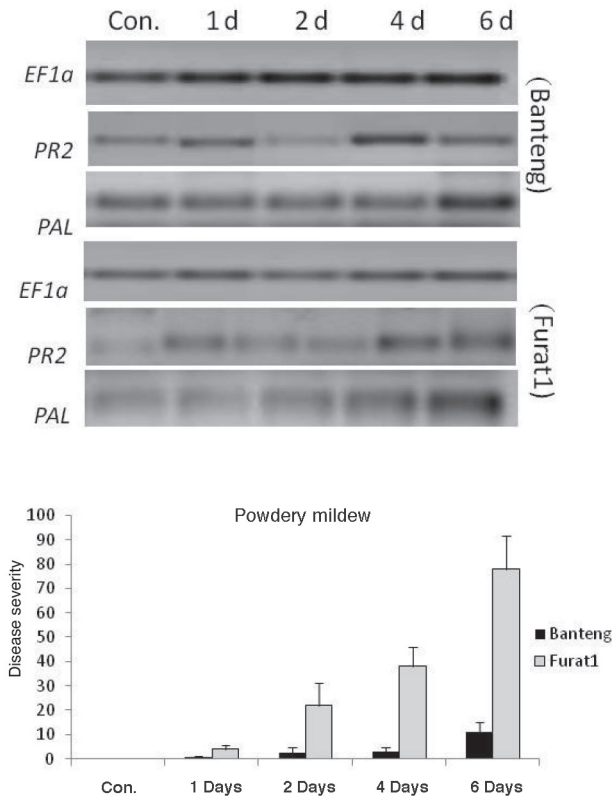


Fig. 2. Disease severity and gene expression by RT-PCR in barley resistant (cv. Banteng) and susceptible (cv. Furat1) plants inoculated with *B. graminis* at 1–6 days

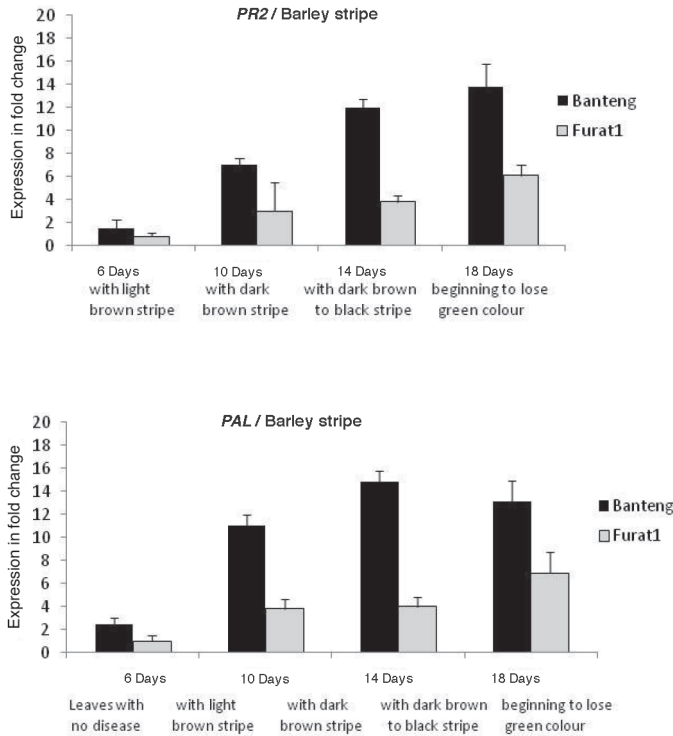


Fig. 3. Relative expression profile of *PR2* and *PAL* genes in the barley resistant (cv. Banteng) and susceptible (cv. Furat1) plants during infection with *Pg* pathogens

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

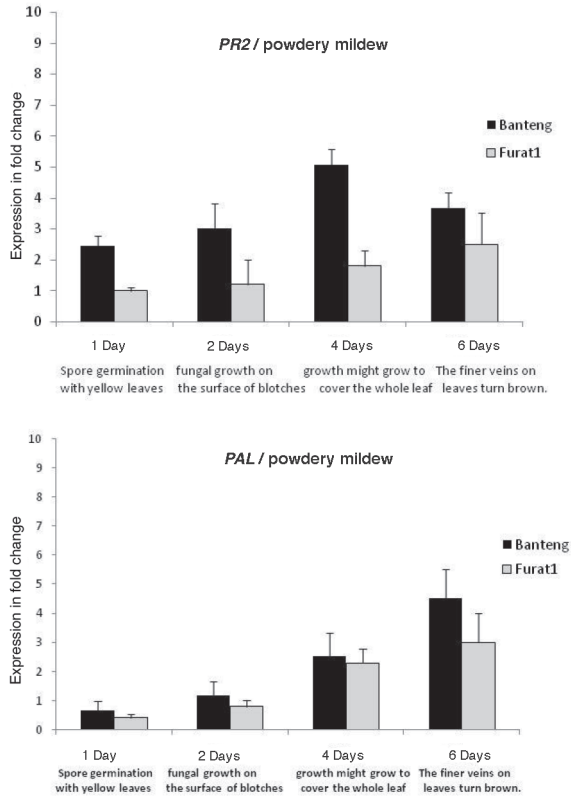


Fig. 4. Relative expression profiles of *PR2* and *PAL* genes in the barley resistant (cv. Banteng) and susceptible (cv. Furat1) plants during infections with *Bg* pathogens

*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- $\beta$ -glucanase which hydrolyses the  $\beta$ -*O*-glycosidic bond of  $\beta$ -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, PR2 presented difference in expression levels between the *Pg* and *Bg* infected plants and the control ( $P = 0.05$ ), with values being higher in plants infected with the seed-borne pathogen *Pg*. Similarly, the PR gene accumulation was recorded in barley leaf tissues infected with *Cochliobolus sativus* (Arabi et al., 2015). Furthermore, previous works indicate that 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 PR2 and PAL expression were found upon barley challenged with seed-borne (*Pg*) and foliar (*Bg*) pathogens, with values being consistently higher in plants inoculated with *Pg*. 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, 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 – *Pg* – *Bg* interactions. This may be an effective complementary option for leaf stripe and powdery mildew diseases management in barley.

## Acknowledgements

The authors thank the Director General of AECS and the Head of Molecular Biology and Biotechnology Department for their much appreciated help throughout the period of this research.

## Literature

- Akiyama, T., Carstens, M. I. and Carstens, E. (2009): Excitation of mouse superficial dorsal horn neurons by histamine and/or PAR-2 agonist: potential role in itch. *J. Neurophysiol.* 102, 2176–2183.
- Arabi, M. I. E. and Jawhar, M. (2012): Expression of resistance to *Blumeria graminis* in barley genotypes (*Hordeum vulgare* L.) under field and controlled conditions. *J. Plant Biol. Res.* 2, 107–112.
- Arabi, M. I. E., Jawhar, M., Al-Safadi, B. and Mirali, N. (2004): Yield responses of barley to leaf stripe (*Pyrenophora graminea*) under experimental conditions in Southern Syria. *J. Phytopathol.* 152, 519–523.
- Arabi, M. I. E., Al-Daoude, A., Shoab, A. and Jawhar, M. (2015): Accumulation of transcripts abundance after barley inoculation with *Cochliobolus sativus*. *Plant Pathol. J.* 31, 72–77.
- Boyd, L. A., Ridout, C., O’Sullivan, D. M., Leach, J. E. and Leung, H. (2013): Plant-pathogen interactions: disease resistance in modern agriculture. *Trends Genet.* 29, 233–240.
- Carver, T. L. W. and Thomas, B. J. (1990): Normal germling development by *Erysiphe graminis* on cereal leaves freed of epicuticular wax. *Plant Pathol.* 39, 367–375.
- Chaman, M. E., Copaja, S. V. and Argandoña, V. H. (2003): Relationships between salicylic acid content, Phenylalanine Ammonia-Lyase (PAL) activity, and resistance of barley to aphid infestation. *J. Agric. Food Chem.* 51, 2227–2231.
- Chahre, P., Gurr, S. J. and Spanu, P. (2000): Stable transformation of *Erysiphe graminis*, an obligate biotrophic pathogen of barley. *Nat. Biotechnol.* 18, 205–207.
- Dean, R., van Kan, J. A. L., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J. J., Dickman, M., Kahmann, R., Ellis, J. and Foster, G. D. (2012): The top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* 13, 414–430.



- Delogu, G., Porta-Puglia, A. and Vannace, G. (1989): Resistance of winter barley varieties subjected to nature of *Pyrenophora graminea*. J. Genet. Breed. 43, 61–66.
- Derveaux, S., Vandesompele, J. and Hellemans, J. (2010): How to do successful gene expression analysis using real-time PCR. Methods 50, 227–230.
- Ebrahim, S., Usha, K. and Singh, B. (2011): Pathogenesis related (PR) proteins in plant defense mechanism: Science against microbial pathogens: Communicating current research and technological advances. Sci. Against Microb. Pathog. 2, 1043–1054.
- Ghannam, A., Alek, H., Doumani, S., Mansour, D. and Arabi, M. I. E. (2016): Deciphering the transcriptional regulation and spatiotemporal distribution of immunity response in barley to *Pyrenophora graminea* fungal invasion. BMC Genom. 17, 256.
- Haegi, A., Bonardi, V., Dall’Aglia, E., Glissant, D., Tumino, G., Collins, N. C., Bulgarelli, D., Infantino, A., Stanca, A. M. and Delledonne, M. et al. (2008): Histological and molecular analysis of Rdg2a barley resistance to leaf stripe. Mol. Plant Pathol. 9, 463–478.
- Häffner, E., Karlovsky, P., Splivallo, R., Traczewska, A. and Diederichsen, E. (2014): ERECTA, salicylic acid, abscisic acid, and jasmonic acid modulate quantitative disease resistance of *Arabidopsis thaliana* to *Verticillium longisporum*. BMC Plant Biol. 14, 71–85.
- Hammouda, A. M. (1986): Modified technique for inoculation in leaf stripe of barley. Acta Phytopathol. et Entomol. Hung. 21, 255–259.
- Kralik, P. and Ricchi, M. (2017): A basic guide to real time PCR in microbial diagnostics: Definitions, parameters and everything. Front. Microb. 8, 108.
- Livak, K. J. and Schmittgen, T. D. (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. Methods 25, 402–408.
- Moseman, J. G. and Baenziger, P. S. (1981): Genes conditioning resistance of *Hordeum spontaneum* to *Erysiphe graminis* f. sp. Hordi. Crop Sci. 21, 229–232.
- Nayanakantha, N. M. C., Rawat, S., Ali, S. and Grover, A. (2016): Differential expression of defense-related genes in *Sinapis alba* and *Brassica juncea* upon the infection of *Alternaria brassicae*. Trop. Agri. Res. 27, 123–136.
- Opalski, K. S., Schultheiss, H., Kogel, K.-H. and Huckelhoven, R. (2005): The receptor-like MLO protein and the RAC/ROP family G-protein RACB modulate actin reorganization in barley attacked by the biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei*. Plant J. 41, 291–303.
- Pagán, I. and García-Arenal, F. (2018): Tolerance to plant pathogens: Theory and experimental evidence. Int. J. Mol. Sci. 19, 810.
- Peyraud, R., Dubiella, U., Barbacci, A., Genin, S., Raffaele, S. and Roby, D. (2017): Advances on plant pathogen interactions from molecular toward systems biology perspectives. The Plant J. 90, 720–737.
- Platenkamp, R. (1976): Investigations on the infections pathway of *Drechslera graminea* in germinating barley. Copenhagen: Royal Veterinary and Agricultural University.
- Samsatly, J., Copley, T. R. and Jabaji, S. H. (2018): Antioxidant genes of plants and fungal pathogens are distinctly regulated during disease development in different *Rhizoctonia solani* pathosystems. PLoS ONE 13(2): e0192682.
- Tayeh, C., Randoux, B., Tisserant, B., Khong, G., Jacques, P. and Reignault, P. (2015): Are ineffective defence reactions potential target for induced resistance during the compatible wheat-powdery mildew interaction? Plant Physiol. Biochem. 96, 9–19.
- Vásquez, A. H., Salinas, P. and Holuigue, L. (2015): Salicylic acid and reactive oxygen species interplay in the transcriptional control of defense genes expression. Front Plant Sci. 6, 171.

