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Cooperative functioning of *mla* & *Rac1* genes and salicylic acid in resistant barley challenged with *Blumeria graminis*

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Abstract

Powdery mildew, caused by the fungal pathogen *Blumeria graminis* (*Bg*) is one of the most important foliar diseases of barley globally. Barley plants have evolved complex signaling pathways during defense response against *Bg*, including plant hormones such as salicylic acid (SA) and pathogenesis-related proteins. Therefore, to better understand the defense mechanisms of resistant barley plants attacked by *Bg*, cooperative functioning between *mla1*, *mla6*, *mla12*, *Rac1* genes and SA was evaluated at early time points of infection. mRNA was isolated 1, 2, 3, 4 and 6 days post inoculation and used for cDNA synthesis. The expression of *mla* and *Rac1* genes was quantified using Quantitative reverse transcriptase PCR (qRT-PCR). SA measurement was performed by a high-performance liquid chromatography (HPLC) system. Data showed a remarkable increase in the expression patterns of *mla* and *Rac1*genes accompanied with an increase in SA level in infected plants 1 day post inoculation (dpi) when compared with non-infected plants. The most outstanding differences were observed in *mla1* and *Rac1* expressions which were 6.27 and 3.55 folds respectively higher, 4 dpi of barley *Bg* interaction, and were accompanied with an elevated SA level (610 ng/g). According to findings, this study might increase our understanding for a deeper molecular research on the cooperative functioning of *mla* & *Rac1* genes and SA in resistant barley responses against *Bg*.

Keywords: Barley, defense response, PCR (qPCR), powdery mildew, *Rac1*, *mla* genes, salicylic acid

1. Introduction

Blumeria graminis f sp *hordei* (*Bg*), is a biotrophic pathogen which causes powdery mildew of barley (*Hordeum vulgare* L.), a disease responsible for heavy crop losses [1, 2]. The infection stages are well documented and each stage is a potential recognition point with the possible release of pathogen or plant-derived signaling molecules [3]. During the infection, barley induced defence responses known to be regulated by different signalling pathways, including plant hormones such as salicylic acid (SA) and pathogenesis-related proteins. However, our understanding of these defensive mechanisms is still limited. On the other hand, the recent development of modern genetics and genomics tells us that studying plant resistance genes is the best way to breed for *Bg* high-resistance [4].

The interaction of barley and *Bg* has been widely studied and many resistance genes (*mla* genes) have been described [5]. Genetic variants of the *mla* locus have been reported worldwide and thus supply a unique experimental system in which the evolution of the allelic diversity of *R* genes in monocot species could be explored [6]. *Rac1* has been molecularly characterized as a gene encoding nucleotide-binding site leucine-rich repeat (NBS-LRR) proteins and confers resistance against *Albugo candida* pathogen in Arabidopsis [7], and enhance drought and salt tolerance in *Nicotiana benthamiana* [8]. However, its expression during barley infection with *Bg* is still poorly understood. Quantitative reverse transcriptase PCR (qRT-PCR) has been considered as an effective method for measuring gene relative expression levels post fungal infection [9].

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Furthermore, SA is a key defense signalling molecule against plant fungal pathogens, and its output can be replaced with the expression profiling of phytohormone-responsive marker genes [10]. In our previous work, we found that knocking out *PR2*, *PAL* and *LSD1* genes in barley resistant plants increased SA content and enhanced resistance to *Bg* [11]. These findings draw our attention to the expression patterns of some antifungal genes such as *mla* and *Rac1* as well as SA content during barley- *Bg* interactions. Therefore, in the current study and in order to complete the wider picture of gene activity drawn by Al-Daoude et al. (2019) [11], the changes in SA and induction of some known defense-related genes *viz., mla1, mla6, mla12* and *Rac1*genes during barley - *Bg* interactions were investigated for enhancing our genetic and molecular understanding of plant fungal resistance.

2. Materials and Methods

2.1. Host genotype

After several years of greenhouse and laboratory screenings, the German barley cv. Banteng has proved to be the most resistant genotype to all *Bg* isolates tested so far [12], therefore, it was used in this study. Seeds were sown in 20-cm pots filled with sterilized peat moss and arranged in three replicates for each genotype/disease at 20°C, with a 16 light/8 h dark cycle.

2.2. Inoculation

Seedlings were inoculated with virulent *Bg* conidiospores of the mildew obtained from a single colony isolate (Pt1m) by shaking susceptible spreader sporulating plants above them [13]. The non-inoculated control plants were kept under clean growth chamber. At seedling (GS 32) and adult plant (GS 80) stages**,** mildew infections were scored according to the scale of Moseman and Baenziger (1981) [14].

2.3. SA quantification

Fresh barley leaves were collected 1, 2, 3, 4 and 6 dpi, and free SA was extracted in 1.5 ml tubes using the protocol described by Trapp et al (2014) [15], with some modifications. Briefly, 100 mg of barley leaves were dried overnight in a freeze drier at −42°C. The extraction was done by adding 1.0 mL of ethyl acetate, dichloromethane, isopropanol, MeOH:H_2O (AppliChem GmbH, Germany) into each tube containing dry leaves. Samples were 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 a speed vac. After drying, 100 µL of MeOH was added to each sample, homogenized under vortex and centrifuged at 16,000 g and 4°C for 10 min. SA measurement was performed by a high-performance liquid chromatography (HPLC) system (Agilent Technologies, Germany) connected to a fluorescent detector using an excitation wavelength (λ_{EX}) of 300 nm and an emission wavelength (λ_{EX}) of 410 nm as described by Verberne et al. (2002) [16]. Six replicates were performed for each time point. Data were analyzed using the standard deviation and t-test methods.

2.4. RNA isolation and cDNA synthesis

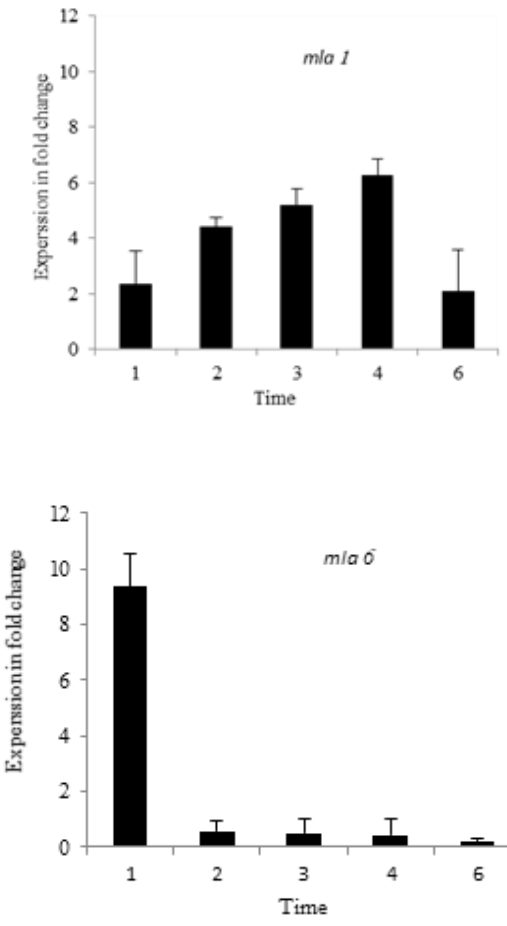
Primary leaves were collected at 1, 2, 3, 4 and 6 dpi and homogenized with a tube pestle in liquid nitrogen. mRNA was extracted with the Nucleotrap mRNA mini kit (Macherey-Nagel, MN, Germany) following the manufacturer's instructions. At the same time points, samples from mock inoculated plants were collected as control. Mock inoculation was done by spraying pathogen-free water on the plants. RNA was used for cDNA synthesis with the Quanti Tect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. cDNA was stored at −20 °C.

2.5. Quantitative real-time PCR (qPCR)

The expression of *mla1, mla6, mla12* and *Rac1* genes was quantified using qRT- PCR in Step One Plus, 96 well using SYBR Green Master kit (Roche, USA) according to Derveaux et al. (2010) [9]. The sequence information for all RT-PCR primers is given in Table 1. Expression measurement of each gene was conducted in triplets with three biological replicates. Relative expression for each gene was calculated using the average cycle threshold (CT), with the ΔCT value determined by subtracting the average CT value of genes from the CT value of *EF1α* gene. Finally, the equation $2^{-\Delta\Delta CT}$ was used to estimate the relative expression levels [17]. Standard deviations were calculated among the three biological replicates. Tukey's test was performed to test whether the expression levels at different time points are different or not. The assumption of coincidence was tested using the ANOVA procedure implemented in the software package Statistica 6.1 (StatSoft, Inc. STATISTICA (2001) Version 6. http://www.statsoft.com).

3. Results and discussion

In order to investigate the biochemical and physiological changes during *Bg* infection of resistant barley plants, the total SA content and the induction of some known defense-related genes *viz.*, *mla1*, *mla6*, *mla12*, and *Rac1* genes were assayed. Data revealed a significant increase in *mla* and *Rac1*genes expression patterns which was accompanied by an increase in SA level contents of infected plants 1 day post inoculation (dpi) in compared with non-infected plants (Figures 1 and 2).



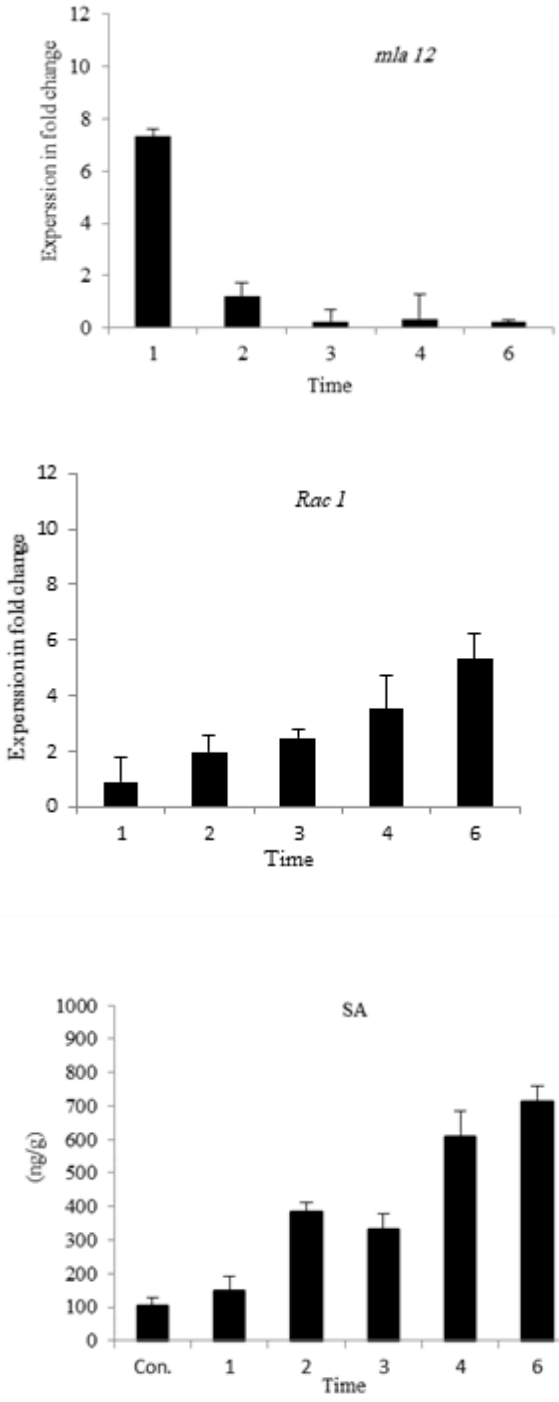


Figure 1. Relative expression profiles of *mla, Rac1* genes and SA content in the resistant barley cv. Banteng during the time course following infections with *B. graminis*. Error bars indicate the standard error (Mean ± SD, *n* = 3). Data are normalized to Elongation factor 1α (EF-1α) gene expression level (to the calibrator, Control 0 h, taken as 0)

Figure 1. Continuation

Data also showed that *mla1* and *Rac1* expressions were 6.27 and 3.55 folds respectively higher 4 dpi of barley *Bg* interaction, which was accompanied with a high SA content (610 ng/g) (Figure 1). This might reflect the strategy of this biotrophic pathogen to cope with barley plant cell death-associated defense [18], since a biotroph such *Bg* requires sufficient time to thwart host defenses and suppress programmed cell death [19]. Panstruga

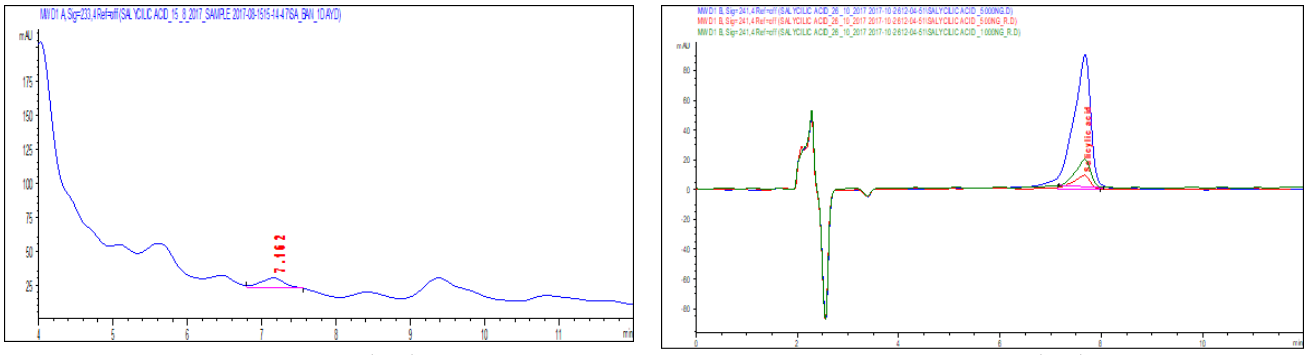


Figure 2. UV spectra for SA (A), and chromatograms of fluorescence detected SA (B) from the resistant barley cv. Banteng leaves after *B. graminis* inoculation

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

Gene	Accession No.	Sequence	Amplified fragment (bp)
EF1α	AT1G07920	TGGATTTGAGGGTGACAACA	167
		CCGTTCCAATACCACCAATC	
Rac 1	AY522496	GCGATGATGTTGTCTCCCAC	195
		CCGTGAGCATCCCTTGAAAC	
mla1	Gu245961	ACCATGCACTGGGGATTGTA	119
		AGACTCAACGACGCGGAAAA	
mla6	AJ302293	CACAACGGCTGCTAGTCATC	248
		CCTCGCTCCACCACAATAGA	
mla12	AY196347	TCATGCTTCCGGACCATTTTT	185
		GGAGATGAAGTAGGAGCCGC	

(2003) and Doehlemann et al. (2008) [20, 21] reported that the biotrophic *Uromycesvignae* and hemibiotrophic *Mycosphaerella graminicola* fungi have suppressed the host defenses during the biotrophic phase of infection. Moreover, figure 1 showed the increase in SA level up to 6 dpi that may be explained by SA role in the regulation of physiological and biochemical processes during the entire lifespan of barley plants [22]. Therefore, SA accumulation has been considered as a useful marker of elevated defense responses which is connected with hypersensitive cell death [23].

The coordinate increase in *mla1, Rac1* and SA takes place well after the contact between conidia and the leaf surface, but before the formation of an elongating secondary hyphae. Therefore, we predict that recognition occurs during the invasion of the host cell and the formation of *Bg* haustoria. There are two possible scenarios that could explain the increase in *mla1*, *Rac1* and SA expressions. The increasing of *mla* levels might be due to the increasing of transcript accumulation in cells surrounding the site of infection and not in the attacked cell itself, since the attacked cell dies quickly through the hypersensitive cell death. It is known that only a fraction of the leaf cells touched with *Bg* conidia, and it is possible that a signal came from the attacked cell induced the surrounding cells to heighten their sensitivity to the avirulence signal via the activation of *mla*, *Rac1*and SA. This might also explain the increase in *mla6* and *mla12* levels directly 1 dpi (Figure 1). These results are in agreement with those of [6], who reported that *mla1* normally confers rapid and absolute resistance, whereas other, *mla* genes confer an intermediate response. On the other hand, Mahlert et al. (2006) [24] reported that *Rac1* is required for pathogenicity of *Ustilago maydis*, and Rac GTPases play an

important role in *Claviceps purpurea* development [25].

The higher activities of *mla1* and *Rac1* and the higher level of SA in infected Banteng leaf tissues compared with control may explain the high level of resistance seen in this barley cultivar [12]. Our study demonstrated that *mla*, *Rac1* genes and SA signaling pathways probably work together in the activation of resistant barley defense responses to *Bg* attack.

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Compliance with Ethics Requirements. Author declares that he respect the journal's ethics requirements. Author declares that he 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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