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Full Length Research Paper

Expression of *WRKY* and *MYB* genes during infection with powdery mildew in cucumber (*Cucumis sativus* L)

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The expression change pattern of three transcription genes (*WRKY30, WRKY6* and *MYB*) in two cucumber lines with different powdery mildew resistance (resistant line 'JIN5-508' and susceptible line 'D8') were investigated during the infection process with powdery mildew using real-time quantitative polymerase chain reaction (RT-PCR). Gene expression analysis during different time points revealed that the expression ratio of *WRKY30* was 10.08585 in D8 and 5.117667 in JIN5-508, respectively, and for *WRKY6*, the expression ratio was 5.396152 in D8 and 3.787322 in JIN5-508, respectively, and for *MYB*, the expression ratio was 14.17324 in D8 and 10.70195 in JIN5-508, respectively. Additionally, the time point of the highest relative expression ratio for the three genes was different in the two cucumber lines according to their resistance to powdery mildew, whereas the susceptible line D8 was earlier than the resistant line JIN5-508 in responding to the powdery mildew infection. We suggest that the three genes' expressions induced by powdery mildew pathogen is related to the disease resistance, and the response of susceptible line is earlier and higher than the resistant line, which may have interactions between the three genes and other resistant genes.

Key words: Cucumber, powdery mildew, gene expression pattern.

INTRODUCTION

Powdery mildew caused by *Podosphaera xanthii* is one of the most important diseases of cucumber under greenhouse condition. It reduces yield by decreasing the quality and fruits harvested due to plant vigor alteration (Agrios, 1988).

WRKY genes play important roles in plant responses to biotic and abiotic stresses as well as in plant developmental processes. Abiotic and biotic stresses are major external factors influencing the expression of *WRKY* genes (Eulgem et al., 2000; Ulker and Somssich, 2004). The expression of more than 70% of the *Arabidopsis WRKY* genes is influenced by various stresses, particularly by pathogen-related stimuli (Dong et al., 2003; Hahlbrock et al., 2003).

Many publications have provided conclusive genetic proof that Arabidopsis *WRKY* factors are crucial regulators of the defense transcriptome and disease resistance. *AtWRKY*52/RRS1 was shown to confer resistance towards the bacterium *Ralstonia solanacearum*, but the encoded protein is quite exceptional and appears to act as an R protein (Deslandes et al., 2002).

Several studies reported the importance of *AtWRKY70*, which appears to affect the balance between signaling branches promoting salicylic acid (SA)-dependent and suppressing jasmonic acid (JA)-dependent responses (Li et al., 2004, 2006). Moreover, *AtWRKY70* is required for both basal defense and full R-gene (RPP4)-mediated disease resistance against the oomycete *Hyaloperonospora parasitica* (Knoth et al., 2007). Similarly, mutants compromised in *AtWRKY33* were more susceptible to infection by *Botrytis* cinerea and *Alternaria brassicicola* (Zheng et al., 2006). Several *WRKY* factors act as negative regulators of resistance.

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For instance, basal plant resistance triggered by a virulent *Pseudomonas syringae* strain was enhanced in *AtWRKY7* and *AtWRKY11*/*AtWRKY17* insertional mutants (Journot-Catalino et al., 2006; Kim et al., 2006), thereby also revealing partly redundant functions for these closely related TFs.

A subgroup IIa of *WRKY* genes, comprising *AtWRKY18*, *AtWRKY40* and *AtWRKY60*, play important and partly redundant functions in regulating plant disease resistance. Xu (2006) showed that *AtWRKY18*/*AtWRKY40* and *AtWRKY18*/*AtWRKY60* double mutants were more resistant to *P. syringae* DC3000 but more susceptible to *B. cinerea* infection. *AtWRKY18*/*AtWRKY40* double mutants were also highly resistant to an otherwise virulent powdery mildew, *Golovinomyces orontii* (Shen et al., 2007). In both studies, single *AtWRKY* mutants behaved similar to wild-type plants. Interestingly, *AtWRKY18* was also identified as a positive regulator required for full systemic acquired resistance (SAR), but here, *AtWRKY40* did not seem to be involved (Wang et al., 2006). Differences in the experimental set-ups employed by Xu (2006) and Wang (2006) may be responsible for the apparent discrepancy observed in the *At*wrky18 mutant when challenged by virulent *P. syringae* strains. Xu (2006) used 10-fold higher bacterial inoculum that may have masked the effect on basal resistance caused by loss-of *AtWRKY18* function. In barley, two IIa *WRKY* members were shown to suppress basal defense to virulent *Blumeria graminis* in silencing and transient overexpression experiments (Shen et al., 2006; Eckey et al., 2004). These results demonstrate that subgroup IIa members can have both positive and negative roles in plant defense. Consistent with this, *AtWRKY18* overexpression alone resulted in enhanced basal *P. syringae* resistance, while combined overexpression of *AtWRKY18* with other IIa *WRKYs* had reverse of this effect (Xu et al., 2006).

AtWRKY6 was first reported to be associated with senescence-and defense-related processes, and it could activate the expression of its target gene SIRK (a receptor-like protein kinase) in the process of senescence (Robatzek and Somssich, 2002). Their studies reveal that *WRKY6* can function both as a positive and negative regulator of transcription, and in particular, they identified one potential direct target gene very likely encoding an important signaling component of leaf senescence and defense response.

Several plant *MYB* genes were reported to be involved in cell death and defense response. A tobacco mosaic virus- and SA-inducible tobacco *MYB* gene (*MYB1*) was identified to be associated with viral infection and hypersensitive cell death (Yang and Klessig, 1996). At least, two *Arabidopsis MYB* genes were also shown to be induced by bacterial pathogens and associated with cell death (Kranz et al., 1998; Daniel et al., 1999). Overexpression of *AtMYB30* in *Arabidopsis* and tobacco was shown to promote hypersensitive cell death and enhance disease resistance to fungal infection (Vailleau et al., 2002). A blast- and JA-induced rice gene (*JAMYB*) that is closely associated with fungal infection and host cell death was isolated and characterized (Lee et al., 2001). It indicated that *R2R3-MYB* genes play a significant role in developmental processes in higher plants (Jin and Martin, 1999; Stracke et al., 2001).

Investigation of gene expression changes during imposition of stress is the first step towards identifying the relation with defense genes. In our study, we found that the two kinds of genes (*WRKY* and *MYB*) respond to the stress of powdery mildew infection in cucumber and the expression ratio was different between resistant and susceptible cucumber lines.

MATERIALS AND METHODS

The cucumber line D8 (highly susceptible) and JIN5-508 with high resistant to powdery mildew were grown in culture media (sphagnum : perlite = 8:2). Seedlings of three leaves stage were inoculated with powdery mildew spores using the spray inoculation method. The infected leaves as spores source were collected from infected cucumbers, and spores were used to make a suspension solution with density of 20 spore / sight (10 × 10 fold) (Tang et al., 2003). The leaves were collected at 0, 4, 8, 16, 24, 48, 72 and 96 h after inoculation, transferred to liquid nitrogen and stored at -80°C for RNA isolation.

RNA isolation and reverse transcription-PCR

Total RNA was extracted from cucumber leaves according to the method described in Xu et al. (2004). The first strand of cDNA was synthesized by using PrimeScript^{TM} 1st Strand cDNA Synthesis Kit (TaKaRa Company). Sequence data of genes used in this study can be found in the GenBank/EMBL data libraries under the following accession numbers: *WRKY30* (FJ036895), *WRKY6* (FJ036899), *MYB* (FJ036890.1) and *ACTIN* (AAZ74666). According to the EST sequences, the gene-specific primers were designed (Table 1), and the predicted amplified fragments were 231 bp in length for *WRKY30*, 152 bp for *WRKY6* and 305 bp for *MYB*. *Actin* gene was used as a control, its primers sequences are also showed in Table 1. The composition of reaction mixture was as follows: 2 µl of cDNA, 2.5 µl primer mixture, 2.5 µl 10 × PCR buffer (including $MgCl_2$), 2 µl of 2.5 mM dNTPs, 0.2 µl of 5U/µL rTaq DNA polymerase and 15.8 µl dH_2O to make final volume of 25 µl. The programs for PCR were as follows: preamplification denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 53 to 54°C for 30 s, extension at 72°C for 1 min, and a final extension of 72°C for 10 min. The amplified products were electrophoresised in 1% agarose gel and photographed under UV light.

Quantitative real-time PCR and gene expression analysis

Quantitative real-time PCR analysis was carried out by SYBR Green I chimeric fluorescence method (ABI PRISM 7500 real-Time PCR System). The data were collected using 7500 system sequence detection system software (version 3.0). The preparation of PCR reactive solution was operated in accordance with the

Table 1. Primers used for real time RT-PCR assays.

Gene	Accession number	Primer pair
WRKY30	FJ036895	F: *CATCTTCACCCTTCTTCAT* R: *CGCATCTCTGCTTCTACTG*
WRKY6	FJ036899	F: *GAGGAGTTGATAGTGGTGG* R: *TTCTTGCTCTGATTTGGTT*
MYB	FJ036890.1	F: *AGTGTTAGGCGTGGGAATA* R: *AGAGGTGGGTGTGGTGGTT*
ACTIN	AAZ74666	F: *TGGACTCTGGTGATGGTGTTA* R: *CAATGAGGGATGGCTGGAAAA*

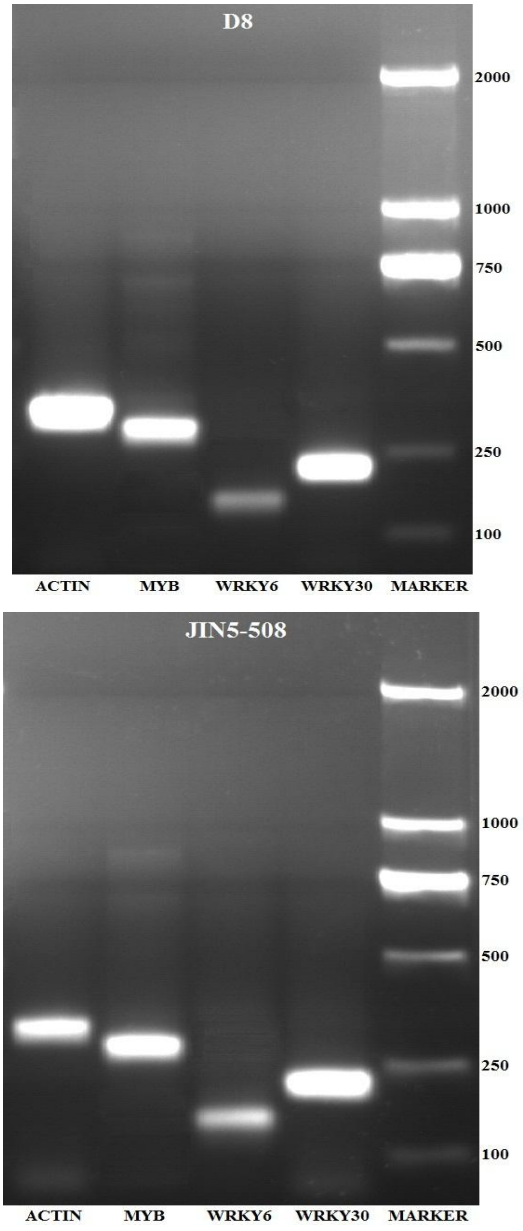

Figure 1. The specific bands of *MYB, WRKY6 and WRKY30*.

manual of SYBR ExScript^{TM} RT-PCR Kit PCR reagent kit (TaKaRa) and it contained 2× SYBR *Premix Ex Taq* 12.5 µl, 50 × ROX reference Dye II 0.5 µl, 2 µl cDNA solution as a template, 1 µl mix solution of target gene primer (with actin as internal control), and 9 µl dH_2O to make a final volume of 25 µl. The specific amplification procedure was as follows: stage 1: preamplification denaturation 95°C for 5 min; stage 2: 40 cycles of denaturation at 95°C for 15 s, annealing at 53°C for 15 s, and extension at 72°C. A melting curve analysis was performed after amplification. The quantitative results were automatically calculated by 7500 system sequence detection software (version 3.0). To minimize sample variations, mRNA expression of the target gene was normalized relative to the expression of the housekeeping gene actin. The quantification of mRNA levels was based on the method of Livak and Schmittgen (2001). The threshold cycle (Ct) value of actin was subtracted from that of the gene of interest to obtain a ΔCt value. The Ct value of untreated control sample was subtracted from the ΔCt value to obtain a ΔΔCt value. The fold changes in expression level relative to the control were expressed as $2^{-\Delta\Delta Ct}$.

RESULTS

By using genomic DNA of D8 and JIN5-508 as a template, a specific band for each pair of primers was obtained through PCR amplification with the specific primers designed (Figure 1). In order to determine the exact expression quantity of the two kinds of genes, a real-time quantitative PCR analysis was performed.

Three genes were expressed in 8 time points after infection with powdery mildew for the two cucumber lines, but with different expression levels. For *WRKY30*, the peak level was found at the time point of 24 h after inoculation in D8 line, and at the time point of 96 h in JIN5-508 line. For *WRKY6*, the peak level was also found at the time point of 24 h after inoculation in D8, and at the time point of 72 h after inoculation in JIN5-508. For *MYB*, the highest expression level was found at the time point of 8 h after inoculation in D8, and at the time point of 72 h in JIN5-508.

As shown in Figure 2, the highest relative expression ratio of *WRKY30* in D8 line at the time point of 24 h after inoculation was 10.08585, and the highest ratio of *WRKY30* in JIN5-508 was 5.117667 at the time point of 96 h.

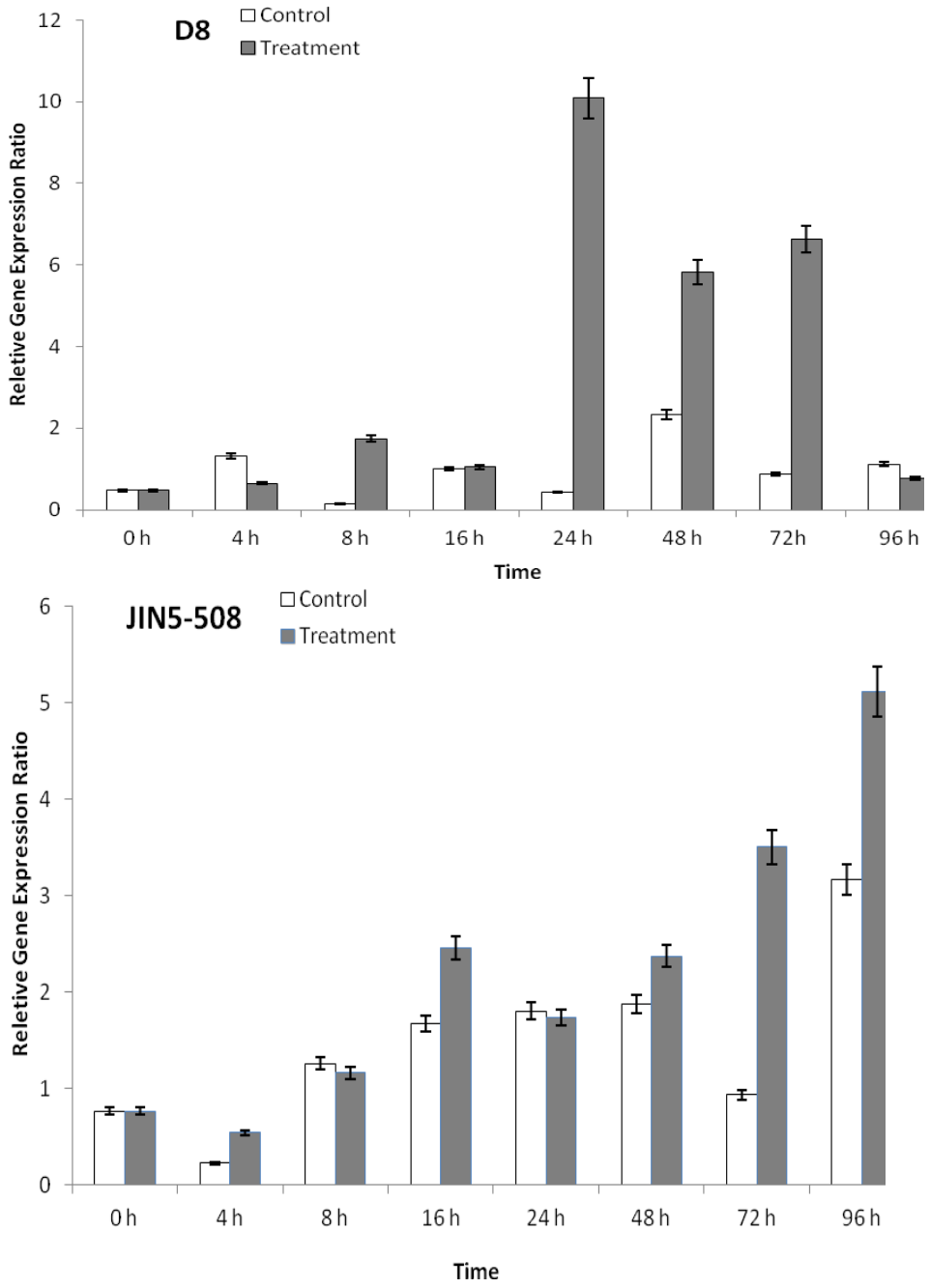

Figure 2. Relative expression analysis of *WRKY30* in different time points after powdery mildew infection by quantitative RT-PCR in D8 and JIN5-508.

For *WRKY6*, the highest relative expression ratio was 5.396152 in D8 at the time point of 24 h, and 3.787322 in JIN5-508 at the time point of 72 h (Figure 3). For *MYB*, the highest relative expression ratio in D8 line was 14.17324 at the time point of 8 h after inoculation, for line JIN5-508, it was 10.70195 at the time point of 72 h (Figure 4).

DISCUSSION

Majority of the analyzed *WRKY* genes responded to pathogen attack and to the endogenous signal molecule salicylic acid (Eulgem and Somssich, 2007; Guo et al., 2004). The results of our study show that *WRKY30* and *WRKY6* were induced by the infection of powdery mildew

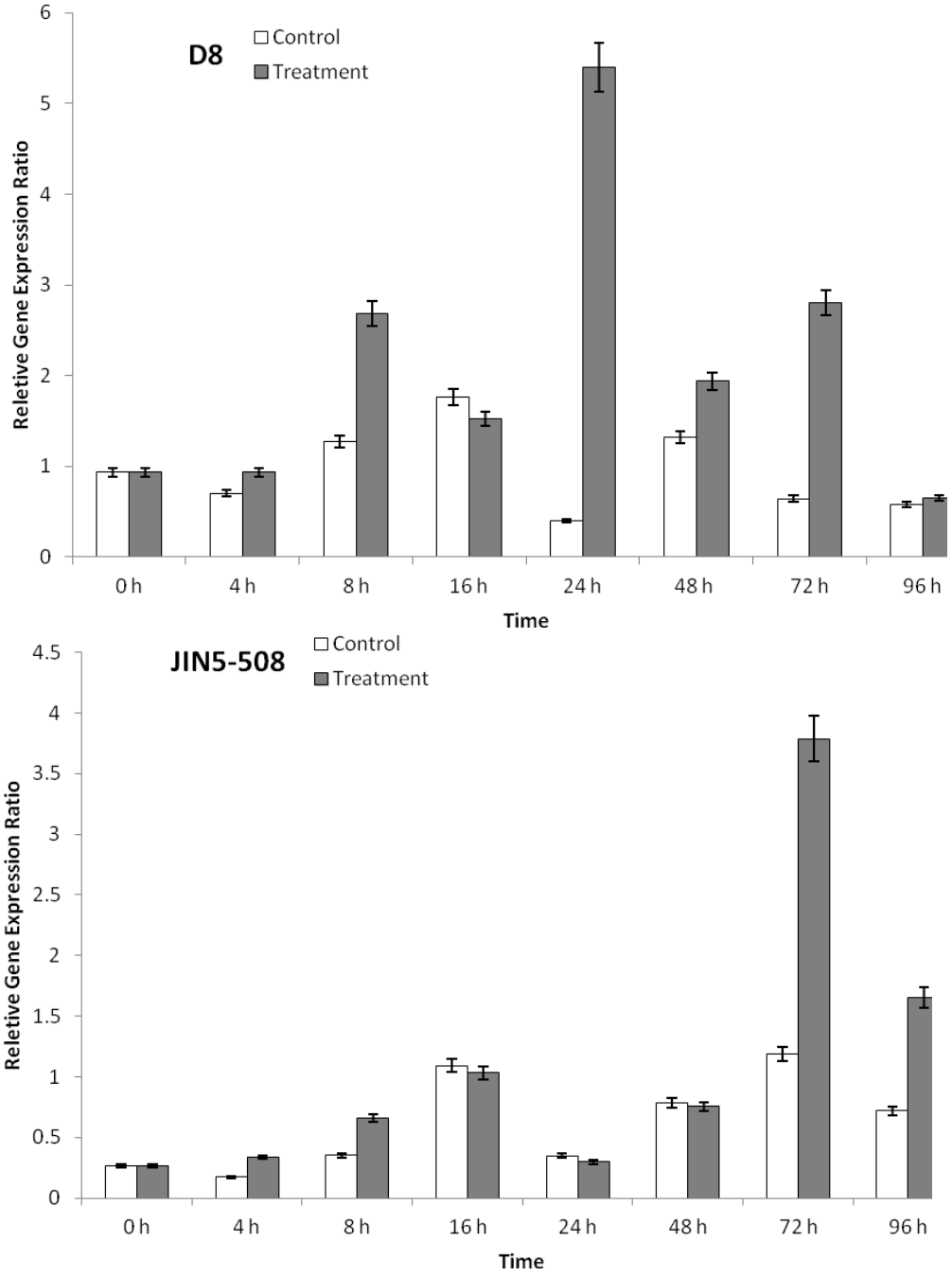

Figure 3. Relative expression analysis of *WRKY6* in different time points after powdery mildew infection by quantitative RT-PCR in D8 and JIN5-508.

pathogen, and they both showed higher expression levels than the control treatments in most of the time points (Figures 2 and 3).

The plant disease resistance and susceptibility are governed by the combined genotypes of host and pathogen, and depend on a complex exchange of signals and responses occurring under given environmental conditions (Yang et al., 1997). In relation with plant defense, the *WRKY* proteins regulate a number of defense-related genes, including pathogenesis-related (PR) genes, through interaction with the W-box (C/T)TGAC(T/C) in the promoter regions of these genes

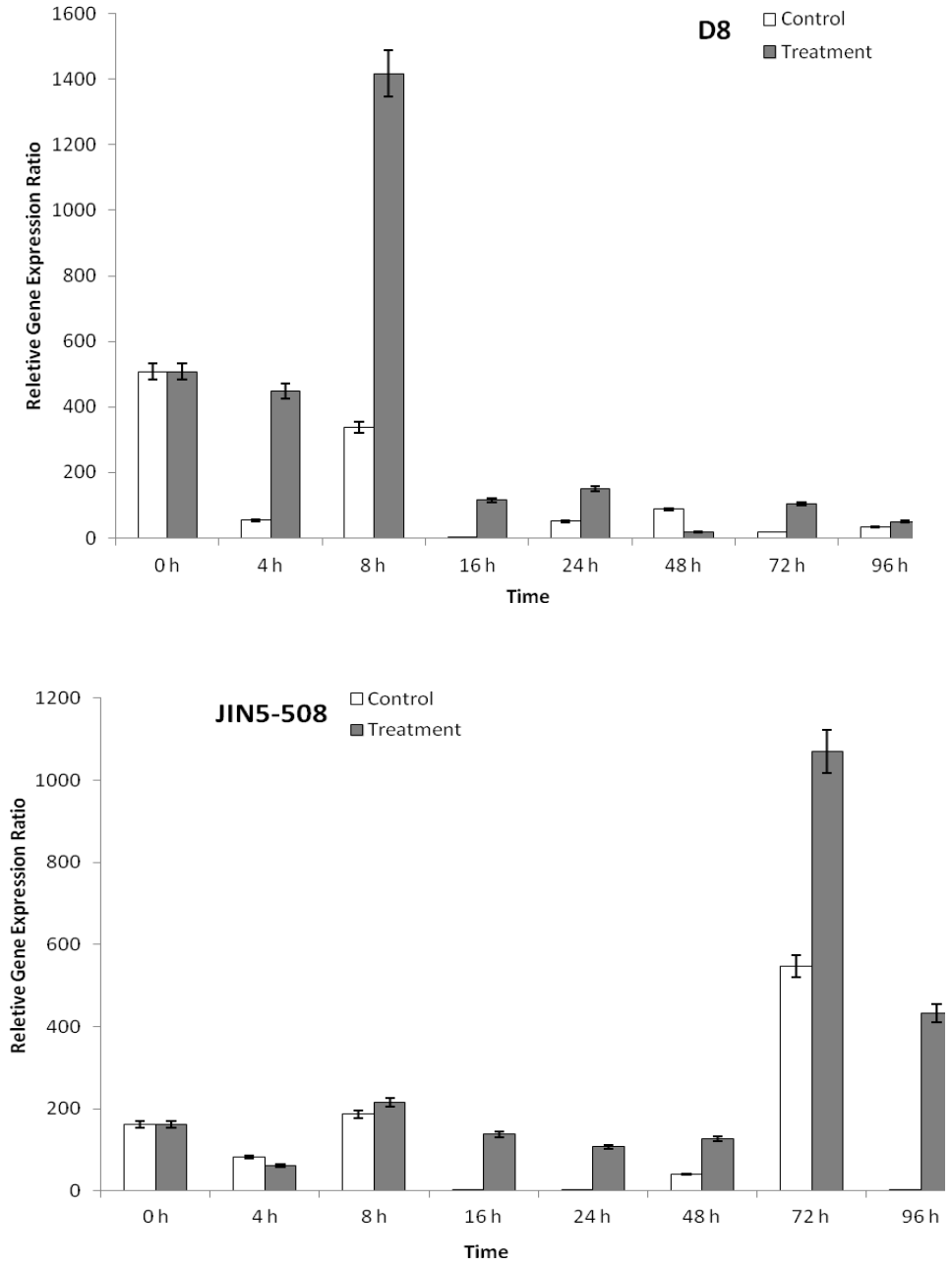

Figure 4. Relative expression analysis of *MYB* in different time points after powdery mildew infection by quantitative RT-PCR in D8 and JIN5-508.

(Rushton et al., 1996; Du and Chen, 2000; Eulgem et al., 2000; Ulker and Somssich, 2004). In the present study, we found that *WRKY30* and *WRKY6* were expressed differently in resistant and susceptible cucumber lines, which are maybe due to *WRKY* regulation of defense-related genes. The two *WRKY* genes of our study were constitutively expressed under the attack of powdery mildew pathogen, but the transcript level differed markedly in different time points in the two cucumber lines, whereas the expression reached the highest level in D8 at the time point of 24 h after inoculation (Figures 2 and 3). In view of this, it could be concluded that *WRKY30* and *WRKY6* were activated earlier in the response of plants to the powdery mildew infection in D8 (susceptible). A study on parsley by Rushton (1996) suggest that three cDNA clones encoding the W-box

binding proteins belong to the *WRKY* family of plant transcription factors, and their mRNA levels were up- or down-regulated on treatment with a fungal protein elicitor, they play a role in signaling parsley (pathogen related) *PR-1* gene activation. Our results show that *WRKY30* and *WRKY6* expression levels in JIN5-508 were induced also but after long time of infection relatively; at the time point of 96 h for *WRKY30* and 72 h for *WRKY6* (Figures 2 and 3), so it could be concluded that the two genes have slow effect on the response of JIN5-508 (resistant) to the infection with powdery mildew.

Plant *MYB* genes regulate diverse developmental processes and plant responses to environmental stimuli (Stracke et al., 2001), such as cell fate determination (Lee and Schiefelbein, 1999) and biotic and abiotic stresses (Mengiste et al., 2003; Jung et al., 2008). Our experiment reveal that *MYB* gene induced by the pathogen of powdery mildew, and the expression levels in most time points was higher in treatments than control. Also, *MYB* was activated early in D8 (at 8 h after inoculation) and late in JIN5-508 (at 72 h), and the susceptible line (D8) was earlier in the response of plants to the powdery mildew infection than resistant line (JIN5-508) (Figure 4). Consistent with our findings in *MYB*, Lee et al. (2001) also reported that a gene *JaMYB* encoding a *MYB* transcription factor was isolated from rice, expression of the *JA*MYB gene was induced after infection with *Magnaporthe grisea* in resistant and susceptible interactions and the expression level was much higher in susceptible interactions. *JaMYB* was activated rapidly by jasmonic acid or wounding.

The expression changes of these three genes indicate that there is a relationship between these genes and pathogenesis-related (PR) genes during infection with powdery mildew, and they may play a role in resistance to this disease which is different between susceptible (D8) and resistant plants (JIN5-508).

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