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Note

Leaf age and time of inoculation contribute to nonhost resistance to *Pyricularia oryzae* in *Arabidopsis thaliana*

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Abstract The outcome of plant-pathogen interactions varies both with developmental stage and time of day. Rice blast caused by *Pyricularia oryzae* (syn. *Magnaporthe oryzae*) is a devastating disease of rice. The mechanisms of resistance to *P. oryzae* have been extensively studied and the rice-*P. oryzae* pathosystem has become a model system in plant-microbe interaction studies. However, the mechanisms of resistance to *P. oryzae* in nonhost remain poorly understood. To determine whether leaf age and time of inoculation would affect nonhost resistance (NHR) to *P. oryzae* in *Arabidopsis thaliana*, Columbia-0 (Col-0) and *penetration2-1* (*pen2-1*) plants were inoculated with *P. oryzae*. The rate of entry of *P. oryzae* into Arabidopsis *pen2-1* old leaves was significantly higher than that into young leaves after inoculation at dusk. However, there was no difference in the rates after inoculation at dawn. These results suggest that leaf age and time of inoculation are involved in nonhost resistance to *P. oryzae* in Arabidopsis.

Key words: *Arabidopsis thaliana*, nonhost resistance, *Pyricularia oryzae*.

For a plant disease to occur, pathogen, host plant and environmental conditions must interact. The susceptibility of host may change both with time of day and developmental stage (Roden and Ingle 2009). The resistance to disease also varies with developmental stage (Develey-Rivière and Galiana 2007). Young tissues formed later in development are highly susceptible, whereas the older tissues remain asymptomatic. Further, the resistance of a leaf can vary greatly with its age, the position of the leaf, and the age of the plant. For example, in rice, old leaves were more resistant to *Pyricularia oryzae* (syn. *Magnaporthe oryzae*) as compared to young leaves (Roumen 1992). Immature leaves that were still extending were more susceptible than mature, fully extended leaves to *Xanthomonas campestris* pv. *oryzae* and resistance of rice to *X. c. oryzae* increased with plant age (Koch and Mew 1991). It is evident that leaves of different age and positions have different physiological characteristics, which may interfere with the expression of resistance.

Light is involved in a full resistance responses in plants (Roden and Ingle 2009). Circadian clock integrates environmental signals to regulate plant physiology (Seo and Mas 2015). Recently, the circadian clock has been shown to affect plant responses to biotic cues (Bhardwaj et al. 2011; Wang et al. 2011; Zhang et al. 2013). The circadian clock allows plants to anticipate regular changes in the environment, such as light and dark, and biotic challenges such as pathogens.

Rice blast caused by *P. oryzae* is a devastating disease of rice. The mechanisms of resistance to *P. oryzae* have been extensively studied, and the rice-*P. oryzae* pathosystem has become a model system in plant-microbe interaction studies (Ebbole 2007; Koga 2001). However, the mechanisms of resistance to *P. oryzae* in nonhost remain poorly understood. Disease resistance shown by an entire plant species to all genetic variants of a non-adapted pathogen species is the most common form of plant immunity and termed NHR (Lipka et al. 2008). We have found that *penetration2* (*pen2*) mutant allowed increased penetration into epidermal cells by *P. oryzae* in Arabidopsis (Maeda et al. 2009). Upon inoculation onto *pen2-1* plants, *P. oryzae* conidia germinated and produced appressoria which attempted penetration of the epidermal cells. Some of them could penetrate leaf epidermal cells. This led to the accumulation of autofluorescent compounds in the challenged epidermal cell and the HR-like cell death (Maeda et al. 2009). *PEN2* encodes an atypical myrosinase that metabolize indolic glucosinolate in defense responses (Bednarek et al. 2009; Clay et al. 2009; Lipka et al. 2005).

So far, we have studied NHR to *P. oryzae* in Arabidopsis (Maeda et al. 2009; Nakao et al. 2011). In our experiments, Arabidopsis plants were grown on MS plates under short-day conditions (9 : 15 L : D) at 22°C

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(100 μmol $m^{-2}s^{-1}$ fuorescent illumination) in a growth room for 3 weeks. Then, the plants were transferred to soil and grown under short-day conditions (9 : 15 L : D) at 22°C (100 μmol $m^{-2}s^{-1}$ fuorescent illumination) in a growth room for 4–5 weeks. *P. oryzae* isolate Hoku 1 (race 007) was incubated on oatmeal agar media at 25°C and the inoculum was prepared. To inoculate *P. oryzae*, 5-μl droplets (4×10^4 spores/ml) were applied to leaves of Arabidopsis plants, which were then kept under short-day conditions (9 : 15 L : D) at 22°C (100 μmol $m^{-2}s^{-1}$ fuorescent illumination) in a growth room with saturating humidity until harvested. However, we have not done inoculation at a fixed time. For example, we inoculated *P. oryzae* at dawn in one experiment and also at dusk in another experiment. To quantify cell entry, we examined the germinated fungal sporelings that developed appressoria on six leaves from six independent plants per experiment and genotype (minimum of 100 appressoria/leaf evaluated). Penetration success of *P. oryzae* was detected by the occurrence of autofluorescence or hyphal elongation at infection sites using fluorescence and bright-field microscopy. Cell entry on each plant genotype was quantified in three independent experiments. From these experiments, we have noticed that the penetration ratio of *pen2* mutant could vary between plants or even leaves within the same plants. We also noticed that time of inoculation could affect the penetration ratio of *pen2* plants. In this study, we examined whether leaf age and time of inoculation would affect NHR to *P. oryzae* in Arabidopsis.

Plant growth conditions were basically the same as that described above. Briefly, Arabidopsis plants were grown on MS plates and the plants were transferred to soil and grown for 4 weeks until they produced their seventeenth true leaf. To compare their penetration resistance, old leaf (leaf number 8) and young leaf (leaf number 13) from a series of rosettes were inoculated at dawn and dusk. Leaves are numbered from oldest to youngest. Plants used in this study are Col-0 (wild-type) and *pen2-1* (Lipka et al. 2005) (Col-0 background). To inoculate *P. oryzae*, 5-μl droplets (4×10^4 spores/ml) were applied to leaves of Arabidopsis plants, which were then kept under short-day conditions (9 : 15 L : D) at 22°C (100 μmol $m^{-2}s^{-1}$ fuorescent illumination) in a growth room with saturating humidity until harvested. Cell entry was quantified in six leaves from six independent plants. A minimum of 100 infection sites were inspected per leaf. Data were collected from three independent experiments. Data were compared using Tukey's highly significant difference (HSD) tests. Calculations were performed on three data sets ($n=3$) and $p<0.05$ indicated statistically significant effects.

To determine whether leaf age and time of inoculation would affect NHR to *P. oryzae* in Arabidopsis, Col-

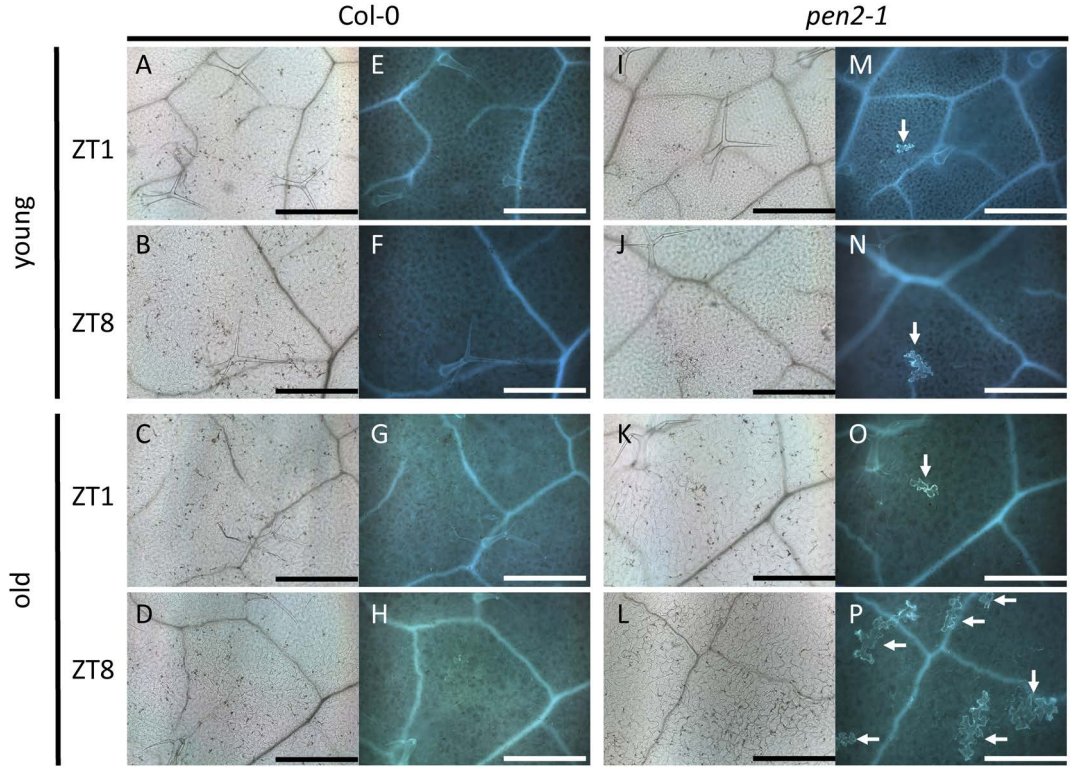

Figure 1. Plant response to *P. oryzae* infection. Light microscopic view of infection sites of Col-0 (wild-type, A–D) and *pen2-1* (I–L) plants at 72 hpi. (E–H and M–P), Cell death associated autofluorescence at infection sites in (A–D and I–L) as visualized by fluorescence microscopy. Zeitgeber Time is the time relative to dawn; ZT1 (1 h after lights on) or ZT8 (1 h before lights off), two times of day associated with drastic changes of light regime. White arrow indicates a penetrated epidermal cell. old, leaf number 8; young, leaf number 13. Scale Bar, 0.5 mm.

0 and *pen2-1* plants were inoculated with *P. oryzae*. Then, we harvested leaves of infected plants at 72 h post inoculation (hpi) and examined them microscopically. The accumulation of autofluorescent compounds in the challenged epidermal cell was used as marker for penetration of *P. oryzae* (Figure 1). To test the influence of leaf age, we studied the resistance in the young leaves and the old leaves of Arabidopsis plants (Figures 1, 2). Further, to test the influence of time of inoculation, we performed infection experiments at Zeitgeber Time 1 (Zeitgeber Time is the time relative to dawn; ZT1 is 1 h after lights on) or ZT8 (1 h before lights off), two times of day associated with drastic changes of light regime (Figure 2A). Upon inoculation onto the Col-0 plants at ZT1 (dawn), the majority of conidia failed to penetrate epidermal cells of young and old leaves (Figures 1, 2). However, in ZT8 (dusk) infection, the penetration ratio of old leaves was slightly higher than that of young leaves, although there were no significant differences between them (Figures 1, 2). Upon inoculation onto the *pen2-1* plants at ZT1 (dawn), the penetration ratio of the old leaves was similar to that of the young leaves (Figures 1, 2). However, in ZT8 (dusk) infection, the penetration ratio of old leaves was significantly higher than that into young leaves (Figures 1, 2).

Next, to determine whether time of inoculation had an effect on spore germination and appressorium formation of *P. oryzae*, we examined the spore germination rates and the appressorium formation rates at 72 hpi on old leaves of Col-0 and *pen2-1* plants. To quantify the rates, we examined the fungal sporelings on six leaves from six independent plants per experiment and genotype (minimum of 100 conidia/leaf evaluated). Experiments were repeated three times. The spore germination rate on Col-0 plants inoculated at ZT1 was lower than the rate on Col-0 plants inoculated at ZT8. However, there were no significant differences between ZT1 and ZT8 for the appressorium formation rates (Figure 3). Further, there were no significant differences between ZT1 and ZT8 for the spore germination rates and for the appressorium formation rates on *pen2-1* plants (Figure 3). These results suggest that time of inoculation had little effect on the appressorium formation rates of *P. oryzae* and the outcome of the interaction is mainly due to the effect of time of inoculation on Arabidopsis.

Taken together, NHR to *P. oryzae* varies with time of inoculation under diurnal conditions in old leaves and old leaves become more susceptible to *P. oryzae* penetration after inoculation at ZT8 (dusk) in *pen2-1* plants. Further, it also suggests that leaf age and time of inoculation acted synergistically in their effects on NHR against *P. oryzae* in *pen2-1* plants.

Decreased resistance in *pen2-1* plants after inoculation at ZT8 (dusk) compared with ZT1 (dawn) (Figures 1, 2) suggests the role of the length of the light/dark

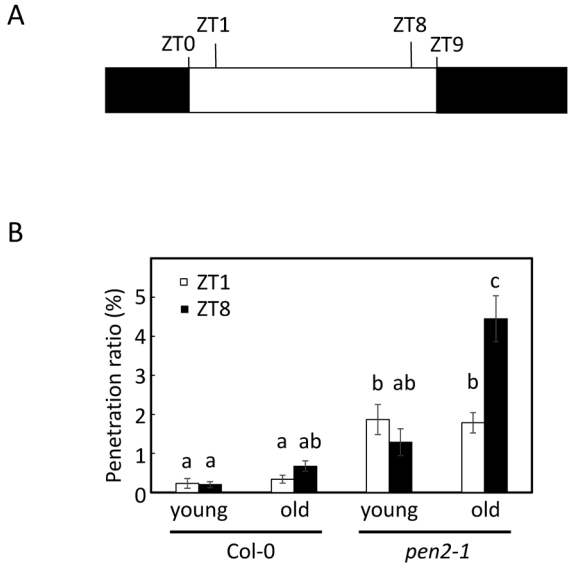

Figure 2. Quantitative analysis of penetration resistance to *P. oryzae* in Arabidopsis plants. (A) Time scheme used in this study. The white box indicates the light period and black boxes indicate dark periods. Zeitgeber Time is the time relative to dawn; ZT1 (1 h after lights on) or ZT8 (1 h before lights off), two times of day associated with drastic changes of light regime (B) Mean frequency of *P. oryzae* penetration into Col-0 (wild-type) and *pen2-1* plants at 72 h post inoculation (hpi) expressed as percentage of the total number of infection sites. Values are means±standard errors, $n=3$ independent experiments. Bars sharing the same lowercase letters are not significantly different ($p>0.05$). old, leaf number 8; young, leaf number 13; white bar, ZT1 infection; black bar, ZT8 infection.

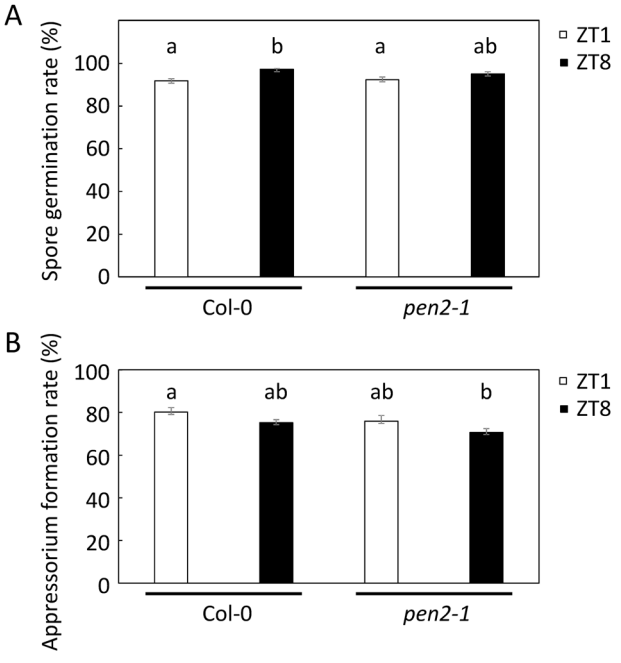

Figure 3. Effect of time of inoculation on *P. oryzae* development. (A) Spore germination rates at 72 hpi. (B) Appressorium formation rates at 72 hpi. white bar, ZT1 infection; black bar, ZT8 infection. Rates are expressed as percentage of the total number of conidia. Values are means±standard errors, $n=3$ independent experiments. Bars sharing the same lowercase letters are not significantly different ($p>0.05$).

period following infection and/or the plant clock in regulating time-of-day differences in NHR to *P. oryzae* in Arabidopsis. The importance of light with respect to the outcome of plant-pathogen interactions is becoming increasingly apparent (Roden and Ingle 2009). In addition, there is growing realization that circadian rhythms may play an important role in plant immunity (Bhardwaj et al. 2011; Wang et al. 2011; Zhang et al. 2013). The circadian clock provides essential timing information and a major time-setting mechanism (zeitgeber) in clock synchronisation is light (Oakenfull and Davis 2017). Thus, light and/or the circadian clock may play key roles in NHR to *P. oryzae* in Arabidopsis.

However, decreased penetration resistance to *P. oryzae* in *pen2-1* plants was only seen in old leaves (Figures 1, 2). Recently, it was shown that the older leaves had a shorter circadian period than the younger leaves in Arabidopsis, which suggests that each leaf in an Arabidopsis has a different circadian period depending on its age (Kim et al. 2016). Thus, the circadian period of old leaves might affect NHR to *P. oryzae* in Arabidopsis.

Furthermore, in Arabidopsis, younger leaves were more resistant to the pathogen *Pseudomonas syringae* pv. *tomato* DC3000 when plants were sprayed with the bacteria (Zipfel et al. 2004). These results suggest the involvement of developmental regulation on resistance to some pathogens, including *P. oryzae*, in Arabidopsis. In contrast, it has been shown that penetration resistance to *P. oryzae* in rice was higher in old leaves than young leaves (Roumen 1992). This discrepancy may suggest the involvement of different developmental regulation on resistance to *P. oryzae* between rice and Arabidopsis. Plants synthesize secondary metabolites to defend themselves against attacking organisms (Dixon 2001). The level of the metabolites, including glucosinolates, may change during leaf development, and these changes might affect the NHR in Arabidopsis.

In conclusion, leaf age and time of inoculation are involved in NHR to *P. oryzae* in Arabidopsis. Future studies will be required to reveal the genetic and mechanistic requirements for NHR to *P. oryzae* in Arabidopsis. Further, it will be interesting to study the effects of leaf age and time of inoculation in resistance responses to other pathogens in Arabidopsis. These studies may eventually be useful to improve resistance in plants.

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