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Original Paper

The possible roles of *AtERF71* in the defense response against the *Fusarium graminearum*

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Abstract The ethylene (ET) signaling pathway is involved in plant immunity and contributes to the disease tolerance of plants to necrotrophic phytopathogens. Ethylene response factors (ERFs) are known to play important roles in the transcriptional regulation of defense genes by ET. In the present study, we analyzed the function of *AtERF71* belonged to group VII ERF family in disease resistance against a hemibiotrophic fungal phytopathogen, *Fusarium graminearum*. When conidia solutions were dropped onto intact leaves of Arabidopsis plants, both *ein2-1* and *ein3-1* mutants showed enhanced disease resistance against *F. graminearum* compared with the wild type. This finding suggested that the ET signaling pathway was involved in the resistance to *Fusarium* entry into the leaf epidermis in Arabidopsis plants. We discovered that the *AtERF71* expression was significantly induced by inoculation with *F. graminearum*. This induction of *AtERF71* was suppressed in the *ein3-1* mutant. Enhanced disease resistance was observed in the leaves of the *aterf71* mutant when compared with wild type. In addition, the expression levels of the JA/ET-responsive *PDF1.2* gene were significantly down-regulated in the *aterf71* mutant after inoculation with *F. graminearum*. Taken together, these results indicate the possible involvement of *AtERF71* in disease tolerance to *F. graminearum* in Arabidopsis plants.

Key words: Arabidopsis, disease resistance, ethylene response factor, *Fusarium*.

Introduction

Plants face the risk of interference by pathogens including bacteria, fungi, and viruses at all times. Plant pathogens are broadly classified into the two following groups on the basis of their lifestyles: biotrophs, which absorb nutrients from the living tissues of host plants, and necrotrophs, which kill the host tissues and then feed on the dead cells (Glazebrook 2005). However, both properties characterize the hemibiotrophic fungus, *Fusarium graminearum*, which is one of the causal pathogens of Fusarium head blight (FHB), a severe disease that afflicts wheat and barley crops worldwide. FHB causes losses in not only cereal production but also food quality. Specifically, this quality loss entails the contamination of grains with mycotoxins (McMullen et al. 1997). Furthermore, grains contaminated with mycotoxin can cause serious health problems when ingested by humans and animals (Desjardins and Proctor 2007; Zain 2011). Despite several approaches being attempted to control FHB, commercial cereal cultivars showing high FHB resistance remain unavailable (McMullen et al. 2012).

It is established that *F. graminearum* can infect both the leaves and flower organs of *Arabidopsis thaliana* (Makandar et al. 2010; Urban et al. 2002). Therefore, this plant has been investigated for signaling pathways that may lead to plant disease resistance against *Fusarium* species such as *F. graminearum*. This resistance was positively controlled via the salicylic acid (SA)-dependent signaling pathway in Arabidopsis and wheat plants (Makandar et al. 2010, 2012). On the contrary, ethylene (ET) and jasmonic acid (JA) negatively regulated host plant resistance against *F. graminearum* (Chen et al. 2009; Makandar et al. 2010). Although ET is a simple gaseous hormone, it plays multiple roles in regulating plant growth and development, such as vegetative growth, the senescence of leaves, flowers,

Abbreviations: AP2, APETALA2; DPI, Day Post Inoculation; ET, Ethylene; EIN, Ethylene-Insensitive; ERF, Ethylene Response Factor; FHB, Fusarium Head Blight; HPI, Hour Post Inoculation; JA, Jasmonic Acid; *PDF1.2*, Plant Defensin1.2; PR, Pathogenesis related; RT-qPCR, Reverse Transcriptase-quantitative Polymerase Chain Reaction; SA, Salicylic Acid; T-DNA, Transfer DNA; TPB, Trypan Blue.

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and fruits (Iqbal et al. 2017), and adaptation to abiotic stresses, such as water-deficits (Gu et al. 2007) and salinity stress (Tao et al. 2015). The perception of ET by its receptors leads to the activation of downstream transcription factors, namely ET-insensitive 3 (EIN3) and ethylene response factor (ERF) (Huang et al. 2016; van Loon et al. 2006). The AP2/ERF superfamily constitute one of the largest plant transcription factors (Nakano et al. 2006) since they are characterized by conserved AP2/ERF DNA-binding domains comprising 57–66 amino acids (Okamuro et al. 1997). The ERFs regulate the transcription of the ET-responsive downstream genes via the GCC-box or related *cis*-elements (Ohme-Takagi and Shinshi 1995). It was recently reported that some ERFs belonging to group VII of AP2/ERF family are involved in both biotic and abiotic stress responses, with *RAP2.12*, *RAP2.2*, and *RAP2.3* active in low oxygen, oxidative, and osmotic responses, respectively (Papdi et al. 2015). *RAP2.2* also participates in disease resistance to *Botrytis cinerea*, in that *RAP2.2* overexpression induced the expression of *PDF1.2* and *PR3*, which led to increased resistance to this fungal pathogen (Zhao et al. 2012).

In a prior study, we reported that a nicotine amide mononucleotide (NMN) pretreatment suppressed the ET signaling pathway and enhanced disease resistance against *F. graminearum* (Miwa et al. 2017). Microarray analysis findings showed that expression of seven *AtERF* genes was down-regulated by NMN-pretreatment of leaves inoculated with *F. graminearum*. Among them, *AtERF71* was reportedly involved in several abiotic stress responses, such as the osmotic stress response and hypoxia (Park et al. 2011). In this study, we analyzed the roles of *AtERF71* in disease resistance to the hemibiotrophic pathogen, *F. graminearum*. Expression of *AtERF71* was highly induced by inoculation with *F. graminearum*; but *AtERF71* expression was significantly down-regulated in the *ein3-1* mutant, thus indicating that *EIN3* was involved in the regulation of the *AtERF71*. Finally, we showed that *AtERF71* regulates disease tolerance against *F. graminearum* in leaves.

Materials and methods

Plant materials and growth conditions

The Colombia-0 (Col-0) ecotype of *A. thaliana* (L.) Heynh was used as the wild type. Two ET-insensitive mutants, *ein2-1* and *ein3-1*, and the T-DNA insertional *aterf71* mutant (SALK 052858) were obtained from the Arabidopsis Biological Resource Center. The T-DNA insertion and homozygous genotype were confirmed by PCR that used the *LBb1* primer (5′-GCG TGG ACC GCT TGC TGC AAC T-3′), *aterf71*_T-DNA_LP (5′-AAG AAA GCG TTA TGG TTC AAA TG-3′) and *aterf71*_T-DNA_RP (5′- CGA CGG TGT TTA GTG TGT TTG-3′). Arabidopsis seeds were sown onto soil, and incubated at 4°C in the dark for 2 days, after which they were grown at 22°C under a 16-h/8-h light/dark cycle.

Fungal materials and growth conditions

This study used the *F. graminearum* strain H3 (Asano et al. 2012). The *F. graminearum* conidia were prepared as described previously (Miwa et al. 2017). The conidia were collected by centrifugation and washed with a phosphate-buffered saline solution at least three times. To calculate the concentration of conidia they were counted using a hemocytometer.

Inoculation assays of Fusarium graminearum

The drop inoculation method was used in this study. Specifically, 5 μl of conidia solutions (5×10^{5} conidia ml^{-1}) with 0.01% (v/v) silwet L77 were dropped onto the surface of 3-week-old Arabidopsis leaves. The inoculated leaves were then incubated at 22°C under high humidity in plastic boxes. To maintain this condition of high humidity, the boxes were covered with cling wrapping. After 3 days, these wraps were removed to reduce the humidity and the inoculated plants incubated for 2 days. At 3 days post inoculation (DPI), the inoculated leaves were photographed and then harvested for further analysis. Thirty-two to forty inoculated leaves in each genotype or time point were divided into 3–4 groups, and then subjected to the fungal gDNA quantification and expression study.

Scoring of disease symptoms

Symptoms of disease were scored based on observations of the inoculated leaves. The disease severity of inoculated leaves were classified into four categories (Miwa et al. 2017). A total of 32–40 inoculated leaves per genotype were used for this disease scoring.

Trypan blue (TPB) staining

Following Tsutsui et al. (2009), the hyphae of inoculated leaves were stained with trypan blue and these TPB-stained leaves were de-stained by a chloral hydrate solution. The resulting leaves were mounted onto a glass slide with 50% glycerol for observation under a microscope (OLYMPUS BX-50; Olympus Optical Co., Tokyo, Japan).

DNA isolation and quantification of F. graminearum gDNA

Genomic DNA (gDNA) was isolated with a Nucleon Phytopure Kit (GE Healthcare, Tokyo, Japan). The amounts of fungal gDNA in inoculated leaves were quantified by qPCR, as described previously (Miwa et al. 2017). The primer sets for the Arabidopsis *Act2/8* and *Fusarium EF-1α* genes were used to quantify the plant gDNA and fungal gDNA, respectively (Miwa et al. 2017).

RT-PCR and RT-qPCR analyses

Total RNA of the wild type and mutant leaves were extracted with an Agilent Plant RNA Isolation Mini Kit (Agilent Technologies, CA, USA), according to the manufacturer's instructions. The cDNA was prepared using 1 μg of total

RNAs and the PrimeScript RT Reagent Kit (Takara Bio, Shiga, Japan). RT-PCR was carried out with Quick Taq HS DyeMix (TOYOBO Co., Ltd., Osaka, Japan), and the RT-qPCR analysis was performed as described by Miwa et al. (2017) with the following pairs of primers for *AtERF71*: *AtERF71*_Fw (5′-GTC TGG CTT GGC ACA TTC AAA AC-3′) and *AtERF71*_Re (5′-CCA TCA GGT CCT CCG ATA AGC TC-3′); other primer sets as described previously.

Results

ET signaling negatively regulates resistance to Fusarium entry into the leaf epidermis in Arabidopsis plants

ET-insensitive mutants, such as *ein2-1* and *ein3-1*, have been reported to show enhanced disease resistance against *F. graminearum* when wounded sites of detached leaves in aseptically grown plants were inoculated with conidia solutions containing 75 μM of DON (Chen et al. 2009). In this study, the conidia solutions without trichothecenes were dropped onto the intact leaves of soil-grown wild type plants, as well as the *ein2-1* and *ein3-1* mutants. Most of the wild type leaves exhibited severe disease symptoms, as shown in Figure 1A, 1B, Supplementary Figure S1A, and S1B, whereas the disease severity was reduced in the leaves of *ein2-1* and *ein3-1* mutants. In fact, fungal gDNA significantly decreased in the leaves of *ein2-1* and *ein3-1* mutants compared with that of wild type plants (Figure 1C, Supplementary Figure S1C). These results suggested that ET signaling negatively regulates plant resistance to *Fusarium* entry into the leaf epidermis of Arabidopsis plants.

Next, we examined the expression of the JA/ET-responsive *PDF1.2* gene in the leaves of *ein3-1* mutant inoculated with *F. graminearum*. Compared with the wild type, the *PDF1.2* gene were significantly down-regulated in the *ein3-1* mutant (Figure 2). The EIN3 was involved in the regulation of this gene expression after inoculation with *F. graminearum*. Our prior study had shown that seven *AtERF* genes are down-regulated by NMN-pretreatment of leaves inoculated with *F. graminearum*, with *AtERF71* clearly down-regulated (Miwa et al.

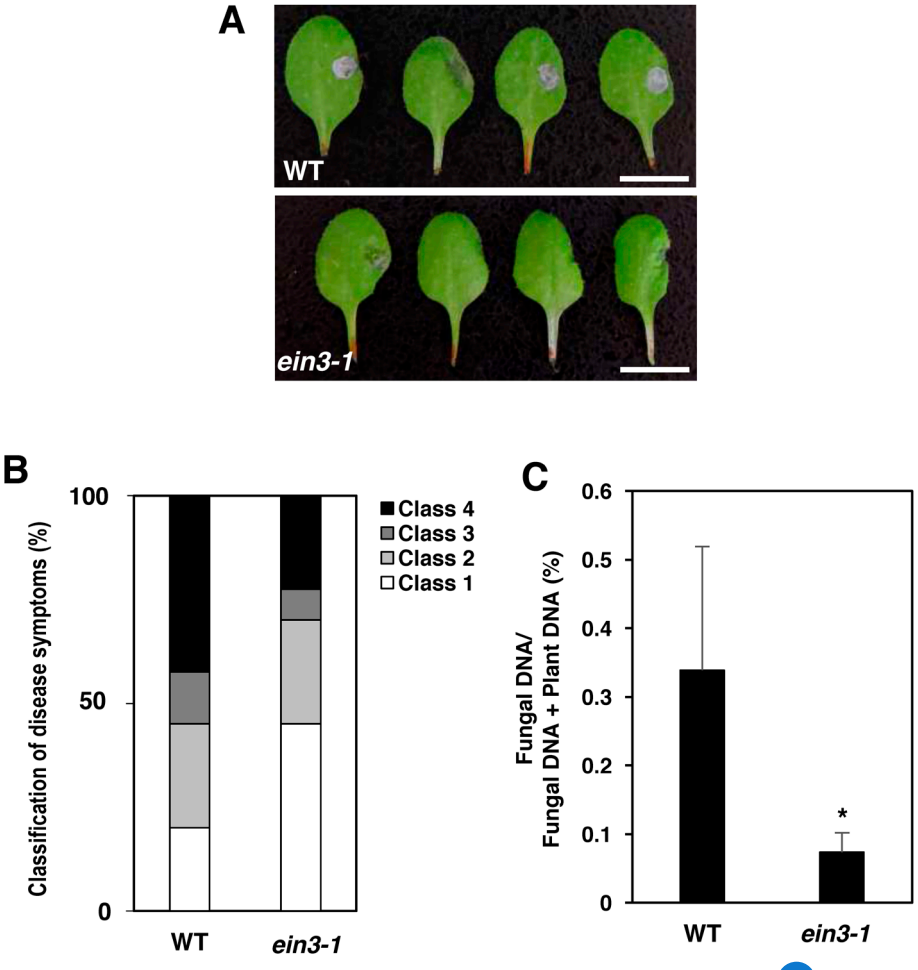

Figure 1. Enhanced disease resistance of the *ein3-1* mutant. (A) Photographs of representative inoculated leaves of the wild type and *ein3-1* mutant Arabidopsis plants at 5 dpi. (B) Disease symptoms were evaluated by classifying the visible symptoms in *F. graminearum*-inoculated leaves. Class 1: normal, Class 2: color change, Class 3: partial aerial mycelium, Class 4: expanded aerial mycelium. (C) The gDNA amount of *F. graminearum* in the inoculated leaves were quantified by qPCR. Error bars indicate one standard deviation (n=3–4). The scale bars represent 1 cm. Student's t-test: * $0.01 < p < 0.05$.

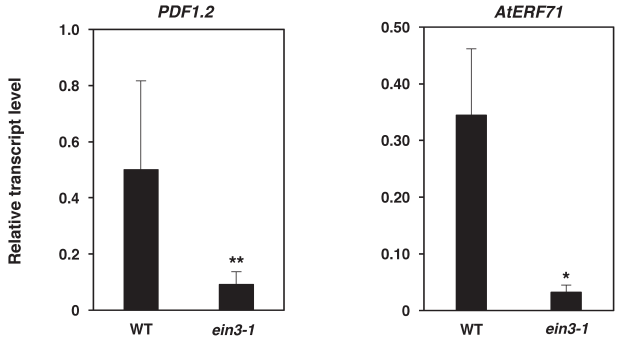

Figure 2. Expression of *AtERF71*, and *PDF1.2* of the *ein3-1* mutant. RT-qPCR analysis of *AtERF71*, and *PDF1.2* expression in the *F. graminearum*-inoculated leaves of the *ein3-1* mutant and wild type Arabidopsis. The *ACTIN2/8* gene was used as the reference gene. Data show the mean for each genotype. Error bars indicate one standard deviation (n=3–4). Student's t-test: * $0.01 < p < 0.05$, ** $p < 0.01$.

2017). Hence, we examined whether or not *AtERF71* expression is regulated by EIN3. Figure 2 shows that the *AtERF71* gene expression was significantly suppressed in the inoculated leaves of *ein3-1* mutant when compared with those of wild type. This result suggested that EIN3 was also involved in the expression of *AtERF71* after inoculation with *F. graminearum*.

Expression of AtERF71 was induced by inoculation with F. graminearum.

We also analyzed the expression pattern of *AtERF71* in the Arabidopsis leaves after inoculation with *F. graminearum*. Leaves of the 3-week-old wild-type plant were used to investigate *AtERF71* gene expression after the drop inoculation with *F. graminearum*. A time course study at 0, 6, 24, 48, 72, and 120 h post inoculation (hpi) was performed by the RT-PCR analysis. Figure 3A shows that the expression of *AtERF71* did not change from 0 to 24 hpi, however, *AtERF71* expression was induced at 48 hpi, and then maintained at a high level until 120 hpi. In addition, the induction of *AtERF71* by inoculation with *F. graminearum* was confirmed using RT-qPCR. As shown in Figure 3B, the expression of *AtERF71* significantly increased at 72 hpi. This result also suggested that *AtERF71* participates in the plant defense response against *F. graminearum*.

The aterf71 mutant displayed an enhanced resistance phenotype against F. graminearum in leaves of Arabidopsis plants

We tested whether or not *AtERF71* contributed to disease resistance to *F. graminearum*. For this purpose, the T-DNA insertional *aterf71* mutant was used (Figure 4A). Its leaves and those of the wild type were drop-inoculated with the conidia of *F. graminearum*. Large lesions and expanded aerial hyphae were often observed in the wild type plants at 5 dpi (Figure 4B). However, the lesions and aerial hyphae of the *aterf71* mutant were restricted to the

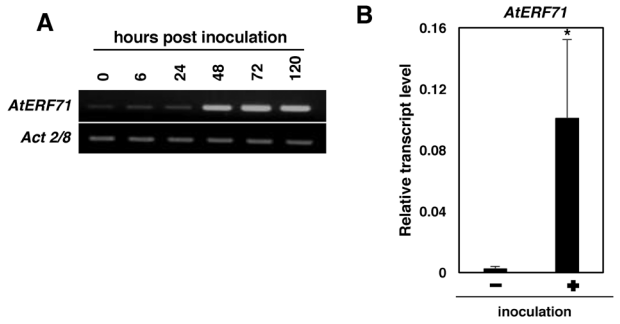

Figure 3. Expression of the *AtERF71* gene was induced by the inoculation of *F. graminearum*. Leaves of 3-week-old wild type Arabidopsis plants were inoculated with conidia solutions and then incubated for various lengths of time. Expression levels of the *AtERF71* gene were analyzed by RT-PCR (A) and RT-qPCR (B). The *ACTIN2/8* gene was used as the reference gene. The non-inoculated leaves of the wild type served as a control in (B). Error bars indicate one standard deviation (n=4). Student's t-test: * $0.01 < p < 0.05$.

inoculation site (Figure 4C). TPB-stained dead cells were decreased in the *aterf71* mutant compared with those of the wild type (Figure 4D, E). As shown in Figure 4F, the scoring of disease symptoms indicated the absence of an expanded aerial mycelium in the *aterf71* mutant, whereas approximately 40% of the inoculated wild type showed an expanded aerial mycelium. Furthermore, we quantified the amounts of fungal genomic DNA in the inoculated Arabidopsis leaves using qPCR. Figure 4G shows that the amount of fungal gDNA decreased in the *aterf71* mutant compared with that of the wild type plant. Thus, the *aterf71* mutant plant showed enhanced disease resistance to *F. graminearum* in its leaves.

Expression of JA/ET-responsive gene was suppressed in the aterf71 mutant

We further monitored the expression of some defense marker genes in the leaves of the *aterf71* mutant inoculated with *F. graminearum*. As shown in Figure 5, in the *aterf71* mutant the expression of ET-responsive *PDF1.2* gene were significantly down-regulated. In contrast, the expression of the SA-inducible *PR1* did not significantly alter between the *aterf71* mutant and wild type after inoculation with *F. graminearum*. These findings suggest that *AtERF71* positively regulates the ET-responsive genes in Arabidopsis leaves inoculated with *F. graminearum*.

Discussion

It is established that the ET signaling pathway positively regulates disease resistance to various necrotrophic pathogens such as *Botrytis cinerea* (van Loon et al. 2006). Therefore, the constitutive expression of some activator-type *ERFs* could confer an enhanced disease resistance against these necrotrophic pathogens. In contrast, we showed that the knockout mutant of *AtERF71* induced

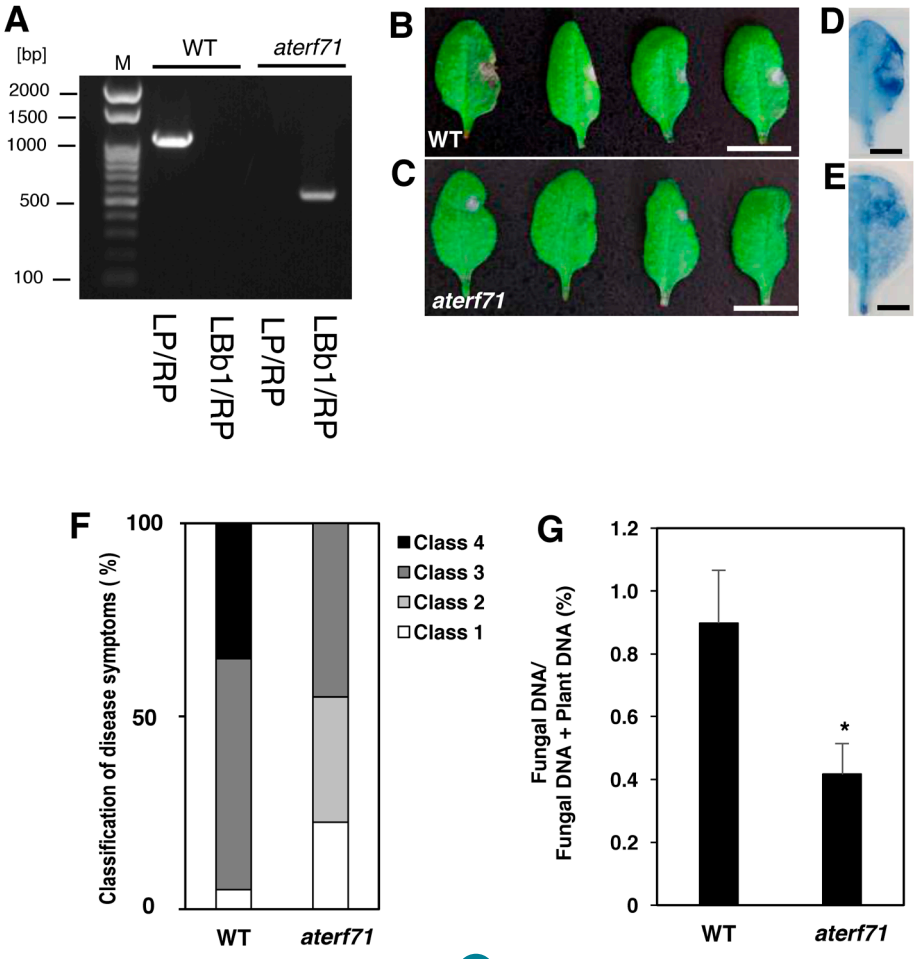

Figure 4. Enhanced disease resistance of the *aterf71* mutant. (A) Insertion of the T-DNA was verified by PCR in the *aterf71* mutant. Photographs of representative Arabidopsis leaves in (B) wild type and (C) *aterf71* at 5 dpi. (D and E) TP staining of *F. graminearum*-inoculated leaves. (F) Disease severity were evaluated by the classification of disease symptoms in *F. graminearum*-inoculated leaves (n=32–40). Class 1: normal, Class 2: color change, Class 3: partial aerial mycelium, Class 4: expanded aerial mycelium. (G) The gDNA amount of *F. graminearum* in the inoculated leaves were quantified by qPCR. Error bars indicate one standard deviation (n=3–4). The scale bars represent 1 cm (B, C), 0.5 cm (D, E). Student's t-test: * $0.01 < p < 0.05$.

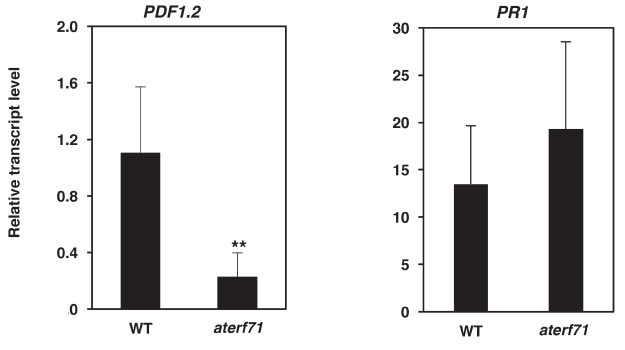

Figure 5. Expression of *PDF1.2*, and *PR1* of the *aterf71* mutant. RT-qPCR analysis of *PDF1.2*, and *PR1* expression levels in the *graminearum*-inoculated leaves of the Arabidopsis wild type and the *aterf71* mutant. *ACTIN* was used as the reference gene. Data show the mean for each genotype. Error bars represent one standard deviation (n=3). Student's t-test: ** $p < 0.01$.

disease resistance to *F. graminearum*. As stated above, the SA signaling pathway positively regulates disease resistance against *F. graminearum* in both Arabidopsis and wheat plants (Makandar et al. 2010), whereas the ET signaling pathway negatively regulates it (Chen et al. 2009). Conversely, the *aterf71* mutant contributed to its enhanced disease resistance against *F. graminearum*. The constitutive expression of *ERF1* (*AtERF100*), belonging to the group IX ERF family, activates the ET signaling pathway and strengthened disease resistance to some necrotrophic pathogens, namely *Botrytis cinerea*, *Plactophanerella cucumerina*, and *Fusarium oxysporum* (Berrocal-Lobo and Molina 2004; Berrocal-Lobo et al. 2002). However, the *ERF1*-overexpressed plants reduced disease tolerance against a bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (Berrocal-Lobo et al. 2002); hence negative cross talk between the SA and ET signaling pathways was observed in the *ERF1*-overexpressed plants. Similarly, other group IX ERF family proteins were also found to positively regulate plant tolerance against necrotrophic pathogens (Huang et al. 2016). In addition, the overexpression of *RAP2.2* (*AtERF75*), a member of the group VII ERF

family, also activated expression of *PDF1.2* and enhanced disease resistance against *Botrytis cinerea* (Zhao et al. 2012). It is likely that the group VII ERF family proteins have redundant functions in biotic and abiotic stress adaptations in plants. However, the *aterf71* single mutant could induce the disease tolerance to *F. graminearum* and suppress the expression of *PDF1.2* after pathogen inoculation. As shown in Figure 5, *PR1* gene expression in the wild type did not differ from the *aterf71* mutant after inoculation with *F. graminearum*. The cross talk between the SA and ET signaling pathways was not observed at this time point in the inoculated leaves of the *aterf71* mutant. However, the further studies such as time course study and mutant analysis are necessary to examine this cross talk.

In this study, we analyzed the involvement of *AtERF71* gene in the disease resistance against *Fusarium graminearum*. We found that the expression of *AtERF71* gene is significantly induced by inoculation of *F. graminearum*. As stated above, the Arabidopsis genome has a large number of ERF family genes. Many ERFs are belonged to group VII family. However, we revealed that the knock-down of single *ERF* gene contributed the disease resistance against *F. graminearum*. In addition, the *AtERF71* gene was also involved in the expression of JA/ET-responsive *PDF1.2* genes in leaves after inoculation with *F. graminearum*. Our findings are very important steps to understand molecular mechanism of disease resistance and to improve disease injury by *Fusarium* species in plants.

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