The *Arabidopsis thaliana* gene AtERF019 negatively regulates plant resistance to *Phytophthora parasitica* by suppressing PAMP-triggered immunity

Wenqin Lu | Fengyan Deng | Jinbu Jia | Xiaokang Chen | Jinfang Li | Qujiang Wen | Tingting Li | Yuling Meng | Weixing Shan

**State Key Laboratory of Crop Stress Biology for Arid Areas and College of Plant Protection, Northwest A&F University, Yangling, China**

**State Key Laboratory of Crop Stress Biology for Arid Areas and College of Life Sciences, Northwest A&F University, Yangling, China**

**Institute of Plant and Food Science, Department of Biology, Southern University of Science and Technology, Shenzhen, China**

**State Key Laboratory of Crop Stress Biology for Arid Areas and College of Horticulture, Northwest A&F University, Yangling, China**

**State Key Laboratory of Crop Stress Biology for Arid Areas and College of Agronomy, Northwest A&F University, Yangling, China**

**Correspondence**
Weixing Shan and Yuling Meng, State Key Laboratory of Crop Stress Biology for Arid Areas and College of Agronomy, Northwest A&F University, Yangling, Shaanxi 712100, China. Email: wxshan@nwafu.edu.cn; mengyuling@nwafu.edu.cn

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**Abstract**
*Phytophthora* species are destructive plant pathogens that cause significant crop losses worldwide. To understand plant susceptibility to oomycete pathogens and to explore novel disease resistance strategies, we employed the *Arabidopsis thaliana*–*Phytophthora parasitica* model pathosystem and screened for *A. thaliana* T-DNA insertion mutant lines resistant to *P. parasitica*. This led to the identification of the resistant mutant 267-31, which carries two T-DNA insertion sites in the promoter region of the *ethylene-responsive factor 19* gene (*ERF019*). Quantitative reverse transcription PCR (RT-qPCR) assays showed that the expression of *ERF019* was induced during *P. parasitica* infection in the wild type, which was suppressed in the 267-31 mutant. Additional *erf019* mutants were generated using CRISPR/Cas9 technology and were confirmed to have increased resistance to *P. parasitica*. In contrast, *ERF019* overexpression lines were more susceptible. Transient overexpression assays in *Nicotiana benthamiana* showed that the nuclear localization of *ERF019* is crucial for its susceptible function. RT-qPCR analyses showed that the expression of marker genes for multiple defence pathways was significantly up-regulated in the mutant compared with the wild type during infection. Flg22-induced hydrogen peroxide accumulation and reactive oxygen species burst were impaired in *ERF019* overexpression lines, and flg22-induced MAPK activation was enhanced in *erf019* mutants. Moreover, transient overexpression of *ERF019* strongly suppressed INF-triggered cell death in *N. benthamiana*. These results reveal the importance of *ERF019* in mediating plant susceptibility to *P. parasitica* through suppression of pathogen-associated molecular pattern-triggered immunity.

**Keywords**
*Arabidopsis thaliana*, oomycete, *Phytophthora parasitica*, susceptibility, transcription factor
1 | INTRODUCTION

The “plant destroyer” Phytophthora causes devastating disease in a large number of crops and forest seedlings worldwide. For example, potato late blight caused by Phytophthora infestans can lead to severe decreases in production and also serious economic losses (Haverkort et al., 2008). P. sojae, P. ramorum, P. parasitica, and P. capsici can also cause important agricultural diseases such as soybean root rot and oak stagnation (Tyler, 2002; Grünwald et al., 2012; Lamour et al., 2012; Meng et al., 2014; Kamoun et al., 2015; Panabères et al., 2016).

Plants have sufficient weapons to repel pathogen attacks, but need to recognize the pathogen in time, which mainly occurs through two different systems. One is referred to as PAMP-triggered immunity (PTI), which is activated by transmembrane pattern-recognition receptors (PRRs) through recognition of pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) (Jones and Dangl, 2006; Boller and Felix, 2009; Bigeard et al., 2015; Boutrot and Zipfel, 2017) and initiates a series of immune responses, including reactive oxygen species (ROS) bursts, callose deposition, biosynthesis of phytohormones (such as salicylic acid [SA], jasmonate [JA], and ethylene [ET]), and the expression of a large number of defence-related genes (Zipfel et al., 2004; Naito et al., 2008). The other system is called effector-triggered immunity (ETI), which is based on the specific recognition between pathogen effectors and plant resistance (R) proteins, according to the gene-for-gene theory. This recognition leads to a rapid and localized hypersensitive response (HR, cell death) at infection sites and inhibits pathogen colonization (Jones and Dangl, 2006).

Although priming of immunity responses when pathogens attack is the key to resistance, excessive and inappropriate defence responses interfere with the growth of a plant. To mitigate the trade-off between growth and defence, plants have evolved a series of mechanisms to negatively regulate defence pathways. For example, the rice Pigm locus confers durable resistance to the fungus Magnaporthe oryzae without yield penalty; this is achieved through epigenetic regulation of two antagonistic receptors, PigmR and PigmS, encoded by this locus (Deng et al., 2017). PigmR confers broad-spectrum resistance, whereas PigmS competitively attenuates PigmR homodimerization to suppress resistance (Deng et al., 2017). In addition, growth-related hormones, auxin, brassinosteroids (BRs), and gibberellins (GAs) can directly or indirectly negatively regulate PTI-mediated defence (Yamada, 1993; Chen et al., 2007; Albrecht et al., 2012; Jaillais and Vert, 2012). However, these negative regulatory pathways can be hijacked by effectors secreted by pathogens to promote infection (Jones and Dangl, 2006). For example, plant cinnamyl alcohol dehydrogenase 7 (CAD7), which is involved in the negative regulation of plant resistance to Phytophthora pathogens including P. infestans, P. parasitica, and P. capsici, is a common target of multiple AVR3a-like effectors from Phytophthora pathogens. These effectors suppress PTI responses by stabilizing CAD7 (Li et al., 2019). In potato, StVIK and StKRBP1 are targeted by the RXLR effectors P17316 and P04089 from P. infestans, respectively, to facilitate invasion (Wang et al., 2015; Murphy et al., 2018). Therefore, appropriate manipulation of negative regulators of plant immunity has the potential to improve broad-spectrum disease resistance.

To explore the mechanisms by which negative regulators suppress plant resistance to pathogens, we used a model compatible system between Arabidopsis thaliana and the oomycete pathogen P. parasitica (Wang et al., 2011b) to screen for A. thaliana T-DNA insertion mutants resistant to P. parasitica infection. We identified an erf019 mutant that showed less susceptibility to P. parasitica. Our analysis revealed that ERF019 negatively regulates plant defence responses to Phytophthora pathogens by suppressing PAMP-triggered immunity, thus acting as an important regulator in balancing plant disease resistance and growth.

2 | RESULTS

2.1 | Identification of an erf019 mutant, 267-31, that limits colonization of P. parasitica

To identify genes that negatively regulate defence against P. parasitica infection, we screened nearly 10,000 independent Arabidopsis T-DNA insertion lines (Zhang et al., 2005) to identify mutants involved in limiting the colonization of P. parasitica. This led to the identification of the mutant 267-31 (Figure 1a,b). In comparison to wild-type Col-0, growth of the pathogen, P. parasitica Pp016 (Wang et al., 2011b; Zhang et al., 2011), was much more restricted in 267-31 at 3 days postinoculation (dpi) (Figure 1a). Consistent with this finding, quantification of P. parasitica colonization in infected Arabidopsis leaves revealed less colonization in 267-31 (Figure 1b).

Thermal asymmetric interlaced (TAIL)-PCR (Liu et al., 1995) was applied to obtain sequences flanking the T-DNA insertion sites in 267-31. Sequence analysis showed that there were two T-DNA insertion sites located 309 and 376 bp upstream of the ERF019 translation start codon, respectively (Figure 1c). The two T-DNA insertion fragments were adjacent and in opposite orientations. Quantitative reverse transcription (RT) PCR (RT-qPCR) analysis showed that the ERF019 transcript levels were dramatically lower in 267-31 than in the wild-type Col-0 (Figure 1d). Thus, ERF019 might play a negative role in resistance against P. parasitica.

2.2 | ERF019 contributes to plant susceptibility to P. parasitica

To confirm that ERF019 contributes to plant susceptibility to P. parasitica, we used the CRISPR/Cas9-mediated genome-editing tool to knock out the ERF019 gene. Two target sites in the exon of ERF019 were chosen (Figure 1e), and the corresponding sgRNA/Cas9 vectors were transformed into the wild-type Col-0 via Agrobacterium tumefaciens-mediated transformation. The mutations at the target sites in the CRISPR/Cas9 transformants were examined using PCR and DNA sequencing analysis, which showed that approximately 90% of T₃ transformants carried mutations at one sgRNA target site...
FIGURE 1 The erf019 mutants of Arabidopsis thaliana limit Phytophthora parasitica colonization. (a) Trypan blue staining showing the disease symptoms of the erf019 T-DNA insertion mutant 267-31 and the wild-type Col-0 infected with P. parasitica strain Pp016. The concentration of zoospore suspensions was adjusted to 200 zoospores/µl. Detached leaves of 4-week-old A. thaliana seedlings were drop-inoculated with 10 µl P. parasitica zoospores (200 zoospores/µl) and photographed at 3 days postinoculation (dpi). (b) Quantitative reverse transcription PCR (RT-qPCR) quantification of pathogen colonization. Total genomic DNA from P. parasitica-infected regions was isolated at 2 dpi. Quantitative PCR (qPCR) with primers specific for the A. thaliana UBC9 gene (AtUBC) and the P. parasitica UBC gene (PpUBC) was used to determine P. parasitica biomass in infected plant tissues. The relative P. parasitica biomass was calculated by PpUBC/AtUBC and normalized using the value of Col-0. (c) Two T-DNAS were inserted in the promoter region of ERF019. (d) RT-qPCR analysis to quantify the expression of ERF019 in 2-week-old seedlings of Arabidopsis mutant 267-31 and wild-type Col-0. UBC9 was used as the internal control. Bars represent standard errors from three biological replicates and asterisks indicate statistical significance based on t test (**p < .01). (e) Targeted indel mutations at the ERF019 gene. Representative sequences of CRISPR/Cas9-based knockout mutant alleles identified from transgenic plants expressing sgRNA targeting ERF019. (f) Disease symptoms of CRISPR/Cas9-based knockout mutants and the wild-type Col-0 infected with P. parasitica. The concentration of zoospore suspensions was adjusted to 200 zoospores/µl. Detached leaves of 4-week-old Arabidopsis plants were drop-inoculated with 20 µl P. parasitica zoospores (200 zoospores/µl) and photographed at 2 dpi. (g) Pathogen colonization in knockout lines. Detached leaves of 4-week-old Arabidopsis were drop-inoculated with 20 µl zoospores of P. parasitica transformant 1121, which stably expresses green fluorescent protein (GFP), and visualized under a fluorescence microscope at 2 dpi. Green fluorescence indicates P. parasitica hyphae, autofluorescence from leaf tissue is visible as red signal. The white bars indicate 500 µm. (h) Quantification of P. parasitica biomass in inoculated leaves of knockout lines by qPCR. Error bars represent SD, and asterisks indicate statistical significance based on t test (*p < .05; **p < .01). Similar results were obtained from at least three individual experiments.
while no mutations were found at the other target site. Of three individual homozygous knockout lines chosen for further analysis, two contain a 1-bp insertion (ko13-4 and ko58-7) and one contains a 4-bp deletion (ko26-14) in the coding region of ERF019 (Figure 1e), which results in a frameshift mutation and predicted truncated protein (Figure S1). Phenotypic observations revealed that none of these three erf019 knockout lines had obvious morphological abnormalities (Figure S2), suggesting ERF019 is not essential for plant growth and development. When inoculated with 1121, a transformant of Pp016 stably and widely expressing ER-rendered green fluorescent protein (GFP) under control of the constitutive Hsp70 promoter of Bremia lactucae (Zhang et al., 2011), the CRISPR/Cas9-edited mutants had attenuated P. parasitica leaf colonization, with significantly smaller water-soaked lesions compared with those in the wild-type Col-0 (Figure 1f). Microscopic observation showed that fewer GFP-expressing hyphae colonized the CRISPR/Cas9-edited mutants compared with the wild-type Col-0 (Figure 1g). In addition, quantification of P. parasitica biomass revealed that CRISPR/Cas9-edited mutants exhibited limited colonization of P. parasitica (Figure 1h).

To further clarify the function of ERF019 in plant susceptibility, we transformed an ERF019-overexpression (OE) construct, in which ERF019 expression is under the control of constitutive cauliflower mosaic virus (CaMV) 35S promoter, into the Col-0 background. Three ERF019-OE lines, OE71, OE72, and OE74, were selected for further analysis following confirmation by RT-qPCR analysis that the ERF019 transcript levels were significantly increased in these lines (Figure 2d). Infection assays with P. parasitica revealed that ERF019-OE plants were more susceptible than the wild-type Col-0. Two days after pathogen infection, ERF019-OE lines developed much larger water-soaked lesions than the wild-type Col-0 (Figure 2a). Heavier hyphal colonization was also visible in ERF019-OE plants when infected with the P. parasitica transformant 1121, which stably expresses GFP (Figure 2b). In addition, both a trypan blue staining assay and a quantification of P. parasitica biomass revealed that ERF019-OE plants exhibited enhanced disease susceptibility to P. parasitica (Figure 2c,e). These results confirmed that ERF019 negatively regulates plant resistance to P. parasitica.

**FIGURE 2** The ERF019-overexpression (ERF019-OE) Arabidopsis thaliana plants were more susceptible to Phytophthora parasitica. (a) Disease symptoms of ERF019-OE plants infected with 10 µl P. parasitica zoospores (200 zoospores/µl) and photographed at 2 days postinoculation (dpi). The concentration of zoospore suspensions was adjusted to 200 zoospores/µl using microscopy. (b) Pathogen colonization on ERF019-OE plants. Detached leaves of 4-week-old A. thaliana plants were drop-inoculated with 10 µl zoospores of P. parasitica transformant 1121, which stably expresses green fluorescent protein (GFP), and visualized under a fluorescence microscope at 2 dpi. Green fluorescence indicates Phytophthora hyphae. (c) Trypan blue staining of ERF019-OE plants infected with P. parasitica. Plant cells infected by pathogen were coloured. (d) Quantitative reverse transcription PCR analysis showed the accumulation of ERF019 transcripts in rosette leaves of ERF019-OE plants. Data represent the ratio of ERF019 expression between ERF019-OE plants and wild-type Col-0. UBQ9 was used as the internal control. Bars represent standard errors from three biological replicates and asterisks indicate statistical significance based on t test (**p < .01). (e) Quantification of P. parasitica biomass in inoculated leaves of ERF019-OE plants by quantitative PCR. Error bars represent SD, and asterisks indicate statistical significance based on t test (*p < .05; **p < .01). Similar results were obtained from at least three individual experiments.
2.3 | ERF019 expression is induced on P. parasitica infection

To examine whether the expression of ERF019 is responsive to P. parasitica infection, we used RT-qPCR to measure the ERF019 expression levels in P. parasitica-inoculated leaves at different time points. The results showed that in Col-0, ERF019 expression was highly induced at 3 hours postinoculation (hpi) and slightly induced at 6 and 12 hpi compared with that in the uninfected leaves and the mock-inoculated controls. The expression levels of ERF019 at 24 hpi were decreased to a level similar to that in the mock-inoculated controls. These observations indicate that ERF019 is responsive to P. parasitica infection. In contrast, the expression level of ERF019 in the 267-31 mutant was significantly lower than that in the wild type, both in the uninfected and infected leaves, though the mutant still produced few detectable transcripts (Figure 3).

2.4 | Nuclear localization of ERF019 is required for its susceptibility function

ERF019 contains an APETALA2 (AP2) domain and is, therefore, predicted as a member of the ethylene-responsive factor (ERF)/AP2 transcription factor family. We transiently co-expressed the ERF019-GFP fusion construct with the nucleus marker H2B-mCherry in Nicotiana benthamiana leaves and found that ERF019-GFP can be observed in both nucleus and cytoplasm (Figure S3). To confirm that the nuclear localization of ERF019 is necessary for its function in promoting pathogen infection, we altered the subcellular localization of ERF019 by fusing it with a nuclear export sequence (NES). A construct expressing ERF019 fused with a mutant NES (nes) was used as a control. We first transiently expressed the ERF019-GFP-nes and ERF019-GFP-nes chimeric proteins under the control of the CaMV 35S promoter in N. benthamiana leaves and observed the subcellular localization by fluorescence microscopy. ERF019-GFP-nes was clearly exported to the cytosol and was barely detectable in the nucleus, while ERF019-GFP-nes displayed a localization pattern similar to that of ERF019-GFP (Figure S4), which indicates that the NES was functional. Next, to examine whether the nuclear export of ERF019 affects its role in plant susceptibility, we transiently expressed these fusion proteins in N. benthamiana leaves and challenged the leaves with P. parasitica. Notably, leaves transiently expressing ERF019-GFP displayed significantly larger infection lesions compared with those expressing the FLAG-GFP control (Figure 4), which is consistent with the role of ERF019 as a negative regulator of plant immunity. ERF019-GFP-nes, but not ERF019-GFP-nes, lost its ability to enhance P. parasitica colonization of N. benthamiana (Figure 4). These results suggest that the nuclear localization of ERF019 is required for its function in promoting colonization of P. parasitica.

2.5 | Expression of defence marker genes is up-regulated in the erf019 mutant

Because ERF019 negatively regulates plant resistance to P. parasitica, we examined the potential role of ERF019 in known defence pathways by measuring the expression of marker genes associated with these pathways in 267-31 and the CRISPR/Cas9-edited erf019 mutant lines (ko13-4, ko26-14). ICS1 (Isochorismate synthase1) and PAL1 (Phenylalanine ammonia lyase 1) are involved in the synthesis of SA (Wildermuth et al., 2001), and PR1 (Pathogenesis-related gene 1) is a well-established marker gene for the SA signalling pathway (Uknes et al., 1992). LOX2 (Lipoxygenase2) is involved in the synthesis of jasmonic acid (JA) (Sasaki et al., 2001), and PDF1.2 (Plant defensin gene 1.2) and VSP2 (Vegetative storage protein 2) are genes that respond to JA (Pieterse et al., 2009). FRK1 (Flg22-induced receptor-like kinase 1) is a core gene that is induced by a conserved 22 amino acid epitope from bacterial flagellin (flg22), and is frequently used to monitor PTI (Shan et al., 2008). ACS2 and ACS6, two members of the ACS gene family that encode 1-amino-cyclopropane-1-carboxylase synthase, are involved in the ethylene biosynthesis (Van der Straeten et al., 1999) and EIN2 (Ethylene-insensitive protein 2) is an important regulator in the ET signalling pathway (Alonso et al., 1999) and ERF6 (Ethylene-responsive factor 6) is an ET-related signalling gene (Moffat et al., 2012). An RT-qPCR assay showed that the expression of defence-related genes was altered in

![Figure 3](image-url) Quantitative reverse transcription PCR analysis for the expression of ERF019 in Arabidopsis thaliana Col-0 and the T-DNA insertion mutant 267-31 at different time points after Phytophthora parasitica inoculation. AtUBC9 was used as the internal control. Bars represent standard errors from three biological replicates and asterisks indicate statistical significance based on t test (*p < .05; **p < .01; ns, not significant). 0 h, uninfected leaves; hpi, hours postinoculation.
the erf019 mutants compared with the wild-type Col-0. In P. parasitica-inoculated plants, the expression levels of ICS1, PR1, VSP2, LOX2, PDF1.2, and FRK1 in 267-31 and the CRISPR/Cas9-edited lines were significantly higher than that in the wild-type Col-0 (Figure 5). The expression of PAL1 was down-regulated on infection, and showed similar level between mutants and the wild-type Col-0 (Figure 5). However, for the marker genes in the ET signalling pathway, the expression levels of ACS6, EIN2, and ERF6 appeared similar between mutants and the wild type, although ACS2 was induced to higher levels in mutants at some time points (Figure 5). Taken together, these results indicate that ERF019 may play an important role in the SA and JA defence signalling pathways but not the ET signalling pathway.

2.6 | ERF019 suppresses PTI responses

Perception of flg22 triggers a series of immunity responses, including an oxidative burst, rapid and transient accumulation of ROS, and the activation of MAP kinases. ERF019 was reported to be highly induced by flg22 (Sano et al., 2014; Huang et al., 2019), which suggests that ERF019 may be involved in flg22-triggered immunity. To test this hypothesis, we used 3,3′-diaminobenzidine-tetrahydrochloride (DAB) staining to detect hydrogen peroxide accumulation in Col-0, the erf019 mutant, and ERF019-OE lines on flg22 treatment. Although no obvious difference was observed between the erf019 mutant and Col-0, our results revealed that the flg22-induced accumulation of hydrogen peroxide was impaired in ERF019-OE lines (OE71, OE72, and OE74) (Figure 6a). We also monitored the flg22-induced accumulation of ROS in erf019 mutants and ERF019-OE lines. The results of this analysis were similar to those of DAB staining: ROS accumulation was impaired in ERF019-OE lines but not in the erf019 mutants (Figure 6a). Moreover, we also found that the activation of MAPK3, MAPK4, and MAPK6, on flg22 treatment, was much stronger in both 267-31 and CRISPR/Cas9 mutants (ko13-14, ko26-14, and ko58-7) than in Col-0 (Figure 6c,d), and the PAMP-triggered MAPK activation was compromised in ERF019-OE lines.
These results suggest that ERF019 negatively regulates PAMP-triggered immunity. To further demonstrate whether ERF019 negatively regulates PTI triggered by Phytophthora elicitors, we tested whether it could inhibit INF1-induced necrosis. We first transiently overexpressed the ERF019 protein in N. benthamiana leaves using A. tumefaciens-mediated transformation and found that ERF019 did not induce necrosis after monitoring for up to 7 days postinfiltration (Figure S5). Next, we co-infiltrated mixtures of A. tumefaciens cultures carrying constructs of elicitors and either ERF019 or FLAG-GFP into 5-week-old N. benthamiana leaves. An HR in N. benthamiana leaves was observed 4 days postinfiltration, and the responses were classified into three categories according to the degree of response: no cell death, partial cell death, and full cell death (Figure 7a). Interestingly, ERF019 expression significantly suppressed the HR induced by INF1 compared with the control FLAG-GFP (Figure 7b,c). We also co-infiltrated ERF019 with the proapoptotic protein elictor Bax and found that ERF019 did not significantly inhibit Bax-induced cell death (Figure 7b,c). These results suggest that ERF019 specifically suppresses Phytophthora elictor INF1-triggered cell death in N. benthamiana. Taken together,

**Figure 5** Defence marker gene expression in the erf019 mutants of Arabidopsis thaliana. Transcript levels of defence-related marker genes in the erf019 mutants (267-31, ko13-4, and ko26-14) and Col-0 were evaluated by quantitative reverse transcription PCR (RT-qPCR) at different times postinoculation. RT-qPCR data are presented as relative transcript level for genes: ICS1 and PAL1, two marker genes for salicylic acid (SA) biosynthesis; PR1, a marker for the SA signalling pathway; LOX2, a marker for jasmonic acid (JA) biosynthesis; PDF1.2 and VSP2, involved in the JA signalling pathway; FRK1, a marker gene for the PAMP-triggered immunity (PTI) pathway; ACS2 and ACS6, two marker genes for ethylene (ET) biosynthesis; and ERF6 and EIN2, involved in the ET signalling pathway. AtUBC9 was used as the internal control and transcript levels relative to Col-0 plants are displayed. Bars represent SE from three biological replicates and asterisks indicate statistical significance based on a two-tailed t test (*p < .05; **p < .01). 0 h, uninfected leaves.
we demonstrated that ERF019 negatively regulates plant resistance by inhibiting PTI.

3 DISCUSSION

The plant diseases caused by Phytophthora spp. pose a great threat to agriculture, highlighting the importance of studies on the mechanisms of plant resistance. Previous studies based on forward genetics or map-based cloning technology have characterized dozens of R genes, which are commonly used in crop resistance breeding. However, R gene-mediated resistance, also described as effector recognition-based resistance, has been frequently overcome by new pathogen races. In contrast, disabling plant disease susceptibility genes (negative regulators of plant resistance) may provide a novel way to achieve durable and broad-spectrum resistance. For example,
loss-of-function of the MLO gene confers broad-spectrum resistance to the powdery mildew fungus (Jorgensen, 1992; Büschges et al., 1997; Piffanelli et al., 2002). The mlo allele has been introduced into European spring barley cultivars, and this has provided robust resistance for nearly four decades (Jorgensen, 1992; Lyngkjær and Carver, 2000), suggesting the great potential of disabling negative regulators in improving crop disease resistance.

Here, we showed the successful use of the compatible system between P. parasitica and A. thaliana to identify negative regulators. We identified the T-DNA insertion mutant 267-31, which was demonstrated to be resistant to P. parasitica without obvious inhibition of growth (Figure 1). Multiple erf019 frameshift mutants generated by CRISPR/Cas9 technology consistently showed enhanced resistance to P. parasitica, while ERF019 overexpression lines were more susceptible (Figures 1 and 2). These results indicate that ERF019 negatively regulates plant resistance to P. parasitica.

The ERF family is a large family of transcription factors in plants, with up to 122 members in Arabidopsis and 139 members in rice (Nakano et al., 2006). ERFs are involved in diverse developmental processes and various responses to environmental stimuli, such as pathogen attack, drought, salt, wounding, UV irradiation, and extreme temperature (Tsutsui et al., 2009; Liu et al., 2012; Licausi et al., 2013; Maruyama et al., 2013). ERF019 was classified into phylogenetic Group II of the Arabidopsis ERF family (Nakano et al., 2006). There are 15 members in this group, which are further classified into three subgroups: IIa, IIb, and Iic. Most genes in subgroups IIA and IIB have been shown to play crucial roles in biotic and abiotic stress responses. For example, overexpression of DEAR1, a subgroup IIA gene, rendered Arabidopsis more resistant to Pseudomonas syringae infection and less tolerant to freezing (Tsutsui et al., 2009). Furthermore, all six genes of subgroup IIA induce cell death in tobacco (Ogata et al., 2013). ERF15, a subgroup IIB gene, has been reported to be a negative regulator of salt and drought tolerance (Lee et al., 2015). Here, we found that the subgroup IIC gene ERF019 negatively regulates plant resistance to P. parasitica. It has also been reported that overexpression of ERF019 delays plant growth and senescence, enhances drought resistance, and increases plant susceptibility to Botrytis cinerea and P. syringae (Scarpeci et al., 2017; Huang et al., 2019). These observations suggest that ERF family subgroup II members may play important roles in plant resistance and abiotic stress, and functional analysis of their orthologous genes in crops will provide potential gene resources for breeding for disease resistance.
Plant PTI responses, including ROS burst, callose deposition, MAP kinase activity, and defence gene induction, are critical for plants to repel pathogen attacks (Bigeard et al., 2015). We show here that the induction of the PTI-related marker gene FRK1 in the 267-31 mutant on P. parasitica infection was stronger than that in the wild type Col-0 (Figure 5). Meanwhile, flg22-induced activation of the MPK3, MPK4, and MPK6 was enhanced in both the 267-31 mutant and erf019 knockout lines when compared with Col-0, and was compromised in ERF019-OE lines (Figure 6). The flg22-induced ROS burst and hydrogen peroxide accumulation were also significantly suppressed in the leaves of ERF019-OE plants (Figure 6), which is consistent with the previous report that flg22-induced callose deposition was significantly impaired in ERF019-OE plants (Huang et al., 2019). Furthermore, we showed that transient overexpression of ERF019 in N. benthamiana suppresses Phytophthora PAMP elicitor INF1-activated cell death (Figure 7). These results demonstrate the critical role of ERF019 in negatively regulating PTI responses.

Plant cell death plays a central role in interactions with hemibiotrophic pathogens, such as Phytophthora species, considering that these pathogens initially develop haustoria to acquire nutrients from living host cells and then subsequently switch to a necrotrophic lifestyle, resulting in the death of the host plant (Lamour et al., 2012). INF1-like proteins are a family of secreted elicitors, which exist widely in Phytophthora. It is reasonable to hypothesize that ERF019 may negatively regulate plant resistance by suppressing cell death, which facilitates the growth of P. parasitica during plant infection. Meanwhile, ERF019 cannot inhibit Bax-induced cell death, like P. sojae effector Avh238 that was reported to inhibit INF1- but not Bax-induced cell death (Wang et al., 2011a). Bax is a proapoptotic member and can translocate into the mitochondrial membrane and trigger the apoptotic process, some features of which resemble plant programmed cell death (Ihara-Ohori et al., 2007). However, compared to Bax-induced cell death, the recognition of INF-1 and the downstream pathway of INF1-triggered cell death may possess some unique features, some of which may be regulated by ERF019. Because the overexpression of ERF019 leads to attenuation of PTI responses, and the silencing of N. benthamiana receptor-like kinase gene SERK3, which encodes a homolog of Arabidopsis BAK1 and plays a key role in PTI by suppressing INF1-induced cell death (Chaparro-Garcia et al., 2011), it is likely that ERF019 suppresses INF1-activated cell death by interfering with the PTI signalling pathway.

Interestingly, the negative regulator ERF019 was induced during P. parasitica infection. Moreover, ERF019 was also highly induced by flg22 (Huang et al., 2019). These observations suggested that ERF019 can be induced during PTI, which in turn inhibits PTI. Previous researchers showed that plant recognition of PAMPs induces both positive and negative PTI signalling pathways. For example, the PAMP-induced MEKK1, MEKK1/2, and MPK4 signalling cascades negatively mediate plant defence responses (Ichimura et al., 2006; Mészáros et al., 2006; Suarez-Rodriµguez et al., 2007; Gao et al., 2008; Qiu et al., 2008; Pitzschke et al., 2009). Thus, ERF019 may be involved in a negative feedback loop that balances growth and resistance.

Our results also showed that the SA biosynthesis-related gene ICS1, SA signalling pathway marker gene PR1, JA signalling marker gene VSP2 and PDF1.2 as well as JA biosynthesis-related gene LOX2 were up-regulated in the erf019 mutant, indicating that the SA and JA signalling pathways are coupled through ERF019. It has been reported that SA is responsible for plant defence against biotrophs, whereas JA or ET is responsible for defence against necrotrophs (Bostock, 2005). However, both the SA and JA signalling pathways have been shown to contribute to basal resistance against P. parasitica (Attard et al., 2010). Interference with SA, JA, or ET signalling in the eds1, eds5, pad4, sid2, ein2, etr1, and jar1 mutants and NahG transgenic plants enhanced plant susceptibility to P. parasitica (Attard et al., 2010). Moreover, the Arabidopsis thaliana Resistant to Phytophthora 5 gene (AtRTP5), which encodes a WD40 repeat domain-containing protein, has been reported to negatively regulate plant resistance to P. parasitica by interfering with the JA and SA signalling pathways (Li et al., 2020). In addition, ERF019 was shown to be induced by OPDA, a cyclopentenone precursor of JA (Taki et al., 2005). The function of ERF019 may be repressed by NINJA (the transcriptional co-repressor Novel INteractor of JAZ), a negative regulator of JA signalling, through protein–protein interaction (Huang et al., 2019). These results suggest a potential role of ERF019 in the JA signalling pathway, which is subjected to complex positive and negative regulation and is coupled with the SA signalling pathway.

Loss of function of a negative regulator of plant resistance may constitutively activate defence responses and reduce plant fitness (Tian et al., 2003; Denancé et al., 2013; Huot et al., 2014). For example, loss of function of the MPK4 gene results in a dwarf phenotype, which is accompanied by elevated SA levels and constitutive expression of pathogenesis-related genes (Petersen et al., 2000). The mek1 mutant and mkk1 mkk2 double mutant also display constitutive defence responses and reduced plant growth (Gao et al., 2008). However, erf019 plants exhibit resistance to P. parasitica without altered plant growth. Consistent with this phenotype, RT-qPCR results showed that the expression of ERF019 was low in adult rosette leaves under normal conditions (Figure 3), suggesting that ERF019 may not be necessary for plant growth. Furthermore, the expression of defence-related marker genes was just slightly up-regulated in the erf019 mutant in the absence of pathogen infection (Figure 5). In addition, after flg22 treatment, activation of MAP kinase was stronger in the erf019 mutants than in the wild-type Col-0 and attenuated in the ERF019-OE plants when compared to the wild-type Col-0 after 10 min of treatment (Figure 6c,d,e). These observations show that PTI responses seem to be amplified in the erf019 mutant without strong constitutive induction of the expression of pathogenesis-related genes, thus increasing resistance without influencing growth.

ERF019 contains a conserved AP2/ERF DNA-binding domain at the N-terminus. There was no conserved motif identified at the C-terminus of ERF019 based on multiple sequence alignment.
analyses of ERF family members (Nakano et al., 2006). The poten-
tially accumulated proteins in the CRISPR/Cas9-edited erf019 mu-
tant lines, which are predicted to be truncated due to the frameshift
mutations from the codon for the 109th amino acid, lost ability to
negatively regulate resistance, indicating a key role of the C-terminus
of ERF019 in immune function. The regions outside the DNA-
binding domain in ERF proteins are generally involved in protein
modification and protein–protein interactions, and are important
for their nuclear localization and transcriptional activities (Nakano
et al., 2006; Licausi et al., 2013). For example, the C-terminal acti-
vation domain, but not the N-terminal DNA binding domain of ERF
protein TINY, interacts with and antagonizes BRASSINOSTEROID
INSENSITIVE1-ETHYL METHANESULFONATE SUPPRESSOR1 (BES1)
in the regulation of drought response (Xie et al., 2019). The tran-
scriptional repressor Novel INteractor of JAZ (NINJA) interacts
with ERF019 and represses its function (Huang et al., 2019). We
speculate that the C-terminus of ERF019 mediates interaction by
other unknown protein factors to regulate plant immunity. Future
efforts to identify the ERF019-interacting proteins will be useful in
understanding the underlying mechanisms of ERF019 in regulating
plant immunity.

Based on our study, we propose that ERF019 plays an important
role in the negative feedback loop that balances growth and resist-
ance on pathogen infection by suppressing PTI and SA/JA defence
responses (Figure 8). Further identification of target genes regulated
by ERF019 will provide insights into the mechanisms of the negative
feedback loop. Identification of loss-of-function alleles of ERF019
and its homologs in crops is a potential strategy for breeding crops
with durable resistance.

4 | EXPERIMENTAL PROCEDUREES

4.1 | Plant materials and growth conditions

The Arabidopsis T-DNA insertion lines were generated (Zhang et al.,
2005) and kindly provided by Dr Jianru Zuo. Arabidopsis ecotype
Col-0 and T-DNA insertion mutants used in this study were grown
at 23°C with 14 hr of light per 24 hr. N. benthamiana plants were also
grown at 23°C with 14 hr of light per 24 hr.

4.2 | Pathogen growth and infection assays

P. parasitica strain Pp016 was originally isolated from diseased to-
bacco plants in Queensland, Australia (Wang et al., 2011b; Zhang
et al., 2011), and 1121 is a transformant of P. parasitica Pp016 stably
expressing ER-rendered GFP under the control of the constitutive
Hsp70 promoter of B. lactucae. The transformant 1121 remained
pathogenic on A. thaliana and tobacco plants, similar to the wild-type
strain Pp016 (Zhang et al., 2011). The GFP in P. parasitica 1121 is
constitutively and widely expressed in the cytoplasm, allowing easy
monitoring during colonization of host plants. P. parasitica culture
conditions, zoospore production, and the detached leaf inocula-
tion assays were performed as described (Wang et al., 2011b). For
the pathogenicity assay, the detached fully expanded apical leaves
from approximately 4-week-old A. thaliana plants were wounded
with a toothpick and the zoospore suspensions were adjusted to a
concentration of 200 zoospores/µl using microscopy and applied
as droplets at the wounding sites to ensure infection. To observe
the lesion size more clearly, trypan blue was used to stain the death
plant cells in lesions of inoculated leaves as described (Li et al., 2019).
A. thaliana leaves infected with the P. parasitica transformant 1121
were observed with the OLYMPUS BX51 fluorescence microscope
(with excitation at 450–480 nm and emission at 515 nm) to detect
P. parasitica hyphae (green fluorescence) at 2 dpi. For pathogen bio-
mass, three biological replicates were performed with at least eight
leaves per replicate. Primers used for pathogen biomass were listed
in Table S1. Disease severity was evaluated based on the lesion sizes
on detached leaves and the extent of pathogen colonization.

4.3 | TAIL-PCR and RT-qPCR assays

TAIL-PCR was performed as described (Liu et al., 1995). For RT-qPCR
assays, three biological replicates were used. Total RNA from the whole
leaves before and after P. parasitica inoculation was extracted using
TRizol reagent (Invitrogen). One microgram of total RNA was used to
perform reverse transcription using the PrimeScript RT Reagent Kit
with gDNA Eraser (TAKARA). For real-time qPCR analysis, 0.5 µl of the
first-strand cDNA reaction products was used as template in a reac-
tion with Ultra SYBR Mixture (CWBO) under the following conditions:
95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 30 s. The
fold changes in target gene expression were normalized using UBC9 as the internal control. Primers used for RT-qPCR are listed in Table S1.

4.4 | CRISPR/Cas9-based knockouts and overexpression of ERF019 in Arabidopsis

For the 35S::ERF019 construct, full-length ERF019 was directionally cloned into pKANNIBAL (Wesley et al., 2001) and then subcloned into the binary vector pART27 (Gleave, 1992). For the CRISPR/Cas9-based knockouts, two 19-bp sgRNA oligonucleotides targeting the exon of ERF019 were inserted in the psgR-Cas9 vector to create deletion mutants as previously described (Feng et al., 2013, 2014). Annealed 19-bp sgRNA oligomers were inserted into the BbsI site of the psgR-Cas9 vector. Based on the psgR-Cas9 vector, the second pATU6-sgR cassette was amplified by PCR after the insertion of target oligomers and ligated into the KpnI/EcoRI site of the above psgR-Cas9 vector. The cassette was then transferred into the binary vector pCXSN. The generated binary vectors were transformed into A. tumefaciens GV3101. A. tumefaciens cells carrying 35S::ERF019 and sgRNAs/Cas9 constructs were transformed into wild-type Col-0 via the floral-dip method (Zhang et al., 2006). 35S::ERF019 transformants were screened on 1/2 Murashige and Skoog (MS) agar plates containing 50 µg/ml kanamycin. sgRNAs/Cas9 transformants were screened on 1/2 MS agar plates containing 50 µg/ml hygromycin.

4.5 | A. tumefaciens infiltration assays

A. tumefaciens GV3101 containing constructs was cultured at 28°C and 200 rpm for approximately 24 hr in Luria Bertani (LB) medium with appropriate antibiotics. The A. tumefaciens cells were collected by centrifugation, resuspended in infiltration buffer (10 mM MES, 10 mM MgCl2, 0.2 mM acetylseryngone, pH 5.6), and adjusted to the appropriate OD600 before being infiltrated into 5-week-old N. benthamiana leaves (the OD600 was generally 0.3 for confocal subcellular localization assays and 0.15–0.4 for infection assays). For infection assays, A. tumefaciens GV3101 cells carrying constructs were suspended at a concentration of OD600 = 0.4, and then inoculated with P. parasitica 2 days after infiltration. For co-expression assays, A. tumefaciens GV3101 cells carrying constructs were suspended at an appropriate OD600 and mixed before infiltration (for INF1 OD600 = 0.15, for Bax OD600 = 0.3, for FLAG-GFP and ERF019 OD600 = 0.4). Symptom development was monitored visually from 2 to 6 days after infiltration depending on the cell death activator.

4.6 | Subcellular localization assays

A. tumefaciens GV3101 cell cultures carrying constructs expressing FLAG-GFP, ERF019-GFP-NES, ERF019-GFP-nes, ERF019-GFP or nucleus marker H2B-mCherry were collected by centrifugation and resuspended in infiltration buffer (10 mM MES, 10 mM MgCl2, 0.2 mM acetylseryngone, [pH 5.6]) at OD600 = 0.4 and then the cells carrying GFP constructs were co-infiltrated with those expressing the nucleus marker H2B-mCherry into 5-week-old N. benthamiana leaves. Three days after infiltration, the N. benthamiana leaves were visually inspected under an Olympus FV3000 confocal microscope with excitation wavelengths of 488 nm for GFP and 587 nm for mCherry.

4.7 | Detection of hydrogen peroxide

Hydrogen peroxide was detected in Arabidopsis rosette leaves infiltrated with 1 µM flg22 as described previously (Daudi and O’Brien, 2012).

4.8 | Oxidative burst measurements

ROS was measured in 30-day-old Arabidopsis seedlings. In brief, the leaf disks were cut from 30-day-old mature leaves with a sharp 5-mm puncher and were floated in sterile ultrapure water in culture dishes overnight. The next day, the leaf disks were transferred into 96-well plates and 100 µl Luminol Enhancer (CW BIO. CW0049M) and 100 µl of 20 µg/ml horseradish peroxidase (Aladdin) were added into each cell. Then, 5 µl of 41 µM flg22 was immediately added into each cell to a final concentration of 1 µM. Luminescence was measured using a TECAN Infinite M200 PRO (TECAN); 40 cycles (1 min per cycle) were used for the measurement.

4.9 | MAPK activity assays

Arabidopsis seedlings were grown on vertical 1/2 MS plates at 23°C with 14 hr of light per 24 hr and then were transferred into liquid MS and incubated overnight with minimum rotation speed (40 rpm). The next day, the seedlings were treated with 1 µM flg22 and frozen in liquid nitrogen. Total proteins were extracted with glycerol-Tris-EDTA-NaCl buffer (10% glycerol, 25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, 0.1% Tween 20, 0.1% NP-40, 2% [v/v] PVPP, 0.1 mM DTT, 1 × inhibitor cocktail, 1 × phosphatase inhibitor cocktail 2 and 1 × phosphatase inhibitor cocktail 3). The protein concentration was measured using the Super-Bradford Protein Assay Kit (CW BIO. CW0013S). Equal amounts of total protein were loaded on a 10% SDS-PAGE gel. Anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP rabbit mAb antibody (Cell Signaling Technology) was used to detect the phosphorylation state of MPK3, MPK4, and MPK6.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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