Phosphate-induced resistance to pathogen infection in Arabidopsis

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SUMMARY

In nature, plants are concurrently exposed to a number of abiotic and biotic stresses. Our understanding of convergence points between responses to combined biotic/abiotic stress pathways remains, however, rudimentary. Here we show that MIR399 overexpression, loss-of-function of PHOSPHATE2 (PHO2), or treatment with high phosphate (Pi) levels is accompanied by an increase in Pi content and accumulation of reactive oxygen species (ROS) in Arabidopsis thaliana. High Pi plants (e.g., miR399 overexpressors, pho2 mutants, and plants grown under high Pi supply) exhibited resistance to infection by necrotrophic and hemibiotrophic fungal pathogens. In the absence of pathogen infection, the expression levels of genes in the salicylic acid (SA)- and jasmonic acid (JA)-dependent signaling pathways were higher in high Pi plants compared to wild-type plants grown under control conditions, which is consistent with increased levels of SA and JA in non-infected high Pi plants. During infection, an opposite regulation in the two branches of the JA pathway (ERF1/PDF1.2 and MYC2/VSP2) occurs in high Pi plants. Thus, while pathogen infection induces PDF1.2 expression in miR399 OE and pho2 plants, VSP2 expression is downregulated by pathogen infection in these plants. This study supports the notion that Pi accumulation promotes resistance to infection by fungal pathogens in Arabidopsis, while providing a basis to better understand interactions between Pi signaling and hormonal signaling pathways for modulation of plant immune responses.

Keywords: Arabidopsis thaliana, Colletotrichum higginsianum, immune response, jasmonic acid (JA), microRNA399 (miR399), phosphate (Pi), PHOSPHATE 2, Plectosphaerella cucumerina, reactive oxygen species (ROS), salicylic acid (SA).

INTRODUCTION

In nature, plants are simultaneously exposed to a combination of biotic and abiotic stresses that are diverse in time and space, which requires proper integration and interactions between different stress response pathways. Exposure to a single stress might, however, impact the plant response to another stress (Kissoudis et al., 2014; Nejat & Mantri, 2017; Pandey et al., 2017). For instance, plant immune responses and disease resistance can be altered in plants exposed to drought or high salinity (Atkinson & Urwin, 2012; Bostock et al., 2014; Yasuda et al., 2008). Inappropriate supply of mineral nutrients (e.g., nitrogen supply) might also impact disease severity (Ballini et al., 2013; Snoeijers et al., 2000). However, the effect of combined abiotic and biotic stress factors might vary depending on the nature of these interactions, and the plant response to simultaneously or sequentially applied stresses cannot be simply inferred from responses to individual stresses (Coolen et al., 2016; Nobori & Tsuda, 2019; Pandey et al., 2017; Prasch & Sonnewald, 2013). As stress responses are costly, when facing multiple stresses simultaneously, plants need to prioritize their stress responses for efficient use of finite resources, in accordance with the optimal defense theory (ODT) (Meldau et al., 2012). According to the ODT, stress responses are prioritized in the most valuable parts (Keith & Mitchell-Olds, 2017), and
recent findings indicate that Arabidopsis plants spatially separate contrasting stress responses in leaves of different ages (e.g., young leaves exhibit higher biotic stress responses but lower abiotic stress responses compared with old leaves) (Berens et al., 2019; Wolinska & Berens, 2019). To date, little information is available on the molecular mechanisms by which biotic and abiotic stress responses are differentially prioritized in plants and how they adapt to conflicting stresses for optimal responses.

To defend themselves against pathogens, plants have evolved an innate immune system in which many interconnected processes are involved (Jones & Dangl, 2006). Pathogen-induced pathways are defined principally according to the molecules recognized by the host plant (Boller & Felix, 2009; Couto & Zipfel, 2016; Jones & Dangl, 2006; Thomma et al., 2011; Upson et al., 2018). Plants recognize pathogen-associated molecular patterns (PAMPs) by receptors at the plasma membrane, which triggers the induction of multiple cascades leading to the induction of immune responses, referred to as PAMP-triggered immunity (PTI). Components of PTI include the reinforcement of cell walls, accumulation of reactive oxygen species (ROS), activation of phosphorylation cascades, production of antimicrobial compounds, and accumulation of pathogenesis-related proteins, among others (Andersen et al., 2018). Some successful pathogens can overcome PTI by delivering effectors into plant cells that suppress PTI, thus leading to disease susceptibility. In turn, some plants have evolved another immune response in which microbial effectors (or host proteins modified by effectors) are recognized by intracellular receptor proteins encoded by resistance genes (Han, 2019). This recognition triggers a rapid and robust defense response, the so-called effector-triggered immunity (ETI) (Jones & Dangl, 2006). ETI is often accompanied by a hypersensitive response (HR) at the infection site, a form of programmed cell death (Thakur et al., 2019). However, some PAMPs (e.g., the bacterial HrpZ protein) can also induce HR in plants (Chang & Nick, 2012).

Among ROS, H$_2$O$_2$ is relatively stable and is an important molecule regulating plant immunity (Torres et al., 2006). H$_2$O$_2$ might have a direct antimicrobial role against the invading pathogen and also provokes cross-linking of cell wall components to arrest pathogen invasion. ROS also function as molecules for the activation of defense mechanisms, and triggers localized cell death around the infection site (Torres et al., 2002). Phytohormones, together with ROS, provide important signals to help orchestrate plant responses to abiotic and biotic stresses. Immune responses are largely coordinated by the phytohormones salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) (Aerts et al., 2021). Plant hormones do not function independently, but synergistic or antagonistic interactions between hormone pathways ultimately drive the fine-tuning of plant defense responses. At the organismal level, interactions between hormone signaling pathways might also balance trade-offs between conflicting biotic and abiotic stress responses (i.e., prioritization of responses in leaves of Arabidopsis plants) (Berens et al., 2019; Wolinska & Berens, 2019). Sugar and ROS have also been proposed as candidate signaling molecules to regulate prioritization between biotic and abiotic stress responses (Wolinska & Berens, 2019).

Plant microRNAs (miRNAs) are a class of small RNA molecules that mediate post-transcriptional gene silencing through sequence complementarity with cognate target transcripts (Bartel, 2004; Xie et al., 2005). They are transcribed from MIR genes as long precursor transcripts that adopt a stem-loop structure by self-complementarity that are processed by a RNase III DICER-like, typically DCL1, to produce a double stranded duplex, the miRNA-3p/miRNA-5p duplex (previously named as miRNA/miRNA*) (Borges & Martienssen, 2015). The functional strand of the duplex is selectively loaded into an ARGONAUTE protein, the effector protein of the RNA-induced silencing complex (RISC), which mediates post-transcriptional gene silencing by cleaving the target transcripts or by translational inhibition (Brodersen et al., 2008; Llave et al., 2002). The important role of plant miRNAs in diverse developmental processes and adaptation to environmental stresses is well documented (Chen, 2009; Seo et al., 2013; Song et al., 2019; Staiger et al., 2013).

The first plant miRNA demonstrated to be involved in immunity was the Arabidopsis miR393. Here, perception of the elicitor flg22 induces miR393 accumulation and down-regulation of regulation of nutrient homeostasis in plants (Paul et al., 2015). Perhaps the best-known example is miR399,
which is involved in the control of phosphate (Pi) homeostasis in Arabidopsis plants (Chiou et al., 2006). Under limiting Pi conditions, miR399 accumulation increases and causes repression of its target gene, PHOSPHATE2 (PHO2), encoding an E2 ubiquitin-conjugating enzyme responsible for degradation of phosphate transporters (Chiou et al., 2006; Fujii et al., 2005; Huang et al., 2013; Kraft et al., 2016; Liu et al., 2012). Hence, miR399 accumulation in response to Pi starvation relieves negative post-transcriptional control of Pi transporters and promotes uptake of Pi in Arabidopsis plants. The miR399/PHO2 regulatory module has been also recognized as a regulator of Pi homeostasis in rice (Oryza sativa) (Chien et al., 2017; Puga et al., 2017).

In this work, we investigated whether miR399 plays a role in disease resistance in Arabidopsis. We show that miR399 overexpression and loss-of-function of PHO2 causes an increase in Pi levels, these plants also exhibiting resistance to infection by necrotrophic (Plectosphaerella cucumerina) and hemibiotrophic (Colletotrichum higginsianum) fungal pathogens. Growing Arabidopsis plants under high Pi supply also increases resistance to infection by these pathogens. In the absence of pathogen infection, plants that overaccumulate Pi (e.g., miR399 overexpressors, pho2 mutants, and wild-type plants grown under high Pi supply) showed ROS accumulation, increased SA and JA levels, and upregulation of genes involved in SA- and JA-dependent defense pathways. Pathogen infection was found to be associated with a higher production of ROS in high Pi plants. Of note, during pathogen infection, induction of the ERF1 branch of the JA pathway and repression of the MYC2/VSP2 branch occur in high Pi plants. Overall, our results support the notion that an increase in Pi content has an impact on hormone networks regulating Arabidopsis defense and promotes resistance to pathogen infection in Arabidopsis. These results are markedly different from those recently reported in rice (Campos-Soriano et al., 2020), where miR399 overexpression and high Pi supply were found to enhance susceptibility to infection by the rice blast fungus Magnaporthe oryzae. These findings illustrate the need of investigating the effects of nutrient supply on the expression of immune responses and disease resistance on a case-by-case basis.

RESULTS

Resistance to infection by fungal pathogens in Arabidopsis plants overexpressing MIR399

In Arabidopsis, the miR399 family comprises six members, MiR399a-f. Of these, miR399f has identical mature sequences in Arabidopsis and rice. Previous studies indicated that transgenic Arabidopsis and rice plants overexpressing miR399f overaccumulate Pi in leaves (Campos-Soriano et al., 2020; Chiou et al., 2006). In this work, we investigated whether miR399f overexpression, and subsequent Pi accumulation, has an effect on resistance to pathogen infection in Arabidopsis. To this end, transgenic plants overexpressing MIR399f (hereinafter referred to as miR399 OE plants) were generated. Compared to wild-type plants, miR399 OE lines accumulated precursor and mature miR399 sequences. The observed increase in miR399 abundance in miR399 OE lines was accompanied by a decrease in PHO2 transcript level and overaccumulation of Pi in rosette leaves (Figure 1a).

The miR399 OE lines were challenged with the fungus P. cucumerina, the causal agent of the sudden death and blight disease in many dicotyledonous species. The Arabidopsis–P. cucumerina pathosystem is a widely used model system for studies on disease resistance against necrotrophic fungi (Sánchez-Vallet et al., 2012; Ton & Mauch-Mani, 2004). At the time of pathogen inoculation (3-week-old plants), no obvious phenotypic differences were observed between miR399 OE plants and wild-type plants (Figure S1a,b). However, at a later developmental stage, the miR399 and pho2 plants displayed symptoms of Pi excess (e.g., chlorosis on mature leaves of adult plants), most probably, because of Pi accumulation overtime (results not shown; similar results were previously reported by Choui et al., 2006).

Upon pathogen challenge, wild-type plants were severely affected by P. cucumerina, while the miR399 OE plants consistently exhibited enhanced resistance (Figure 1b). While 75–85% of miR399 OE plants survived at 7 dpi, only 20% of the wild-type plants were able to overcome infection (Figure 1c, left panel). Quantitative PCR (qPCR) measurement of fungal DNA confirmed that less fungal biomass was present in leaves of miR399 OE plants compared with leaves of wild-type plants infected with P. cucumerina (Figure 1c, right panel), which is consistent with the phenotype of resistance that is observed in miR399 OE plants.

Trypan blue staining was used to visualize both fungal structures and dead cells in the fungal-infected leaves of wild-type and miR399 OE plants. Whereas extensive fungal growth occurred in leaves of wild-type plants, no fungal growth could be observed in leaves of miR399 OE plants (Figure 1d). Instead, scattered groups of dead cells were visualized in P. cucumerina-infected miR399 OE plants (Figure 1d, right panels).

Since P. cucumerina is a necrotrophic fungus, we hypothesized that the effect of miR399 overexpression on disease resistance might be dependent on the lifestyle of this pathogen. Accordingly, we investigated resistance of miR399 OE plants to infection by the hemibiotrophic fungus C. higginsianum. This fungus causes the anthracnose leaf spot disease on Brassica species, as well as A. thaliana (O’Connell et al., 2004). The miR399 OE plants showed resistance to C. higginsianum infection relative to wild-
miR399 OE #1

Figure 1. Resistance of miR399 OE plants to infection by the necrotrophic fungus *P. cucumerina*. Homozygous miR399 OE lines (#1, #4, and #10) and wild-type plants were grown in soil for 3 weeks and then assayed for disease resistance. Three independent experiments were carried out with at least 12 plants per line in each experiment. (a) Accumulation of precursor (pre-miR399) and mature miR399 sequences was determined by RT-qPCR and stem-loop RT-qPCR, respectively. Expression of the miR399 target *AdPHO2* was determined by RT-qPCR using specific primers for *P. cucumerina* DNA was performed by qPCR using specific primers for *P. cucumerina* tubulin1 (At5g05620) gene (At5g05620) with *At5g05620* sequences was determined by RT-qPCR and stem-loop RT-qPCR, respectively. Expression of the miR399 target *AdPHO2* was determined by RT-qPCR using specific primers for *P. cucumerina* tubulin1 (At5g05620) gene (At5g05620) was used to normalize transcript levels (relative expression). The accumulation of free Pi in leaves is shown (right panel). Bars represent mean ± SEM of three biological replicates with at least three plants per replicate (t-test, *P* ≤ 0.05, **P** ≤ 0.01, ***P** ≤ 0.001). (b) Plants were spray-inoculated with *P. cucumerina* spores (5 X 10^6 spores mL^-1^). Pictures were taken at 7 days post-inoculation (dpi). (c) Survival ratio of wild-type and miR399 OE plants at 7 dpi. Quantification of *P. cucumerina* DNA was performed by qPCR using specific primers for *P. cucumerina* tubulin1 at 7 dpi. Values of fungal DNA were normalized against the *Arabidopsis* *UBIQUITIN21* gene (Atg25760). Comparisons have been made relative to wild-type plants. Data are mean ± SEM (n = 7) (t-test, *P* ≤ 0.05, **P** ≤ 0.01). (d) Trypan blue staining of *P. cucumerina*-infected leaves of wild-type and miR399 OE plants (7 dpi). h, hyphae. Arrows and arrowheads indicate fungal hyphae and dead cells, respectively. Higher magnifications are shown (wild type and miR399 OE, right panels). Bars represent 300 μm.

Phosphate-induced disease resistance in Arabidopsis

Type plants, as revealed by quantification of diseased leaf area and fungal biomass (Figure 2a). As observed in infection assays with *P. cucumerina*, the *C. higginsianum*-infected miR399 OE plants exhibited groups of dead cells in their leaves (Figure 2b).

Collectively, these results demonstrate that miR399 overexpression enhances resistance to infection by necrotrophic (*P. cucumerina*) and hemibiotrophic (*C. higginsianum*) fungal pathogens. Hence, it is likely that miR399-mediated resistance in Arabidopsis does not depend on the lifestyle of the fungus. A pattern of cell death occurs in the fungal-infected miR399 OE plants.

**Pho2** mutants exhibit resistance to infection by fungal pathogens

miR399 targets and suppresses the expression of PHO2, encoding the ubiquitin-conjugating enzyme that mediates the degradation of Pi transporter proteins (Chiou et al., 2006; Fuji et al., 2005; Huang et al., 2013). A loss-of-function allele of *PHO2* was previously described (Aung et al., 2006; Delhaize & Randall, 1995). This mutant allele possesses a single nucleotide mutation that causes premature termination and loss of ubiquitin conjugation activity of PHO2 (Figure S2a). pho2 plants resemble miR399 OE plants in that they both show Pi overaccumulation in leaves resulting from increased Pi uptake from roots and root-to-shoot translocation (Aung et al., 2006) (Figure S2b). We therefore hypothesized that loss-of-function of *PHO2* might result in a similar disease phenotype as suppression of PHO2 by overexpression of miR399.

The *pho2* plants were then examined for pathogen resistance. No phenotypic differences were observed between *pho2* and wild-type plants at the time of inoculation (Figure S2c). Consistent with the disease phenotype observed in miR399 OE plants, the *pho2* mutant exhibited resistance to infection by *P. cucumerina* (Figure 3a), which was confirmed by quantifying the survival ratio of the infected plants and the amount of fungal biomass (Figure 3b). Trypan blue staining revealed a pattern of dead cells in leaves of *pho2* mutants that have been inoculated with *P. cucumerina* spores (Figure S3c), as was also observed in miR399 OE plants. Finally, the *pho2* plants also showed resistance to *C. higginsianum* infection as compared with wild-type plants (Figure 3d). Taken together, these results presented support the notion that both *pho2* and miR399 OE plants accumulate Pi in leaves and exhibit resistance to infection by fungal pathogens with a necrotrophic or hemibiotrophic lifestyle.

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MIR399 expression is upregulated during fungal infection and treatment with fungal elicitors

To gather further support for the involvement of MIR399 in Arabidopsis immunity, we investigated whether fungal infection or treatment with fungal elicitors provokes alterations in MIR399 expression in wild-type plants. The accumulation of MIR399 precursor transcripts was examined by reverse transcription qPCR (RT-qPCR), while mature MIR399 abundance was determined by stem-loop RT-qPCR. Differences among mature MIR399 sequences are found at their 3' terminal region (miRBase, https://www.mirbase.org/). This fact allowed us to design MIR399-specific primers for stem-loop RT-qPCR analysis. The PCR-amplified products were further confirmed through DNA sequencing. Fungal-responsiveness of MIR399f was examined at 48 and 72 h post-inoculation (hpi) with P. cucumerina spores. An increase in the accumulation of both precursor and mature MIR399 sequences was clearly observed in the response of wild-type plants to pathogen infection (Figure 4a). When examining the PHO2 expression behavior in response to infection, an opposite profile was observed between MIR399f and PHO2 at the earliest time of infection here assayed (48 hpi with P. cucumerina spores). At 72 hpi, however, the accumulation of PHO2 transcripts in fungal-infected wild-type plants was similar to that in mock-inoculated plants (Figure 4a).

In PTI, the activation of defense mechanisms relies on the detection of PAMPs (commonly referred to as elicitors). Accordingly, we investigated whether MIR399 accumulation is affected by treatment with a crude preparation of
Figure 3. Resistance to infection by fungal pathogens in pho2 plants. Three-week-old mutant plants were inoculated with fungal *P. cucumerina* spores. Three independent experiments were carried out with similar results with at least 12 plants per genotype. (a) Disease phenotype of wild-type and pho2 plants upon inoculation with *P. cucumerina* spores (5 × 10⁵ spores ml⁻¹). Pictures were taken at 7 days post-inoculation (dpi). (b) Survival ratio of wild-type and pho2 plants at 7 dpi (left panel). Quantification of *P. cucumerina* DNA was carried out using specific primers for *P. cucumerina* β-tubulin at 7 dpi (right panel). Values are fungal DNA levels normalized against the Arabidopsis UBIQUITIN21 gene (At5g25760). Data are mean ± SEM (n = 7). (t-test, **P ≤ 0.01, ***P ≤ 0.001). (c) Trypan blue staining of *P. cucumerina*-infected leaves and visualization of cell death and fungal growth. h, hyphae. Arrows and arrowheads indicate fungal hyphae and dead cells, respectively. Bars represent 200 μm. (d) Disease phenotype of wild-type and pho2 plants at 8 dpi with *C. higginsianum* spores (4 × 10⁶ spores ml⁻¹).
elicitors obtained by autoclaving and sonicating *P. cucumerina* mycelium. Similar to *P. cucumerina* infection, elicitor treatment induced the accumulation of both miR399 precursor and mature sequences (Figure 4b). Furthermore, an opposite profile was observed between MIR399 and PHO2 expression at 90 min of elicitor treatment (e.g., upregulation of MIR399f and downregulation of PHO2), but this anticorrelation in expression patterns was not observed at 120 min of elicitor treatment. The observation that pathogen infection and treatment with fungal elicitors are accompanied by upregulation of MIR399 expression suggests that miR399 might function in PTI. Taking into account that miR399 has been shown to negatively regulate PHO2 via the canonical mechanism of cleavage of PHO2 transcripts, results here presented point to the existence of regulatory mechanisms for the control of PHO2 expression other than miR399-guided cleavage of PHO2 transcripts during pathogen infection or treatment with fungal elicitor (as inferred from results obtained at 72 hpi with *P. cucumerina* spores or 120 min of elicitor treatment). Supporting this possibility, it was recently reported that PHO2 might be modulated by regulatory mechanisms independent of miR399-directed regulation in the response of Arabidopsis plants to salt stress (Pegler et al., 2021). In that study, no anticorrelation between miR399 accumulation and PHO2 transcript abundance was reported. Whether additional factors are involved in the regulation of PHO2 during pathogen infection deserves further investigation.

**Stimulation of ROS production in Arabidopsis plants containing increased levels of Pi**

One of the hallmarks of host-pathogen interactions is the overproduction of ROS as a plant defense mechanism, the so-called oxidative burst. ROS include various forms of reactive molecules, such as superoxide radicals (O$_2^-$), hydroxyl radicals (OH$^-$), and hydrogen peroxide (H$_2$O$_2$). Of these, H$_2$O$_2$ might act as both signaling molecule for the activation of plant immune responses and antimicrobial agent (Torres et al., 2006). *Respiratory Burst Oxidase Homolog D* (*RBOHD*), a member of the Arabidopsis Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase gene family, has been shown to be responsible for ROS production after pathogen infection (Kadota et al., 2015; Torres et al., 2002). Furthermore, ROS may promote cell death and limit pathogen spread.
Knowing that miR399 OE and pho2 plants exhibit a pattern of cell death upon pathogen infection, we examined ROS accumulation in miR399 OE and pho2 plants, both in the presence and in the absence of pathogen infection. Histochimical detection of H$_2$O$_2$ was carried out using the fluorescent probe 2',7'-dichlorofluorescein diacetate (H$_2$DCFDA). H$_2$DCFDA was previously shown to detect different forms of ROS, mainly H$_2$O$_2$ but also hydroxyl radicals and superoxide anions (Fichman et al., 2019). Compared with wild-type plants, a higher level of ROS accumulation could be observed in leaves of miR399 OE and pho2 plants compared to wild-type plants in the absence of pathogen infection (Figure 5a, upper panels).

**Plecostheraella cucumerina** infection further increased ROS levels in miR399 OE and pho2 plants (Figure 5a, lower panels). Discrete regions accumulating ROS, most probably, correspond to infection sites. Similar results were observed by 3,3'-diaminobenzidine (DAB) staining of *P. cucumerina*-infected leaves (Figure S3). Moreover, H$_2$DCFDA staining revealed a higher level of ROS accumulation in high Pi plants (e.g., miR399 OE and pho2 plants) that have been treated with *P. cucumerina* elicitors compared with elicitor-treated wild-type plants (Figure S4).

Additionally, we examined ROS generated in miR399 OE, pho2, and wild-type plants in response to treatment with *P. cucumerina* elicitors using the luminol method. This study confirmed a higher production of ROS after elicitor treatment in leaves of miR399 OE and pho2 plants compared to that in wild-type plants (Figure S5a). Collectively, these findings support ROS accumulation in leaves of miR399 OE and pho2 plants in the absence of pathogen infection. During infection with *P. cucumerina* or treatment with elicitors obtained from this fungus, miR399 OE and pho2 plants produced higher levels of ROS than wild-type plants.

In concordance with results obtained by histochimical detection of ROS and measurement of ROS production using the luminol assay, the miR399 OE and pho2 plants exhibited upregulation of RBOHD in the absence of pathogen infection compared with wild-type plants (Figure 5b, black bars). *Plecostheraella cucumerina* infection further induced RBOHD expression in all the genotypes (wild-type, miR399 OE, and pho2 plants), but its expression reached higher levels in miR399 OE and pho2 plants than in wild-type plants (Figure 5b, gray bars). Then, ROS accumulation in miR399 OE and pho2 plants can be explained, at least partially, by an increased RBOHD expression. However, other factors causing an increase in ROS accumulation cannot be discarded.

The observation that both miR399 OE and pho2 plants accumulated Pi and ROS, these plants also exhibiting enhanced disease resistance (Figures 1 and 2), prompted us to investigate whether ROS production and disease resistance are affected by Pi supply in Arabidopsis. To address this question, wild-type plants were grown in vitro on media at different Pi concentrations (0.05, 0.1, and 0.25 mM Pi, hereinafter referred to as P$_{0.05}$, P$_{0.1}$, and P$_{0.25}$ plants). As expected, measurement of Pi content confirmed that increasing Pi supply to wild-type plants results in higher leaf Pi content (Figure 5c). Most importantly, increasing Pi supply was accompanied by an increase in ROS accumulation as revealed by H$_2$DCFDA staining of leaves from Pi-treated plants (Figure 5d). Similarly, luminol assays demonstrated that treatment with Pi fosters ROS production in wild-type plants, with ROS production correlating well with Pi concentration (Figure S5b). Upon pathogen inoculation, high Pi plants consistently displayed enhanced resistance to infection by either *P. cucumerina* or *C. higginsianum* (Figure 5e,f, respectively).

**Pi-induced resistance to pathogen infection in Arabidopsis is associated with modulation of the SA- and JA-dependent defense pathways**

As previously mentioned, SA and JA play a critical role in the transcriptional reprogramming of Arabidopsis plants in response to infection (Cao et al., 2006; Yang et al., 2015; Chae et al., 2017). In Arabidopsis plants overaccumulate Pi, leading to an increase in ROS production, with ROS accumulation contributing to the transcriptional reprogramming of Arabidopsis plants in response to infection (Yang et al., 2015; Chae et al., 2017). The upregulation of RBOHD in the absence of pathogen infection compared with wild-type plants (Figure 5b, black bars) suggests that Pi-induced resistance to pathogen infection in Arabidopsis is associated with modulation of the SA- and JA-dependent defense pathways.

**Figure 5.** Enhanced accumulation of ROS and disease resistance in Arabidopsis plants that overaccumulate Pi. (a) In situ histochemical detection of ROS in leaves of miR399 OE and pho2 plants. Plants were spray-inoculated with *P. cucumerina* spores ($5 \times 10^6$ spores ml$^{-1}$) or mock-inoculated. Visualization of H$_2$O$_2$ accumulation was carried out using the fluorescent probe H$_2$DCFDA at 2 days post-inoculation (dpi). Bars represent 200 µm. (b) Expression of RBOHD in mock-inoculated and *P. cucumerina*-inoculated plants at 24 hpi (black and gray bars, respectively). The expression values were normalized to the Arabidopsis ubiquitin21 gene (At5g25760). Three biological replicates, each with three plants per replicate, were examined. Different letters correspond to statistically significant differences (ANOVA followed by Tukey’s HSD test; P < 0.05). (c) Free Pi content in plants that have been grown under different conditions of Pi supply. Plants were grown on agar plates for 1 week and transferred to fresh agar plates with medium containing different concentrations of Pi (0.05, 0.1, or 0.25 mM). Plants were allowed to continue growth for one more week and then inoculated with fungal spores. Pi content was determined at the time of inoculation with fungal spores. (d) ROS accumulation in wild-type Arabidopsis (Col-0) plants that have been grown under different Pi supply conditions, that is, 0.05, 0.1, 0.25, or 2 mM Pi (P$_{0.05}$, P$_{0.1}$, P$_{0.25}$, and P$_{2}$ respectively). ROS were detected using H$_2$DCFDA. Representative images are shown. Bars correspond to 1 mm. (e) Resistance to infection by *P. cucumerina* in Pi-treated Arabidopsis plants. Appearance of plants at 7 dpi with *P. cucumerina* spores ($4 \times 10^6$ spores ml$^{-1}$; left panel). Representative results from one of three independent infection experiments that gave similar results are shown. Right panel, fungal biomass determined at 3 dpi by qPCR analysis using specific primers for *P. cucumerina* β-tubulin and normalized to the Arabidopsis ubiquitin21 gene (At5g25760). (f) Resistance of Pi-treated Arabidopsis plants to infection by *C. higginsianum*. Disease symptoms of Arabidopsis plants at 12 dpi with *C. higginsianum* spores ($5 \times 10^6$ spores ml$^{-1}$; right panel). Fungal biomass at 7 dpi as determined by qPCR analysis using specific primers for *C. higginsianum* Internally transcribed spacer 2 (ITS2). Means of three biological replicates, each one from a pool of at least three plants, are shown in (e) and (f) (right panels); t-test, *P* ≤ 0.05, **P** ≤ 0.01, ***P*** ≤ 0.001.

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response to pathogen infection (Pieterse et al., 2012). A pathogen-induced accumulation of ROS is also required for the induction of SA-dependent defenses, indicating that ROS and SA are intertwined in a complex regulatory network (Herrera-Vásquez et al., 2015; Wang et al., 2014). To get deeper insights into the mechanisms underlying disease resistance in miR399 OE and pho2 plants, we investigated the expression of defense genes linked to the SA and JA pathways in these plants. The marker genes of the SA-mediated defense response here examined were: Pathogenesis-Related 1 (PR1), Non-expressor of Pathogenesis-Related genes 1 (NPR1), and Phytoalexin Deficient4 (PAD4) (Jirage et al., 1999). We found that in the absence of pathogen infection, PR1, NPR1, and PAD4 expression was upregulated in both miR399 OE and pho2 plants compared with wild-type plants (Figure 6a, black bars). PR1, NPR1, and PAD4 expression further increased in response to P. cucumerina infection in miR399 OE and pho2 plants, but not in wild-type plants (Figure 6a, gray bars).

Regarding the JA pathway, two branches are documented in Arabidopsis: the MYC2 branch, which is regulated by AtMYC2 (a basic helix–loop–helix–leucine zipper transcription factor), and the ERF branch, which is regulated by AtERF1 (a member of the APETALA/ERF transcription factor family) (Lorenzo et al., 2004). Plant Defensin 1.2 (PDF1.2) is commonly used as marker of the ERF branch, whereas the MYC branch is marked by the induction of Vegetative Storage Protein 2 (VSP2) (Lorenzo et al., 2004; Pieterse et al., 2012; Wasternack & Hause, 2013; Zhang et al., 2017). The ERF branch and the MYC branch of the JA signaling pathway have been reported to repress each other (Aerts et al., 2021; Lorenzo et al., 2004; Wasternack & Hause, 2013).

Compared with wild-type plants, the miR399 OE and pho2 plants showed higher expression of transcription factor and defense marker genes associated to the ERF1/PDF1.2 and MYC2/VSP2 branches of the JA signaling pathway in the absence of pathogen infection (Figure 6b,c, black bars). Pathogen infection further induced ERF1/PDF1 expression in wild-type, miR399 OE, and pho2 plants (Figure 6b, gray bars). Of note, while MYC2/VSP2 expression was not significantly affected by pathogen infection in wild-type plants, their expression was found to be repressed in miR399 OE plants. Pathogen infection downregulated VSP2 expression in pho2 plants, but not in wild-type plants (Figure 6c, gray bars). Together, these findings support the notion that resistance to pathogen infection in plants accumulating Pi (i.e., miR399 OE and pho2 plants) is associated with a higher expression of SA- and JA-regulated genes under non-infection conditions. A pathogen-induced superactivation of genes involved in the SA pathway and the ERF1 branch of the JA pathway occurs in these plants, while pathogen infection represses the MYC2 branch of the JA signaling pathway.

Further, we measured the levels of the phytohormones SA and JA in miR399 OE and pho2 plants. Compared with wild-type plants, miR399 OE and pho2 plants accumulated significantly higher levels of SA under normal growth conditions (e.g., in the absence of pathogen infection) (Figure 7a, upper left panel, black bars). This observation is consistent with the expression pattern of SA-responsive defense genes found in miR399 OE and pho2 plants (see Figure 6). Upon pathogen infection, however, there were no significant differences in SA levels among wild-type, miR399 OE, and pho2 plants (Figure 7a, upper left panel, gray bars). We also noticed that SA glucoside (SAG, the storage form of SA) accumulated at substantially higher levels in the fungal-infected miR399 OE and pho2 plants compared with the fungal-infected wild-type plants (Figure 7a, upper right panel). These observations point to a strict control of the SA level in miR399 OE and pho2 plants.

In the absence of pathogen infection, miR399 OE and pho2 plants accumulated more JA than wild-type plants (Figure 7a, lower left panel, black bars). JA content further increased during infection in wild-type and miR399 OE plants (Figure 7a, lower left panel, gray bars). The level of 12-oxophytodienoic acid (OPDA), a JA precursor, in miR399 OE and pho2 plants did not differ significantly from that in wild-type plants (Figure 7a).

Additionally, we investigated whether Pi treatment modulates SA and JA signaling pathways. Increasing Pi supply results in a gradual increase in SA and JA levels (as well as SAG and OPDA levels) (Figure 7b). These results are in concordance with the upregulation of PR1, NPR1, and PDF1.2 in response to Pi treatment (Figure S6).

Collectively, these results support the notion that in the absence of pathogen infection, miR399 overexpression, loss-of-function of PHO2, and Pi treatment result in higher levels of SA and JA and subsequent upregulation of SA- and JA-dependent defense gene expression. A Pi-mediated modulation of the SA and JA signaling pathways may contribute to the phenotype of disease resistance that is observed in Arabidopsis plants overaccumulating Pi, namely miR399 OE, pho2, and wild-type plants grown under high Pi supply.

DISCUSSION

Nutrients play crucial roles in normal plant growth and development, and nutrient imbalance might also have a substantial impact on the predisposition of plants to resist pathogen attack. Depending on the identity of the interacting partners, nutritional imbalances caused by either nutrient excess or deficiency may determine the outcome of the interaction, resistance or susceptibility (Veresoglou et al., 2013). On the other hand, although miRNA-mediated regulation of gene expression in processes involved in either nutrient stress or immune responses is well documented, less effort has been made to investigate miRNA
function in plant immunity under nutrient stress conditions, in particular Pi stress.

In this study, we provide evidence that increasing Pi content in Arabidopsis results in enhanced disease resistance. Several pieces of evidence support this conclusion. On the one hand, we show that Pi accumulation caused by miR399 overexpression, loss-of-function of PHO2, or treatment with Pi confers resistance to infection by fungal

Figure 6. Expression of defense genes in miR399 OE and pho2 plants. Transcript levels were determined by RT-qPCR in mock-inoculated or P. cucumerina-inoculated plants at 24 h post-inoculation (black and gray bars, respectively). The expression values were normalized to the Arabidopsis β-tubulin2 gene (At5g62690). (a) Expression of genes involved in the SA pathway (PR1, NPR1, PAD4). (b) Expression of genes in the ERF branch of the JA pathway (ERF1, PDF1.2). (c) Expression of genes in the MYC branch of the JA pathway (MYC2, VSP2). Three independent experiments (with 12 plants per genotype) were examined, with similar results. Bars represent mean ± SEM. Different letters represent statistically significant differences (ANOVA followed by Tukey's HSD test; \( P < 0.05 \)).
Pathogens with necrotrophic (*P. cucumerina*) and hemibiotrophic (*C. higginsianum*) lifestyles. On the other hand, resistance in Arabidopsis plants overaccumulating Pi correlated well with upregulation of defense-related genes under normal growth conditions (i.e., in the absence of pathogen infection). Additionally, we show that *P. cucumerina* infection is accompanied by an increase in miR399 accumulation. Not only pathogen infection, but also treatment with fungal elicitors results in higher levels of miR399. The observed elicitor-responsiveness of MIR399...
expression might be indicative that miR399 functions in PTI. Altogether, these findings support the notion that miR399 overexpression has a positive impact on resistance to pathogen infection in Arabidopsis and reinforce the notion that miR399 plays a dual role in plants by controlling Pi homeostasis and disease resistance.

Another finding of this study is that, under non-infection conditions, overaccumulation of Pi in Arabidopsis leaves is accompanied by production of ROS, their levels increasing significantly during pathogen infection. Hence, ROS accumulation in miR399 OE and pho2 plants might be responsible for the pattern of cell death observed in these plants during pathogen infection, a response that is reminiscent of the pathogen-induced HR. In contrast, the fungal-infected wild-type plants did not exhibit cell death and showed extensive fungal growth. Additionally, elevated levels of ROS might contribute to the activation of immune responses in high Pi plants leading to a phenotype of disease resistance. From this point of view, it might be interesting to determine whether Pi-induced ROS accumulation can be generalized to other plant species.

At the time of inoculation with fungal spores (3-week-old plants), miR399 OE and pho2 plants accumulated 3–4 times more Pi than wild-type plants. By this developmental stage, plant growth is not compromised in plants accumulating this level of Pi. It is tempting to hypothesize that this increase in Pi content and/or ROS levels is perceived by the host plant as a stressful situation and that the plant responds to these signals with the induction of defense gene expression. An increase in Pi content might eventually increase intracellular and extracellular Pi levels, increasing ATP levels. Here, it should be mentioned that ATP has been shown to function as a DAMP signal after release into the extracellular space upon cellular damage and that extracellular ATP enhances plant defense against pathogens through the activation of JA signaling (Tanaka et al., 2014; Tripathi et al., 2018). Further studies are needed to establish whether ATP levels are altered in high Pi plants.

Gene expression analysis revealed regulation of the SA and JA defense signaling pathways in Arabidopsis plants overaccumulating Pi in leaves. Thus, high Pi plants (miR399 OE and pho2 plants) showed activation of SA-regulated and JA-regulated genes under non-infection conditions. SA and JA were reported to play a positive role in the regulation of resistance to P. cucumerina infection in Arabidopsis (Berrocal-Lobo et al., 2002; Sánchez-Vallet et al., 2012). Previous studies also revealed that PDF1.2 induction was associated to resistance to infection by necrotrophic fungi, including P. cucumerina, in Arabidopsis (Berrocal-Lobo et al., 2002; Thomma et al., 1998). Consistent with the upregulation of genes involved in the SA and JA signaling pathways, SA and JA accumulated in non-infected high Pi plants. In other studies, a feedback loop between SA and ROS production (e.g., H2O2) has been reported in which ROS are involved both upstream and downstream of SA in the plant defense response to pathogen infection (Herrera-Vásquez et al., 2015). We hypothesize that Pi content and possibly also ROS accumulation might influence defense hormone signaling.

Interestingly, a different regulation in the two branches of the JA pathway was observed in high Pi plants during pathogen infection. Whereas ERF1 and PDF1.2 expression is further increased during infection in miR399 OE and pho2 plants (infected versus non-infected plants), VSP2 expression diminished in miR399 OE and pho2 plants (infected versus non-infected plants). We envisage that this differential regulation might be due to still unknown factors that cooperate in an antagonistic manner in the regulation of PDF1.2 and VSP2 expression during infection with the fungal pathogen P. cucumerina. In line with this, a negative correlation between the ERF1 and MYC2 branches has been previously described in the Arabidopsis response to different attackers such as pathogens and phytophagous insects (Lorenzo et al., 2004; Pieterse et al., 2012; Wasternack & Hause, 2013; Zhang et al., 2017). Here, necrotrophic pathogens preferentially activate the ERF branch, while the MYC2 branch is activated by insect herbivory and wounding. A specialization in the host plant for modulation of each branch of the JA signaling pathway might exist. It will be of interest to explore whether Pi accumulation has an effect on plant–insect interactions in Arabidopsis.

Clearly, interactions between defense-related hormone pathways provide the plant with a powerful regulatory potential to control defense responses (Aerts et al., 2021; Zheng et al., 2012). However, the type of induced response that is effective for disease resistance appears to vary depending on the host plant and pathogen identity. Although there are exceptions, pathogens with a biotrophic or hemibiotrophic lifestyle (such as P. syringae) are generally more sensitive to SA-dependent responses, whereas necrotrophic pathogens are commonly deterred by JA/ET-dependent defenses (Glazebrook, 2005). The observation that high Pi plants (e.g., miR399 OE, pho2, and plants grown under high Pi supply conditions) show enhanced resistance to necrotrophic (P. cucumerina) and hemibiotrophic (C. higginsianum) fungal pathogens makes it unlikely that the pathogen lifestyle determines disease resistance in high Pi plants.

Based on the results obtained in this study, a model is proposed to describe possible mechanisms underlying disease resistance in high Pi Arabidopsis plants (Figure 8). According to our model, miR399 overexpression and loss-of-function of PHO2, as well as growing plants under high Pi supply, increases Pi accumulation, ROS production, and SA and JA accumulation. A higher expression of SA- and JA-dependent defense responses in Arabidopsis plants
transgenic expression of a phytoplasma effector (SAP11) in Arabidopsis was found to trigger PSRs that are mainly dependent on PHR1 (Lu et al., 2014). The SAP11 transgenic plants overaccumulated Pi in leaves and were more susceptible to P. syringae pv. tomato DC3000 infection (Lu et al., 2014). The PSR system also appears to control root colonization by the endophytic fungus Colletotrichum tofieldiae in Arabidopsis (Ferligianni et al., 2021; Hiruma et al., 2016). Very recently, Dindas et al. (2022) described a negative regulation of Pi transport by immune signaling in Arabidopsis. In citrus plants, Pi content was associated to symptomology in Huanglongbing (HLB) disease, where Pi deficiency favors development of HLB symptoms (Zhao et al., 2013). In plant-insect interactions, Pi deficiency was found to induce the JA signaling pathway to enhance resistance to insect herbivory in a process that is partially under the control of PHR1 (Khan et al., 2016). Collectively, results here presented together with those found in the literature in other pathosystems support the existence of connections between Pi homeostasis and immune signaling. These mechanisms should operate in a coordinated manner to properly balance nutrient responses and plant immunity.

Results from our study also raise intriguing questions about the impact of Pi content on disease resistance in different pathosystems. In the present study we show that an increase in Pi content positively regulates Arabidopsis immune responses, which, in turn, enhances resistance to infection by fungal pathogens. Contrarily, in rice plants, a higher Pi content caused by miR399 overexpression or high Pi fertilization was found to negatively regulate defense gene expression, thus increasing susceptibility to infection by the blast fungus M. oryzae (Campos-Soriano et al., 2020). In other studies, Pi deficiency was found to enhance resistance to Verticillium dahliae in cotton (Gossypium hirsutum) (Luo et al., 2021) and insect herbivory in Arabidopsis (Khan et al., 2016). Together, our results and those previously reported support the notion that Pi content might positively or negatively regulate disease resistance depending on the interacting partners. It is tempting to speculate that different plants might have evolved divergent mechanisms to adapt to Pi alterations which, in turn, would determine a different effect of Pi in the regulation of immune responses. It is likely that integration of Pi stress (either deficiency or excess) and immune responses might vary depending on the host plant and the type of pathogen, the outcome of the interaction being also dependent on the role of the defense hormones SA and JA in that particular interaction. Alternatively, Pi might affect growth and/or pathogenicity of a fungal pathogen, either by creating a less favorable environment for pathogen growth or by reducing the production of pathogen virulence factors. Clearly, these aspects deserve further investigation.
Finally, it is worth mentioning that one of the major challenges plants face is defending against pathogen infection under continuous changes in nutrient availability, particularly P_i availability. Results here presented set the basis for further research to elucidate the exact mechanisms by which P_i signaling and pathogen-induced signaling interact with each other in plants. Further studies, however, should be conducted on a case-by-case basis in different plant-pathogen interactions. A better understanding of these mechanisms will allow the development of novel strategies to improve disease resistance in plants.

EXPERIMENTAL PROCEDURES

Plant material and infection assays

_Arabidopsis thaliana_ (ecotype Columbia-0 [Col-0]) plants were grown in a mixture of soil:perlite:vermiculite (2:1:1) and modified Hoagland half strength medium, under neutral photoperiod (12 h light/12 h dark), at a relative humidity of 60% and a temperature of 22°C ± 2°C. The fungus _P. cucumerina_ was grown on Potato Dextrose Agar plates with chloramphenicol (34 μg ml−1). _Coleotrichium higginsianum_ was grown on Oatmeal agar plates in darkness. Fungal spores were collected by adding sterile water to the surface of the mycelium and adjusted to the desired final concentration using a Bürker counting chamber.

For infection experiments in soil-grown plants, 3-week-old plants were spray-inoculated with a spore suspension of _P. cucumerina_ (5 x 10^6 spores/ml) or mock-inoculated. _Plectosphaerella cucumerina_ inoculated and mock-inoculated plants were maintained under high humidity and plant survival was assessed at 7 dpi. For infection with _C. higginsianum_, the fungal spores were locally inoculated (4 x 10^6 spores ml−1; 10 μl per leaf and five leaves per plant). The lesion area of _C. higginsianum_-infected leaves was measured with ImageJ software (National Institute of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij/) at 7 dpi. Three independent experiments were performed with at least 12 plants per genotype in each experiment. For _in vitro_ experiments, 2-week-old Arabidopsis plants were spray-inoculated with _P. cucumerina_ (4 x 10^6 spores ml−1) or locally inoculated with _C. higginsianum_ (5 x 10^6 spores ml−1). Fungal biomass was determined by quantitative PCR (qPCR) using specific primers for the corresponding fungus; the Arabidopsis _UBQUITIN21_ (At5g25780) gene was used as the internal control (Soto-Suarez et al., 2017). PCR primers are listed in Table S1. Statistically significant differences were determined by the t-test.

Elicitor treatments were performed by spraying 3-week-old plants with an elicitor extract obtained from the fungus _P. cucumerina_ (300 μg ml−1) as previously described (Casacuberta et al., 1992). Three independent experiments were performed with at least 12 plants per genotype in each experiment.

For Pi treatment experiments, plants were grown _in vitro_ on meshes placed on agar plates with modified Hoagland half strength medium containing 0.25 mM KH₂PO₄ for 1 week. Seedlings were then transferred to fresh agar medium at the desired concentration of Pi (0.05, 0.1, 0.25, or 2 mM Pi). The plants were allowed to continue growing for one more week under each Pi regime. The _in vitro_-grown plants were then inoculated with a spore suspension of _P. cucumerina_ or _C. higginsianum_ as above.

The _pho2_ mutant, previously named _ubc24_ (UBQUITIN-CONJUGATING ENZYME 24; At2g33770; Col-0 background) was obtained from the Arabidopsis Biological Resource Center (ABRC, ref. CS8508). A point mutation in the sixth exon (from G2539 to A, relative to the translational start codon) causes an early termination at the beginning of the UBC domain, thus resulting in the loss of the ubiquitin-conjugating activity of PHO2 in the _pho2_ mutant (Aung et al., 2006).

Plant tissue staining

For trypan blue staining, leaves were fixed by vacuum infiltration for 1 h in ethanol:formaldehyde:acetic acid (90:3:5.5 v/v/v), stained with lactophenol blue solution for 1 h, and then washed with chloral hydrate for 15 min. Leaves were placed on glass slides with glycerol and observed using a Leica DM6 microscope under bright field conditions.

For H₂DCFDA staining, Arabidopsis leaves were placed on a solution of H₂DCFDA (at a concentration of 10 μM), vacuum infiltrated during 5 min, and then maintained in darkness for 10 min. Two washes with distilled water were performed. Photographs were taken on a Leica DM6 microscope to visualize green fluorescence. DAB staining was also used to examine H₂O₂ levels. For this, Arabidopsis plants were immersed in a DAB solution (1 mg ml−1) for 30 min with vacuum treatment, maintained during 4 h in darkness under agitation, washed with 95% ethanol for 30 min at 75°C, and observed using a Leica DM6 microscope under bright field illumination.

ROS production in response to treatment with _P. cucumerina_ elicitors (300 μg ml−1) was measured using the luminol method described by Smith and Heese (2014). Measurements were carried out on a CentroXS3 LB 960 Microplate reader (Berthold Technologies, Bad Wildbad, Germany).

Generation of transgenic Arabidopsis plants

For _MIR399F_ overexpression, the miR399F precursor sequence was cloned under the control of the Cauliflower mosaic virus (CMV) 35S promoter in the PMDC32 plasmid (Chiou et al., 2006). The plant expression vector was transferred to _Agrobacterium tumefaciens_ strain GV301. Arabidopsis Col-0 plants were transformed using the floral dip method. Homozygosis was achieved by antibiotic selection. Segregation analysis confirmed transgene inheritance in successive generations of transgenic lines.

Measurements of Pi content and chlorophyll content

The Pi content of Arabidopsis plants was determined as previously described (Versaw & Harrison, 2002). Chlorophylls were extracted and quantified spectrophotometrically (SpectraMax M3, Molecular Devices, San Jose, CA, USA) as previously described (Lichtenthaler & Buschmann, 2001).

Gene expression analyses

Total RNA was extracted using TRIzol reagent (Invitrogen, Waltham, MA, USA). First-strand cDNA was synthesized from DNase-treated total RNA (1 μg) with reverse transcriptase and oligo-dT (High Capacity cDNA reverse transcription kit, Applied Biosystems, Waltham, MA, USA). RT-qPCR was performed in optical 96-well plates using SYBR® green in a LightCycler 480 (Roche, Basel, Switzerland). Primers were designed using Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The _β-tubulin2_ gene (At5g05620) was used to normalize the transcript levels in each sample. Primers used for RT-qPCR and stem-loop RT-qPCR are listed in Table S1. Accumulation of mature miR399F was determined by stem-loop RT-qPCR (Varkonyi-Gasic et al., 2007). DNA sequencing confirmed the specificity of the PCR-amplified products. Two-way analysis of variance (ANOVA) followed by Tukey's
Phosphate-induced disease resistance in Arabidopsis

CONFLICT OF INTEREST

The authors declare that they do not have competing interests.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the published article and its supporting material.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Phenotypical analysis of miR399 OE plants.

Figure S2. Analysis of pho2 mutant plants under non-infection conditions.

Figure S3. ROS accumulation in leaves of high Pi plants (miR399 OE, pho2 plants) under non-infection conditions.

Figure S4. ROS accumulation in leaves of high Pi plants (miR399 OE, pho2 plants) in response to treatment with P. cucumerina elicitors as determined by DAB staining.

Figure S5. ROS production in leaves of miR399 OE, pho2, and wild-type plants in response to treatment with P. cucumerina elicitors as determined using the luminol assay. ROS production in wild-type plants that have been grown under different Pi supply was also determined using the luminol assay.

Figure S6. Expression of defense-related genes in wild-type Arabidopsis plants that have been grown under different Pi supply (0.05, 0.1, or 0.25 mM).

Table S1. List of oligonucleotides.

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