the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2022) **110,** 452–469 doi: 10.1111/tpj.15680

Phosphate-induced resistance to pathogen infection in Arabidopsis

Beatriz Val-Torregrosa[1] , Mireia Bundó[1] , Héctor Martín-Cardoso[1] , Marcel Bach-Pages[1] , Tzyy-Jen Chiou[2] , Victor Flors[3] and Blanca San Segundo[1,4,*]

[1]*Centre for Research in Agricultural Genomics (CRAG) CSIC-IRTA-UAB-UB, Campus Universitat Autònoma de Barcelona (UAB), Bellaterra (Cerdanyola del Vallés), Barcelona, Spain,*

[2]*Agricultural Biotechnology Research Center, Academia Sinica, Taipei 115, Taiwan,*

[3]*Departamento de Ciencias Agrarias y del Medio Natural, Escuela Superior de Tecnología y Ciencias Experimentales, Universitat Jaume I, Castelló, Spain, and*

[4]*Consejo Superior de Investigaciones Científicas (CSIC), Barcelona, Spain*

Received 13 February 2021; revised 30 December 2021; accepted 17 January 2022; published online 21 January 2022
*For correspondence (e-mail blanca.sansegundo@cragenomica.es).

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 Harpin HrpZ protein) can also induce HR in plants (Chang & Nick, 2012).

Among ROS, H_2O_2 is relatively stable and is an important molecule regulating plant immunity (Torres et al., 2006). H_2O_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-5p/miRNA-3p 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 auxin receptors, resulting in resistance to bacterial pathogens (Navarro et al., 2006). Since then, other miRNAs controlling diverse processes have been shown to be involved in Arabidopsis immunity, either ETI or PTI (Huang et al., 2016; Jagadeeswaran et al., 2009; Seo et al., 2013; Shivaprasad et al., 2012; Staiger et al., 2013). Some examples are miR160, miR396, miR398, miR400, miR472, miR773, miR844, miR858, miR863, and miR156 (Boccara et al., 2014; Camargo-Ramírez et al., 2017; Lee et al., 2015; Li et al., 2010; Niu et al., 2016; Park et al., 2014; Salvador-Guirao et al., 2018; Yin et al., 2019). Depending on their target gene, these miRNAs can function as positive or negative regulators in fine-tuning immune responses. Even though pathogen infection has been shown to induce alterations in the expression of a plethora of Arabidopsis miRNAs, the mechanistic role of these miRNAs in processes underling immunity is often unclear. Additionally, most studies on miRNAs involved in plant immunity have been carried out in the interaction of Arabidopsis plants with the bacterial pathogen *Pseudomonas syringae*, and less is known on miRNAs involved in resistance against fungal pathogens.

On the other hand, distinct miRNAs have been associated with 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 Chiou 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-

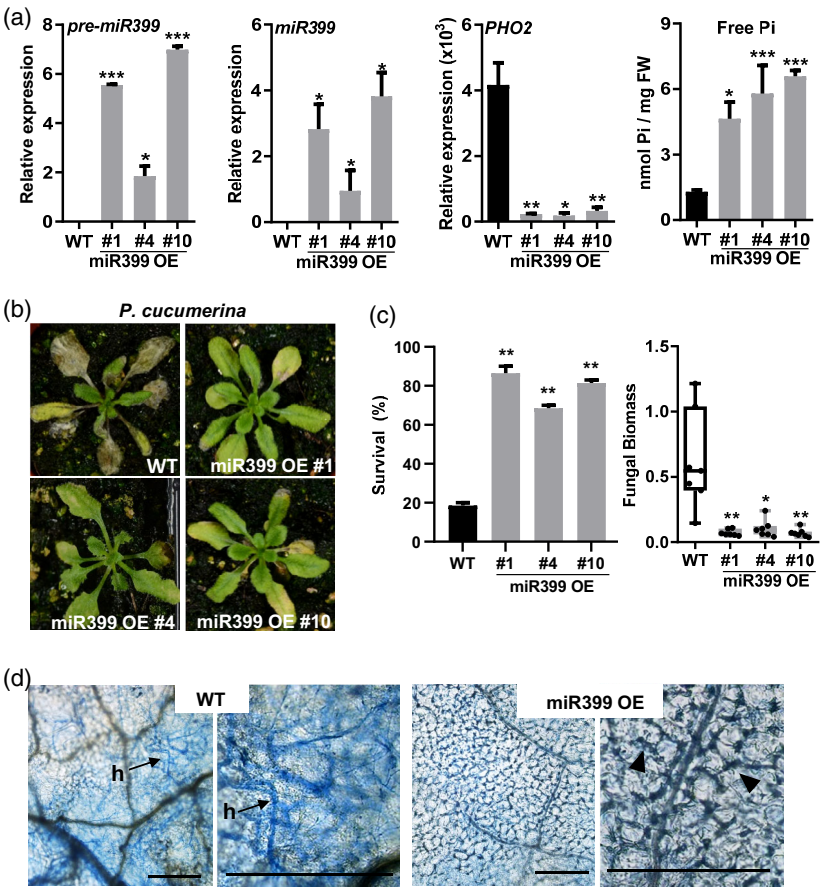


Figure 1. Resistance of miR399 OE plants to infection by the necrotrophic fungus *P. cucumerina*. Homozygous miR399 OE lines (#1, #4, and #10) and wild-type plants were grown in soil for 3 weeks and then assayed for disease resistance. Three independent experiments were carried out with at least 12 plants 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 *AtPHO2* was determined by RT-qPCR. The Arabidopsis *β-tubulin2* 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, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$). (b) Plants were spray-inoculated with *P. cucumerina* spores (5×10^5 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 β-tubulin* at 7 dpi. Values of fungal DNA were normalized against the Arabidopsis *UBIQUITIN21* gene (At5g25760). Comparisons have been made relative to wild-type plants. Data are mean ± SEM ($n = 7$) (*t*-test, $*P \leq 0.05$, $**P \leq 0.01$). (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.

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 mutant plants 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; Fujii 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 3c), 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, results here 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.

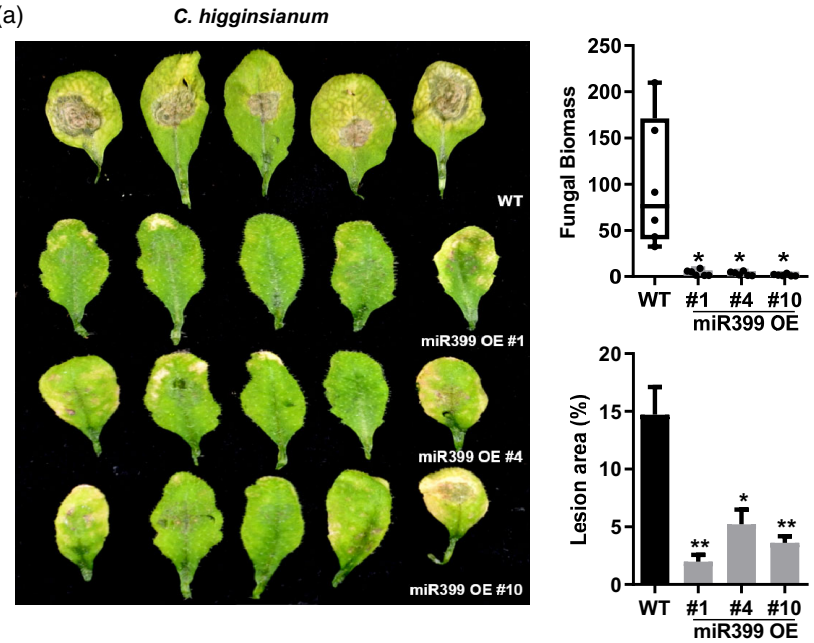


Figure 2. Resistance of miR399 OE plants to infection by the hemibiotrophic fungus *C. higginsianum*. Leaves were locally inoculated with a spore suspension at 4×10^6 spores ml^{-1}. Results are from one out of three independent experiments performed with three independent miR399 OE lines (lines 1, 4, and 10) and wild-type plants which gave similar results. At least 12 plants per genotype were assayed in each experiment. (a) Disease symptoms at 7 days post-inoculation (dpi) with fungal spores. The diseased leaf area was quantified using ImageJ software. Quantification of *C. higginsianum* DNA was carried out by qPCR using specific primers for the *C. higginsianum Internally transcribed spacer 2* (*ITS2*) gene at 7 dpi. Values are fungal DNA levels normalized against the Arabidopsis *UBIQUITIN21* gene (At5g25760). Comparisons have been made relative to wild-type plants. Histograms show the mean ± SEM (*t*-test, $*P \leq 0.05$, $**P \leq 0.01$). (b) Trypan blue staining of *C. higginsianum*-infected leaves of wild-type and miR399 OE plants at 7 dpi. h, hyphae. Arrows and arrowheads indicate fungal hyphae and dead cells, respectively. Higher magnifications of these regions are shown (right panels). Bars represent 100 μm.

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 miR399f 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 miR399f-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

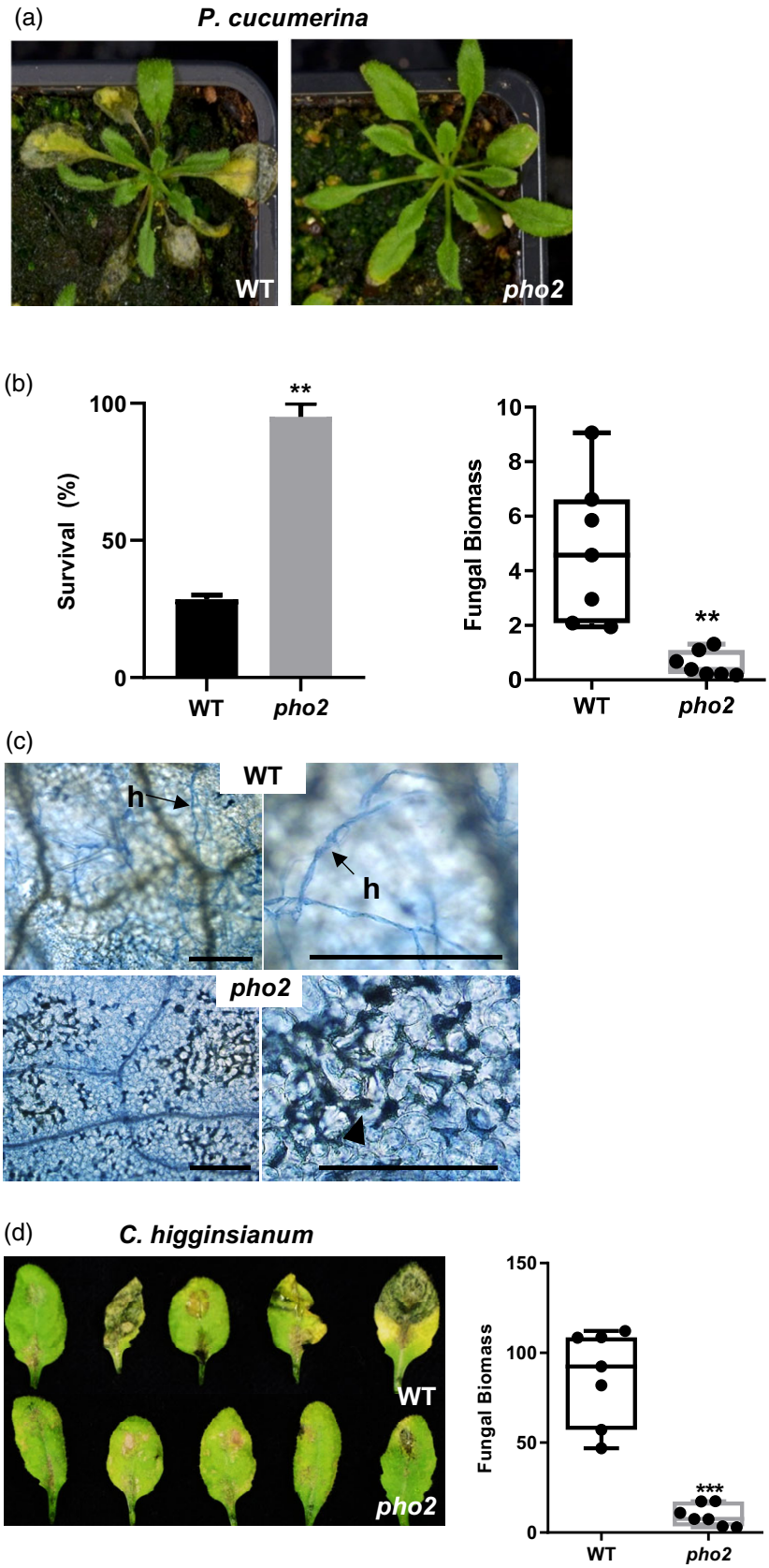


Figure 3. Resistance to infection by fungal pathogens in *pho2* plants. Three-week-old mutant plants were inoculated with fungal *P. cucumerina* spores. Three independent experiments were carried out with similar results with at least 12 plants per genotype. (a) Disease phenotype of wild-type and *pho2* plants upon inoculation with *P. cucumerina* spores (5×10^5 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, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$). (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 (4×10^6 spores ml^{-1}).

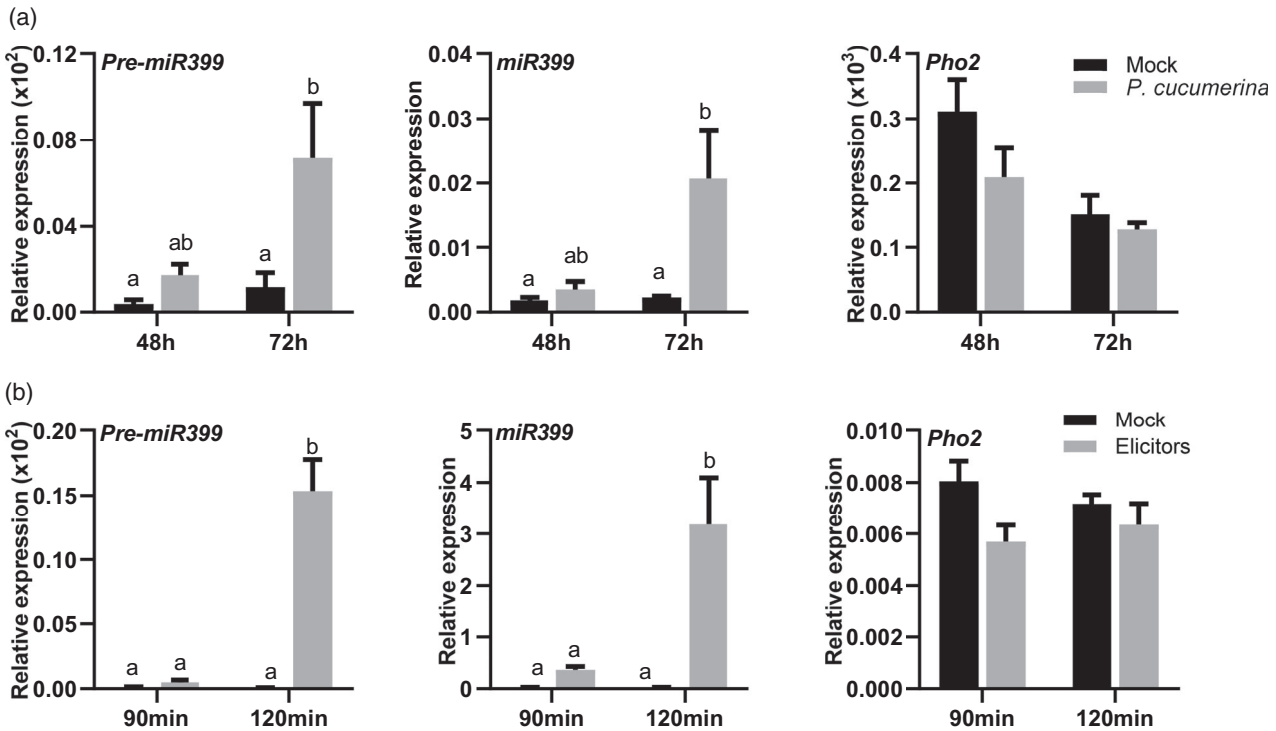


Figure 4. Acumulation of precursor (pre-miR399), mature (miR399), and *PHO2* transcripts in wild-type plants in response to inoculation with *P. cucumerina* spores or treatment with *P. cucumerina* elicitors. The accumulation of pre-miR399f and mature miR399f sequences was determined by RT-qPCR and stem-loop RT-qPCR, respectively, at the indicated times after inoculation with fungal spores (a) or elicitor treatment (b). At 48 and 72 hpi with *P. cucumerina* spores, pre-miR399 levels increased 4.5- and 6.0-fold, respectively. At the same time points, miR399f levels increased 2.0- and 9.0-fold. Elicitor treatment elevated the level of pre-miR399f accumulation 5.0- and 267-fold at 90 and 120 min of treatment, respectively. MiR399f levels increased 20- and 147-fold in response to elicitor treatment (90 and 120 min, respectively). Four biological replicates and three technical replicates per time point were assayed. Statistically significant differences were determined by ANOVA followed by Tukey's HSD test, where different letters represent statistically significant differences.

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 ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2). Of these, H_2O_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. Histochemical detection of H_2O_2 was carried out using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (H_2DCFDA). H_2DCFDA was previously shown to detect different forms of ROS, mainly H_2O_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). *Plectosphaerella 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_2DCFDA 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.

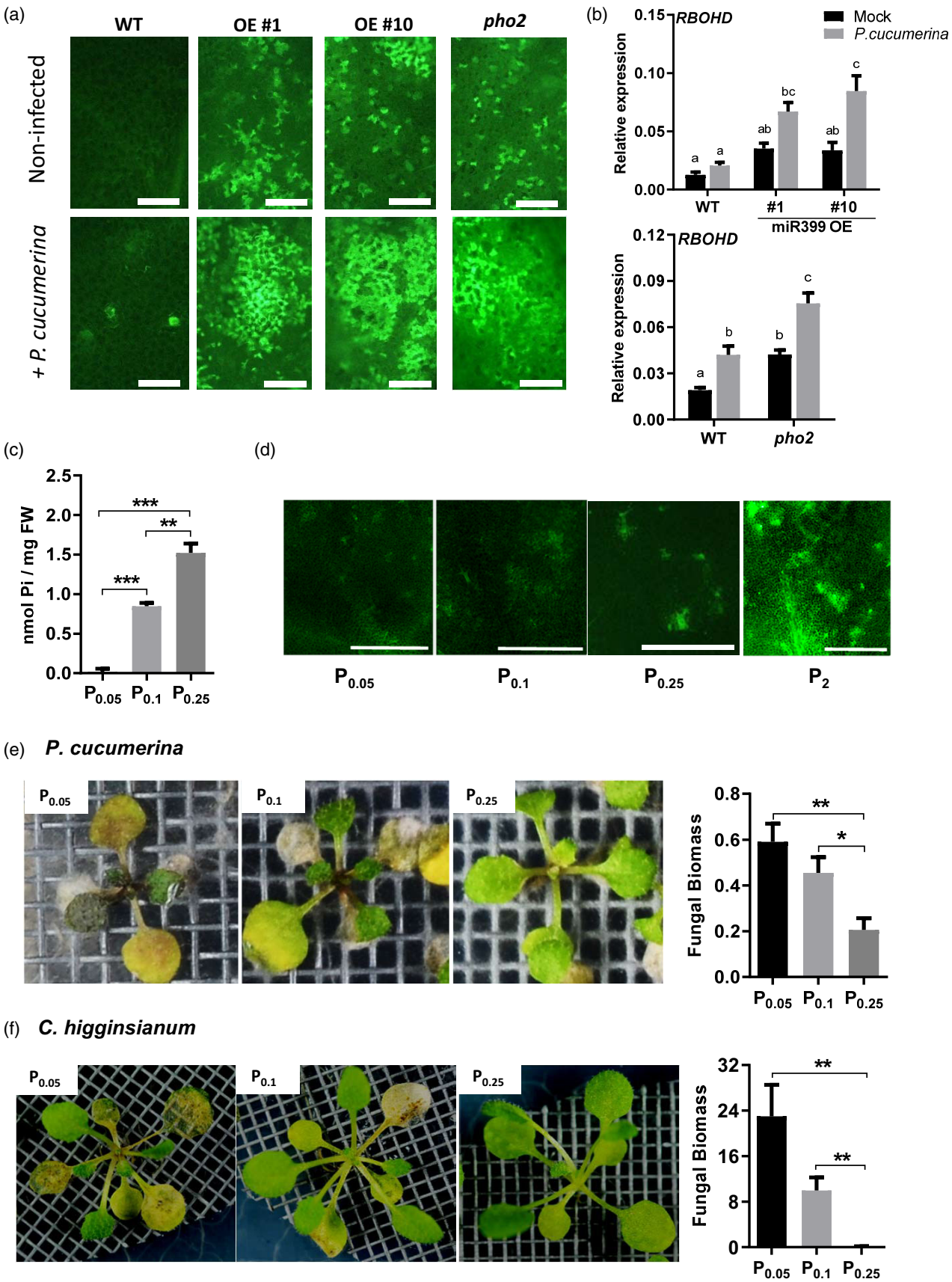
In concordance with results obtained by histochemical detection of ROS and measurement of ROS production using the luminol assay, the miR399 OE and *pho2* plants exhibited upregulation of *RBOHD* in the absence of pathogen infection compared with wild-type plants (Figure 5b, black bars). *Plectosphaerella 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_2DCFDA 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

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 (5×10^5 spores ml^{-1}) or mock-inoculated. Visualization of H_2O_2 accumulation was carried out using the fluorescent probe H_2DCFDA 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 β-tubulin2* gene (At5g62690). Three biological replicates (with three plants per replicate) were examined. Different letters represent 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_2DCFDA. 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 (4×10^6 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 (5×10^5 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, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$).



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 points 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 pathogen 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

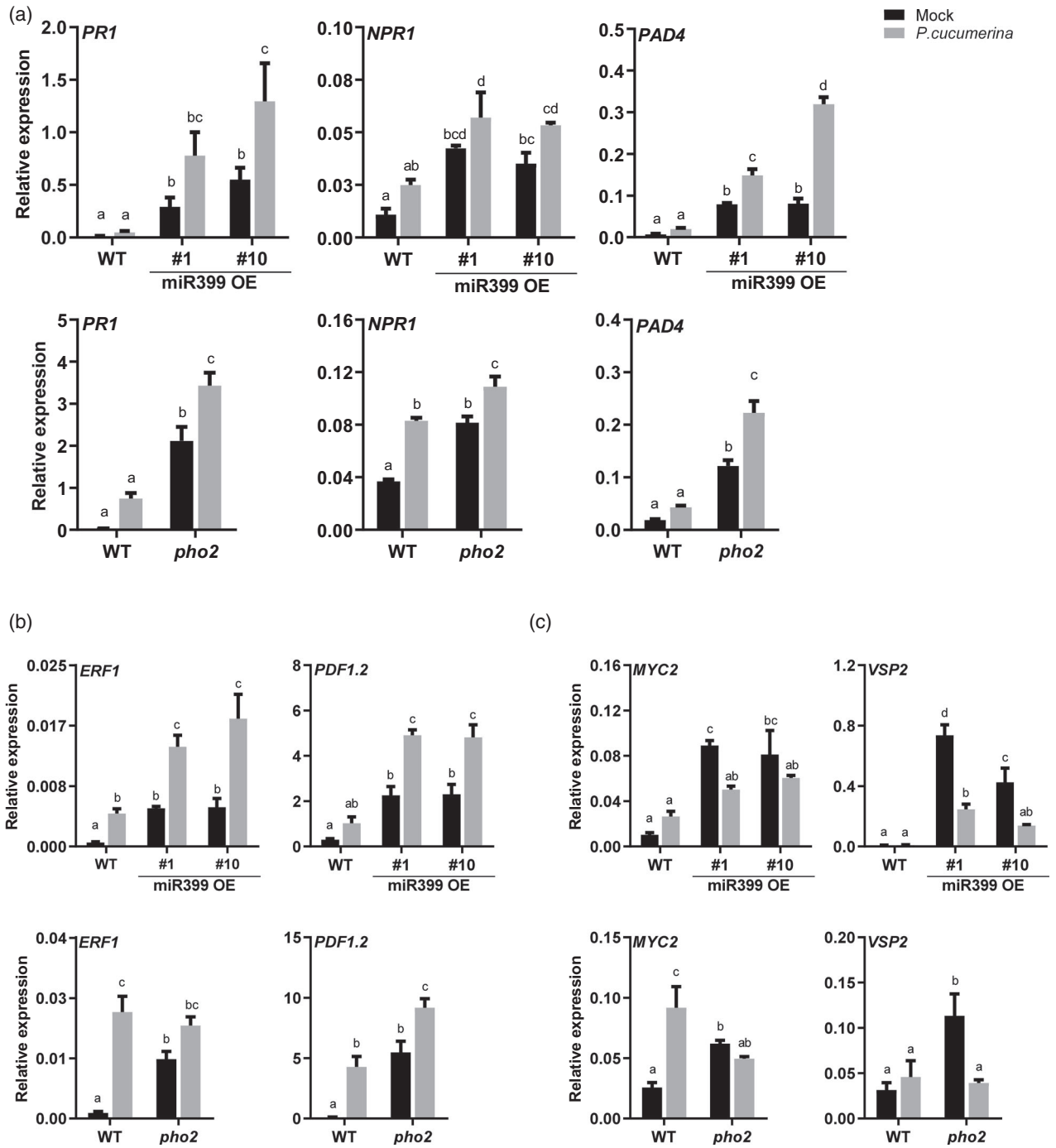


Figure 6. Expression of defense genes in miR399 OE and *pho2* plants. Transcript levels were determined by RT-qPCR in mock-inoculated or *P. cucumerina*-inoculated plants at 24 h post-inoculation (black and gray bars, respectively). The expression values were normalized to the Arabidopsis *β-tubulin2* 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; $P < 0.05$).

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

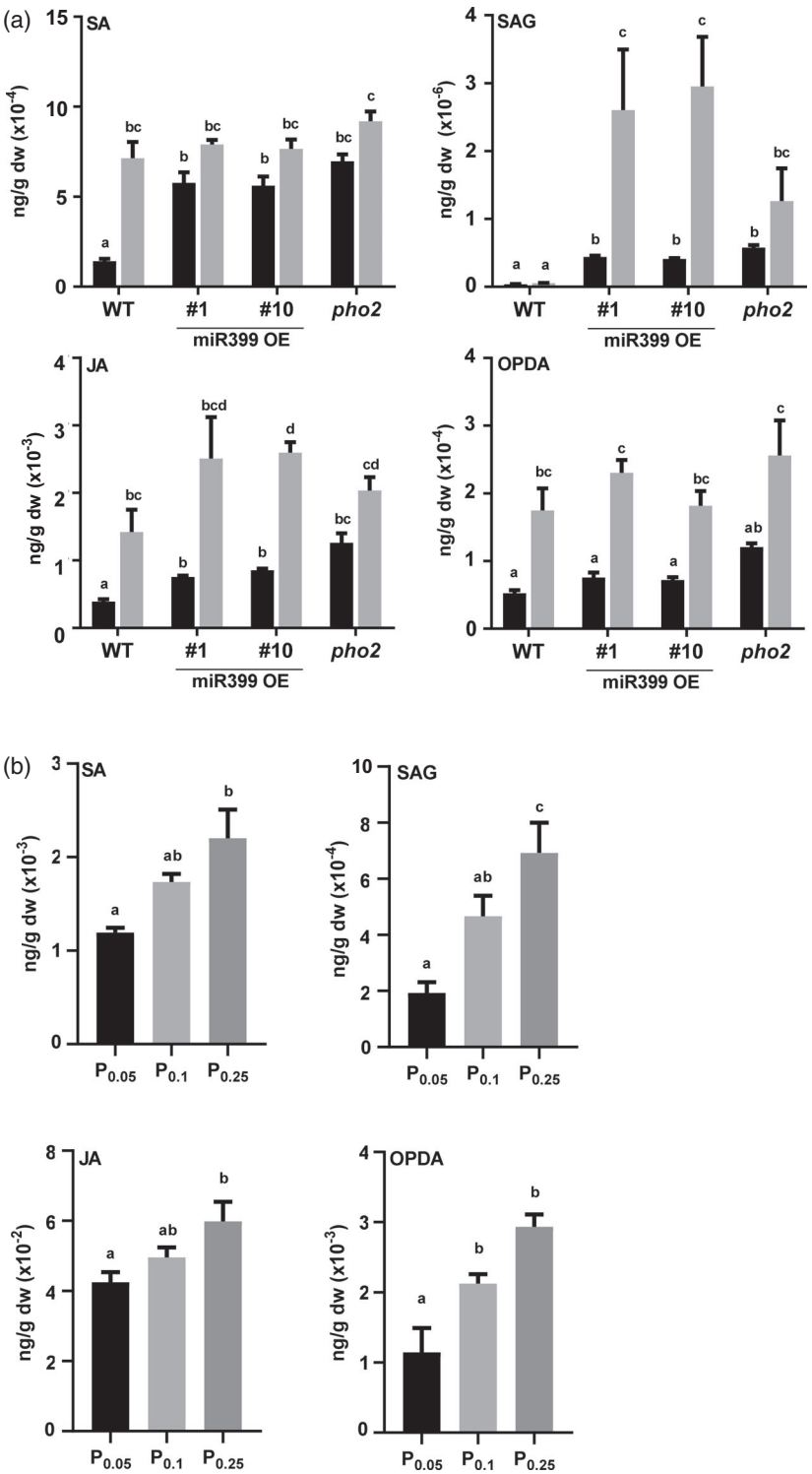


Figure 7. Levels of SA, SAG, JA, and OPDA in leaves of Arabidopsis plants that overaccumulate Pi (miR399 OE, *pho2*, and wild-type plants). (a) Hormone levels were measured in mock-inoculated and *P. cucumerina*-inoculated *miR399 and pho2* plants at 48 hpi. (b) Hormone levels in wild-type plants that have been grown at the indicated Pi supply conditions (0.05, 0.1, and 0.25 mM Pi). Plants were grown as indicated in Figure 5. 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 feed-forward loop between SA and ROS production (e.g., H_2O_2) 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

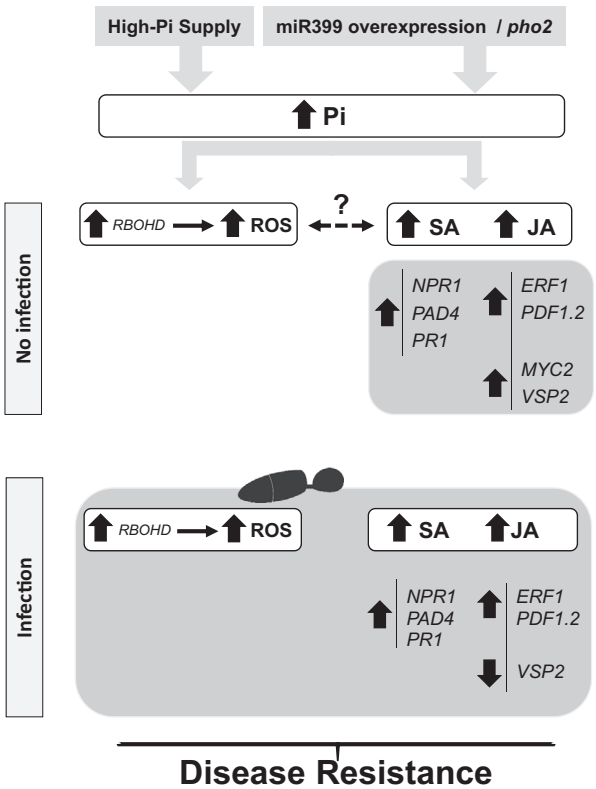


Figure 8. Proposed model to explain how Pi content and defense responses are integrated for modulation of resistance to infection by fungal pathogens in Arabidopsis. Treatment with high Pi, *MIR399* overexpression, and loss-of-function of *PHO2* would trigger Pi accumulation, which, in turn, would increase ROS and hormone (SA and JA) levels in Arabidopsis leaves for the induction of genes involved in the SA and JA signaling pathways. Upon pathogen infection, the expression of genes involved in the SA pathway and the ERF1/PDF1.2 branch of the JA pathway would be further induced, while genes in the MYC2/VSP2 branch of the JA pathway are repressed. The interplay between Pi, ROS, and hormones would allow the plant to mount an effective immune response. Although crosstalk between ROS and hormonal pathways has been described (Herrera-Vásquez et al., 2015; Xia et al., 2015), further investigation is needed to elucidate (i) the exact mechanisms by which Pi content modulates ROS production and hormone content and (ii) how these signaling pathways communicate with each other in the coordinated regulation of Pi homeostasis and immune responses.

accumulating Pi would allow the plant to mount a successful defense response upon encountering a pathogen.

The existence of links between components of the phosphate starvation response (PSR) and disease resistance has been previously described (Chan et al., 2021). For instance, enhanced resistance to infection by the bacterial pathogen *P. syringae* pv. tomato DC3000 and the oomycete pathogen *Hyaloperonospora arabidopsidis* was reported in *phr1* mutant plants, PHR1 being the master transcriptional regulator of the Arabidopsis PSR (Castrillo et al., 2017). This study also led the authors to propose that *PHR1* might fine-tune JA responses under Pi starvation in specific biological contexts, rather than being a regulator of the JA signaling pathway (Castrillo et al., 2017). In other studies, 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 (Frerigmann 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 diverse 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 Pi availability. Results here presented set the basis for further research to elucidate the exact mechanisms by which Pi 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}). *Colletotrichum 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* (5×10^5 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 (4×10^6 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* (4×10^6 spores ml^{-1}) or locally inoculated with *C. higginsianum* (5×10^5 spores ml^{-1}). Fungal biomass was determined by quantitative PCR (qPCR) using specific primers for the corresponding fungus; the Arabidopsis *UBIQUITIN21* (At5g25760) gene was used as the internal control (Soto-Suárez 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 KH_2PO_4 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* (UBIQUITIN-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 G_{2539} 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 (80:3.5:5 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 H_2DCFDA staining, Arabidopsis leaves were placed on a solution of H_2DCFDA (at a concentration of 10 μM), vacuum infiltrated during 5 min, and then maintained in darkness for 10 min. Two washes with distillated water were performed. Photographs were taken on a Leica DM6 microscope to visualize green fluorescence. DAB staining was also used to examine H_2O_2 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 miR399 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

honestly significant difference (HSD) test was used to analyze RT-qPCR data.

Hormone determination

The rosettes of 3-week-old wild-type (Col-0), miR399 OE, and *pho2* plants were analyzed by LC–MS for SA, SAG, JA, and OPDA content as previously described (Sánchez-Bel et al., 2018). Briefly, 30 mg of freeze-dried material was extracted with MeOH:H_2O (30:70) containing 0.01% of HCOOH and a mixture of 10 μg L^{-1} of the internal standards SA-d5 and dehydro-JA (Sigma-Aldrich, St. Louis, MO, USA). Following extraction, samples were centrifuged (25 000 *g*, 15 min) and filtered through regenerated cellulose filters. An aliquot of 20 μl was injected into a UPLC system (Waters Acquity) interfaced with a Xevo TQ-S Mass Spectrometer (Waters, Milford, MA, USA). Hormones were quantified by contrasting with an external calibration curve of pure chemical standards of SA, SAG, JA, and OPDA. Sample separation was performed with an LC Kinetex C18 analytical column with a particle size of 5 μm, 2.1 × 100 mm (Phenomenex, Torrance, CA, USA). Chromatographic and MS conditions were as described in Sánchez-Bel et al. (2018). At least six biological replicates were analyzed per genotype and condition, each replicate consisting of leaves from at least three independent plants. The plant material was lyophilized prior to analysis. Two-way ANOVA followed by Tukey's HSD test was used to analyze data.

ACCESSION NUMBERS

β-Tubulin 2 (At5g62690); *PHO2* (At2g33770); *PR1* (At2g14610); *NPR1* (At1g64280); *PAD4* (At3g52430); *ERF1* (At3g23240); *PDF1.2* (At2g26020); *MYC2* (At1g32640); *VSP2* (At5g24770); *RBOHD* (At5g47910); *Ubiquitin 21* (At5g25760).

ACKNOWLEDGMENTS

We thank A. Molina and R.J. O'Connell for providing the *P. cucumerina* and *C. higginsianum* strains and Glòria Escolà for assistance in parts of this work. We acknowledge support of the publication fee by the CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI). This research was supported by the Ministry of Science, Innovation and Universities/FEDER/Innovation-State Research Agency (AEI) (grant RTI2018-101275-B-I00) to BSS, the Research Promotion Plan from the Universitat Jaume I (UJI-B2019-2), and the Scientific Instrumentation Central Service from the Universitat Jaume I to VF. We acknowledge financial support from the Spanish Ministry of Science/AEI through the "Severo Ochoa Programme for Centres of Excellence in R&D" SEV-2015-0533 and CEX2019-000902-S, and the CERCA Programme/"Generalitat de Catalunya". BVT was a recipient of a fellowship from the Ministry of Economy, Industry and Competitiveness/AEI (BES-2016-076289). MBP was a recipient of a scholarship from La Caixa Foundation.

AUTHOR CONTRIBUTIONS

BSS and TJC devised the research project. MB, BVT, and BSS designed the experiments and analyzed the data. BVT conducted most experiments and prepared the figures. MBP performed infection experiments with *P. cucumerina*. HMC collaborated in expression analyses and ROS detection. VF performed hormone analyses. BSS and BVT wrote the article with further contributions from MB. All authors supervised and complemented the writing. BSS agrees to be responsible for contact and ensures communication.

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 and upon infection with *P. cucumerina* as determined by DAB staining.

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 H_2DCFDA 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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