the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2024) **119,** 828–843 doi: 10.1111/tpj.16800

Phosphate deficiency increases plant susceptibility to *Botrytis cinerea* infection by inducing the abscisic acid pathway

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Received 4 October 2023; accepted 18 April 2024; published online 28 May 2024.
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SUMMARY

Plants have evolved finely regulated defense systems to counter biotic and abiotic threats. In the natural environment, plants are typically challenged by simultaneous stresses and, amid such conditions, crosstalk between the activated signaling pathways becomes evident, ultimately altering the outcome of the defense response. As an example of combined biotic and abiotic stresses, inorganic phosphate (Pi) deficiency, common in natural and agricultural environments, can occur along with attack by the fungus *Botrytis cinerea*, a devastating necrotrophic generalist pathogen responsible for massive crop losses. We report that Pi deficiency in *Arabidopsis thaliana* increases its susceptibility to infection by *B. cinerea* by influencing the early stages of pathogen infection, namely spore adhesion and germination on the leaf surface. Remarkably, Pi-deficient plants are more susceptible to *B. cinerea* despite displaying the appropriate activation of the jasmonic acid and ethylene signaling pathways, as well as producing secondary defense metabolites and reactive oxygen species. Conversely, the callose deposition in response to *B. cinerea* infection is compromised under Pi-deficient conditions. The levels of abscisic acid (ABA) are increased in Pi-deficient plants, and the heightened susceptibility to *B. cinerea* observed under Pi deficiency can be reverted by blocking ABA biosynthesis. Furthermore, high level of leaf ABA induced by overexpression of *NCED6* in Pi-sufficient plants also resulted in greater susceptibility to *B. cinerea* infection associated with increased spore adhesion and germination, and reduced callose deposition. Our findings reveal a link between the enhanced accumulation of ABA induced by Pi deficiency and an increased sensitivity to *B. cinerea* infection.

Keywords: Arabidopsis, phosphate deficiency, *Botrytis cinerea*, abscisic acid, defense.

INTRODUCTION

In the natural environment, plants face both biotic and abiotic stresses, and there is growing evidence that a plant's response to each individual stress is a poor predictor of the global response when stresses are combined (Leisner et al., 2023; Saijo & Loo, 2020). Phosphorus (P) is an important nutrient for plant growth and development in agricultural and natural ecosystems. Plants acquire P as soluble inorganic phosphate (Pi), which is incorporated into a myriad of essential biomolecules, such as nucleic acids, phospholipids, phosphoproteins, and sugar phosphates (Poirier et al., 2022). Given the strong chemical tendency of Pi to produce insoluble complexes with cations (e.g., calcium [Ca^{+2}]) and metals (e.g., iron [Fe^{+3}] and aluminum [Al^{+3}]) that are abundant in the soil, the amount of soluble Pi available to roots for uptake is very low, typically below 10 μM. This stands in contrast to the typical intracellular Pi concentration of approximately 10 mM. To cope with Pi deficiency, plants activate diverse adaptations and signaling pathways, collectively termed the P starvation response, inducing changes at the developmental, biochemical, and transcriptomic levels to increase the acquisition of external Pi and optimize internal Pi use to sustain growth and reproduction (Paz-Ares et al., 2022; Poirier et al., 2022). To acquire Pi from the soil, plants rely on the PHOSPHATE TRANSPORTER 1 (PHT1) family of Pi/H^{+} co-transporters located on the plasma membrane of root epidermal and cortical cells (Wang, Wang, et al., 2021). The Pi acquired by the root is translocated to the aerial tissues by PHOSPHATE 1 (PHO1), a Pi exporter localized at the root pericycle and xylem parenchyma cells, which loads Pi into the xylem (Arpat et al., 2012; Hamburger et al., 2002; Poirier et al., 1991). The systemic P starvation response is primarily modulated by the transcription factors PHOSPHATE STARVATION RESPONSE 1 (PHR1), which control the expression of Pi-transporter genes and numerous Pi

deficiency–responsive genes (Bustos et al., 2010; Rubio et al., 2001).

Plants are surrounded by potential biological threats. Among them, invading pathogens are classified as necrotrophs, biotrophs, or hemibiotrophs according to their mode of acquiring nutrients from the host plant. Necrotrophs secret toxins and enzymes to kill the host and consume the released nutrients; by contrast, biotrophs do not secrete toxins and feed from the live host for an extended period. The hemibiotrophs have an initial biotrophic phase followed by a necrotrophic one (Horbach et al., 2011). *Botrytis* is the first and most extensively studied genus of necrotrophic fungi, of which *B. cinerea* is responsible for major crop losses by virtue of the wide range of plant species that it can infect and feed upon (Elad et al., 2016). Infection by *B. cinerea* starts when the conidia (spores) land on the plant surface and, if humidity and temperature levels are favorable, they will attach, germinate, and invade the plant tissue by producing cell wall-degrading enzymes, toxins, oxalic acid, and reactive oxygen species (ROS) (Holz et al., 2007; Nakajima & Akutsu, 2014; Veloso & Van Kan, 2018). After overcoming the plant's constitutive defenses, such as cell wall, cuticle, and preformed antimicrobial compounds commonly known as phytoanticipins, the invading pathogens must survive the induced defenses, such as the production and export of defense-related secondary metabolites, the accumulation of ROS causing cell death at the infection site, callose deposition at the attempted site of penetration, the biosynthesis of defense phytohormones, and the expression of defense-related genes (Ghozlan et al., 2020; Piasecka et al., 2015).

The defense response against a particular combination of biotic and abiotic stresses depends largely on the balanced crosstalk between phytohormones (Ku et al., 2018). The immune response is contingent upon the type of attacker and is orchestrated mainly by three phytohormones: jasmonic acid (JA), ethylene (ET), and salicylic acid (SA). The defense against wounding and herbivory relies on JA and JA-isoleucine (JA-Ile) conjugates, while JA together with ET are essential for defense against necrotrophic pathogens, such as *B. cinerea* (AbuQamar et al., 2017). The SA-activated signaling pathway is generally considered to play a central role in defense against biotrophic pathogens (Aerts et al., 2021).

The interplay between Pi deficiency and the biotic stress response has been demonstrated in different plant species, with the majority of reports pointing to low Pi having a positive influence on the immune system (Chan et al., 2021; Pazhamala & Giri, 2023). In line with this, Pi deficiency was demonstrated to induce JA and JA-Ile biosynthesis in the shoots and roots of Arabidopsis (*Arabidopsis thaliana*), leading to the activation of the JA-signaling pathway and ultimately enhancing the response to wounding and herbivory by the cotton leafworm *Spodoptera littoralis* (Khan et al., 2016). This study showed that increased resistance to insect herbivory triggered by Pi deficiency was also found in tomato (*Lycopersicum esculentum*) and tobacco (*Nicotiana benthamiana*), highlighting the evolutionary conservation of this signaling interaction.

Among the defense phytohormones, abscisic acid (ABA) is a well-characterized positive player in the response against abiotic stresses, such as cold, osmotic, salt, and drought stress (Zhang, Zhu, et al., 2022). By contrast, the role of ABA in response to biotic stress is more ambivalent, displaying a positive or negative influence depending on the nutritional state of the plant, the lifestyle of the attacking pathogen, and its strategy of host penetration (Checker et al., 2018). One of the most common ABA-mediated responses is the rapid closure of stomata to thwart the attempted invasion by pathogens that use the plant's natural openings for penetration, as is the case for the hemibiotroph *Pseudomonas syringae* (Melotto et al., 2006). Regarding the effect of ABA after infection with necrotrophic and hemibiotrophic pathogens, studies have shown that mutants defective in ABA biosynthesis or signaling are more susceptible to *Alternaria brassicicola* but are surprisingly more resistant to *B. cinerea* and *Fusarium oxysporum* infection, highlighting the contrasting effect of ABA in pathogen defense (Anderson et al., 2004; Audenaert et al., 2002; Mondal et al., 2020). Furthermore, ABA has been found to have an antagonistic function to that of JA and ET, as treatment with ABA suppresses the activation of defense gene expression mediated by JA and ET (Anderson et al., 2004).

Here, we show that Pi deficiency makes Arabidopsis plants more susceptible to infection by *B. cinerea* despite them adequately activating their JA and ET signaling pathways for defense and producing the secondary metabolites camalexin and ROS. This increased susceptibility in Pi-deficient plants correlated with a higher number of spores attaching to the leaf surface and an increased frequency of spore germination. Furthermore, we observed a reduction in callose deposition in Pi-deficient plants challenged with *B. cinerea*. Remarkably, Pi deficiency resulted in high ABA levels in the leaves, and the increased susceptibility of the Pi-deficient mutant *pho1* to *B. cinerea* infection could be rescued by blocking ABA biosynthesis. Collectively, our data highlights a link between Pi deficiency, ABA, and susceptibility to *B. cinerea* infection.

RESULTS

Pi-deficient plants are more susceptible to infection by *B. cinerea*

To test the influence of Pi deficiency on plant susceptibility to *B. cinerea*, we grew wild-type Columbia-0 (Col-0)

Arabidopsis plants for 4–5 weeks in a clay-based substrate irrigated with Pi-sufficient (1 mM) or Pi-deficient (10 μM) solutions and then infected them with a *B. cinerea* spore suspension. We evaluated their phenotypes by measuring the lesion area and quantifying fungal biomass 48 hours post inoculation (hpi). Despite having elevated basal levels of JA (Figure S1; Khan et al., 2016), the Pi-deficient plants were significantly more susceptible to *B. cinerea* infection than the Pi-sufficient plants (Figure 1A; Figure S2A).

We continued our analysis with the *pho1* mutant, which is defective in root-to-shoot Pi transfer and thus has constitutive Pi deficiency in the shoot even when grown in soil that contains an adequate amount of available Pi (Arpat et al., 2012; Hamburger et al., 2002; Poirier et al., 1991), making this mutant an excellent proxy for plants grown under Pi-deficient conditions. We used two null alleles of *pho1* (*pho1-2* and *pho1-3*) generated using ethyl methanesulfonate mutagenesis (Hamburger et al., 2002). The shoots of both *pho1* mutants display all the hallmarks of phosphate deficiency: low shoot Pi, reduction in biomass, accumulation of anthocyanins, and the expression of genes associated with Pi deficiency (Poirier et al., 1991; Rouached et al., 2010). Defense responses are dependent on the developmental stage of the plant (Hu & Yang, 2019); therefore, we first assessed the developmental progression of the *pho1* mutants compared to Col-0 by quantifying the number of leaves produced by plants grown in short-day conditions in soil until the appearance of trichomes on the abaxial surface of the rosette leaves. This feature marks the transition from the juvenile to the adult vegetative phase (Kerstetter & Poethig, 1998). The vegetative phase change occurred at leaf number 8 in both Pi-sufficient Col-0 and the Pi-deficient *pho1* mutants, but the *pho1* mutants required approximately 5 weeks of growth to reach that stage while Col-0 required 4 weeks (Figure S3). To ensure that we were studying the defense response of plants at comparable developmental stages, we grew all Pi-deficient plants, including both *pho1* mutants and the Pi-deficient Col-0 plants, 1 week ahead of the Pi-sufficient plants. All the defense experiments were performed after 4 and 5 weeks of growth for the Pi-sufficient and Pi-deficient plants, respectively.

To assess the susceptibility of *pho1* mutants to infection by *B. cinerea*, we analyzed the lesion size and the quantity of fungal biomass in the rosette leaves of Col-0 and the two *pho1* mutants grown in soil at 48 hpi. While the lesions induced by *B. cinerea* infection were consistently larger in soil-grown plants than in plants grown in clay substrate supplemented with nutrient solution with high or low Pi (Figure 1A,B), soil-grown Pi-deficient *pho1* mutants were more susceptible to the infection than the soil-grown Pi-sufficient Col-0 control (Figure 1B,C; Figure S2B), making this mutant a suitable model for Pi deficiency in our analyses.

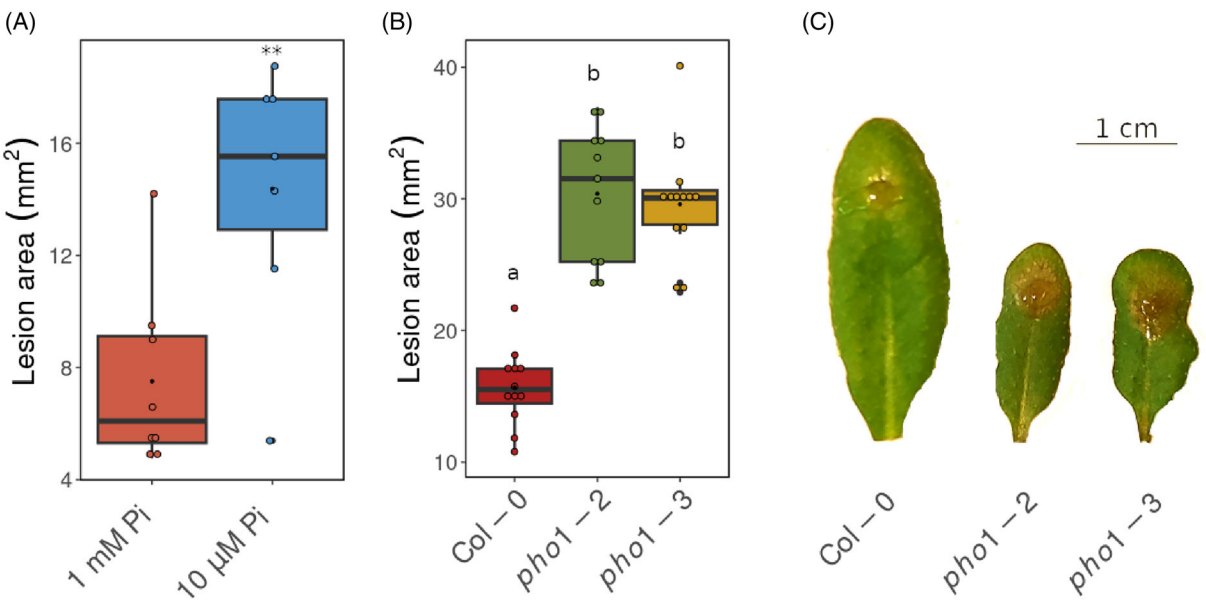

Figure 1. Inorganic phosphate (Pi) deficiency increases susceptibility to *Botrytis cinerea* in Arabidopsis.
(A, B) Susceptibility to *B. cinerea* infection was measured as the lesion area at 48 hours post inoculation (hpi) in wild-type Col-0 plants grown for 4–5 weeks in a clay-based substrate fertilized with 1/4 MS medium containing 1 mM or 10 μM Pi (A); and in Col-0, *pho1-2*, and *pho1-3* plants grown in soil for 4–5 weeks (B). Each dot represents the average lesion size assessed in three leaves per plant. Asterisks (A) or different letters (B) denote statistical differences determined using a Student's *t*-test (**$P < 0.01$) or one-way ANOVA with Tukey's test ($P < 0.05$), respectively. The experiments were performed twice (A) or three times (B) with similar results.
(C) Representative pictures of *B. cinerea*-infected *leaves at 48 hpi.*

The late defense mechanisms are properly activated in the *pho1* mutant upon *B. cinerea* infection

To investigate the possible causes for the increased susceptibility of Pi-deficient plants to *B. cinerea* infection, we first measured the expression of three key defense-response genes in soil-grown Col-0 and *pho1-2* shoots: *BOTRYTIS-SUSCEPTIBLE 1* (*BOS1*), encoding an R2R3MYB transcription factor required for delaying *B. cinerea* growth in infected plants; *PLANT DEFENSIN 1.2 A* (*PDF1.2A*), encoding an antifungal peptide in the plant defensins family; and *WRKY DNA-BINDING PROTEIN 33* (*WRKY33*), encoding a transcription factor essential for defense against necrotrophic pathogens (Birkenbihl et al., 2012; Mengiste et al., 2003; Penninckx et al., 1996). All three genes showed low transcript levels under a mock treatment but were upregulated in both Col-0 and the *pho1-2* mutant shoots at 48 hpi with *B. cinerea* spores (Figure 2A–C), indicating that the mutants had an adequate transcriptional response to *B. cinerea* infection. Additionally, upon infection with *B. cinerea*, *ETHYLENE RESPONSE FACTOR 1* (*ERF1*), encoding a regulator of the synergistic response to ET and JA under pathogen attack (Lorenzo et al., 2003), was more strongly upregulated in *pho1-2* than in Col-0, as well as in the mock-treated *pho1-2* mutant

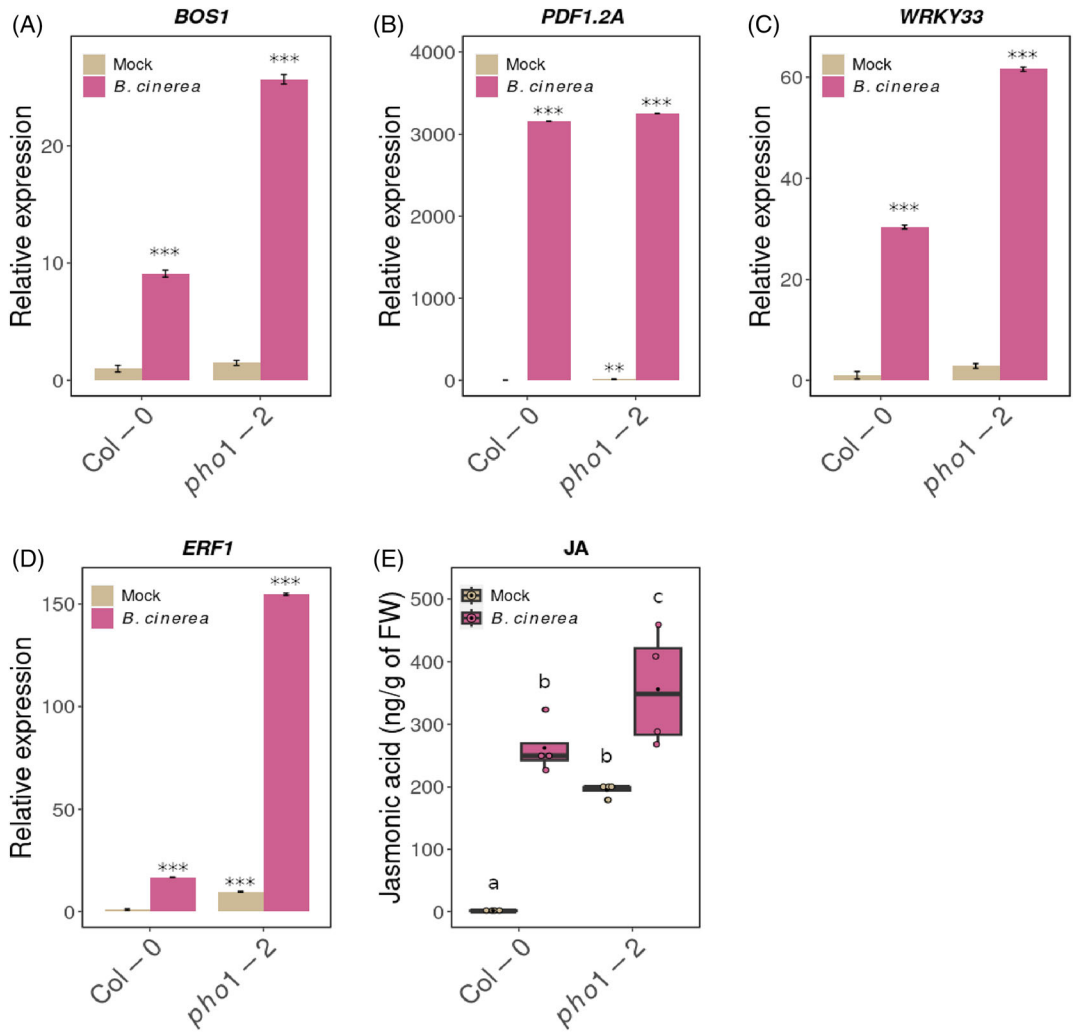

Figure 2. Late defense gene responses in the *pho1-2* mutant infected with *Botrytis cinerea*.
(A–D) Fold changes in the transcript abundance of (A) *BOS1*, (B) *PDF1.2A*, (C) *WRKY33*, and (D) *ERF1*. Total RNA was isolated from 4- to 5-week-old shoots of Col-0 and *pho1-2* at 48 hours post inoculation (hpi) with a mock or *B. cinerea* spore suspension. Transcript levels were normalized to the expression of *ACTIN2*. Bars represent the mean ± SEM of three biological replicates that were analyzed in triplicate. Asterisks denote a statistical significance from the Col-0 plants subjected to the mock treatment, determined using two-way ANOVA with Dunnett's test (**$P < 0.01$; ***$P < 0.001$).
(E) Jasmonic acid (JA) content in the shoots of 4- to 5-week-old Col-0 and *pho1-2* plants. Samples from four independent whole rosettes were taken at 48 hpi with a mock or *B. cinerea* spore suspension. Different letters denote a statistical significance, determined using a two-way ANOVA with Tukey's test. All gene expression experiments were performed three times, while the JA quantification was performed twice; similar results were detected for all replicates.

compared with Col-0 (Figure 2D). Moreover, the levels of JA in the shoot were significantly elevated in the *pho1-2* mutant compared with Col-0 under the mock treatment, as expected for Pi-deficient plants (Khan et al., 2016), and were further increased in *pho1-2* mutant plants at 48 hpi with *B. cinerea* (Figure 2E). Together, these data indicate that the increased sensitivity of Pi-deficient *pho1-2* mutant to *B. cinerea* infection is not caused by a reduction in the activation of the JA or ET pathway nor by the failure to activate the key defense genes following infection.

B. cinerea spore adhesion and germination are enhanced by Pi deficiency

The *B. cinerea* infection process starts with the attachment and germination of spores on the leaf surface (Holz et al., 2007). To investigate the state of these early events in Pi-deficient plants, we first performed trypan blue staining to visualize spores and germinating tubes on the leaf surface. We examined soil-grown Col-0 and *pho1-2* leaves at 15 hpi and quantified the intensity of the staining as number of blue pixels, revealing more intense staining on the *pho1-2*-inoculated leaves (Figure 3A,B). Accordingly, both the spore attachment (Figure 3C), measured as number of spores per mm^2 of leaf area at 8 hpi, and spore germination rate (Figure 3D), quantified at 15 hpi, were higher on the *pho1-2* leaf surface than on Col-0. Similarly, for Col-0 plants grown in clay-based substrate supplemented with the Pi-sufficient and Pi-deficient nutrient solutions, the Col-0 plants grown under Pi-deficient conditions displayed a higher rate of spore attachment and germination than

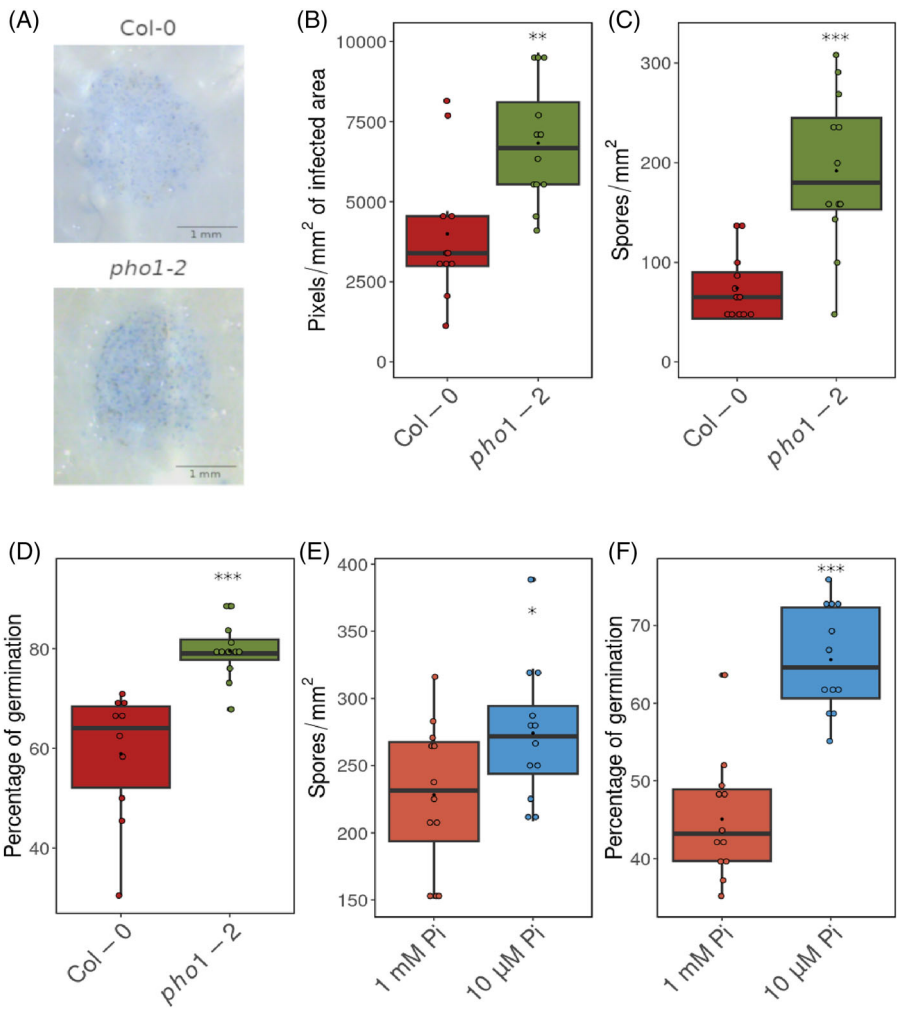

Figure 3. *Botrytis cinerea* spore attachment and germination are enhanced in Pi-deficient plants.
(A–F) Plants were inoculated with a drop of *B. cinerea* spore suspension, and the leaves of 7–12 independent plants from each genotype were detached at 15 hours post inoculation (hpi) (A, B, D, F) or 8 hpi (C, E) and stained with trypan blue. The leaves were photographed using a bright field microscope (A) to quantify the number of blue pixels (B), to count the number of *B. cinerea* spores present in the inoculated area of the leaf (C, E), and to count the number of germinated spores (D, F). The plants were grown for 4–5 weeks either in soil (A–D) or in a clay-based substrate supplemented with 1/4 MS medium containing 1 mM or 10 µM inorganic phosphate (Pi) (E, F). In all plots, each dot represents the average of three leaves. Asterisks denote a statistical significance, determined using a Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). All experiments were performed at least twice with similar results.

those grown in the Pi-sufficient conditions (Figure 3E,F). These data indicated that shoot Pi deficiency impacts the early events of the *B. cinerea* infection process.

The expression of secondary defense metabolites genes and the accumulation of ROS are induced in *pho1* mutants upon *B. cinerea* infection

To counteract the early stages of fungal infection, plants produce and export defense-related secondary metabolites to the leaf surface, mainly the phytoalexin camalexin and glucosinolates in the Brassicaceae family, and they also produce ROS and deposit callose at the infection site (Ghozlan et al., 2020). To assess the state of activation of these lines of defense in the *pho1* mutant compared to Col-0, we first quantified camalexin at 24 and 48 hpi with the mock solution or *B. cinerea* spore suspension. The camalexin content increased in both genotypes following infection; the contents were similar between the two genotypes at 24 hpi but higher in *pho1-2* at 48 hpi (Figure 4A). Furthermore, *PENETRATION 3* (*PEN3*) and *PLEIOTROPIC DRUG*

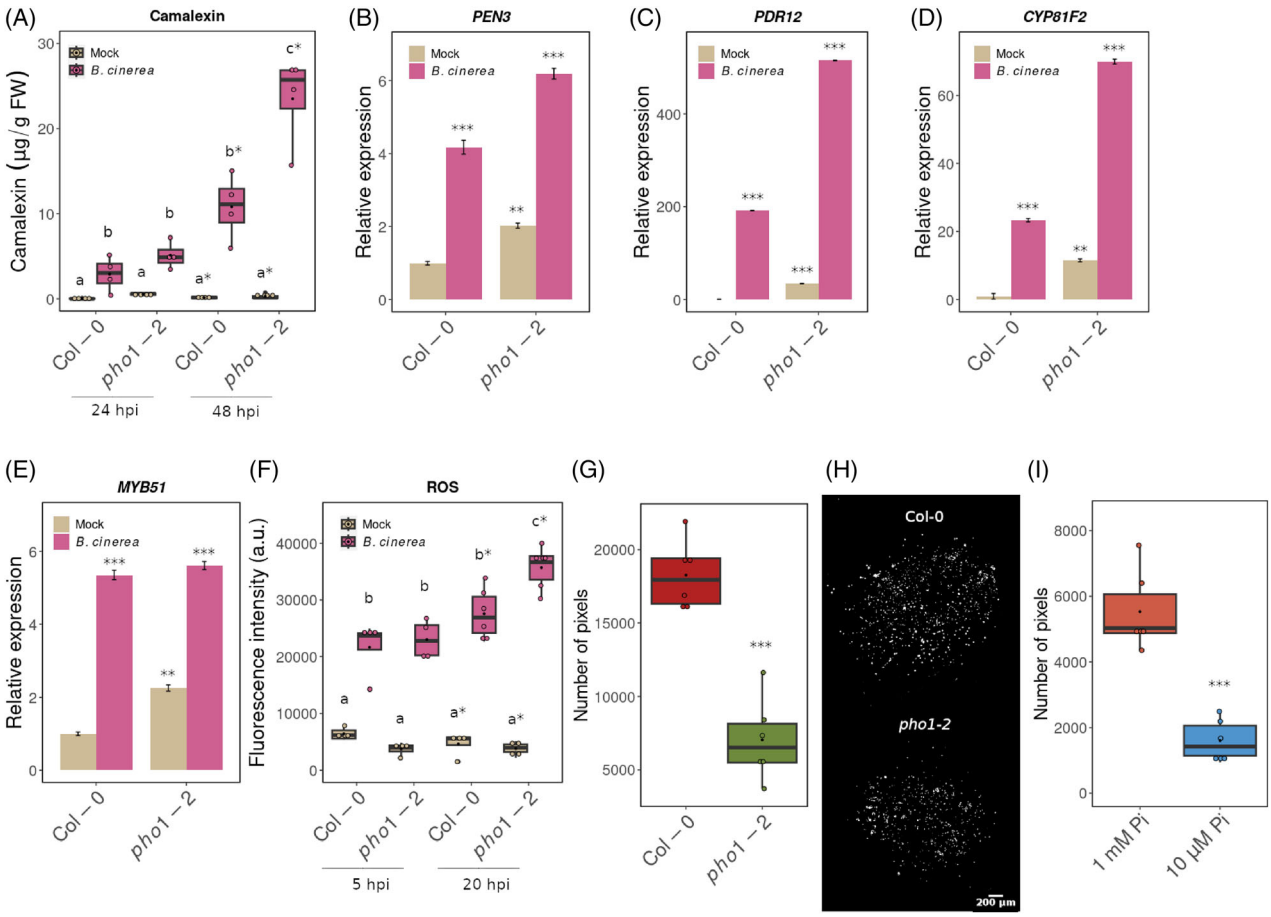

Figure 4. Early responses to *Botrytis cinerea* infection in Pi-deficient plants.
Plants were grown for 4–5 weeks either in soil (A–H) or in a clay-based substrate supplemented with nutrient solution containing 1 mM or 10 µM inorganic phosphate (Pi) (I).
(A) Camalexin content was quantified in shoot samples from four independent whole rosettes taken at 24 and 48 hours post inoculation (hpi) with a mock or *B. cinerea* spore suspension. Different letters denote a statistical significance determined using a two-way ANOVA with Tukey's test ($P < 0.05$). Letters without an asterisk indicate statistical significance for the 24 hpi dataset. Letters with and asterisk indicate statistical significance for the 48 hpi dataset.
(B–E) Fold changes in the transcript abundance of *PEN3* (B), *PDR12* (C), *CYP81F2* (D), and *MYB51* (E). Total RNA was isolated from 4- to 5-week-old shoots at 15 hpi with the mock or *B. cinerea* spore suspension. The transcript levels were normalized to the expression of *ACTIN2*. Bars represent the mean ± SEM of three biological replicates that were analyzed in triplicate. Asterisks denote a statistical significance from the reference plants (Col-0 subjected to the mock treatment), determined using a two-way ANOVA with Dunnett's test (**$P < 0.01$; ***$P < 0.001$).
(F) Reactive oxygen species (ROS) production was quantified in arbitrary units (a.u.) at 5 and 20 hpi with the mock or *B. cinerea* spore suspension, determined by staining the samples with H_2DCFDA. Each dot represents the average of three leaves. Different letters denote a statistical significance determined using a two-way ANOVA with Tukey's test ($P < 0.05$). Letters without an asterisk indicate statistical significance for the 5 hpi dataset. Letters with and asterisk indicate statistical significance for the 20 hpi dataset.
(G, I) Callose deposition, quantified as the number of pixels in the inoculation area at 15 hpi with the *B. cinerea* spore suspension and after aniline blue staining. Asterisks denote a statistical difference, determined using Student's *t*-test (***$p < 0.001$).
(H) Representative pictures of callose deposition in the infected leaves of the indicated genotypes.

RESISTANCE 12 (*PDR12*), encoding two members of the ATP binding cassette transporter protein subfamily G (ABCG) that function redundantly to export camalexin in Arabidopsis (He et al., 2019), were strongly upregulated as early as 15 hpi with *B. cinerea* in both Col-0 and the *pho1-2* mutant, with higher expression levels in both mock- and *B. cinerea*-inoculated *pho1-2* plants compared with Col-0 (Figure 4B,C).

Next, we quantified the expression of two genes involved in glucosinolate biosynthesis: *CYTOCHROME P450, FAMILY 81, SUBFAMILY F 2* (*CYP81F2*), encoding a cytochrome P450 monooxygenase, and *MYB DOMAIN PROTEIN 51* (*MYB51*), encoding a transcription factor that regulates the expression of glucosinolate biosynthetic genes (Bednarek et al., 2009; Clay et al., 2009). *CYP81F2* and *MYB51* were strongly upregulated in both Col-0 and *pho1*-2 at 15 hpi with *B. cinerea*, with a higher expression level in mock-inoculated *pho1-2* plants compared with Col-0 (Figure 4D,E). After *B. cinerea* inoculation, *CYP81F2* transcript levels were higher in the *pho1-2* mutant than in Col-0 (Figure 4D).

We then quantified ROS production by staining mock- or *B. cinerea*-inoculated leaves 5 and 20 hpi with carboxy-H_2DCFDA. Under the mock treatment, both Col-0 and *pho1-2* produced comparable levels of ROS, and both genotypes increased ROS production in response to *B. cinerea* infection. However, at 20 hpi, ROS accumulation was significantly higher in *pho1-2* than in Col-0 (Figure 4F).

Altogether, these data indicate that the biosynthesis of camalexin, glucosinolates, and ROS upon infection with *B. cinerea* is not compromised by Pi deficiency.

Aberrant callose deposition and increased susceptibility to *B. cinerea* in the *pho1* mutant is dependent on ABA

To assess callose deposition in *B. cinerea*-infected plants, we stained leaves of *pho1-2* and Col-0 taken at 15 hpi with aniline blue. Despite the higher level of spore attachment and germination in *pho1-2* compared with Col-0, callose deposition was significantly reduced in the infected *pho1-2* leaves (Figure 4G,H). This occurred despite the mutant's adequate upregulation of *POWDERY MILDEW RESISTANT 4* (*PMR4*), encoding the stress-induced callose synthase (Nishimura et al., 2003), as measured at 15 hpi with *B. cinerea* (Figure S4). Moreover, we also observed this defect in callose deposition at 15 hpi with *B. cinerea* in Col-0 plants grown in a clay-based substrate under Pi deficiency compared with the Pi-sufficient condition (Figure 4I).

Abscisic acid mediates several abiotic stress response, and has antagonistic roles with the JA and ET signaling pathway (Cao et al., 2011). In the context of callose deposition, ABA has been reported as having both positive and negative roles (De Torres-Zabala et al., 2007; Ton & Mauch-Mani, 2004). We thus evaluated the potential involvement of this phytohormone in the susceptibility to *B. cinerea* infection under Pi deficiency. First, we quantified the levels of endogenous ABA in the shoots of Col-0 and the *pho1* mutants grown in soil for 4–5 weeks. Under normal conditions, the ABA levels were significantly higher in both *pho1* mutants than in Col-0 (Figure 5A). To further validate these results, we introgressed into the *pho1-3* mutant the *6xABRE_R:erGPF* synthetic promoter reporter that recapitulates the transcriptional response of a tissue to the endogenous ABA levels (Wu et al., 2018). We observed a higher activation of the reporter in leaf epidermal cells of the *pho1-3 6xABRE_R:erGFP* line compared with that of Col-0 *6xABRE_R:erGFP* (Figure 5B). We then examined the expression level of ABA biosynthesis genes, namely *ABA DEFICIENT 1* (*ABA1*), *ABA DEFICIENT 2* (*ABA2*), *ALDEHYDE OXIDASE 3* (*AAO3*) and *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*), and ABA signaling genes, namely *ABA INSENSITIVE 4* (*ABI4*) and *SNF1-RELATED PROTEIN KINASE 2.6* (*SnRK2.6*). We found a significant increase in the expression of all the analyzed genes in both *pho1* mutants compared to Col-0 (Figure 5C). We also detected an increase in ABA levels, signaling activation of the fluorescent reporter, and a significant increase in the expression level of ABA biosynthesis and signaling genes in Col-0 *6xABRE_R:erGFP* plants grown in a clay-based substrate under Pi-deficient condition compared with Pi-sufficient condition (Figure 5D–F).

To assess whether the elevated content of ABA in the *pho1* mutant was associated with the higher sensitivity to *B. cinerea* infection, we produced *pho1-2 aba2-1* double mutants, in which a mutation in *ABA DEFICIENT 2* (*ABA2*) strongly reduces the level of endogenous ABA (Schwartz et al., 1997). Analysis of the area of infection and quantification of fungal biomass at 48 hpi with *B. cinerea* revealed that blocking ABA biosynthesis in the *pho1* mutant decreased the susceptibility to *B. cinerea* to a level similar to the infected Col-0 plants (Figure 6A; Figure S2C). Furthermore, the ABA signaling sextuple mutant *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* strongly impaired in ABA perception (Gonzalez-Guzman et al., 2012) also showed an enhanced resistance to *B. cinerea* compared to Col-0 when plants were grown under the Pi-deficient condition (Figure 6B; Figure S2D). While spore attachment to the leaf surface was not significantly different between *pho1-2 aba2-1* and *pho1-2* (Figure 6C), *B. cinerea* spore germination was strongly decreased on the *pho1-2 aba2-1* mutant compared with the *pho1-2* single mutant (Figure 6D). Additionally, callose deposition in the *pho1-2 aba2-1* mutant at 15 hpi was partially restored to the Col-0 level (Figure 6E,F). These results suggested that Pi-deficient plants were more susceptible to *B. cinerea* as a consequence of the increased endogenous levels of ABA. To further test this hypothesis and determine if Pi deficiency was essential or dispensable for the increased susceptibility to *B. cinerea* infection in the context of high endogenous ABA level, we employed

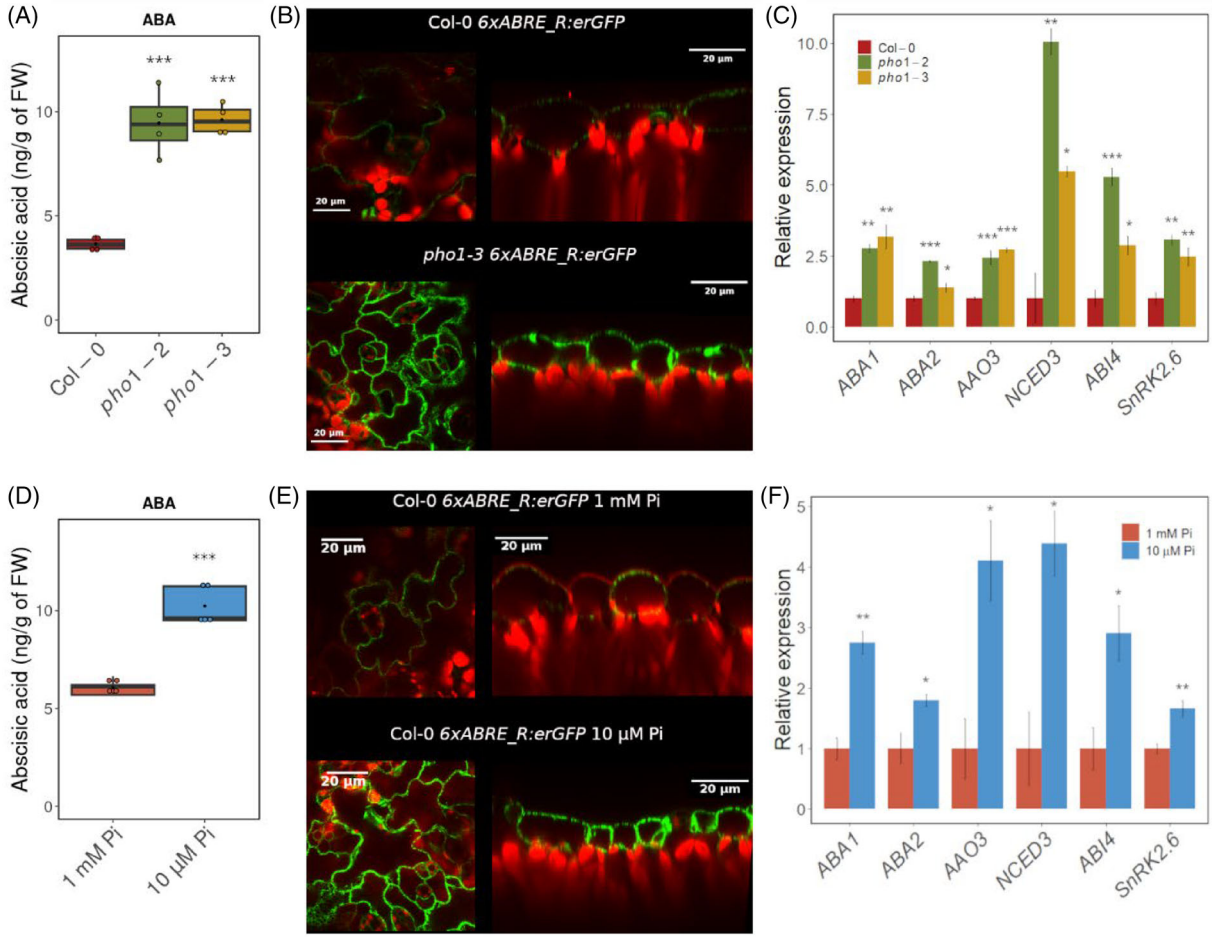

Figure 5. Pi deficiency is associated with an increase in endogenous abscisic acid (ABA) levels.
The plants were grown for 4–5 weeks either in soil (A–C) or in a clay-based substrate supplemented with nutrient solution containing 1 mM or 10 µM Pi (D–F).
(A, D) ABA content in the shoot was quantified using liquid chromatography-mass spectrometry.
(B, E) Confocal images showing the expression pattern of the *6xABRE_R* reporter in propidium iodide-stained leaves. Both the leaf surface (left) and a cross-section (right) are shown.
(C, F) Fold changes in transcript abundance of the indicated genes. Total RNA was isolated from 4- to 5-week-old shoots. The transcript levels were normalized to the expression of *ACTIN2*. Bars represent the mean ± SEM of three biological replicates that were analyzed in triplicate. Asterisks denote statistical differences, determined using a one-way ANOVA with Dunnett's test (A, C) or Student's *t*-test (D, F) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

the transgenic line *35S:NCED6-1* expressing the ABA biosynthesis gene *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 6* (*NCED6*) under the control of the constitutive cauliflower mosaic virus (*CaMV*) *35S* promoter (Lefebvre et al., 2006). This line accumulates higher levels of ABA than Col-0 under Pi-sufficient condition without substantial changes in leaf Pi content (Figure 7A; Figure S5). Line *35S:NCED6-1* was significantly more susceptible to the infection by *B. cinerea* under Pi-sufficient condition compared to Col-0 (Figure 7B; Figure S2E). Moreover, spore attachment and germination were both significantly increased and callose deposition was significantly reduced in the *35S:NCED6-1* line compared to Col-0 grown under Pi-sufficient conditions (Figure 7C–E). Finally, to test whether ABA can directly influence *B. cinerea* germination, ABA was supplied to a spore suspension. Germination rate of *B. cinerea* spores in liquid medium was significantly enhanced by the exogenous addition of ABA (Figure 7F; Figure S6).

Taken together, these results revealed that the high levels of endogenous ABA in the shoots of Pi-deficient plants is the key determinant in their increased susceptibility to *B. cinerea* and that a direct effect of ABA on spore germination is likely one of the contributing factors to this phenotype.

The transcription factor PHR1 is a crucial node in the Pi-deficiency and immune responses (Bustos et al., 2010; Chan et al., 2021). To assess the involvement of PHR1 in the response of Pi-deficient plants to *B. cinerea*, we evaluated the lesion size of the double mutant *pho1 phr1-1* at 48 hpi. We observed no significant difference between the susceptibility of the *pho1 phr1-1* double mutant and the

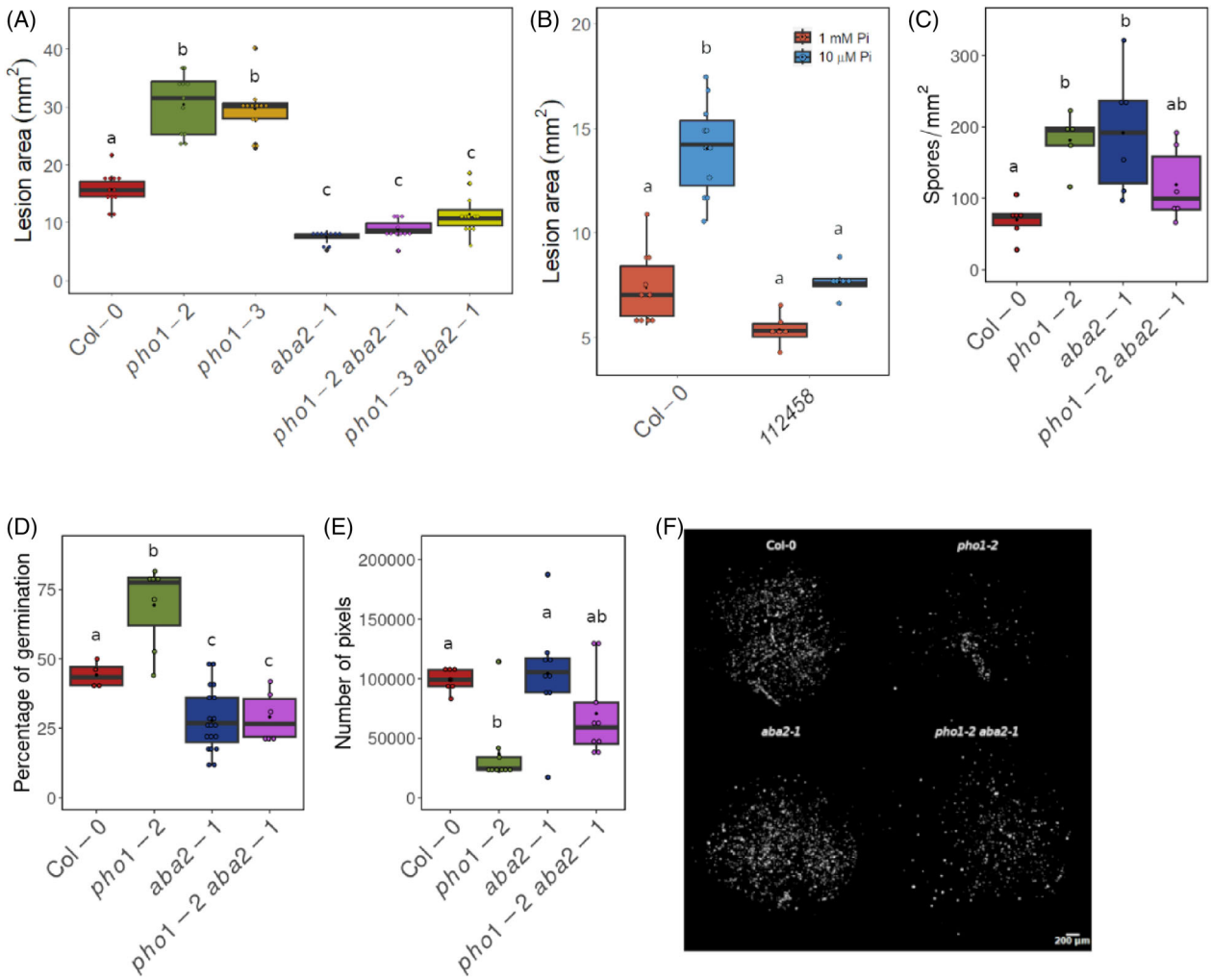

Figure 6. The phenotype of susceptibility to *Botrytis cinerea* under Pi deficiency can be reverted by blocking abscisic acid (ABA) biosynthesis or ABA signaling.
(A, B) Lesion area was measured at 48 hours post inoculation (hpi) with a *B. cinerea* spore suspension in 7–12 independent plants of the indicated genotypes. The single *aba2-1* mutant and *pho1 aba2-1* double mutants (A) are affected in ABA biosynthesis while the sextuple mutant *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* (abbreviated as *112458*) is impaired in ABA signaling. Each dot represents the average lesion size assessed in three leaves per plant.
(C, D) The indicated genotypes were assessed for spore attachment at 8 hpi (C) and spore germination at 15 hpi (D) after trypan blue staining. Each dot represents the average of three leaves per plant.
(E) Callose deposition was quantified as the number of pixels in the inoculation area at 15 hpi after aniline blue staining.
(F) Representative pictures of callose deposition in the infected leaves of the indicated genotypes. For panels (A–E), different letters denote a statistical significance determined using a one-way ANOVA with Tukey's test ($P < 0.05$).

pho1 single mutants (Figure 7G), indicating that the response to *B. cinerea* under Pi deficiency is not primarily under the control of PHR1 in Arabidopsis.

DISCUSSION

Plants have evolved an immune system finely orchestrated by phytohormones that activate specific responses to different types of environmental stresses. When plants are subjected to a combination of multiple stresses, the crosstalk between the various activated phytohormonal pathways determines the outcome of the global response (Atkinson & Urwin, 2012). The Pi-deficiency stress response is tightly interconnected with the immune defense response (Chan et al., 2021; Pazhamala & Giri, 2023), which is underscored by the fact that, under Pi-limited conditions, plants enhance the production of the defense-related phytohormones SA and JA, and of defense-related secondary metabolites, such as phenylpropanoids and glucosinolates (Khan et al., 2016; Morcillo et al., 2020; Pant et al., 2015). In agreement with this, the majority of reports to date highlight the greater immune response of Pi-deficient plants. This is exemplified by the heightened resilience to insect herbivory observed in Arabidopsis, tomato, tobacco (Khan et al., 2016), and young ribwort plantain (*Plantago lanceolata*) plants grown under low-Pi conditions (Qu et al., 2021). Other studies have

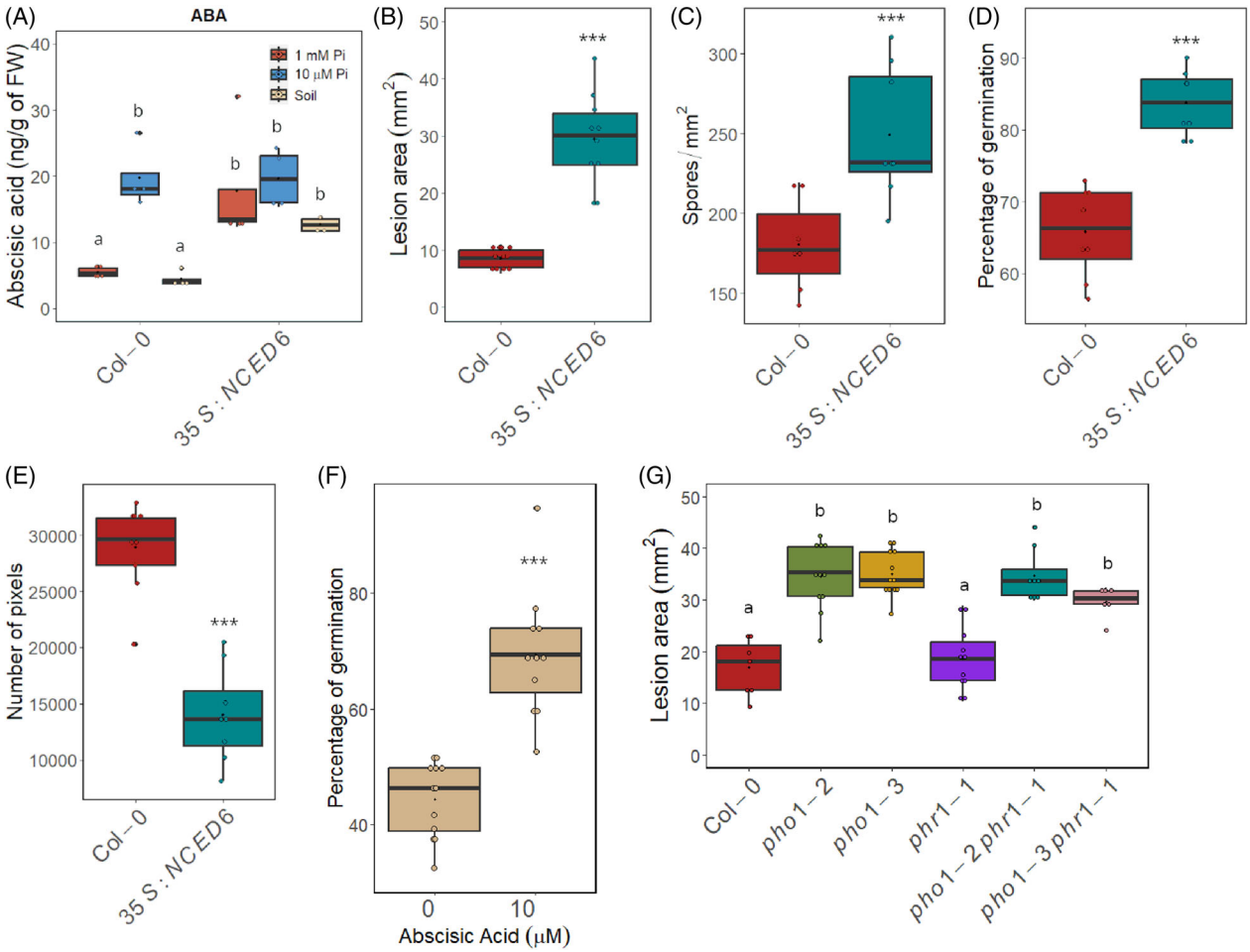

Figure 7. High abscisic acid (ABA) level in the absence of Pi deficiency leads to increased susceptibility to *Botrytis cinerea* infection.
(A) ABA content in the shoot of the indicated genotypes was quantified using liquid chromatography-mass spectrometry. Plants were grown for 4–5 weeks in a clay-based substrate supplemented with nutrient solution containing 1 mM or 10 µM Pi or were grown in soil.
(B, G) Lesion area was measured at 48 hours post inoculation (hpi) with a *B. cinerea* spore suspension in 7–12 independent plants of the indicated genotypes grown in soil. Each dot represents the average lesion size assessed in three leaves per plant.
(C, D) The indicated genotypes were assessed for spore attachment at 8 hpi (C) and spore germination at 15 hpi (D) after trypan blue staining. Each dot represents the average of three leaves per plant.
(E) Callose deposition was quantified as the number of pixels in the inoculation area at 15 hpi after aniline blue staining.
(F) *In vitro* germination of *B. cinerea* spores was assessed after 4 h in germination medium with or without 10 µM ABA. Different letters (A, G) or asterisks (B–F) denote statistical differences, determined using a one-way ANOVA with Tukey's test ($P < 0.05$) or Student's *t*-test, respectively (***$P < 0.001$).

shown enhanced resistance to the vascular fungus *Verticillium dahliae* in Pi-deficient cotton (*Gossypium hirsutum*) plants (Luo et al., 2021), and elevated protection against bacterial pathogens in Pi-deficient rice (*Oryza sativa*) (Kong et al., 2021) and Arabidopsis (Scheible et al., 2023). By contrast, plants over-accumulating Pi, such as the *pho2* mutant, which is deficient in an E2 ubiquitin-conjugating enzyme, have shown increased resistance to the necrotrophic fungus *Plectosphaerella cucumerina* and the hemibiotrophic fungus *Colletotrichum higginsianum* (Val-Torregrosa et al., 2022).

Several studies have highlighted the important role of the JA pathway in mediating resistance against *B. cinerea* (Ghozlan et al., 2020). The Arabidopsis JA-insensitive *coronatine insensitive 1* (*coi1*) and JA-resistant *jasmonate resistant 1* (*jar1*) mutants are impaired in JA signal perception and JA-Ile biosynthesis, respectively, and each showed increased susceptibility to *B. cinerea* (Ferrari et al., 2003; Thomma et al., 1998). Conversely, the Arabidopsis *fatty acid oxygenation upregulated 2* (*fou2*) and *overexpressor of cationic peroxidase 3* (*ocp3*) mutants, which have increased endogenous JA levels, showed an increased resistance to *B. cinerea* (Bonaventure et al., 2007; Coego et al., 2005). Considering that Pi deficiency leads to increased JA biosynthesis and activation of the JA-signaling pathway (Khan et al., 2016), it was surprising to observe that Pi deficiency was associated with an enhanced sensitivity to *B. cinerea* in the present study.

Numerous defense responses associated with *B. cinerea* infection were similar or even enhanced in the Pi-deficient *pho1* mutant compared with Col-0, including camalexin and ROS production and the activation of numerous defense-related genes important to *B. cinerea* resistance, including *BOS1* and *WRKY33* (Birkenbihl et al., 2012; Mengiste et al., 2003). The only defense response that we found to be severely altered in the *pho1* mutant was callose deposition. Callose is a β-(1,3)-D-glucan polysaccharide that is actively deposited in response to biotic and abiotic stresses. Particularly during pathogen attack, callose is deposited between the plasma membrane and the cell wall, in structures called papillae, to stop pathogen penetration (Wang, Li, et al., 2021). The inhibition of callose deposition in tomato by treatment with 2-deoxy-D-glucose increases its susceptibility to *B. cinerea* infection, highlighting the importance of this defense response (Asselbergh & Höfte, 2007). Here, we showed that the increased susceptibility to *B. cinerea* in Pi-deficient plants can be correlated to the heightened adhesion and germination of *B. cinerea* spores on the leaf surface, as well as to the reduction in callose deposition. The fact that reduction in callose formation in Pi-deficient plants occurred despite the appropriate activation of the *PMR4* gene indicates that the mechanisms involved in callose production are likely complex and not dependent only on *PMR4* expression.

The apparent paradox that Pi deficiency increases the sensitivity of Arabidopsis to *B. cinerea* infection despite the activation of many classical defense responses can likely be attributed to the increased spore load on the leaf surface resulting from increased attachment and germination. Indeed, density of germinating spores from the initial inoculum has been proposed to be a critical factor in determining the outcome of the *Botrytis*-plant host interaction process (Veloso & Van Kan, 2018). A higher spore density leads to higher production of virulence compounds by the germinating fungus, resulting in an accelerated attack that can overpower the plant defense response, making the susceptibility scenario unavoidable.

The increased susceptibility to *B. cinerea* infection and the reduced callose deposition in Pi-deficient plants were dependent on their elevated basal levels of ABA. The role of ABA in callose deposition is complex: depending on the plant species, the nature of the invading pathogen, and the plant endogenous nutritional balance, ABA can either have a positive or a negative impact on callose deposition (Asselbergh & Höfte, 2007; De Torres-Zabala et al., 2007; Ton & Mauch-Mani, 2004). While adding exogenous ABA to plants grown under low levels of sucrose induced callose deposition upon treatment with chitosan, ABA negatively influenced callose deposition in plants grown in high concentrations of sucrose and/or vitamins (Luna et al., 2011). High shoot sucrose levels are associated with Pi deficiency (Hammond & White, 2011), and are found in *pho1-2* plants (Figure S7). Whether a crosstalk between sucrose metabolism and ABA is involved in the response to *B. cinerea* and callose deposition under Pi deficiency deserves further investigation.

In agreement with the results presented in this work, ABA is generally regarded as a negative regulator of the immune response to necrotrophic pathogens, including *B. cinerea*. Mutants of genes involved in ABA biosynthesis in tomato and Arabidopsis display an enhanced immunity toward *B. cinerea* (Audenaert et al., 2002; L'Haridon et al., 2011). Moreover, mutants of *WRKY33*, encoding a transcription factor crucial for camalexin biosynthesis and for the transcriptional activation of JA-responsive genes, are more susceptible to infection by *B. cinerea*. This phenotype has been directly linked to an increase in ABA levels in the *wrky33* mutant as a consequence of the elevated expression of *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) and *NCED5*, encoding enzymes involved in ABA biosynthetic pathway (Birkenbihl et al., 2012; Liu et al., 2015). Increased susceptibility to *B. cinerea* was also observed in Arabidopsis plants pretreated with high concentrations of salt in the growth medium (Haller et al., 2020). This phenotype is proposed to be a direct consequence of salt stress-induced ABA accumulation, as treatment with increasing concentrations of ABA mimics the decreased resistance observed under salt stress.

Intriguingly, some phytopathogenic fungi are capable of biosynthesizing ABA or stimulating the host's ABA biosynthesis as an invasion mechanism. Notably, a pathogenic strain of the fungus *Colletotrichum tofieldiae* relies upon the activation of the host's ABA signaling pathways through the expression of a putative fungal ABA biosynthesis gene cluster (Hiruma et al., 2023). Similarly, the hemibiotrophic fungus *Magnaporthe oryzae* requires ABA to effectively infect rice roots, as evidenced by the impaired lesion formation of a fungal strain with impaired ABA biosynthesis, or under conditions where *M. oryzae* is incapable of activating ABA biosynthesis genes in rice. Furthermore, exogenous treatment of *M. oryzae* with ABA promotes spore germination and appressoria formation *in vitro* (Spence et al., 2015). Our data suggest that ABA could have a similar impact in *B. cinerea* infection, as Pi-deficient plants and transgenic lines overexpressing the ABA biosynthesis gene *NCED6* and that contain high levels of endogenous ABA present a more favorable environment for spore germination compared with their Pi-sufficient counterparts. Moreover, spore germination is promoted when ABA is applied directly to *B. cinerea* spores *in vitro*.

Our findings provide insight into the intricate interplay between Pi deficiency and ABA signaling. Some early work showing a connection between Pi deficiency and ABA included the enhanced stomatal response of cotton plants (Radin, 1984) and the greater transport of ABA from root to shoot in castor bean (*Ricinus communis*) under Pi-deficient

conditions (Jaschke et al., 1997). Some genes belonging to the *PHO1* family are also repressed by ABA (Ribot et al., 2008); for example, the down-regulation of *PHO1* expression by ABA is mediated by the transcription factor ABA INSENSITIVE 5 (ABI5), which binds to the *PHO1* promoter to repress its expression (Huang et al., 2017). Recently, enhanced ABA levels in Pi-deficient seedlings were implicated in the upregulation of *PHT1;1* and *PHT1;4* via ABI5 binding to their respective promoters (Zhang, Li, et al., 2022). ABI5 was also shown to participate in the Pi-deficiency-induced anthocyanins accumulation by directly binding and activating the expression of the *Chalcone Synthase* (*CHS*) gene (Song et al., 2024). The current work shows that Pi deficiency resulted in the enhanced expression of several ABA biosynthetic and signaling genes, and that this upregulation likely contributes to the observed increased accumulation of ABA in mature shoots and the enhancement of the ABA signaling pathway in the epidermal cells of Pi-deficient plants. Enhancement of ABA production and activation of the ABA signaling pathway are key factors in the increased susceptibility to *B. cinerea* infection in Pi-deficient plants. This is supported by several experimental evidence, namely: (i) an enhanced resistance of the *pho1-2 aba2-1* mutant compared to the single *pho1-2* mutant, (ii) the increased resistance of the signaling sextuple mutant *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* under Pi-deficient conditions, (iii) the enhanced susceptibility of the line *35S:NCED6-1* over-accumulating ABA in plants grown under Pi-sufficient conditions.

Pi deficiency leads to strong changes in the root system architecture, including reduction of primary root growth and increasing in the density and length or root hairs (Péret et al., 2011). High ABA also reduces primary root growth in Arabidopsis via an increase in the quiescence of the quiescent center and the suppression of stem cell differentiation (Zhang et al., 2010). Recent work has also shown that ABA influences primary root growth by modulating ABI4-mediated cell cycle and auxin-related regulatory pathways (Luo et al., 2023). It will be interesting to study if changes in local root ABA concentration as a result of Pi deficiency influence the root architecture.

Pi deficiency is known to influence the root microbiome, both in the context of beneficial microbes, such as arbuscular mycorrhizae and root endophytes, as well as fungal and bacterial pathogens (Castrillo et al., 2017; Hacquard et al., 2016; Paries & Gutjahr, 2023). PHR1 is a key transcription factor playing a central role not only in the Pi-deficiency response but also in plant immunity and in modulating the interaction between plants and microbes (Castrillo et al., 2017). PHR1 represses the defense signaling pathways, leading to increased resistance of the Arabidopsis *phr1* mutant to infection by *P. syringae* as well as by the oomycete pathogen *Hyaloperonospora arabidopsidis* (Castrillo et al., 2017; Scheible et al., 2023). Notably, PHR1 participates in the JA-signaling pathway with PHR1 in Arabidopsis and its rice ortholog OsPHR2 positively modulating the activity of MYC2, a transcription factor that acts as a central hub for the JA-signaling cascade, both by activating *MYC2* transcription and physically associating with the MYC2 protein (He et al., 2023; Kong et al., 2021). The lack of these interactions in the rice *phr2* mutant results in its increased susceptibility to infection by the hemibiotroph *Xanthomonas oryzae* (Kong et al., 2021). Conversely, immune defense signaling pathways can in turn alter the Pi-deficiency responses; for example, the Arabidopsis JASMONATE ZIM-DOMAIN (JAZ) proteins, which are negative regulators of the JA-signaling pathway, directly bind to the *PHR1* promoter and repress its transcriptional activation, while in rice, OsJAZ11 physically interacts with the Pi sensor SPX DOMAIN GENE 1 (OsSPX1) to regulate Pi homeostasis (Pandey et al., 2021; Sun et al., 2023). Given the role of PHR1 in both immunity and the Pi-deficiency response, it is striking that the sensitivity of the *phr1* mutant to *B. cinerea* infection was similar to that of Col-0 and that the enhanced sensitivity of *pho1* mutants to *B. cinerea* was unchanged in the *pho1 phr1* double mutant. Future studies aimed at understanding the molecular links between Pi deficiency and enhanced ABA biosynthesis and signaling will be important to complete our understanding of this important relationship.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

All Arabidopsis (*A. thaliana*) plants used in this study were in the Col-0 background. The *aba2-1*, *pho1-2*, *pho1-3*, *phr1-1*, and *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* mutants, and the transgenic lines *35S:NCED6-1* and *6xABRE_R:erGFP* reporter line were described previously (Gonzalez-Guzman et al., 2012; Hamburger et al., 2002; Lefebvre et al., 2006; Léon-Kloosterziel et al., 1996; Rubio et al., 2001; Wu et al., 2018). Plants were grown in soil or in a clay-based substrate (www.seramis.com) irrigated with phosphate-free 1/4-strength Murashige and Skoog (MS; Caisson, Smithfield, UT, USA; CAT No. MSP11) nutrients supplemented with 0.5% (w/v) 2-(*N*-morpholino) ethanesulfonic acid and 1 mM or 10 μM KH_2PO_4 (pH 5.7). The growth chamber conditions were 22°C and 60% relative humidity with an 8-h-light (100 μE m^{-2} sec^{-1} of white fluorescent light)/16-h-dark photoperiod.

Fungal culture and *B. cinerea* bioassays

The *B. cinerea* B05.10 strain was grown and maintained in potato dextrose broth (PDB) agar (24 g PDB L^{-1} [Sigma-Aldrich, Saint-Louis, MO, USA; P6685], 1.5% w/v Bacto agar [BD, Basel, Switzerland; 214010]). The spores were collected in distilled water after 7–10 days of growth at 22°C on agar plates. The spore suspension was filtered and diluted to a final concentration of 3.5×10^5 spores ml^{-1} in PDB, except for the germination assay where a concentration of 3.5×10^4 spores ml^{-1} was used. The *B. cinerea* spore suspension or PDB mock solution was applied to plants as a spray or droplet inoculation. For the droplet inoculations, 5 μl of spore suspension was applied to the adaxial surface of three fully expanded leaves per plant. To avoid desiccation, the

inoculated plants were incubated under a plastic dome to maintain high humidity. To assess the infection rate at 48 hpi, two methods were employed, namely measure of the infection area and quantification of fungal genomic DNA (gDNA). To assess the infection area, the infected leaves from between 7 and 12 plants per genotype were detached and photographed, and the lesion area was measured using ImageJ software v1.54f (http://imagej.nih.gov/ij). The quantification of fungal biomass was performed as previously described (Gachon & Saindrenan, 2004). In brief, the infected leaves of two to three plants were sampled together using a cork borer of an appropriate size to span the infected area. The samples were flash-frozen and grinded. gDNA was extracted by adding 500 μl of DNA Extraction Buffer (200 mM Tris HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS), followed by precipitation by mixing the supernatant with one volume of cold isopropanol. After centrifugation, the pellet was washed with two volumes of 70% ethanol and resuspended in 100 μl of distilled water. The qPCR analysis was performed using 1 μl of gDNA and SYBR Select Master Mix (Thermo Fisher Scientific, Waltham, MA, USA; CAT 4472908) with primer pairs specific to *B. cinerea* β-*TUBULIN* gene. The Arabidopsis gene *SHIKIMATE KINASE 2* was used for data normalization. The primer sequences are listed in Table S1. The relative gDNA levels were determined using the comparative cycle threshold (Ct) method. Four biological replicates for every genotype or growth condition were analyzed in triplicate.

To visualize *B. cinerea* spore attachment and germination, the inoculated leaves were stained with trypan blue solution (30 ml lactic acid, 60 ml 50% v/v glycerol, 30 ml phenol, 6 mg trypan blue) for 30 min at room temperature, cleared in 95% ethanol at 55°C, and stored in 75% ethanol until visualized. To observe spore attachment, the detached leaves were vigorously shaken in distilled water to remove unattached spores prior to staining. The stained leaves were photographed using a Leica THUNDER microscope equipped with a DMC5400 20 Megapixel color CMOS camera (Leica Microsystems, Wetzlar, Germany). The images were analyzed with ImageJ. To assess spore germination *in vitro* under ABA treatment, *B. cinerea* spores were incubated in PDB medium or PDB containing 10 μM ABA at 22°C for 4 h, and germination was observed under a bright field using a Leica THUNDER microscope. The spores were considered to have germinated when the length of the germinating tube was at least two times the spore diameter.

RNA isolation and reverse transcription quantitative PCR

Total RNA was isolated using the ReliaPrep RNA Miniprep Systems (Promega, Madison, WI, USA) following the manufacturer's instructions. cDNA was synthesized from 1 μg RNA using the SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) and oligo-d$(T)_{18}$, following the manufacturer's instructions. The qPCR analysis was performed using SYBR Select Master Mix (Thermo Fisher Scientific) with primer pairs specific to the genes of interest and *ACTIN2* was used for data normalization. The primer sequences are listed in Table S1. The relative transcript levels were determined using the comparative cycle threshold (Ct) method. Three biological replicates were analyzed in triplicate for every condition.

Phytohormone quantification

Jasmonic acid and ABA were quantified following the protocol from Glauser et al. (2014), with slight modifications. In brief, between two and three rosettes per genotype per treatment were harvested together and immediately flash-frozen in liquid nitrogen. The tissue was ground into a fine powder, and 50 mg was placed in a 1.5-ml Eppendorf tube. The hormones were extracted in a mixture of ethylacetate:formic acid (99.5:0.5, v/v) containing isotopically labeled d_5-JA (1 ng) and d_6-ABA (1 ng) as internal standard solutions. The solvent was fully evaporated under a gentle nitrogen flow, and the sample was resuspended in 200 μl 50% methanol (v/v) in distilled water. The suspension was centrifuged and transferred into a conical glass insert in appropriate vials. For quantification, 2 μl of extract was injected into an ultra-high-pressure liquid chromatography system coupled to a tandem mass spectrometer.

Camalexin quantification

For camalexin quantification, between two and three rosettes per genotype per treatment were harvested together and immediately flash-frozen in liquid nitrogen. The tissue was ground to a fine powder, and 25 mg was placed in a 1.5-ml Eppendorf tube. The tissue powder was mixed with 250 μl methanol:water:formic acid (80:20:0.5, v/v/v). After centrifugation, 125 μl of the mixture was transferred into a conical glass insert in appropriate vials. For the quantification, 1 μl of the extract was injected into an ultra-high-pressure liquid chromatography system coupled to a tandem mass spectrometer.

Sucrose quantification

For the sucrose extraction, between two and three rosettes per genotype were harvested together and immediately flash-frozen in liquid nitrogen. The tissue was ground into a fine powder, and 25 mg was placed in a 2-ml screw-cap Eppendorf tube and mixed with 80% (v/v) ethanol in distilled water. The tubes were placed in an ultrasonic bath at 55°C for 15 min. After centrifugation, 200 μl of the supernatant was mixed with 800 μl of 90% acetonitrile (v/v) in distilled H_2O, and 700 μl was loaded into a high-performance liquid chromatography vial.

Pi quantification

For Pi level determination, the shoots of 4- to 5-week-old plants were submerged in 40 μl of distilled water per mg of fresh weight. The cellular content was extracted by repeated cycles of freezing and thawing and a final incubation of 30 min at 83°C in a water bath. The Pi content was quantified by molybdate assay (Ames, 1966).

ROS and callose staining

Three infected leaves per plant were sampled using a cork borer of an appropriate size to span the *B. cinerea*-inoculated area, and the samples were placed in a microtiter plate. For ROS staining, the leaf samples were floated on 200 μl 10 μM carboxy-H_2DCFDA (Thermo Fisher Scientific; C400) in 70 mM potassium phosphate buffer (pH 8). A gentle vacuum was applied for 5 min, followed by incubation at room temperature in the dark for 10 min. The ROS staining solution was then removed and the leaf samples were washed with 70 mM potassium phosphate buffer (pH 8). The leaf samples were then immediately observed using a Leica THUNDER microscope equipped with a K8 Scientific CMOS fluorescence camera (Leica Microsystems). The green fluorescent protein (GFP) in each sample was excited at 488 nm, and the emission was detected at 496–536 nm.

For callose staining, the leaf samples were placed in a microtiter plate and incubated overnight in 95% ethanol with smooth shaking to remove the chlorophyll. The cleared leaf samples were washed with 70 mM potassium phosphate buffer (pH 8), and after a 15-min incubation, the buffer was replaced with callose staining

solution (0.5 g aniline blue [Sigma-Aldrich; 415049] in 50 ml of 70 mM potassium phosphate buffer, pH 8). The samples were stained for 1–2 h with smooth shaking, then washed with potassium phosphate buffer. The stained samples were immediately observed using a Leica THUNDER microscope equipped with a UV filter (bandpass filter 340–380 nm, longpass filter 425 nm; Leica Microsystems).

Images of ROS and callose staining were analyzed using ImageJ. The quantification methods were similar: the inoculated region was outlined using the circle tool, maintaining the same area used in all analyzed pictures for each experiment. The threshold was adjusted using only the 'Moments' filter, unless the background signal from the autofluorescence of the vasculature was too high. Using the paintbrush tool, the background from the trichome autofluorescence was removed. Finally, the number of black pixels inside the selected area was determined.

Confocal imaging

Confocal laser-scanning microscopy images of the *6xABRE_R:erGPF* reporter lines were obtained using a Leica Stelaris 5 DMi 8 inverted microscope (Leica Microsystems) using LAS X (2020) v4.1.23273. The GFP (in green) was excited at 488 nm, and its emission was detected at 500–530 nm. Propidium iodide (in red) was excited at 561 nm, and its emission was detected at 590–650 nm.

Statistical analysis

The statistical calculations were performed using the tidyverse (https://www.tidyverse.org/packages/) and emmeans (https://cran.r-project.org/web/packages/emmeans/index.html) packages in RStudio (R Core Team). The mean values for each measured parameter were compared using a one-way ANOVA, two-way ANOVA, or Student's *t*-test. Post hoc Tukey's or Dunnett's tests were applied whenever appropriate.

ACKNOWLEDGMENTS

We thank Javier Paz-Ares (CNB-Madrid), Luis Lopez-Molina (University of Geneva), and Pedro L. Rodriguez (Universidad Politécnica de Valencia) for providing the seeds of the *phr1-1*, *aba2-1*, and *pyr1 pyl1 pyl2 pyl4 pyl5 pyl8* mutants, respectively. We are also grateful to José R. Dinneny (Carnegie Institution for Science, Stanford, California, USA) for providing the *6xABRE_R:erGFP* reporter line as well as to Annie Marion-Poll (INRA – INAPG, Institut Jean-Pierre Bourgin, Versailles, France) and Yvan Kraepiel (Sorbonne University, Paris, France) for the *35S:NCED6-1* line. We thank Gaétan Glauser and his team at the Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Neuchâtel, Switzerland for quantifying phytohormones, camalexin, and sucrose. We thank Philippe Reymond and Christiane Nawrath (University of Lausanne) for their critical reading of this manuscript as well as Pedro L. Rodriguez (Universidad Politécnica de Valencia) for useful discussions. This work was supported by a grant from the Swiss National Science Foundation (31003A-182462) to YP. Open access funding provided by Universite de Lausanne.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPORTING INFORMATION

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

Figure S1. Jasmonic acid (JA) content in the shoots of Col-0 plants grown for 4–5 weeks in a clay-based substrate fertilized with 1/4 MS medium containing 1 mM or 10 μM Pi.

Figure S2. Fold change of *B. cinerea* fungal biomass on the indicated *Arabidopsis* genotypes.

Figure S3. Number of rosette leaves produced by the denoted genotypes until the apparition of abaxial trichomes.

Figure S4. Fold change in transcript abundance of *PMR4*.

Figure S5. Analysis of the Pi content in leaves of the indicated genotypes grown in soil for 4–5 weeks under a short-day photoperiod (8 h-light–16 h-dark).

Figure S6. Representative pictures of *B. cinerea* spores germinated *in vitro* with or without the addition of 10 μM ABA to the liquid PDB medium.

Figure S7. Sucrose content in shoots was quantified by high performance liquid chromatography (HPLC).

Table S1. Primer list.

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