Dissecting Phosphite-Induced Priming in Arabidopsis Infected with *Hyaloperonospora arabidopsidis*1[W]

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Phosphite (Phi), a phloem-mobile oxyanion of phosphorous acid (H$_3$PO$_3$), protects plants against diseases caused by oomycetes. Its mode of action is unclear, as evidence indicates both direct antibiotic effects on pathogens as well as inhibition through enhanced plant defense responses, and its target(s) in the plants is unknown. Here, we demonstrate that the biotrophic oomycete *Hyaloperonospora arabidopsidis* (Hpa) exhibits an unusual biphasic dose-dependent response to Phi after inoculation of Arabidopsis (*Arabidopsis thaliana*), with characteristics of indirect activity at low doses (10 mM or less) and direct inhibition at high doses (50 mM or greater). The effect of low doses of Phi on Hpa infection was nullified in salicylic acid (SA)-defective plants (sid2-1, NahG) and in a mutant impaired in SA signaling (npr1-1). Compromised jasmonate (jar1-1) and ethylene (ein2-1) signaling or abscisic acid (aba1-5) biosynthesis, reactive oxygen generation (atrbohD), or accumulation of the phytoalexins camalexin (pad3-1) and scopoletin (f6'/h1-1) did not affect Phi activity. Low doses of Phi primed the accumulation of SA and Pathogenesis-Related protein1 transcripts and mobilized two essential components of basal resistance, Enhanced Disease Susceptibility1 and Phytoalexin Deficient4, following pathogen challenge. Compared with inoculated, Phi-untreated plants, the gene expression, accumulation, and phosphorylation of the mitogen-activated protein kinase MPK4, a negative regulator of SA-dependent defenses, were reduced in plants treated with low doses of Phi. We propose that Phi negatively regulates MPK4, thus priming SA-dependent defense responses following Hpa infection.

Plants deploy an innate immune system including an array of preformed barriers and inducible responses for defense against invading pathogens. A type of induced resistance (IR) is the systemic acquired resistance (SAR) found in adjacent and distal plant parts after infection by a necrotizing pathogen, and it requires salicylic acid (SA; Delaney et al., 1994) and the presence of the defense regulatory protein Nonexpresor of Pathogenesis-Related protein1 (NPR1; Durrant and Dong, 2004). Another type of IR is induced by nonpathogenic growth-promoting rhizobacteria and is called induced systemic resistance, which requires jasmonate (JA) and ethylene (ET; van Loon et al., 1998).

IR is often associated with the priming phenomenon, the augmented capacity to mobilize cellular defense responses following challenge by a broad spectrum of pathogens (Conrath et al., 2002; Conrath, 2011). Thus, inoculation of Arabidopsis (*Arabidopsis thaliana*) leaves with the avirulent strain of *Pseudomonas syringae pv tomato* (Pst) DC3000 carrying the *avrRpm1* gene primes defense responses to subsequent challenge by the virulent strain *Pst* DC3000 (Kohler et al., 2002). Priming is also effective in plants after root inoculation with beneficial rhizobacteria (van Wees et al., 2000; Conrath et al., 2002; Verhagen et al., 2004). Similarly, treatments with natural or synthetic compounds enhance resistance responses only after pathogen challenge. Treatment of plants with SA, riboflavin (vitamin B2), thiamine (vitamin B1), menadione, 2,6-dichloroisonicotinic acid, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH), the nonprotein amino acid β-aminobutyric acid (BABA), or phosphite (Phi) has been shown to prime defenses for augmented responses to pathogens (Kauss et al., 1992; Kauss and Jeblick, 1995; Katz et al., 1998; Zimmerli et al., 2000; Ahn et al., 2007; Borges et al., 2009; Zhang et al., 2009; Eshraghi et al., 2011). Numerous studies have attempted to decipher the molecular components of defense priming (Zimmerli et al., 2000; Kohler et al., 2002; Ton et al., 2005; Zhang et al., 2009). BABA has been shown to prime defenses against *Hyaloperonospora arabidopsidis* (Hpa; formerly *Peroonospora parasitica* or *Hyaloperonospora parasitica*) through an SA- and NPR1-independent signaling pathway (Zimmerli et al., 2000; Ton et al., 2005). BABA-IR involves increased deposition of callose at the site of attempted infection (Zimmerli et al., 2001; Ton and Mauch-Mani, 2004). However, the biochemical and molecular mechanism(s) of priming remained poorly understood until fairly recently. Beckers et al. (2009) demonstrated that defense priming resulted from the accumulation of inactive mitogen-activated protein kinases (MAPKs) following pathogen challenge, leading

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to the development of systemic immunity. The MAPK cascade involves three functionally linked protein kinases, a MAP kinase kinase kinase (MEKK), a MAP kinase kinase (MKK), and a MAP kinase (MPK; Colcombet and Hirt, 2008). The elevated accumulation of inactive forms of the MAPKs MPK3 and MPK6 in Arabidopsis exposed to BTH was proposed as a possible priming mechanism (Beckers et al., 2009). Chromatin modifications and alteration of primary metabolism have also been shown to be linked to priming (Schmitz et al., 2010; Jaskiewicz et al., 2011).

Phi (HPO\textsubscript{3}\textsuperscript{2−}/H\textsubscript{2}PO\textsubscript{3}−), an oxyanion of phosphorous acid (H\textsubscript{2}PO\textsubscript{4}−), is the reduced form of phosphate (Pi) and as such may be considered as a structural analog of Pi. Phi, a phloem-mobile molecule, is especially effective against oomycete diseases (Guest and Bompeix, 1990; Guest and Grant, 1991). The mode of action of Phi is still unknown and debated. At high concentrations, Phi inhibits mycelial growth of Phytophthora species through direct toxicity (Fenn and Coffey, 1984) by inhibiting key phosphorylation reactions (Niere et al., 1994). Phi also activates plant defense responses (Saindrenan and Guest, 1994). Phi-induced resistance in cowpea (Vigna unguiculata) infected with Phytophthora cryptogea was suppressed by the application of a competitive inhibitor of Phenylalanine ammonia lyase and isoflavonoid phytoalexin biosynthesis (Saindrenan et al., 1988). Similarly, Phi-induced resistance and localized cell death are inhibited by quenchers of superoxide anion in Phi-pretreated Arabidopsis seedlings inoculated with zoospores of Phytophthora palmivora (Daniel and Guest, 2006). Phi was recently shown to directly enhance the expression of defense genes and to prime callose deposition and hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) accumulation in Arabidopsis infected with Phytophthora citrophthora (Eshraghi et al., 2011). All these results only provide correlative evidence for an indirect mode of action of Phi, and there is still no conclusive evidence on the mode of action of Phi and its potential target(s) in the plant.

The Hpa-Arabidopsis pathosystem is a suitable model to unravel the physiological and molecular mechanism(s) underlying Phi-IR. Effector-triggered immunity (ETI) in Arabidopsis against Hpa is mediated by the isolate-specific RPP resistance genes through SA-dependent or -independent pathways depending on the isolate used (McDowell et al., 2000; van der Biezen et al., 2000). Among the early signaling events and cellular responses in plants acting downstream of pathogen-associated molecular patterns or avirulence genes is the activation of the MAPK cascade (Asai et al., 2002; Nakagami et al., 2005). Thus, resistance to the Hpa avirulent isolate Hiks1 was compromised in MPK6-silenced Arabidopsis ecotype Columbia (Col-0) plants (Menke et al., 2004), and overexpression of the MKK7 gene enhanced resistance to the Hpa isolate Noco2 (Zhang et al., 2007). Reactive oxygen species (ROS) are part of the early components of defense responses in Arabidopsis. The plasma membrane NADPH oxidase enzyme Arabidopsis respiratory burst oxidase homolog D (ATRBOHD) was shown to be responsible for a stronger oxidative burst in ETI triggered by the Hpa isolate Emco5 (Torres et al., 2002). SA is a component essential in ETI and in basal resistance as the SA induction-deficient2-1 (sid2-1) mutant (Nawrath and Métraux, 1999), and SA hydroxylase (NahG) plants are impaired in resistance to virulent and avirulent isolates of Hpa. In addition, ETI as well as basal defense against Hpa in Arabidopsis involve Enhanced Disease Susceptibility1 (EDS1) and its interactor Phytoalexin Deficient4 (PAD4), two lipase-like proteins that are required for SA accumulation (Jirage et al., 1999; Feyes et al., 2001), as well as NPR1 activation and accumulation of the Pathogenesis-Related protein1 PR1 (Durrant and Dong, 2004).

This study aims to dissect the mechanism(s) underlying Phi-IR in Arabidopsis against Hpa. Here, we show that Phi exerts a dual activity in a concentration-dependent manner with either a priming-inducing activity in the plant or direct inhibition of the pathogen. At low concentrations (10 mM or less), Phi primes defense responses against infection of Arabidopsis Col-0 by the normally virulent Hpa Noco2. Phi-induced priming depends on SA signaling and mobilizes EDS1-PAD4 and NPR1 but is independent of other known pathways of hormone signal transduction. Finally, we identify Phi as a negative regulator of the MAPK MPK4 and present evidence that decreased gene expression, accumulation, and phosphorylation of this kinase are part of Phi-induced priming against Hpa in Arabidopsis.

RESULTS

Phi Pretreatment Renders Arabidopsis More Resistant to Hpa

We inoculated Phi-pretreated Arabidopsis ecotype Col-0 with the virulent Hpa isolate Noco2 to assess the protective effect of Phi. The avirulent isolate Emwa1 interacts with Col-0 through the RPP4 resistance gene (van der Biezen et al., 2002) and was used to compare genetic and chemical-induced resistance. Plants were treated by soil drenching with Phi (5–100 mM) or with MES (mock) 72 h prior to inoculation with Hpa spores. Susceptibility was assessed by counting the number of pathogen spores formed on the leaf surface and by microscopically examining hyphal growth in inoculated leaves at 7 d post inoculation (dpi). There was no difference in the germination rate of inoculated spores on leaves of Phi-pretreated plants compared with mock-pretreated plants (data not shown). Sporulation of Hpa was not affected by 5 mM Phi treatment, whereas 10 and 25 mM Phi treatments proportionately decreased spore numbers, and 50 mM Phi or more inhibited sporulation as much as in the incompatible interaction with the avirulent Emwa1 (Fig. 1A). The effect of Phi treatments on hyphal growth in leaf tissues closely matched the reduction in Hpa spore production (Fig. 1B), indicating that spore number
gives a valid estimate of susceptibility. Soil drenching with 10 mM Phi as soon as 24 h before Hpa inoculation reduced spore number by 35% compared with mock-inoculated plants, but the same concentration applied simultaneous to inoculation or 24 h post inoculation (hpi) had no effect (Supplemental Fig. S1). This indicates that Phi does not exhibit any curative effect once the infection is established in leaf tissues.

The Inhibition of Hpa by Phi Exhibits a Biphasic Dose-Dependent Response Curve

The effectiveness of Phi on Hpa Noco2 infection was analyzed in more detail at concentrations of 5 to 50 mM. Plants were inoculated 72 h after soil drenching with Phi, and spore number was determined at 7 dpi. Response curves were derived from the percentage inhibition of sporulation at each concentration of Phi relative to mock-pretreated plants. The dose-dependent response curve appears biphasic (Fig. 2). In the first phase, sporulation was inhibited linearly from 5 to 12.5 mM Phi, then it stabilized between 12.5 and 22.5 mM at approximately 43% inhibition across the range of doses. In the second phase, inhibition increased linearly to 97% from 22.5 to 50 mM Phi, consistent with a direct dose-related response to a toxicant. This biphasic dose response suggests the additive effect of independent responses to Phi. Thus, in contrast to most conventional fungicides, Phi exhibits a dual mode of action.

The Phi Effect at 10 mM Is Abolished in Arabidopsis Plants Defective in SA Signaling But Not in Mutants Impaired in JA- or ET-Dependent Signals and Abscisic Acid Biosynthesis

Mutants or transgenic plants impaired in signal transduction and biosynthesis pathways involved in IR were used to investigate the role of Phi-induced plant defenses in the inhibition of Hpa Noco2. The effectiveness of Phi against Hpa was first tested in the SA-deficient mutant sid2-1 (Nawrath and Métraux, 1999) treated with 10 mM Phi (Fig. 3), a dose that inhibited sporulation by 35% compared with mock-pretreated plants (Fig. 2). Spore number on the sid2-1 mutant was 2-fold higher (1,200 ± 48 spores mg⁻¹ fresh weight) than on wild-type plants (610 ± 30 spores mg⁻¹ fresh weight; data not shown), confirming...
that suppression of SA production in Col-0 affects basal resistance to *Hpa* isolate Noco2. More importantly, 10 mM Phi failed to reduce spore production on the *sid2-1* mutant, indicating that SA signaling is essential for the 35% reduction in sporulation afforded by Phi in the Col-0 wild type (Fig. 3).

*NPR1* regulates SA-induced defenses downstream of SA and upstream of *PR* genes (Cao et al., 1994). Phi treatment reduced spore number by only 4.6% in *npr1-1* compared with 35% in the wild type (Fig. 3). The response to 10 mM Phi treatment in the mutants *jasmonate resistant*1 (*jar1-1*), *ethylene-insensitive*2-1 (*ein2-1*), and *abscisic acid-deficient*1-5 (*aba1-5*), impaired in ET and JA signaling and asbicsic acid (ABA) biosynthesis, respectively, was not compromised (Fig. 3), indicating that Phi-IR is independent of JA and ET signaling and ABA biosynthesis.

Overall, these findings indicate that 10 mM Phi partly protects Arabidopsis against *Hpa* through SA- and NPR1-dependent defense mechanisms. In NahG plants, the effect of 10 mM Phi is also totally suppressed, whereas it is only partially abolished at 25 mM (Supplemental Fig. S2), suggesting that the protection afforded at this concentration partly resulted from SA-independent factors. The response to increasing Phi was linear up to 50 mM (Supplemental Fig. S2), where sporulation was inhibited at similar levels in wild-type and NahG plants, indicating a direct toxicity to the pathogen. Unless otherwise stated, plants were treated with Phi at 10 mM throughout the following experiments.

**Phi Primes SA Accumulation and *PRI* Expression**

Priming only becomes apparent after pathogen challenge and may be monitored using markers of defense-associated cellular events (Conrath et al., 2002). As the SA signaling pathway was shown to be involved in the Phi-mediated protection of Arabidopsis against *Hpa* Noco2, we analyzed the impact of Phi treatment on the accumulation of free and total SA and on the expression of *PRI*, two important defense responses in our model system. No significant difference was observed in free and total SA levels between mock- and Phi-pretreated plants before inoculation (Supplemental Fig. S3). However, the levels of free SA detected in Phi-pretreated plants were significantly higher than in mock-pretreated plants at 24, 48, and 72 hpi (Fig. 4A). Total SA contents in Phi-pretreated plants were also significantly higher than those in mock-pretreated plants at 24 and 48 hpi (Fig. 4B). Phi-pretreated plants showed higher *PR1* transcription earlier than did mock-pretreated plants, but the levels were similar to mock-pretreated plants at 72 hpi (Fig. 4C). *Plant Defensin1-2* (*PDF1-2*), a biochemical marker of JA- and ET-induced defense responses against necrotrophs (Penninckx et al., 1996; Thomma et al., 1998), was weakly but not differentially induced in mock- and Phi-pretreated plants (Supplemental Fig. S4), confirming that Phi-IR is independent of JA/ET signaling in this pathosystem. Overall, these results indicate that Phi primes SA-dependent defenses for augmented responses against *Hpa*.

**Phi-Induced Priming Is Independent of Phytoalexins and ATRBOHD-Dependent ROS**

ROS produced by the membrane-associated ATRBOH NADPH oxidases play important roles in plant defense and are an early response to penetration of the Arabidopsis epidermis by an avirulent isolate of *Hpa* (Torres et al., 2002; Slusarenko and Schlaich, 2003). However, *atrbohD* mutants were equally responsive to 10 mM Phi as wild-type plants (Fig. 5A), suggesting that Phi-IR is independent of ROS produced by the membrane-bound ATRBOHD. As phytoalexins have been shown to be essential to Phi activity in other plant-oomycete interactions (Saindrenan et al., 1988; Nemestothy and Guest, 1990), we then examined the involvement of camalexin and scopoletin in Phi-induced priming using *phytoalexin deficient*3-1 (*pad3-1*; Böttcher et al., 2009) and *feruloyl-CoA 6′-hydroxylase*1-1 (*f6′h1-1*; Kai et al., 2008) mutants. Phi-IR was not compromised in *pad3-1* and *f6′h1-1* mutants as in Col-0 wild-type plants (Fig. 5A), nor were levels of camalexin and scopoletin accumulation affected by Phi treatment, either before or after inoculation with *Hpa* (Fig. 5, B and C). These results indicate that ATRBOHD-dependent ROS, camalexin, and scopoletin are not components of Phi-induced priming against *Hpa* infection in Arabidopsis.

**Phi Primes Enhanced Expression of *PAD4* and *EDS1**

The interacting *PAD4* and *EDS1* proteins function upstream of SA and are required for SA signaling in ETI and basal resistance (Falk et al., 1999; Jiránek et al.,
1999; Brodersen et al., 2006), particularly in response to *Hpa* (Parker et al., 1996). Mutation in *PAD4* impaired the response of Arabidopsis to Phi treatment after pathogen challenge, indicating that *PAD4* is necessary for full Phi-IR (Supplemental Fig. S5). We monitored *PAD4* and *EDS1* expression in mock- and Phi-pretreated plants before and after inoculation with Noco2 using *Emwa1*-inoculated plants as positive controls. Levels of *PAD4* and *EDS1* were calculated relative to their expression at the time of Phi treatment (i.e. 72 h before inoculation with *Hpa*; Fig. 6). *EDS1* and *PAD4* were similarly expressed at the time of inoculation (0 hpi) in mock- and Phi-pretreated plants. Phi treatment enhanced the expression levels of *PAD4* and *EDS1* at 24 hpi, similar to those observed in Arabidopsis inoculated with *Hpa* *Emwa1*. These data indicate that *PAD4* and *EDS1* genes are primed upon Phi treatment.

**Figure 4.** Effects of Phi treatment on the accumulation of SA and PR1 transcripts in Arabidopsis in response to *Hpa*. Col-0 wild-type plants were treated and inoculated as described in the legend of Figure 3. A, Accumulation of free SA. B, Accumulation of total SA. C, Accumulation of PR1 transcripts normalized to the transcript level of the internal control gene *ACTIN2*. Levels of SA and PR1 transcripts were quantified by HPLC and qRT-PCR, respectively. Values in graphs are means ± so of three replicates. The experiments were repeated twice with similar results. Asterisks indicate data that are significantly different between Phi and MES treatments (Mann-Whitney test; *P* < 0.05). FW, Fresh weight.

**Figure 5.** Relationship between Phi effectiveness and ROS, camalexin, and scopoletin accumulation. Plants were treated and inoculated as described in the legend of Figure 3. A, Phi effectiveness in Col-0 wild-type plants (WT) and *atrbohD*, *pad3-1*, and *f6’ h1-1* mutants. Phi effectiveness was calculated as described in the legend of Figure 2. B, Levels of camalexin. C, Levels of total scopoletin. Values are means ± SE of 15 replicates from five biological independent experiments in A and means ± SD of three replicates in B and C. No significant differences were found between values in A, B, and C (ANOVA, Newman-Keuls test; *P* < 0.05). The experiments in B and C were repeated twice with similar results. Means ± SD of spore numbers quantified on mock-pretreated plants of Col-0 wild-type plants and *atrbohD*, *pad3-1*, and *f6’ h1-1* mutants were 610 ± 75, 575 ± 46, 615 ± 40, and 680 ± 48 spores mg⁻¹ fresh weight (FW), respectively.
Phi Negatively Regulates MPK4 Transcription, MPK4 Protein Accumulation, and Phosphorylation

A genetic interaction between EDS1-PAD4 and the MAPK MPK4 regulates defenses against biotrophs (Brodersen et al., 2006). Loss-of function mpk4 mutants exhibit high levels of SA and PR1 transcripts and have increased resistance to the virulent pathogens Pst DC3000 and Hpa Noco2 (Brodersen et al., 2006). MPK4 expression was the same in mock- and Phi-pretreated plants at 8 and 24 h before inoculation and at the time of inoculation (Supplemental Fig. S6). Inoculation with Hpa induced similar levels of MPK4 expression at 8 hpi in mock- and Phi-pretreated plants (Fig. 7A), but by 10 hpi, MPK4 expression had declined in Phi-pretreated plants relative to mock-pretreated plants (Fig. 7A). The accumulation and phosphorylation state of MPK4 were monitored by western blotting leaf extracts with α-MPK4 and α-p44/42-ERK antibodies, respectively. MPK4 was present in similar levels in Phi- and mock-pretreated plants at 0 hpi (Fig. 7B). Importantly, upon challenge with the virulent pathogen, MPK4 levels significantly decreased at 8 and 10 hpi in Phi-pretreated plants. Phosphorylation of MPK4 was enhanced after inoculation in both mock- and Phi-pretreated plants (Fig. 7B), but to a lesser extent in Phi-pretreated plants (Fig. 7B; Supplemental Fig. S7). The signal that corresponds to the MPK4 protein was not detected in extract prepared from the mpk4 mutant.

MPK3 was recently shown to be the major component for full priming of stress responses in Arabidopsis (Beckers et al., 2009). The involvement of MPK3 in Phi-induced priming to Hpa was tested using the mpk3 mutant. Mutation in MPK3 did not impair the response of Arabidopsis to Phi treatment after pathogen challenge, providing evidence that MPK3 is not required for Phi-IR (Fig. 8). Moreover, Phi did not modify MPK3 transcription, protein accumulation, or the phosphorylation state of MPK3 following pathogen challenge (Supplemental Fig. S8). Altogether, these results suggest that Phi specifically down-regulates MPK4 gene expression, MPK4 protein accumulation, and phosphorylation during the response of Arabidopsis to infection with Hpa Noco2.

DISCUSSION

Phi is extensively used to protect plants against diseases mainly caused by oomycetes (Lobato et al., 2010). The mode of action of Phi is unknown and remains controversial, given evidence for both direct and indirect modes of action (Guest and Grant, 1991). Here, we have dissected Phi-induced defense responses in the Arabidopsis-Hpa pathosystem to decipher the molecular mechanisms of Phi-IR. We show that Phi exerts a dual activity on Hpa infection in a concentration-dependent manner. Phi directly inhibits mycelial growth at high concentrations (50 μM or greater) but at low concentrations (10 μM or less) induces partial protection against Hpa by priming SA-dependent defenses and down-regulating MPK4.

Phi-Induced Protection Is Strictly Dependent on SA Signaling

Soil drenching Col-0 wild-type plants with 10 μM Phi at 72 h before inoculation with Hpa Noco2 reduced
sporulation by 35% compared with mock-treated plants. Importantly, 10 mM Phi was completely ineffective in the sid2-1 mutant (Fig. 3), indicating that Phi inhibits pathogen growth via SA-dependent plant defenses at this concentration. It could be argued that Phi uptake and translocation in the leaves is modified by the absence of a functional SID2 protein, resulting in a lower concentration of the chemical at the site of infection. However, transgenic NahG plants also failed to respond to 10 mM Phi (Supplemental Fig. S2), independently confirming the involvement of SA-dependent defenses. Moreover, the npr1-1 mutant is insensitive to 10 mM Phi (Fig. 3), indicating that a functional NPR1 gene is required for IR and under-scoring the importance of SA signaling in Phi-IR. Fosetyl-Al (aluminum Tris-O-ethyl phosphite; Aliette), an agrochemical that releases Phi, ethanol, and aluminum ions, was shown to protect Arabidopsis against Hpa when sprayed at high concentrations, and its effectiveness was only partially impaired in NahG plants and the non inducible immunity1 (nim1)/npr1 mutant (Molina et al., 1998). However, fosetyl-Al directly induced PR1 expression as a consequence of the chemical toxicity (Molina et al., 1998), confounding the identification of the precise function of Phi as a SAR activator.

ET, JA, and ABA were shown to be involved in activating certain defense responses in Arabidopsis (van Loon et al., 1998; Ton et al., 2009; Ballaré, 2011). Mutants of Arabidopsis affected in JA and ET perception and ABA biosynthesis were not impaired in their responsiveness to 10 mM Phi treatment after Hpa inoculation (Fig. 3). Moreover, transcriptional analysis of the JA- and ET-inducible PDF1.2 gene did not reveal any correlation with Phi-induced responses (Supplemental Fig. S4). Therefore, in this pathosystem, the effectiveness of Phi is strictly dependent on SA signaling.

**Phi Exhibits a Dual Function in a Concentration-Dependent Manner**

The inhibition of Hpa Noco2 by Phi exhibited an unusual biphasic dose-response relationship, suggesting that two independent factors contribute to pathogen restriction in this pathosystem. The first sigmoid in Figure 2 reflects the SA-dependent indirect mode of action of the chemical below 12.5 mM. Interestingly, the Phi effect is abolished in sid2-1 and NahG plants (Fig. 3; Supplemental Fig. S2). The second sigmoid, comprising between 22.5 and 50 mM Phi, reflects the direct toxicity of Phi to the pathogen, as Phi effectiveness is not compromised in NahG plants (Supplemental Fig. S2).

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**Figure 7.** Effects of Phi treatment on gene expression, protein accumulation, and phosphorylation of MPK4 in response to Hpa. A. MPK4 expression. B. Accumulation and phosphorylation state of MPK4 protein. Plants were treated and inoculated as described in the legend of Figure 3. Samples were harvested at 0, 8, and 10 hpi. Two aliquots of leaf tissues were used for the quantification of MPK4 transcripts by qRT-PCR as for PR1 transcripts in the legend of Figure 4. Another aliquot of leaf tissues was used for protein extraction and SDS-PAGE. A polyclonal α-MPK4 antibody was used for the immunodetection of MPK4 protein. α-Phospho-p44/42 ERK antibody was used to check the phosphorylation state of MPK4. A leaf protein extract of the mpk4 mutant was used as a negative control for MPK4. The experiment was repeated three times with similar results. The asterisk indicates data that are significantly different between Phi and MES treatments (Mann-Whitney test; *P < 0.05).

**Figure 8.** Phi effectiveness in mpk3 mutant and Col-0 wild-type (WT) plants against Hpa. Phi treatment, pathogen inoculation, and calculation of Phi effectiveness were performed as described in the legend of Figure 3. Values are means ± SE of 15 replicates from five biological independent experiments. Phi effectiveness was not different between Col-0 and mpk3 plants (Mann-Whitney test; *P < 0.05). Means ± SE of spore numbers quantified in mock-pretreated plants of the Col-0 wild type and the mpk3 mutant were 600 ± 75 and 612 ± 62 spores mg⁻¹ fresh weight, respectively.
S2). Phi treatments between 12.5 and 22.5 mM induce a complex interaction combining indirect and direct modes of action and result in a 43% inhibition of the pathogen across the range of concentrations. This plateau might result from a saturation of Phi target(s) in the plant and/or in the pathogen, or it might indicate the maximal augmented capacity to express the IR (Ahmad et al., 2010). Beyond 22.5 mM Phi, the direct inhibition of mycelial growth overshadows the contribution of Phi-induced plant defenses to pathogen inhibition. Contrasting conclusions on the mode of action of Phi can be explained by understanding this bimodal activity. Phi exerts a dual activity in a concentration-dependent manner through either an SA signaling-dependent activity in the plant or a direct action on the pathogen.

Phi-Induced Resistance to Hpa Is Accomplished by Priming of a Subset of Defense Responses

Some chemicals do not trigger molecular defense responses per se, although they confer resistance to virulent pathogens by enhancing or priming plant capacity to express defense responses. Thus, the synthetic compounds 2,6-dichloroisonicotinic acid, BTH, and BABA and the natural compound SA are all potent inducers of priming and augmented defense-related gene expression and disease resistance at low concentrations (Kauss et al., 1992; Mur et al., 1996; Katz et al., 1998; Zimmerli et al., 2000). Noteworthy, Phi alone at 10 mM did not induce SA accumulation before inoculation with Hpa (Supplemental Fig. S3). However, SA accumulation and PR1 expression were augmented following pathogen challenge (Fig. 4). Thus, this clearly indicates that Phi primes defense responses in Arabidopsis, resulting in enhanced disease resistance to Hpa.

Phi was shown to induce an oxidative burst and phytoalexin accumulation associated with a hypersensitive-like response in cowpea and Arabidopsis infected with P. cryptogea and P. palmivora, respectively (Saindrenan et al., 1988; Daniel and Guest, 2006). The response of atrbohD mutants infected with Hpa to Phi was similar to that in the Col-0 wild type (Fig. 5), indicating that ROS produced from the plasma membrane NADPH oxidase do not contribute to the Phi-IR to Hpa in Arabidopsis. Camalexin and scoopoletin are two phytoalexins that accumulate in Arabidopsis in response to pathogen challenge (Glawischning, 2007; Simon et al., 2010) through PAD3 and F6' H1 activities, respectively (Kai et al., 2008; Böttcher et al., 2009). Recently, it was shown that the disease resistance of Arabidopsis to Phytophthora brassicaceae is established by the sequential action of indole glucosinolates and camalexin (Schlaeppi et al., 2010). However, it is not clear whether camalexin accumulation is a cause or a consequence of IR in the Arabidopsis-Hpa pathosystem (Mert-Türk et al., 2003). The expression of Phi-IR was unaffected in pad3-1 and f6' h1-1 mutants (Fig. 5A) and did not correlate with phytoalexin levels (Fig. 5, B and C), demonstrating that neither phytoalexin contributes to Phi-IR. Recently, it was reported that Phi elicited the accumulation of PR proteins associated with SA and JA/ET signaling pathways in noninoculated leaves of Arabidopsis and primed callose deposition and H2O2 accumulation after inoculation with P. cinnamomi (Eshraghi et al., 2011). It remains puzzling how Phi can prime for SA-inducible PR1 expression but not for ROS production in the Hpa-Arabidopsis interaction and enhanced production of H2O2 in the P. cinnamomi-Arabidopsis interaction. The differences observed between Phi-IR to Hpa and to P. cinnamomi might reflect the different lifestyles of biotrophic and hemibiotrophic oomycete pathogens and/or the recognition of different pathogen-associated molecular patterns by the plant, leading to the activation of different signaling pathways (Baxter et al., 2010).

Phi Mobilizes EDS1 and PAD4 Expression for Priming

EDS1 and PAD4 proteins are essential components of basal resistance and ETI to biotrophic pathogens (Parker et al., 1996; Jirage et al., 1999) and are required for SA accumulation following pathogen challenge (Falk et al., 1999; Jirage et al., 1999; Feys et al., 2001). Our data reveal that PAD4 and EDS1 expression is primed by Phi (Fig. 6). EDS1 and PAD4 are transcriptionally and posttranscriptionally regulated (Feys et al., 2001). Both proteins have been shown to be present in healthy plants (Fey et al., 2001), and after pathogen infection, gene expression changes dependent on EDS1 and PAD4 take place at early time points (Bartsch et al., 2006) before any reported protein up-regulation (Fey et al., 2001; Bartsch et al., 2006). This implies the activation of preexisting EDS1-PAD4 protein complexes and the existence of translational regulatory protein mechanisms. A common regulatory posttranslational modification is phosphorylation (Peck, 2003). However, changes in the phosphorylation state of either PAD4 or EDS1 in challenged plants have not yet been reported. It could be hypothesized that Phi activates preexisting EDS1 and/or PAD4 proteins. Thus, a possible mechanism of Phi-induced priming could be to modify normal EDS1 and/or PAD4 localization or redistribution between cytoplasmic and nuclear compartments after pathogen challenge (Fey et al., 2005; Rietz et al., 2011), as signaling through relocation may be central to EDS1-PAD4 function (Fey et al., 2005; Bartsch et al., 2006). Phi treatment reduced sporulation by only 10% in the pad4 mutants compared with 35% in the Col-0 wild type (Supplemental Fig. S5), underlining that this lipase-like protein is a component of the Phi signal transduction pathway leading to IR. However, the delayed effect of Phi on the enhanced expression of EDS1 and PAD4 after pathogen challenge suggests a more likely target(s) upstream of EDS1-PAD4.

Phi-Induced Priming Involves the Negative Regulation of the MAPK MPK4

The MAPK MPK4 is required for normal plant growth and functions in a variety of physiological
processes (Gao et al., 2008; Kosetsu et al., 2010). MPK4 is a negative regulator of SAR that is upstream of, but dependent on, EDS1 and PAD4 (Petersen et al., 2000). EDS1 and PAD4 are up-regulated in mpk4 mutants (Cui et al., 2010) that are also fully resistant to Hpa and Pst DC3000 (Brodersen et al., 2006; Gao et al., 2008). However, loss-of-function mpk4 mutants exhibit a dwarf phenotype, making it difficult to more directly address the role of MPK4 in Phi-induced priming. The Arabidopsis genome encodes more than 20 MPKs, including MPK3, MPK4, and MPK6, which are involved in innate immunity (Petersen et al., 2000; Asai et al., 2002; Menke et al., 2004). While Phi has no effect on MPK4 expression before inoculation (Supplemental Fig. S6), our data show that Phi induces the down-regulation of MPK4 gene expression, protein level, and phosphorylation at 10 hpi, indicating that Phi-IR is regulated at the transcriptional level (Fig. 7). It is of note that Phi does not affect MPK4 transcript accumulation at 8 hpi, while MPK4 protein levels and protein phosphorylation decreased (Fig. 7; Supplemental Fig. S7). It is possible that Phi induces an immediate degradation of MPK4 protein following pathogen inoculation that leads to later down-regulation of MPK4 gene expression. As MPK4 represses the expression of defense against Hpa (Brodersen et al., 2006), we propose that Phi-induced priming of Arabidopsis against Hpa involves the specific negative regulation of MPK4. BTH priming against the virulent Pst DC3000 strain was shown to be associated with increased activity of MPK3 and MPK6 (Beckers et al., 2009), two closely related proteins exhibiting a high level of functional redundancy (Colcombet and Hirt, 2008). It was assumed that MPK3 was the major component in primed defense gene activation by BTH, while MPK6 served a minor role (Beckers et al., 2009). MPK4 has an opposing effect to MPK3/MPK6 in the regulation of plant defense responses (Nakagami et al., 2005). MPK4 negatively regulates biotic stress signaling, while MPK3 and MPK6 act as positive components of defense responses (Pitzschke et al., 2009). Noteworthy, Phi-IR was not compromised in the mpk3 mutant (Fig. 8) and Phi did not modify MPK3 transcription, protein accumulation, or the phosphorylation state of MPK3 following pathogen challenge (Supplemental Fig. S8), suggesting that Phi-IR is rather linked to MPK4 activity. Future analyses with plants overexpressing MPK4 should help to specify the precise role of this MAPK in Phi-IR.

**Phi-Induced Resistance to Hpa Involves Major Components of SA Signaling**

Our data suggest a model of how Phi primes defense responses against Hpa (Fig. 9). Phi-IR involves EDS1 and PAD4 downstream of MPK4 (Fig. 6). MPK4 negatively regulates SA accumulation (Petersen et al., 2000), and the repression of MPK4 expression/MPK4 activity by Phi following pathogen inoculation results in enhanced accumulation of PAD4 and EDS1 transcripts, leading to augmented levels of SA and enhanced expression of PR1 (Fig. 4). Moreover, Phi primes SA-related defenses in response to Hpa infection through a NPR1-dependent signaling pathway (Fig. 3). Although NPR1-independent defense responses involving the transcription factor WHY1 have been described (Desveaux et al., 2004), we could not detect any change in WHY1 transcript accumulation in Phi-pretreated plants at 24 hpi (data not shown). While we suggest that Phi-induced priming results from the repression of an active MPK, an alternative, but not exclusive, explanation would be that priming could originate from one or more factor(s) upstream of MPK4. MPK4 functions in a cascade that includes the MAP kinase kinase kinase MEKK1 and the MAP kinase kinases M KK1 and M KK2 (Ichimura et al., 1998). Hence, it remains conceivable that Phi may target one of these components of the MAPK module. The single mkk1 and mkk2 T-DNA insertion mutants were as susceptible as the wild-type plants to the virulent strain Hpa Noco2, while mkk1/mkk2 double mutants were found to be highly resistant to the virulent oomycete pathogen (Qiu et al., 2008). Hence, plants expressing a constitutively active version of MKK1 and/or MKK2 would be more appropriate to improve Phi-IR in such a pathosystem.

![Diagram](image-url)

**Figure 9.** Model for priming by Phi for augmented defense responses in Arabidopsis infected with Hpa. Treatment with Phi inhibits the accumulation and phosphorylation of MPK4 after Hpa inoculation. The exact target(s) of Phi could be MPK4 or upstream components of the MAPK cascade as MEKK1 and MKK1/MKK2. Inactivation of MPK4 may also be mediated by Phase (for phosphatase protein[s]). Repression of MPK4 expression/MPK4 activity enhances PAD4 and EDS1 accumulation (dashed line) and triggers SA signaling-dependent priming for enhanced resistance through the defense regulatory protein NPR1.
Phi interferes with the Pi-sensing machinery in plants and suppresses plant responses to Pi deprivation (Varadarajan et al., 2002), but it does not affect cellular metabolism on Pi-sufficient medium (Ticconi et al., 2001). As Phi interferes with Pi metabolism (Danovalt et al., 2008), this suggests that its effect may be at the plant-pathogen interface, where the chemical would disrupt Pi homeostasis. Phosphatase genes are induced by Pi limitation at the transcriptional level (Misson et al., 2005; Thibaud et al., 2010) but also are repressed in high Pi. In Arabidopsis, regulated dephosphorylation and inactivation of MAPKs is mediated by MAPK phosphatases and PP2C-type phosphatases (Andreasson and Ellis, 2010). Inactivation of MPK4 was shown to be mediated by the Tyr-specific phosphatase PTP1 (Huang et al., 2000), the dual specificity (Thr/Tyr) protein phosphatase MKP1 (Ulm et al., 2002), or the PP2C-type phosphatase AP2C1 (Schweighofer et al., 2007). Upon pathogen infection, down-regulation of MPK4 by enhanced protein phosphatase activity derepresses the SA signaling pathway (Fig. 9). Therefore, Phi may prime the plants for augmented defense responses upon biotroph challenge by mimicking Pi starvation and inducing protein phosphatase activity (ies). This possibility can only be resolved by identifying the precise molecular target(s) of Phi.

CONCLUSION

It appears that signaling components like MPK4 and MPK3/MPK6 (Beckers et al., 2009) play a role in Arabidopsis in the priming phenomenon induced by Phi and BTH, respectively. In addition, it was shown that the Arabidopsis impaired BABA-induced setrility1 (ibs1) mutant, affected in a cyclin-dependent kinase-like protein, was impaired in BABA-induced priming of SA-dependent defenses (Ton et al., 2005). Overall, this underscores the importance of protein phosphorylation cascades in priming for enhanced defense responses in Arabidopsis.

MATERIALS AND METHODS

Plant Materials

Arabidopsis (Arabidopsis thaliana) Col-0 and ecotype Wassilewskija-0 were used in this work. The mutants sid2-1 (Wildermuth et al., 2001), jir1-1 (Staswick et al., 2002), ein2-1 (Guzmán and Ecker, 1990), aba1-5 (Koomneef et al., 1982), npr1-1 (Cao et al., 1994), pad3-1 (Glazebrook and Ausubel, 1994), atrbohD (Torres et al., 2002), and pad3-1 (Kai et al., 2008) and transgenic NahG plants (Delaney et al., 1994) were all in the Col-0 background. Plants were grown in controlled-environment chambers under an 8 h/16 h day/night regime with temperatures of 20°C/18°C, respectively; light intensity was 100 μE m⁻² s⁻¹, and relative humidity was 65%. Plants were grown on 12-well plates filled with soil (2.5 mg of seeds per well). Two-week-old plants were used for experiments and pathogen maintenance.

Chemical Treatment

Seedlings were treated with potassium Phi or MES (27 mM; Sigma-Aldrich; http://www.sigmaaldrich.com/) as a control (2 mL per well) at 3 dpi to pathogen inoculation. Phosphorous acid (99%; Sigma-Aldrich) used in this study was partially neutralized with potassium hydroxide prepared in 27 mM MES to yield Phi (HPO₄²⁻/H₂PO₃⁻) at pH 6.3.

Pathogen Maintenance

*Hyaloperonospora arabidopsidis* isolates Noco2 and Emwa1 were obtained from Harald Keller and maintained weekly by transferring spores onto healthy seedlings of Col-0 and Wassilewskija-0 accessions, respectively. Spores were harvested by vortexing infected seedlings in water. Healthy seedlings were inoculated by spraying with spore suspension.

Pathogen Assay

Soil-grown seedlings on 12-well plates were inoculated by spraying with a 2-mL suspension of 5 × 10⁴ spores mL⁻¹. Inoculated seedlings were kept under high relative humidity for 1 dpi, returned to normal conditions, and then placed again at high humidity between 5 and 7 dpi. Spore production was evaluated at 7 dpi. Seedlings of each well were removed, weighed, and then vortexed in 5 mL of water for 10 min to liberate pathogen spores. Spores from three samples of each treatment were counted using a Nageotte chamber, and the means were converted to spore number mg⁻¹ fresh weight.

Histochemical Staining

To visualize pathogen mycelium at the cellular level, infected plants were stained with trypan blue in lactophenol and ethanol at 7 dpi as described by Cao et al. (1998). The seedlings were destained overnight in saturated solution of chloral hydrate and imaged using a light macroscope (AZ100; Nikon).

Quantification of SA, Camalexin, and Scopeolitin

SA, camalexin, and scopeolitin were extracted and quantified as described by Simon et al. (2010). Standards of SA and scopeolitin were from Sigma-Aldrich, whereas authentic camalexin was a kind gift of A.J. Buchala.

Analysis of Gene Expression

Total RNA was extracted from seedling tissue using Extract All Reagent according to the manufacturer’s recommendations (Eurobio; http://www.eurobio.fr/). Samples were subjected to RNase-free DNase I treatment (DNA-free kit; Ambion, Applied Biosystems; http://www.ambion.com/) for 30 min. Total RNA was determined at 260 nm using a spectrophotometer (NanoDrop; Thermo Scientific; http://www.nanodrop.com/) for 30 min. Reverse transcription reaction for cDNA synthesis was performed on 2 μg of RNA using oligo(dT) primers and the ImProm-II kit (Promega; http://www.promega.com/) according to the manufacturer’s instructions. Quantitative reverse transcription (qRT)-PCR experiments were performed with 5 μL of a 1:10 dilution of cDNA and LightCycler 480 SYBR Green 1 Master (Roche; http://www.roche.fr/) according to the manufacturer’s instructions. Relative transcript levels were determined by normalizing the PCR threshold cycle number of each gene with that of the *ACTIN2* reference gene. The qRT-PCR primer sequences used in this work are supplied in Supplemental Table S1.

Protein Extraction and Immunodetection

Fifteen micrograms of total soluble protein extracted from Arabidopsis seedlings was separated by SDS-PAGE (12% acrylamide) as described (Conrath et al., 1997). Equal loading of protein was confirmed by Ponceau S staining. After transfer to a polyvinylidene difluoride membrane, immunodetection of MPK4 and MPK3 was performed with the SNAPKit system (Millipore; http://www.millipore.com/) using anti-rabbit polyclonal antibodies raised against MPK4 and MPK3 (a kind gift from Dr. Hirt) at a 1:1,500 dilution. To detect phosphorylated MPK4 and MPK3, antibodies-phospho-p44/42-ERK antibodies (α-P-MAPKα/β, Cell Signaling Technology; http://www.cellsignal.com/) were used at 1:1,000 dilution (Hesse et al., 2007). Antibody complexed proteins were detected using horseradish peroxidase-conjugated anti-rabbit secondary antibody (Pierce; http://www.piercenet.com/) used at a 1:10,000 dilution and an enhanced chemiluminescence kit (Perkin-Elmer; http://las.perkinelmer.com/).

Statistical Analyses

All experiments were repeated at least two times with similar results. Means of acquired data were compared using ANOVA, Newman-Keuls, or Mann-Whitney test as indicated in the figure legends.
**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Incidence of timing of treatment on Phi effectiveness.

**Supplemental Figure S2.** Phi effectiveness in Col-0 wild-type and NahG plants against Hpa Noco2.

**Supplemental Figure S3.** Effect of Phi treatment on SA accumulation in Arabidopsis before inoculation with Hpa.

**Supplemental Figure S4.** Impact of Phi on PDF1.2 transcription accumulation in Arabidopsis in response to Hpa Noco2.

**Supplemental Figure S5.** Phi effectiveness in Col-0 wild-type and pad4 plants against Hpa Noco2.

**Supplemental Figure S6.** Impact of Phi on MPK4 expression in Arabidopsis before inoculation with Hpa.

**Supplemental Figure S7.** Quantitation of immunodetection signals of MPK4 activity in Figure 7B.

**Supplemental Figure S8.** Effect of Phi treatment on gene expression, protein accumulation, and phosphorylation of MPK3 in response to Hpa Noco2.

**Supplemental Table S1.** Primers used in this work.

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transcriptional responses to phosphate starvation in Arabidopsis. Plant J 64: 775–789