Induction and Suppression of PEN3 Focal Accumulation During *Pseudomonas syringae* pv. *tomato* DC3000 Infection of *Arabidopsis*

Xiu-Fang Xin,^{1,2} Kinya Nomura,² William Underwood,³ and Sheng Yang He^{1,2,4}

¹Department of Plant Biology, Michigan State University, East Lansing 48824, U.S.A.; ²Department of Energy Plant Research Laboratory, Michigan State University, East Lansing; ³Energy Biosciences Institute, University of California, Berkeley 94720, U.S.A.; ⁴Howard Hughes Medical Institute-Gordon and Betty Moore Foundation, Michigan State University, East Lansing

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The pleiotropic drug resistance (PDR) proteins belong to the super-family of ATP-binding cassette (ABC) transporters. AtPDR8, also called PEN3, is required for penetration resistance of *Arabidopsis* to nonadapted powdery mildew fungi. During fungal infection, plasma-membrane-localized PEN3 is concentrated at fungal entry sites, as part of the plant's focal immune response. Here, we show that the *pen3* mutant is compromised in resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000. *P. syringae* pv. *tomato* DC3000 infection or treatment with a flagellin-derived peptide, flg22, induced strong focal accumulation of PEN3-green fluorescent protein. Interestingly, after an initial induction of PEN3 accumulation, *P. syringae* pv. *tomato* DC3000 but not the type-III-secretion-deficient mutant *hrcC* could suppress PEN3 accumulation. Moreover, transgenic overexpression of the *P. syringae* pv. *tomato* DC3000 effector AvrPto was sufficient to suppress PEN3 focal accumulation in response to flg22. Analyses of *P. syringae* pv. *tomato* DC3000 effector deletion mutants showed that individual effectors, including AvrPto, appear to be insufficient to suppress PEN3 accumulation when delivered by bacteria, suggesting a requirement for a combined action of multiple effectors. Collectively, our results indicate that PEN3 plays a positive role in plant resistance to a bacterial pathogen and show that focal accumulation of PEN3 protein may be a useful cellular response marker for the *Arabidopsis*–*P. syringae* interaction.

The ATP-binding cassette (ABC) transporters, found in all organisms, represent one of the largest protein families known, with approximately 130 members in *Arabidopsis thaliana* (Jasinski et al. 2003). Based on phylogeny and structural features, this protein family is classified into several subfamilies, among which the three best-characterized ones are the multi-drug resistance, pleiotropic drug resistance (PDR), and multi-drug resistance-associated protein (Crouzet et al. 2006; Jasinski et al. 2003). ABC transporters are predicted to transport a broad

range of substrates, including toxic compounds, secondary metabolites, inorganic acid, or drugs (Martinoia et al. 2002; Verrier et al. 2008).

Thus far, the PDR subfamily has been found only in plants and fungi but not in animals. They are full-size ABC transporters, consisting of two hydrophobic (trans-membrane domain) and two hydrophilic (nucleotide-binding) domains (Crouzet et al. 2006). A few members of the PDR family in plants have been characterized. For example, earlier studies showed that a PDR protein, SpTUR2, from the plant *Spirodela polyrrhiza* is induced by the plant hormone abscisic acid (ABA) and environmental stress, and could transport sclareol (Smart and Fleming 1996; van den Brûle et al. 2002). NpPDR1 from *Nicotiana plumbaginifolia* was found to be important for pathogen resistance; NpPDR1-silenced plants show increased susceptibility to fungal and oomycete pathogens (Bultreys et al. 2009; Stukkens et al. 2005). The wheat gene *Lr34*, which encodes a putative PDR protein, confers durable resistance to leaf rust, stripe rust, and powdery mildew pathogens in wheat (Krattinger et al. 2009).

In *Arabidopsis*, there are 15 PDR genes. AtPDR12, for example, is regulated by plant defense signaling, contributes to lead resistance, and, more recently, has been shown to transport ABA (Campbell et al. 2003; Kang et al. 2010; Lee et al. 2005). AtPDR8 has been reported to function in transport of heavy metal cadmium and auxin precursor indole-3-butyric acid (IBA) (Kim et al. 2007; Strader and Bartel 2009). In a study to identify *Arabidopsis* genes required for fungal penetration resistance, *pdr8* (i.e., *pen3*) mutants were found to allow higher penetration rates of the nonhost powdery mildew fungus *Blumeria graminis* f. sp. *hordei* on *Arabidopsis* leaves (Stein et al. 2006). The *pen3/pdr8* mutants are also defective in resistance to several other nonadapted pathogens, including *Plectosphaerella cucumerina*, *Erysiphe pisi* (pea powdery mildew), and *Phytophthora infestans* (potato late blight) (Stein et al. 2006). Furthermore, PEN3-green fluorescent protein (GFP) was found to accumulate strongly at penetration sites during powdery mildew infection, and is hypothesized to focally transport defense-related compounds to prevent fungal hyphae growth as a plant defense mechanism (Stein et al. 2006).

PEN3 is also required for focal deposition of defense-associated callose in response to the microbe-associated molecular pattern (MAMP) flg22, suggesting a potentially positive role of PEN3 in plant resistance to bacterial pathogens (Bednarek et al. 2009; Clay et al. 2009). Surprisingly, however, Kobae and colleagues (2006) reported that the *pen3* mutant exhibits

Corresponding author: S. Y. He; Telephone: +1.517.353.9181; E-mail: hes@msu.edu

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elevated basal defense against *Pseudomonas syringae* pv. *tomato* DC3000 under their growth conditions. It is not clear whether the resistance observed is caused directly by *PEN3* acting as a genuine negative regulator of defense or by a secondary, nonspecific effect of the *pen3* mutation under certain growth conditions. In a study to examine a possible involvement of *Arabidopsis* *PEN* genes in resistance to *P. syringae* pv. *tomato* DC3000, we found that the *pen3* mutant is compromised in resistance to *P. syringae* pv. *tomato* DC3000. Furthermore, *PEN3*-GFP protein focally accumulates in response to bacteria or flg22 and, interestingly, this focal accumulation is suppressed by *P. syringae* pv. *tomato* DC3000 in a type III secretion system-dependent manner. Our study affirms a positive role of *PEN3* in plant resistance to a bacterial pathogen, and suggests that focal accumulation of *PEN3* may be a useful cellular marker for the study of *P. syringae* pv. *tomato* DC3000 pathogenesis in *Arabidopsis*.

RESULTS

PEN3 is involved in plant resistance to *P. syringae* pv. *tomato* DC3000 infection.

Intrigued by the earlier publications of somewhat contradicting observations in terms of the role of *PEN3* in plant response to bacteria or bacterial elicitor flg22 (Clay et al. 2009; Kobae et al. 2006), we examined the response of *pen3-1*, which is a loss-of-function mutant (Stein et al. 2006), to infection by wild-type

P. syringae pv. *tomato* DC3000; the Δ CEL mutant, a low-virulence mutant in which several conserved effector genes are deleted (Alfano et al. 2000; DebRoy et al. 2004); and the *hrcC* mutant, which is defective in type III secretion (Yuan and He 1996). Col-0 and *pen3* mutant plants were dip inoculated with bacteria at 1×10^8 CFU/ml and disease symptoms and bacterial numbers were evaluated 4 days later. Compared with Col-0 plants, *pen3* mutant plants showed a higher susceptibility to Δ CEL and *hrcC* bacteria (Fig. 1A; Supplementary Fig. S1). *P. syringae* pv. *tomato* DC3000 multiplied to either a slightly higher level in *pen3* mutant plants than in Col-0 plants (Fig. 1A) or similarly in *pen3* and Col-0 plants (Fig. 1B) but never to a lower level in *pen3* mutant plants compared with Col-0 plants, as was previously observed by Kobae and colleagues (2006).

PEN3 is required for flg22- and benzothiadiazole-induced plant protection against *P. syringae* pv. *tomato* DC3000 infection.

Flg22 and benzothiadiazole (BTH) are well-known inducers of MAMP-triggered immunity and salicylic acid (SA)-triggered immunity, respectively (Boller and Felix 2009; Vlot et al. 2009). We tested whether the *pen3* mutant is compromised in flg22- and BTH-triggered immunity against *P. syringae* pv. *tomato* DC3000 infection. Col-0 and *pen3* plants were pre-treated with 1 μ M flg22, 30 μ M BTH, or H₂O; 24 h later, plants were dip inoculated with *P. syringae* pv. *tomato* DC3000 at 1×10^8 CFU/ml and bacteria were enumerated 3 days after

Fig. 1. *PEN3* has a positive role in plant immunity against *Pseudomonas syringae* pv. *tomato* DC3000. **A**, The *pen3-1* mutant is more susceptible to bacterial infection, compared with Col-0 plants. Plants were dip inoculated with wild-type *P. syringae* pv. *tomato* DC3000, the Δ CEL mutant, or the *hrcC* mutant at 1×10^8 CFU/ml and bacteria were enumerated 4 days after inoculation; * and ** indicate $P < 0.05$ and 0.005 , respectively (two-tailed t test for comparisons of Col-0 and *pen3* mutant plants). **B**, The *pen3* mutants are compromised in flg22- and benzothiadiazole (BTH)-induced immunity against *P. syringae* pv. *tomato* DC3000. H₂O, 30 μ M BTH, or 1 μ M flg22 were sprayed onto plants 24 h before dip inoculation with *P. syringae* pv. *tomato* DC3000 at 1×10^8 CFU/ml. Bacteria in infected plants were enumerated 3 days after inoculation. **C**, Callose deposition is reduced in *pen3* mutant plants. H₂O, *P. syringae* pv. *tomato* DC3000 (1×10^8 CFU/ml), the Δ CEL mutant (1×10^8 CFU/ml), 1 μ M flg22, or 30 μ M BTH was hand-infiltrated into leaves. Callose deposits were counted 15 h after treatment. **D**, Reverse-transcriptase polymerase chain reaction (RT-PCR) shows that the transcript levels of *PR1* and *FRK1* are not compromised in the *pen3* mutant in response to bacterial infection or flg22 or BTH treatment. H₂O, *P. syringae* pv. *tomato* DC3000 (1×10^8 CFU/ml), the Δ CEL mutant (1×10^8 CFU/ml), 1 μ M flg22, or 30 μ M BTH was infiltrated into *Arabidopsis* leaves. Then, 15 h later, leaves were collected for RNA extraction and subjected to RT-PCR analysis. The *ACTIN1* gene was used as a constitutively expressed gene control.

inoculation. As expected, *P. syringae* pv. *tomato* DC3000 grew much less in Col-0 plants after flg22 or BTH treatment. However, *P. syringae* pv. *tomato* DC3000 grew to a much higher level in *pen3* plants compared with Col-0 plants, demonstrating that *PEN3* is required for flg22- and BTH-induced immunity against *P. syringae* pv. *tomato* DC3000 (Fig. 1B).

Callose deposition is a common downstream defense response, which can be triggered by bacterial infection or flg22 or BTH treatment (Clay et al. 2009; Hauck et al. 2003). To determine the role of *PEN3* in callose deposition, we infiltrated *pen3* and Col-0 plant leaves with water, *P. syringae* pv. *tomato* DC3000 (1×10^8 CFU/ml), the Δ CEL mutant (1×10^8 CFU/ml), 1 μ M flg22, or 30 μ M BTH, and callose deposits were counted 15 h later. Callose deposition was significantly decreased in the *pen3* mutant in response to the Δ CEL mutant, flg22, and BTH treatment, whereas *P. syringae* pv. *tomato* DC3000 suppressed callose deposition in both plants (Fig. 1C).

To determine whether *PEN3* is required for defense gene induction, we examined the expression levels of *FRK1* and *PR1*, which are commonly used marker genes for flg22- and SA-triggered immunity, respectively (Boudsocq et al. 2010; Spoel and Dong 2012). Water, *P. syringae* pv. *tomato* DC3000 (1×10^8 CFU/ml), the Δ CEL mutant (1×10^8 CFU/ml), 1 μ M flg22, or 30 μ M BTH was infiltrated into plant leaves, and RNA was collected and subjected to reverse-transcriptase polymerase chain reaction (RT-PCR) to examine the transcript level of *PR1* and *FRK1*. *PR1* and *FRK1* expression was induced by every treatment except water (Fig. 1D). However, neither *PR1* nor *FRK1* showed differential expression in response to different bacteria or flg22 or BTH treatment in the *pen3* mutant versus in Col-0 plants, suggesting that *PR1* and *FRK1* expression is not affected in the *pen3* mutant, despite the compromised plant immunity in this mutant.

PEN3 protein forms strong focal accumulation in response to flg22 treatment.

PEN3 protein was shown to accumulate at fungal penetration sites (Stein et al. 2006). We examined whether *PEN3* focal accumulation also occurs in plants infiltrated with flg22. The transgenic *Arabidopsis* expressing p*PEN3*:*PEN3*-GFP (in the *pen3-1* background) (Stein et al. 2006) was infiltrated with 100 nM flg22 or water (used as control). Confocal microscopy was performed 6 h later to determine the localization pattern of

Fig. 2. Treatment with flg22 induces strong focal accumulation of *PEN3*-GFP protein. **A** to **C**, Confocal laser scanning microscopy using transgenic *Arabidopsis* expressing *PEN3*-GFP shows that *PEN3*-GFP has a uniform plasma-membrane localization pattern at the resting state (H_2O treated). **A** and **B**, epidermal cells; **C**, mesophyll cells. **D** to **F**, *PEN3*-GFP forms strong focal accumulation (indicated by arrows) in response to 100 nM flg22. The flg22 was hand infiltrated into *PEN3*-GFP leaves and images were collected 6 h later. Focal accumulation formed in both **E**, epidermal cells and **F**, mesophyll cells; **D**, a large-area view shows *PEN3*-GFP foci in epidermal cells. Images shown here are z-stacks. Bars = 10 μ m.

PEN3-GFP. Water-treated plants displayed a uniform pattern of *PEN3*-GFP in the plasma-membrane (PM) (Fig. 2A to C), consistent with earlier reports (Kobae et al. 2006; Stein et al. 2006). In contrast, flg22 treatment triggered strong focal accumulation of *PEN3*-GFP, evidenced by distinct, strong signal foci (Fig. 2D to F, arrows). The strong focal accumulation can be visualized in both epidermal cells (Fig. 2E) and mesophyll cells (Fig. 2F). We also examined the localization of another plant PM protein, AUX1-yellow fluorescent protein (YFP) (an auxin influx facilitator fused to YFP) (Bennett et al. 1996), in response to *hrcC* bacteria. AUX1-YFP did not form focal accumulation, suggesting that not all PM proteins form focal accumulation in response to bacteria (Supplementary Fig. S2).

Next, we used a *Heteractis crispata* red fluorescent protein (hcRed)-labeled *hrcC* strain to determine whether the *PEN3* focal accumulation sites are associated with the presence of bacteria. Indeed, hcRed-labeled *hrcC* bacteria were found at or near *PEN3*-GFP accumulation sites (Supplementary Fig. S3), suggesting that the locations of bacteria or locally concentrated pathogen-associated molecular patterns define whether *PEN3* focal accumulation occurs.

***P. syringae* pv. *tomato* DC3000 induces focal accumulation of *PEN3*-GFP, but only transiently.**

We next examined possible focal accumulation of *PEN3*-GFP in the context of bacterial infection. *P. syringae* pv. *tomato* DC3000 and *hrcC* bacteria (at 1×10^8 CFU/ml) were infiltrated into *PEN3*-GFP plant leaves, which were subsequently observed under a confocal microscope at various time points. At early time points, both *P. syringae* pv. *tomato* DC3000 and *hrcC* induced focal accumulation of *PEN3*-GFP, with strong signals observed consistently at 5 h after infiltration (Fig. 3A and B). The strong focal accumulation of *PEN3*-GFP persisted in *hrcC* mutant-infiltrated leaves; however, in

Fig. 3. Dynamic changes of *PEN3* focal accumulation in response to *Pseudomonas syringae* pv. *tomato* DC3000 infection. **A** to **D**, Cellular localization of *PEN3*-GFP responds to *P. syringae* pv. *tomato* DC3000 and the *hrcC* mutant differently. *P. syringae* pv. *tomato* DC3000 and *hrcC* bacteria were hand infiltrated into *PEN3*-GFP leaves at 1×10^8 CFU/ml. **A** and **B**, At 5 h after infiltration, strong focal accumulation of *PEN3*-GFP signal was observed in leaves infiltrated with either bacterial strain. **C** and **D**, However, 10 h after infiltration, *P. syringae* pv. *tomato* DC3000 suppressed *PEN3*-GFP focal accumulation, whereas the *hrcC* mutant did not. Bars = 20 μ m. **E**, Total protein levels of *PEN3*-GFP are not changed in response to infiltration with H_2O or *P. syringae* pv. *tomato* DC3000. H_2O , *P. syringae* pv. *tomato* DC3000, or *hrcC* (at 1×10^8 CFU/ml) was hand-infiltrated and 0.3 g of leaf tissue was collected for each treatment 10 h after infiltration. Total membrane was collected by ultracentrifuge at 100,000 $\times$ g, and subjected to immunoblot analysis with a green fluorescent protein (GFP) antibody (Abcam). Arrow shows the *PEN3*-GFP band with a predicted size of 180 kDa. Asterisk shows a nonspecific band from the same gel, indicating equal protein loading. Lanes were cropped from the same blot with uniform adjustment of brightness and contrast.

P. syringae pv. *tomato* DC3000-treated leaves, accumulation of PEN3-GFP began to disappear after 7 h and, by 10 h after infiltration, almost no focal accumulation could be observed (Fig. 3C and D). The transient focal accumulation pattern of PEN3-GFP during *P. syringae* pv. *tomato* DC3000 infection but not *hrcC* mutant infection provides the first evidence that *P. syringae* pv. *tomato* DC3000 is able to suppress PEN3 focal accumulation in a type III secretion-dependent manner. Although we used high inocula (1×10^8 CFU/ml) to minimize population differences between DC3000 and the *hrcC* mutant, we still observed that the population of the *hrcC* mutant was approximately 10-fold lower than that of *P. syringae* pv. *tomato* DC3000 at 10 h after infiltration (Supplementary Fig. S4). To determine whether the lack of suppression of PEN3 focal accumulation by the *hrcC* mutant at 10 h after infiltration was caused by the lower population level, we adjusted *hrcC* inocula accordingly (at 1×10^9 CFU/ml). However, even at a 10-fold higher inoculum (compared with that of *P. syringae* pv. *tomato* DC3000), the *hrcC* mutant still could not suppress PEN3 focal accumulation, suggesting that high *P. syringae* pv. *tomato* DC3000 population per se does not account for the ability of *P. syringae* pv. *tomato* DC3000 to suppress PEN3 focal accumulation.

To further evaluate changes in PEN3-GFP focal accumulation, the protein level of PEN3-GFP was checked. PEN3-GFP plant leaves were infiltrated with water, *P. syringae* pv. *tomato* DC3000, or *hrcC* bacteria (at 1×10^8 CFU/ml) and leaf tissues were collected 10 h later. Total microsomal fraction was isolated and PEN3-GFP protein level was checked by Western blot. A protein band with predicted size for PEN3-GFP (approximately 180 kDa) was detected by anti-GFP antibody with similar intensity among different samples (Fig. 3E; Supplementary Fig. S5), suggesting that the suppression of PEN3 focal accumulation observed in *P. syringae* pv. *tomato* DC3000 was not due to a decreased amount of PEN3-GFP but, rather, to a change in the pattern of PEN3-GFP localization in the PM.

PEN3 focal accumulation can be suppressed by the effector AvrPto overexpressed in transgenic *Arabidopsis*.

Because we found that flg22 strongly induced focal accumulation of PEN-GFP (Fig. 2D to F) and because *P. syringae* pv. *tomato* DC3000 effector AvrPto has been known to interfere with the function of the flagellin/flg22 receptor FLS2 (Göhre et al. 2008; Xiang et al. 2008), we examined the possibility that AvrPto might be able to suppress the focal accumulation of PEN3-GFP. We first used transgenically expressed AvrPto to address this question. AvrPto transgenic plants

(Hauck et al. 2003) were crossed with PEN3-GFP plants and T1 plants were obtained. AvrPto expression was induced by spraying plants with 30 μ M dexamethasone (DEX). Then, 24 h later, 1 μ M flg22 was infiltrated, and the PEN3-GFP pattern was examined 6 h after flg22 infiltration. Although flg22 induced strong focal accumulation in water-treated AvrPto/PEN3-GFP plants (Fig. 4) or DEX-treated PEN3-GFP plants (Supplementary Fig. S6), greatly reduced focal accumulation of PEN3-GFP was observed in DEX-treated AvrPto/PEN3-GFP plants. This result showed that transgenic overexpression of a single effector, AvrPto, is sufficient to reduce flg22-induced focal accumulation of PEN3-GFP.

Next, we investigated whether AvrPto could suppress focal accumulation of PEN3-GFP when delivered by *P. syringae* pv. *tomato* DC3000 during infection. For this purpose, we used a *P. syringae* pv. *tomato* DC3000 effector deletion mutant, CUCPB5500, in which 18 clustered effector genes were deleted, leaving only 10 nonclustered effector genes, including *avrPto*, in the genome (Kvitko et al. 2009). However, we found that this mutant strain induced strong focal accumulation of PEN3-GFP (Table 1) in a manner that was similar to the *hrcC* mutant, suggesting that none of these 10 effectors, including AvrPto, was sufficient to suppress focal accumulation of PEN3-GFP during infection.

Fig. 4. Overexpression of *Pseudomonas syringae* pv. *tomato* DC3000 effector AvrPto in transgenic *Arabidopsis* suppresses PEN3 focal accumulation. Water or dexamethasone (DEX) (30 μ M) was sprayed onto AvrPto/PEN3 GFP plants to induce AvrPto expression. Then, 24 h later, 1 μ M flg22 was infiltrated into leaves. Confocal images were collected 6 h after flg22 infiltration. Bar = 20 μ m. **A**, AvrPto/PEN3-GFP plants treated with water were used as control. Strong focal accumulation of PEN3-GFP was observed after flg22 treatment. **B**, DEX-induced AvrPto overexpression suppressed PEN3 focal accumulation.

Table 1. Different abilities of *Pseudomonas syringae* pv. *tomato* DC3000 mutant strains to suppress PEN3-GFP focal accumulation

Strains (references)	Effectors genes deleted in the strain	Degree of PEN3 focal accumulation ^a
<i>hrcC</i> (Yuan and He 1996)	Type III secretion system deficient	+++
CUCPB5500 (Cunnac et al. 2011; Kvitko et al. 2009)	<i>hopAA1-1</i> ; <i>hopAA1-2</i> ; <i>hopAO1</i> ; <i>hopAM1-2</i> ; <i>hopC1</i> ; <i>hopD1</i> ; <i>avrE</i> ; <i>hopF2</i> ; <i>hopG1</i> ; <i>hopH1</i> ; <i>hopM1</i> ; <i>hopN1</i> ; <i>hopO1-1</i> ; <i>hopQ1-1</i> ; <i>hopR1</i> ; <i>hopT1-1</i> ; <i>hopU1</i> ; <i>hopV1</i> ; <i>hopX1</i>	+++
CUCPB5539 (Kvitko et al. 2009)	<i>hopAA1-1</i> ; <i>hopAA1-2</i> ; <i>hopAO1</i> ; <i>hopC1</i> ; <i>hopD1</i> ; <i>avrE</i> ; <i>hopG1</i> ; <i>hopH1</i> ; <i>hopM1</i> ; <i>hopN1</i> ; <i>hopQ1-1</i> ; <i>hopR1</i> ; <i>hopV1</i>	+++
CUCPB5459 (Cunnac et al. 2011; Kvitko et al. 2009)	<i>hopAA1-2</i> ; <i>hopAO1</i> ; <i>hopAM1-2</i> ; <i>hopC1</i> ; <i>hopD1</i> ; <i>hopF2</i> ; <i>hopG1</i> ; <i>hopH1</i> ; <i>hopO1-1</i> ; <i>hopQ1-1</i> ; <i>hopR1</i> ; <i>hopT1-1</i> ; <i>hopU1</i> ; <i>hopV1</i> ; <i>hopX1</i>	+++
CUCPB5452 (Kvitko et al. 2009; Wei et al. 2007)	<i>hopAA1-2</i> ; <i>hopAO1</i> ; <i>hopAM1-2</i> ; <i>hopC1</i> ; <i>hopD1</i> ; <i>hopG1</i> ; <i>hopH1</i> ; <i>hopO1-1</i> ; <i>hopQ1-1</i> ; <i>hopR1</i> ; <i>hopT1-1</i> ; <i>hopV1</i> ; <i>hopX1</i>	++
CUCPB5451 (Wei et al. 2007)	<i>hopAA1-2</i> ; <i>hopAO1</i> ; <i>hopC1</i> ; <i>hopD1</i> ; <i>hopG1</i> ; <i>hopH1</i> ; <i>hopQ1-1</i> ; <i>hopR1</i> ; <i>hopV1</i>	+
ΔCEL (Alfano et al. 2000; DebRoy et al. 2004)	<i>HopAA1-1</i> ; <i>avrE</i> ; <i>hopM1</i> ; <i>hopN1</i>	+
CUCPB5448 (Wei et al. 2007)	<i>hopC1</i> ; <i>hopD1</i> ; <i>hopH1</i> ; <i>hopQ1-1</i> ; <i>hopR1</i>	–
CUCPB5138 (Buell et al. 2003; Wei et al. 2007)	<i>hopAM1-2</i> ; <i>hopO1-1</i> ; <i>hopT1-1</i> ; <i>hopX1</i> ; (<i>pDC3000A-B</i>)	–
CUCPB5445 (Wei et al. 2007)	<i>hopC1</i> ; <i>hopH1</i>	–
DC3000 (Ma et al. 1991)	...	–

^a Average number of focal accumulation sites per 1.6 mm² (1.26 by 1.26 mm): +++ = >70, ++ = 40–70, + = 6–40, and – = <5.

Analysis of *P. syringae* pv. *tomato* DC3000 effector deletion mutants in their ability to suppress PEN3 focal accumulation.

We next examined additional *P. syringae* pv. *tomato* DC3000 effector deletion mutants in their ability to suppress PEN3 focal accumulation (Table 1). These strains contained different combinations of clustered effector gene deletions and showed differential virulence on *Arabidopsis* or *N. benthamiana* (Buell et al. 2003; Kvitko et al. 2009; Wei et al. 2007). Bacteria strains were infiltrated into PEN3-GFP leaves at 1×10^8 CFU/ml and images were collected 10 h later. Average number of PEN3-GFP focal accumulation sites per 1.6 mm^2 ($1.26 \text{ by } 1.26 \text{ mm}$) was counted for each strain. We found that these strains possess different abilities of suppressing PEN3-GFP focal accumulation (Table 1). However, this analysis also showed that there is not a specific effector or cluster that is capable of suppressing PEN3-GFP focal accumulation in the context of bacterial infection. For example, whereas CUCPB5448 effectively suppressed PEN3-GFP focal localization, CUCPB5452 could not. This initial result suggested to us that effector clusters I (containing *hopU1* and *hopF2*) and X (containing *hopAM1-2*, *hopX1*, *hopO1-1*, and *hopT1-1*) would be responsible for the suppression. However, CUCPB 5539, which contains only effector clusters I and X, failed to suppress PEN3 accumulation. Thus, a yet-to-be discovered combination of type III effectors is needed to suppress PEN3-GFP focal accumulation during *P. syringae* pv. *tomato* DC3000 infection.

DISCUSSION

In this study, we reevaluated the role of *PDR8/PEN3* in plant interaction with a bacterial pathogen. Contrary to what was observed by Kobae and colleagues (2006), we found that *Arabidopsis pdr8/pen3* mutants are compromised in resistance to *P. syringae* pv. *tomato* DC3000, with the most obvious defect observed in flg22 and BTH protection assays or when *P. syringae* pv. *tomato* DC3000 mutants with reduced virulence (e.g., the *hrcC* or ΔCEL mutant) are used in disease assays. Furthermore, we found that, similar to powdery mildew fungal infection, *P. syringae* pv. *tomato* DC3000 infection and bacterial MAMP (flg22) treatment efficiently induced strong focal accumulation of PEN3-GFP protein, suggesting that this focal localization pattern of PEN3 is a common defense response to fungal and bacterial pathogens. Furthermore, we found that PEN3 focal accumulation is suppressed by *P. syringae* pv. *tomato* DC3000 in a type III secretion-dependent manner. The immune defects displayed in *pen3* mutants and dynamic changes of PEN3 focal accumulation during *P. syringae* pv. *tomato* DC3000 infection are consistent with and extend the initial observation by Clay and colleagues (2009) that PEN3 has a positive role in mediating defense-associated callose deposition in response to a bacterial MAMP flg22.

The different results with respect to responses of *pen3* mutants to *P. syringae* pv. *tomato* DC3000 infection obtained by us and by Kobae and colleagues (2006) warrant some further discussion. Kobae and colleagues found that the *pdr8* T-DNA insertion mutant exhibited chlorotic lesions and hypersensitive-response-like cell death under their laboratory growth conditions, and that *P. syringae* pv. *tomato* DC3000 growth was suppressed in this mutant. However, under our growth condition, we did not observe these phenotypes with the *pen3-1* mutant. The plants looked healthy and were often slightly larger than Col-0 control plants. We speculate that *pdr8/pen3* plants may be more sensitive than Col-0 plants to abiotic or biotic stresses associated with certain growth conditions. When stressed, *pdr8/pen3* plants may activate nonspecific, secondary defense, which could lead to “gain-of-function” resistance to *P. syringae* pv. *tomato*

DC3000. Indeed, Stein and associates (2006) reported that *pen3* mutant plants have heightened sensitivity to abiotic stress, such as high light, and Kobae and colleagues found that *pdr8* mutants grown under nonsterile conditions showed constitutively expressed defense genes, such as *PR-1*, *PR-2*, and *AtrbohD*. Plants grown under our condition did not have elevated expression of defense-related genes, such as *PR-1* and *FRK1* (Fig. 1D). Therefore, obtaining healthy *pdr8/pen3* plants is critical to reveal the enhanced disease susceptibility and avoid stress-induced secondary effects. Interestingly, the effect of plant growth conditions on disease phenotypes is not unique to *pdr8/pen3* mutations and has been shown for other *Arabidopsis* defense-associated mutations (Lu et al. 2010; Nomura et al. 2011; Veronese et al. 2006). Another reason that might contribute to the disparity of the *pen3* plant responses to *P. syringae* pv. *tomato* DC3000 is the different bacterial inoculation methods used. We dip inoculated plants with bacteria, and Kobae and colleagues used infiltration. *PEN3* expression was shown to be enriched in cells near stomata (Kobae et al. 2006). Therefore, it is possible that *PEN3* may have an important defense function at the site of bacterial entry, which would be revealed only by the dip inoculation.

How PEN3 contributes to plant resistance to *P. syringae* pv. *tomato* DC3000 remains to be determined. Knowledge of the substrates of the PEN3 transporter will be an essential step toward understanding its function. It seems reasonable to hypothesize that PEN3, being a member of the PDR family of transporters, may transport different classes of substrates when plants are exposed to different abiotic or biotic stresses. PDR in yeast have been shown to transport a variety of compounds with little or no common features (Bauer et al. 1999; Rogers et al. 2001). PEN3 has been reported to extrude cadmium, contributing to heavy metal resistance, and to mediate the export of auxin precursor IBA (Kim et al. 2007; Strader and Bartel 2009). However, it is not yet clear how cadmium and IBA export would influence *P. syringae* pv. *tomato* DC3000 pathogenesis. PEN3 is also required for callose deposition (Clay et al. 2009), although it is not known how callose deposition alone could restrict bacterial multiplication. Therefore, it remains possible that PEN3 may also transport antimicrobial compounds effective against bacterial pathogens (Lipka et al. 2005; Stein et al. 2006). How PEN3's different transport activities are regulated and coordinated should be an interesting topic of future study.

Focal cellular accumulation of plant defense-associated proteins has emerged as a common theme during fungal infection. Multiple plant proteins have been demonstrated to be redistributed at fungal entry sites. These proteins include PEN1, VAMP721/VAMP722, PEN2, and PEN3 in *Arabidopsis* and barley syntaxin ROR2 and mildew resistance locus O (MLO) (Bhat et al. 2005; Kwon et al. 2008; Lipka et al. 2005; Stein et al. 2006). The mechanisms underlying the redistribution of these proteins are not yet clear. Studies on *Arabidopsis* PEN1 have led to the hypothesis that attempted entry of fungal hyphae triggers formation of specific PM microdomains, to which certain PM proteins, including PEN1, are recruited. The focal nature of PEN1-dependent exocytosis could lead to localized cell-wall defense responses, such as papillae formation, which requires the ARF-GTP exchange factor GNOM (Bhat et al. 2005; Meyer et al. 2009; Nielsen et al. 2012). It would be important for future research to examine the possibility of PEN3 being recruited to PM microdomains or other membrane structures, such as exosomes, in response to flg22 and bacteria. Equally important would be to identify *P. syringae* pv. *tomato* DC3000 effectors that suppress focal accumulation of PEN3-GFP. Although we have shown that transgenically expressed AvrPto could reduce flg22-induced PEN3 focal accumulation, consistent with the ability of this effector to inhibit the function of the flagellin/

flg22 receptor FLS2 (Göhre et al. 2008; Xiang et al. 2008), we have, thus far, failed to identify individual effectors (or combinations of effectors) that could block PEN3-GFP focal accumulation in the context of *P. syringae* pv. *tomato* DC3000 infection. Likely, this is because transgenic expression of AvrPto produced a much higher level of AvrPto protein than that delivered by bacteria during infection. It is also possible that bacteria release a more complex mix of elicitors (compared with flg22 alone) that trigger PEN3-GFP focal accumulation, which requires a more complicated mix of effectors to suppress. We believe that identifying these effectors and studying their suppression mechanisms may provide a powerful means of understanding the general mechanism of focal accumulation of PEN3 and, possibly, other defense proteins. Even without such understanding, however, we believe that the dynamic change of PEN3 localization pattern, as revealed in this study, can be used as a facile cellular marker for study of *Arabidopsis*–*P. syringae* interactions in live cells.

MATERIALS AND METHODS

Plant materials and growth conditions.

The following *A. thaliana* mutants and transgenic plants were used. The *pen3-1* mutant and PEN3:GFP transgenic *Arabidopsis* plants were described by Stein and associates (2006). AvrPto transgenic *Arabidopsis* plants were described by Hauck and associates (2003). All plants are in Col-0 background. Seed were sown in Redi-earth soil and kept at 4°C for 2 days for stratification. Plants were grown with 12 h of light (80 to 100 $\mu\text{E m}^{-2} \text{s}^{-1}$). Five-week-old plants were used for disease assays and confocal microscopy.

Bacterial infection assay.

Plants were grown to 5 weeks old and dip inoculated with *P. syringae* pv. *tomato* DC3000 strains at 1×10^8 CFU/ml. Infected plants were monitored for a 3- to 4-day period, and bacterial populations were determined at day 3 or 4. For flg22 and BTH protection assays, plants were sprayed with H₂O, 30 μM BTH, or 1 μM flg22 24 h before dip inoculation with *P. syringae* pv. *tomato* DC3000 (1×10^8 CFU/ml).

Callose staining.

Callose staining was performed 15 h after bacteria (1×10^8 CFU/ml) or flg22 or BTH infiltration. Staining was done as described previously (Nomura et al. 2011). Averages of values are presented with standard deviations derived from at least four independent leaves for each treatment.

Microsome isolation and protein immunoblot.

Plant leaves were infiltrated with bacteria at 1×10^8 CFU/ml and collected 10 h later. After polytron homogenization, samples were centrifuged at $13,000 \times g$ for 15 min at 4°C. The supernatant was collected and subjected to centrifugation at $100,000 \times g$ for 40 min at 4°C to collect the microsomal fraction.

For immunoblot analysis of PEN3-GFP protein, a microsomal pellet was resuspended in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heated at 50°C for 5 min. Proteins were subjected to SDS-PAGE separation and transferring onto membrane (Immobilon-P membrane; Millipore Corp., Bedford, MA, U.S.A.). An anti-GFP antibody (rabbit polyclonal; Abcam, Cambridge, U.K.) was used for detecting PEN3-GFP.

Laser-scanning microscopy.

PEN3-GFP or AvrPto/PEN3-GFP plants were grown to 5 weeks old and fully expanded leaves were infiltrated with bacteria, flg22, or H₂O. At different time points after treatment, infiltrated leaves were randomly picked for confocal imaging.

Imaging was performed with an Olympus FluoView FV1000 Laser-Scanning Confocal Microscope for GFP visualization at 488 nm. Imaging experiments were conducted with a $\times 40$ oil immersion objective (Fig. 2A, C, and D) or a $\times 10$ dry objective (Figs. 2B and E, 3, 4, and 5). Images were processed using Olympus FluoView Viewer software, version 2.0B.

Quantification of PEN3 focal accumulation sites.

Confocal images (1.26 by 1.26 mm) were collected randomly from plant leaves inoculated with each strain. Focal accumulation sites were counted on each image. Six representative images were analyzed for each strain to obtain the average. At least two experimental repeats were performed for each strain.

RT-PCR analysis of gene expression.

Arabidopsis leaves were infiltrated with H₂O, bacteria at 1×10^8 CFU/ml, 1 μM flg22, or 30 μM BTH and used for RT-PCR analysis. Leaf samples were collected 15 h after infiltration. Total RNA was purified by using the RNeasy Total RNA Isolation System (Promega Corp., Madison, WI, U.S.A.), followed by RT-PCR using the TaKaRa RNA PCR Kit (ver. 3.1; Takara, Tokyo). The following primers were used: *PR1*, forward primer 5'ATGAATTTTACTGGCTATTCTCGA3' and reverse primer 5'CAAACCTCCATTGCACGTGTTTCGA3'; *FRK1*, forward primer 5'TCCAAGCGAAGATGGCGGACTTCG3' and reverse primer 5'CTACCTTGCTCGAGGAACCATCTC3'; and *ACTIN1*, forward primer 5'ATGGCTGATGGTGAAGACATTCAA3' and reverse primer 5'TTAGCTTTCGGGTTAAGCGGTGCC3'.

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