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

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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 lrcC 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.

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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 bacterial elicitor flg22 (Clay et al. 2009; Kobae et al. 2006), to infection by wild-type *P. syringae pv. tomato* DC3000, we examined the response of pen3 mutants to bacteria or bacterial elicitor flg22 (Clay et al. 2009; Kobae et al. 2006). We tested whether the pen3-1 mutant, which is a loss-of-function mutant (Stein et al. 2006), to infection by wild-type *P. syringae pv. tomato* DC3000; the ∆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 ∆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).

**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 pathogenesis in *Arabidopsis*.

**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 pretreated with 1 μM flg22, 30 μM BTH, or H2O; 24 h later, plants were dip inoculated with *P. syringae pv. tomato* DC3000 at 1 × 10^6 CFU/ml and bacteria were enumerated 3 days after inoculation.

**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 ∆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 μM BTH, or 1 μM 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 ∆CEL mutant (1 × 10^8 CFU/ml), 1 μM flg22, or 30 μM 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 ∆CEL mutant (1 × 10^8 CFU/ml), 1 μM flg22, or 30 μM 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 ΔCEL mutant (1 × 10^8 CFU/ml), 1 μM flg22, or 30 μM BTH, and callose deposits were counted 15 h later. Callose deposition was significantly decreased in the *pen3* mutant in response to the ΔCEL 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 ΔCEL mutant (1 × 10^8 CFU/ml), 1 μM flg22, or 30 μM 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 pPEN3: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 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 crispa* 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...
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 × 10^7 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 × 10^7 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 PEN3-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 μM dexamethasone (DEX). Then, 24 h later, 1 μM 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.](image)

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

<table>
<thead>
<tr>
<th>Strains (references)</th>
<th>Effectors genes deleted in the strain</th>
<th>Degree of PEN3 focal accumulation^a</th>
</tr>
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<tbody>
<tr>
<td>hrcC (Yuan and He 1996)</td>
<td>hopA1-1; hopA1-2; hopAO1; hopAM1-2; hopC1; hopD1; avrE; hopF2; hopG1; hopH1; hopM1; hopO1-1; hopQ1-1; hopQ1-2; hopR1; hopT1-1; hopU1; hopV1; HopX1</td>
<td>+++</td>
</tr>
<tr>
<td>CUCPB5500 (Cunnac et al. 2011; Kvitko et al. 2009)</td>
<td>hopA1-1; hopA1-2; hopAO1; hopC1; hopD1; avrE; hopG1; hopH1; hopM1; hopO1-1; hopQ1-1; hopQ1-2; hopR1; hopT1-1; hopU1; hopV1; HopX1</td>
<td>+++</td>
</tr>
<tr>
<td>CUCPB5539 (Kvitko et al. 2009)</td>
<td>hopA1-1; hopA1-2; hopAO1; hopC1; hopD1; avrE; hopG1; hopH1; hopM1; hopO1-1; hopQ1-1; hopQ1-2; hopR1; hopT1-1; hopU1; hopV1; HopX1</td>
<td>+++</td>
</tr>
<tr>
<td>CUCPB5459 (Cunnac et al. 2011; Kvitko et al. 2009)</td>
<td>hopA1-1; hopAO1; hopAM1-2; hopC1; hopD1; hopF2; hopG1; hopH1; hopO1-1; hopQ1-1; hopQ1-2; hopR1; hopT1-1; hopU1; hopV1; HopX1</td>
<td>+++</td>
</tr>
<tr>
<td>CUCPB5452 (Kvitko et al. 2009; Wei et al. 2007)</td>
<td>hopA1-1; hopAO1; hopAM1-2; hopC1; hopD1; hopG1; hopH1; hopO1-1; hopQ1-1; hopQ1-2; hopR1; hopT1-1; hopU1; hopV1; HopX1</td>
<td>+++</td>
</tr>
<tr>
<td>CUCPB5451 (Wei et al. 2007; DCEL (Alfano et al. 2000; DebRoy et al. 2004)</td>
<td>hopA1-1; hopAO1; hopC1; hopD1; hopG1; hopH1; hopQ1-1; hopR1; hopV1; HopX1</td>
<td>++</td>
</tr>
<tr>
<td>CUCPB5448 (Wei et al. 2007)</td>
<td>hopC1; hopD1; hopG1; hopH1; hopQ1-1; hopR1</td>
<td>+</td>
</tr>
<tr>
<td>CUCPB5138 (Buell et al. 2003; Wei et al. 2007)</td>
<td>hopAM1-2; hopO1-1; hopT1-1; hopX1; (pDC3000A-B-)</td>
<td>–</td>
</tr>
<tr>
<td>CUCPB5445 (Wei et al. 2007)</td>
<td>hopC1; hopH1</td>
<td>–</td>
</tr>
<tr>
<td>DC3000 (Ma et al. 1991)</td>
<td>...</td>
<td>–</td>
</tr>
</tbody>
</table>

^a Average number of focal accumulation sites per 1.6 mm^2 (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). Bacterium 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 mm² (1.26 by 1.26 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 acEL 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 Pen1 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 effectors (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::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 μE m⁻² 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 × 10⁸ 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 × 10⁸ CFU/ml).

Callose staining.

Callose staining was performed 15 h after bacteria (1 × 10⁸ 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 × 10⁸ CFU/ml and collected 10 h later. After polytron homogenization, samples were centrifuged at 15,000 × g for 15 min at 4°C. The supernatant was collected and subjected to centrifugation at 100,000 × 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 (Immobilion-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 treatments, 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 ×40 oil immersion objective (Fig. 2A, C, and D) or a ×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 × 10⁸ 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 RNAgent 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′-ATGATTTTTACTGGCTATTCTCGA-3′ and reverse primer 5′-CAAACTCCATTGCACGTGTTCGA-3′; FRK1, forward primer 5′-TCAACGGCAGATGCGGACCTTCG-3′ and reverse primer 5′-CTACCTTGTGCTAGGAACCATC-3′; and ACTIN1, forward primer 5′-ATGGCTGATGGGTAAGACATTCAAA-3′ and reverse primer 5′-TTAGCTTTCGGGTAAGCCGGTGCC-3′.

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