Pathogenic bacteria target plant plasmodesmata to colonize and invade surrounding tissues

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In memory of Dr. James Robert Alfano.

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One-sentence summary: The \textit{Pseudomonas syringae} effector protein HopO1-1 targets and destabilizes plasmodesmata-located proteins to promote disease in plants.

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Abstract

A hallmark of multicellular organisms is their ability to maintain physiological homeostasis by communicating among cells, tissues, and organs. In plants, intercellular communication is largely dependent on plasmodesmata (PD), which are membrane-lined channels connecting adjacent plant cells. Upon immune stimulation, plants close PD as part of their immune responses. Here, we show that the bacterial pathogen \textit{Pseudomonas syringae} deploys an effector protein HopO1-1 that modulates PD function. HopO1-1 is required for \textit{P. syringae} to spread locally to neighboring tissues during infection. Expression of HopO1-1 in Arabidopsis increases the distance of PD-dependent molecular flux between neighboring plant cells. Being a putative ribosyltransferase, the catalytic activity of HopO1-1 is required for regulation of PD. HopO1-1 physically interacts with and destabilizes plant PD-located proteins PDLP7 and possibly PDLP5. Both PDLPs are involved in bacterial immunity. Our findings reveal that a pathogenic bacterium utilizes an effector to manipulate PD-mediated host intercellular communication for maximizing the spread of bacterial infection.
Introduction

Multicellular organisms host a wide array of microorganisms. Although most microbes are beneficial or harmless to their hosts, infections caused by a few pathogenic microorganisms can lead to devastating diseases in animals and plants. Over the past three decades, progress has been made toward understanding how plants defend against pathogens at the molecular and cellular levels (Jones and Dangl 2006; Grant et al., 2006; Nicaise et al., 2009). Plants detect the presence of microorganisms by recognizing microbial signatures such as bacterial flagellin and fungal chitin, collectively known as microbe-associated molecular patterns (MAMPs; Ranf, 2017). Recognition of MAMPs by membrane-bound pattern recognition receptors (PRRs) on the plant cell surface initiates a cascade of signaling events, activating a form of plant innate immunity known as pattern-triggered immunity (PTI; Ranf, 2017; Saijo et al., 2018). To overcome host immunity, pathogenic microbes deliver virulence-intended microbial molecules, collectively called “effectors”, mostly into host cells as a major pathogenesis mechanism (Grant et al., 2006; Le Fevre et al., 2015; Toruno et al., 2016). To counter pathogen virulence, plants have evolved a second set of receptors, mainly intracellular NBS-LRR (NLR) proteins, that recognize individual effectors and activate effector-triggered immunity (Cui et al., 2015). Current models suggest that pattern-triggered immunity and effector-triggered immunity constitute two major forms of cell-autonomous immunity in plants.

In addition to cell-autonomous immunity, uninfected host cells in an infected plant can exhibit immune responses (non-cell-autonomous immunity). Such immune responses in systemic tissues can limit subsequent infections by the same pathogen, a phenomenon known as systemic acquired resistance (SAR; Klessig et al., 2018). This process requires cell-to-cell communication. In plants, communication between cells is achieved through apoplastic and symplastic pathways. In the apoplastic pathway, signaling molecules exit signal-generating cells and enter into the apoplast (i.e., extracellular space). To enable intercellular communication, signaling molecules can enter signal-receiving cells through different means of trafficking (Lim et a., 2016). In the symplastic pathway, on the other hand, signaling molecules move from signal-generating cells to signal-receiving cells by passing through plasmodesmata (PD) (Stahl and Simon, 2013; Lee, 2015; Liu and Chen 2018; Cheval and Faulkner, 2017; Lee, 2014). PD are membrane-lined channels that span the cell walls of neighboring plant cells, providing cytoplasmic, endoplasmic reticulum (ER), and plasma membrane (PM) continuity between adjoining cells. The cytoplasmic sleeve between the two membranes, PM and ER, allows symplastic molecular movement between adjoining plant cells (Lucas et al., 2009). Three-dimensional ultrastructural analyses revealed that there are extensive ER-PM contact sites within the cytoplasmic sleeve (Nicolas et al., 2017).

Being a physical structure allowing the movement of molecules between plant cells, the aperture of PD, which determines the size exclusion limit, was known as a major determinant of PD function (Lucas and Lee., 2004).
The PD aperture is controlled by dynamic deposition and degradation of callose, a plant polysaccharide, at PD within the cell walls. The accumulation and degradation of callose are mediated by callose synthases and \( \beta-1,3 \) glucanases, respectively (De Storme and Geelen, 2014). In addition, PD-localized proteins (PDLPs) are important regulators of callose homeostasis at PD (Lee et al., 2011; Cui and Lee, 2017). Expression of \textit{PDLP5} in Arabidopsis is upregulated upon pathogen infection, coinciding with the accumulation of callose at PD; whereas the \textit{pdlp5} knockout mutant exhibits reduced callose deposition at PD (Lee et al., 2011). These findings suggest that PDLPs are required for pathogen-induced callose deposition at PD.

\textit{Pseudomonas syringae} pv. \textit{tomato} (\textit{Pst}) DC3000 is a Gram-negative bacterial pathogen, which infects not only a crop plant, tomato (\textit{Solanum lycopersicum}), but also the model plant \textit{Arabidopsis thaliana} (Whalen et al., 1991). It injects 36 virulence-associated effector proteins into plant cells through the type III secretion system to modulate plant cellular processes (Wei et al., 2015; Xin et al., 2013; 2018). Using live cell imaging, we discovered that one of the effectors, HopO1-1, is targeted to PD and increases the distance of PD-dependent molecular flux between cells in Arabidopsis. Furthermore, HopO1-1 physically interacts with PDLP7 and PDLP5 and destabilizes these two proteins. We found that this manipulation is linked to the ability of the bacterium to successfully colonize and maximize infection.

**Results**

**HopO1-1 is targeted to the PM and PD in Arabidopsis**

Our work on HopO1-1 was initiated following a systematic subcellular localization study of 32 \textit{Pst} DC3000 effectors. Yellow fluorescent protein (YFP) fusions of effectors (both N- and C-terminal fusion) were generated and transiently expressed in \textit{Nicotiana tabacum} leaves. Most interestingly to us, HopO1-1-YFP was observed as prominent, often symmetrical, punctate spots between two adjacent plant cells (Figure 1A). The distinct localization of HopO1-1 led us to speculate that HopO1-1 might be localized to PD.

To confirm the PD localization, we generated stable transgenic plants expressing HopO1-1 tagged with YFP in Arabidopsis. Using immunoblot analyses, we detected expression of full-length fusion proteins (Supplemental Figure 1B). The \( T_2 \) generation of HopO1-1-YFP transgenic plants was subjected to subcellular localization using confocal laser scanning microscopy. Consistent with transient expression results, HopO1-1-YFP signals were detected in the periphery (with puncta) of Arabidopsis cells, suggesting that the fusion protein targets to both the PM and PD. YFP-HopO1-1 signals, on the other hand, were detected in both the nucleus and the cytoplasm (Figure 1A). HopO1-1-1 contains a putative myristoylation site (N-terminal glycine: G2), which targets a protein to the PM. We thus reasoned that tagging YFP to the C-terminus of HopO1-1 (HopO1-1-YFP fusion protein) is correctly targeted to the right cellular compartment in Arabidopsis, whereas tagging YFP to the N-terminus of HopO1-1 (i.e., YFP-HopO1-1) resulted in its mislocalization to the nucleus and the
cytoplasm. Consistent with this hypothesis, we observed that expression of HopO1-1-YFP resulted in slower plant growth compared to that of wild-type Col-0 or YFP-HopO1-1 (Supplemental Figure 1C). A similar growth defect was also observed in transgenic plants expressing wild-type HopO1-1 without any fusion (Supplemental Figure 1D and 1E), suggesting that HopO1-1-YFP is functionally similar to non-tagged wild-type HopO1-1, whereas YFP-HopO1-1 is likely nonfunctional.

To further validate the PM/PD localization of HopO1-1, we stained the PM of Arabidopsis transgenic plant 35S-HopO1-1-YFP with FM 4-64 (Speth et al., 2009). HopO1-1-YFP signals overlapped with FM 4-64-stained PM (Figure 1B), verifying the PM localization of HopO1-1-YFP. To confirm the PD localization, we labeled callose deposited at PD of 35S-HopO1-1-YFP transgenic plant using aniline blue fluorochrome (Guseman et al., 2012). As expected, HopO1-1-YFP fusion proteins were co-localized with aniline blue-stained PD (Figure 1C). The PD localization of HopO1-1 was further tested by transiently co-expressing HopO1-1-YFP with PDLP5-CFP in Nicotiana benthamiana leaves. HopO1-1-YFP signals were found to overlap with PDLP5-CFP signals at PD (Figure 1D). To further confirm PD association of HopO1-1, we performed plasmolysis with leaves of Arabidopsis transgenic plants expressing HopO1-1-YFP or PDLP7-YFP. These fusion proteins were detected in the periphery with puncta in between plant cells (Supplemental Figure 2A and 2B). After plasmolysis, punctate signals of HopO1-1-YFP and PDLP7-YFP fusion proteins were retained on the cell wall (Figure 1E and Supplemental Figure 1C). These findings confirmed that HopO1-1 is targeted to both the PM and PD in Arabidopsis.

**Role of the ADP-RT domain in HopO1-1 localization to PD in Arabidopsis**

Although several plant and viral proteins are localized to PD, a consensus PD-targeting signal has not emerged and remains largely unknown (Thomas et al., 2008; Caillaud et al., 2014; Yuan et al., 2016). We next examined the domain/sequence of HopO1-1 required for PD localization. As HopO1-1 contains a putative N-myristylation site (G2), we first looked at its role in PD localization. Consistent with its predicted role in membrane-association, G2A mutant abolishes the PM localization as well as the PD localization of HopO1-1 (Figure 1F), suggesting that N-myristilation is essential for PM/PD localization. We next searched for putative functional domains of HopO1-1 using NCBI Conserved Domain Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Amino acids 41–283 (C-terminal end residue) are predicted to encode an ADP-ribosyltransferase (ADP-RT; Supplemental Figure 2A); other than an ADP-RT domain, none of other known targeting signals or transmembrane domains was detected. To determine whether the ADP-RT domain is important for the PM/PD localization, we generated two deletion forms: deletion of the ADP-RT domain (HopO1-11–40-YFP) and deletion of the first 40 amino acids (HopO1-141-end-YFP). Confocal analyses showed that, while amino acids 1–40 of HopO1-1 are sufficient to localize the fusion protein to the PM, there is no detectable PD signals. HopO1-141-end-YFP, on the other hand, was mainly detected in the
nucleus and the cytoplasm (Figure 1F). We further investigated whether the putative catalytic residues of HopO1-1 are involved in the correct subcellular localization by generating a YFP-tagged catalytic mutant of HopO1-1 (HopO1-1\textsuperscript{DD}-YFP), in which two catalytic residues, E247 and E249, were mutated to D. Unlike HopO1-1-YFP plants, HopO1-1\textsuperscript{DD}-YFP transgenic plants lost the ability to slow plant growth (Supplemental Figure 1C), suggesting that the predicted catalytic site residues are required for HopO1-1 function in planta. However, HopO1-1\textsuperscript{DD}-YFP was found in the periphery (with punta) of Arabidopsis cells, similar to HopO1-1-YFP (Figure 1A). Taken together, these results indicate that the PM/PD localization of HopO1-1 likely requires two signals: amino acids 1–40 of HopO1-1 contains the first signal that is needed for localization to the PM, whereas the ADP-RT domain (but not catalytic site residues) is necessary for PD localization.

**HopO1-1 exhibits some mono-ADP-riboysyltransferase (mADP-RT) activity in vitro**

We next performed experiments to directly test whether HopO1-1 is indeed a mono-ADP-riboysyltransferase (mADP-RT), as previously predicted along with HopU1 (Fu et al., 2007). His-MBP-HopO1-1, His-MBP-HopO1-1\textsuperscript{DD}, and His-MBP (a negative control) were expressed in *E. coli* and purified. Additionally, we included His-MBP-HopU1 and His-MBP-HopU1\textsuperscript{DD} as a positive or negative control, respectively (Fu et al., 2007; Figure 2A). Purified proteins were incubated together with a generic substrate, poly-L-arginine, to perform an in vitro ADP-ribosylation assay as described (Fu et al., 2007). Consistent with a previous report (Fu et al., 2007), His-MBP-HopU1 ribosylates the generic substrate in vitro. His-MBP-HopO1-1 produced a significantly higher amount of ADP-ribosylated poly-L-arginine compared to that of His-MBP. Furthermore, the catalytic mutant, His-MBP-HopO1-1\textsuperscript{DD}, has a reduced activity (Figure 2B). However, we noticed that the activity of HopO1-1 is significantly lower than that of HopU1 in this assay. Together, these results suggest that HopO1-1 is likely an active mADP-RT, although we cannot exclude the possibility that it also has another enzymatic activity.

**HopO1-1 contributes to bacterial virulence**

To investigate the virulence function of HopO1-1 in the context of bacterial infection, we generated a *ΔhopO1-1* deletion strain of *Pst* DC3000. The leaves of five-week-old Arabidopsis plants (wild type accession Col-0) were infected with 2 x 10\textsuperscript{8} colony-forming units per milliliter (cfu/ml) of *Pst* DC3000 and the *ΔhopO1-1* mutant using a dip inoculation method. The *ΔhopO1-1* mutant was significantly compromised in virulence compared to that of wild-type *Pst* DC3000 (Figure 3A). In parallel, we also tested the virulence activity of an independent mutant, UNL137, in which *hopO1-1* and the adjacent *hopT1-1* genes are deleted (Guo et al., 2005). As shown in Figure 3B, the double mutant was also compromised in virulence. To investigate whether the PM/PD localization and catalytic activity of HopO1-1 are required for the function of HopO1-1, we conducted complementation experiments with HopO1-1\textsuperscript{G2A} (defective in PM/PD localization) and HopO1-1\textsuperscript{DD} (defective in ADP-RT activity), respectively. Whereas wild-type *hopO1-1* partially complemented UNL137, *hopO1-1*\textsuperscript{G2A}
and hopO1-1DD failed to rescue the pathogenicity of UNL137 (Figure 3B). The results are consistent with the hypothesis that HopO1-1 is targeted to the PM/PD in plants to exert its virulence through its putative ADP-RT activity.

**HopO1-1 alters PD-mediated cell-to-cell molecular trafficking in Arabidopsis**

Given the PD localization of HopO1-1, we hypothesized that HopO1-1 modulates PD-dependent molecular flux between plant cells. To test this hypothesis, we adopted a microparticle bombardment approach. Gold particles were coated with plasmids that express YFP (which can move from transformed cells to adjacent cells through PD in Arabidopsis epidermal cells) and ER-trapped cyan fluorescent protein (ER-CFP; which cannot move beyond transformed cells). Plasmid-coated particles were bombarded into leaves of wild-type Col-0, 35S-HopO1-1, and 35S-HopO1-1DD transgenic plants following the protocol described previously (Thomas et al., 2008; Faulkner et al., 2013; Aung et al., 2017). Fluorescent signals were detected at ~20 hours after bombardment using confocal microscopy. The degree of diffusion of YFP proteins was used to determine PD-dependent molecular flux between Arabidopsis abaxial epidermis cells, whereas transformed cells were marked by nondiffusible ER-CFP. We observed a greater movement of YFP molecules in transgenic plants expressing HopO1-1 compared to that of wild-type Col-0 plants or transgenic plants expressing the HopO1-1DD mutant (Figure 4A). Overall, around 60% of transformed cells led to PD-dependent trafficking of YFP molecules in the wild type and HopO1-1DD, whereas expression of HopO1-1 resulted in PD-dependent trafficking in over 80% of transformed cells (Figure 4B). More strikingly, transgenic expression of HopO1-1 promoted the movement of YFP molecules to more surrounding plant cells (Figure 4A and 4C).

Because HopO1-1 increases the cell-to-cell movement of YFP, we examined whether it also enlarges the size exclusion limit of PD. We built a YFP cancatemer with two or three YFP molecules to increase the size of YFP, resulting in 2xYFP (~54 kDa) or 3xYFP (~81 kDa), respectively (Supplemental Figure 3A). We did not observe an enhanced movement of 2xYFP molecules in transgenic plants expressing HopO1-1 compared to that of wild-type Col-0 (Supplemental Figure 3B). In addition, there was no movement of 3xYFP in either Col-0 or 35S-HopO1-1 leaves (Supplemental Figure 3C). Together, these results suggest that transgenic expression of HopO1-1 increases the distance of PD-mediated molecular flux without drastically increasing the size exclusion limit of PD, as detected by incremental 27-kDa size increases. However, our data cannot exclude the possibility that a small increase of the PD aperture in HopO1-1 trangenic plants might contribute to increasing PD-mediated trafficking of 1xYFP.

**HopO1-1 physically associates with PD-located receptor-like proteins (PDLPs)**

To modulate PD-dependent molecular flux, we hypothesized that HopO1-1 might manipulate one or more PD regulators. We noticed that PDLP5 is involved in bacterial immunity (Lee et al., 2011) through maintaining
callose homeostasis at PD (Cui and Lee., 2016). PDLP5 belongs to the PDLP family, which has 8 members (PDLP1-8), in Arabidopsis (Thomas et al., 2008). To examine whether HopO1-1 targets PDLPs, we tested the physical interaction between HopO1-1 and all eight PDLPs using co-immunoprecipitation (co-IP) analyses in planta. PDLP-YFP with or without HopO1-1-cMyc was transiently expressed in tobacco leaves. Co-IP followed by immunoblot analyses showed that HopO1-1 interacts with PDLP5 and PDLP7 (Figure 5A). We further confirmed these interactions using bimolecular fluorescence complementation (BiFC) assays. Confocal images showed that when HopO1-1:NVen210 was co-expressed together with PDLP5:CVen210 or PDLP7:CVen210, the fluorescent signals could be reconstituted (Figure 5B). PLDP6 and a cytosolic CVen peptide (X:CVen210), on the other hand, do not complement the fluorescent signals when expressed together with HopO1-1 (Figure 5B). Intriguingly, the complemented fluorescent signals between HopO1-1 and PDLP5 were mainly detected on the PM, whereas PD-like puncta were observed when HopO1-1 and PDLP7 were co-expressed. Together, the BiFC results agree with co-IP results, validating that HopO1-1 interacts with PDLP5 and PDLP7 in planta.

PDLPs are type I membrane proteins, which contain a short fragment (7–19 amino acids) of the C-terminal cytoplasmic tail (C-tail; Figure 5C). As HopO1-1 is secreted into plant cells, the interaction between HopO1-1 and PDLPs would likely be mediated in part by the C-tail of PDLPs. To examine whether the C-tail of PDLP7 confers specificity in the PDLP interaction with HopO1-1, we generated two different chimeric forms between PDLP6 and PDLP7 by swapping the transmembrane domain (TMD) plus the C-tail (Figure 5C). Using the above-mentioned co-IP approach, we found that PDLPN7:C6 failed to interact with HopO1-1. PDLPN6:C7, on the other hand, became competent as a HopO1-1-interacting protein (Figure 5D). Together, these results show that the interaction between HopO1-1 and PDLP7 is mediated through the C-tail of PDLP7.

**HopO1-1 affects the stability of PDLP5 and PDLP7**

Having found that HopO1-1 physically associates with PDLP5 and PDLP7, we next investigated whether HopO1-1 affects the levels or molecular weights of the PDLPs in planta. We generated transgenic plants stably expressing PDLP-YFP fusion proteins in wild-type Col-0 or 35S-HopO1-1 background. As the transgenic plants were generated in different backgrounds, we analyzed three independent lines for each construct. The transgenic plants were subjected to confocal imaging to examine levels of PDLP-YFP fusion proteins. As shown in Figure 6A, we noticed that YFP signals from PDLP5-YFP and PDLP7-YFP plants were much dimmer in the 35S-HopO1-1 background, whereas YFP signals in PDLP6-YFP plants were not affected by the expression of HopO1-1. This raised the possibility that HopO1-1 might destabilize PDLP5 and PDLP7. To determine the levels of PDLPs more qualitatively, we performed immunoblot analyses to detect YFP fusion proteins using a GFP antibody. In line with confocal images, we found that expression of HopO1-1 affects the
levels of PDLP5 and PDLP7, while the level of PDLP6 was not drastically affected (Figure 6B; Supplemental Figure 4). However, HopO1-1 does not affect the molecular weights of the PDLPs (Figure 6C).

Given that HopO1-1 affects the protein stability of PDLPs, we next examined whether the PDLPs are degraded through a proteasome-dependent pathway. Arabidopsis transgenic seedlings expressing PDLP-YFP in wild-type Col-0 or 35S-HopO1-1 were treated with MG132, a proteasome inhibitor, and subjected to immunoblot analyses. As shown in Figure 6C, MG132 blocked the degradation of PDLP5-YFP and PDLP7-YFP fusion proteins in 35S-HopO1-1 background. We further showed that HopO1-1 did not affect the transcript levels of PDLP5, PDLP6, and PDLP7 (Supplemental Figure 5A). Collectively, our results suggest that HopO1-1 affects the stability of PDLP5 and PDLP7 through a proteasome-dependent mechanism without affecting the transcript levels.

To directly test possible degradation of PDLP5 and/or PDLP7 in the context of bacterial infection, we infected PDLP-HF (His and Flag epitopes) transgenic plants with different bacterial strains: Pst DC3000, ΔhopO1-1 or hrcC. Infected leaves were harvested at 0, 6, and 12 hours post-infection and subjected to immunoblot analyses. As shown in Figure 6D, the level of PDLP7 is lower at 12 hours post infection with Pst DC3000, whereas ΔhopO1-1 and hrcC mutants do not destabilize PDLP7. By contrast, Pst DC3000 does not degrade PDLP5 (Figure 6D). The findings suggest that PDLP7, but not PDLP5, is a biologically relevant host target of HopO1-1 during infection. As a negative control, the PDLP6 level was not changed in response to Pst DC3000, ΔhopO1-1 or hrcC infection (Figure 6D).

**Putative ribosylation sites are crucial for the degradation of PDLPs**

mADP-RT can modify target proteins by ribosylating arginine (Hassa et al., 2006). PDLP5 and PDLP6 contain one arginine, whereas PDLP7 has two arginine residues at their C-terminal ends (Supplemental Figure 5B). To examine the role of putative ribosylation sites on PDLP7 protein stability, we mutated R280 and R285 into alanine (PDLP7R280A/R285A) and generated transgenic plants stably expressing PDLP7R280A/R285A-YFP in the wild-type Col-0 or 35S-HopO1-1 background. Unlike HopO1-1’s effect on wild-type PDLP7, expression of HopO1-1 does not affect the level of PDLP7R280A/R285A (Figure 6E). Similarly, the expression of PDLP transcripts was not affected by HopO1-1 (Supplemental Figure 5C). This result suggests that the arginine residues at the C-terminus of PDLP7 are important for HopO1-1-dependent protein degradation.

We next performed experiments to determine whether HopO1-1 directly ribosylates PDLP7 to modulate its stability. To test mADP-RT activity of HopO1-1 in planta, we incubated total proteins extracted from Arabidopsis or tobacco with the recombinant His-MBP fusion protein purified from E. coli or the YFP fusion protein enriched from N. benthamiana. Consistent with a previous report, HopU1 ribosylated both Arabidopsis and tobacco proteins (Fu et al., 2007), whereas HopO1-1 did not ribosylate any plant proteins (Supplemental Figure 6A and 6B). We next enriched PDLP-YFP fusion proteins from Arabidopsis transgenic plants stably
expressing PDLP-YPF in wild type Col-0 or 35S-HopO1-1 to detect possible HopO1-1-mediated ribosylation of PDLPs using an anti-pan-ADP-ribose binding reagent (Redditt et al., 2019). Although ribosylated plant proteins were detected in transgenic plants expressing HopU1 (DEX-HopU1), we did not observe any ribosylation on PDLPs (Supplemental Figure 6C). Given that PDLPs are destabilized through a proteasome-dependent pathway (Figure 6C), we treated the transgenic plants with MG132 and subjected them to immunoblot analyses. Again, we did not detect HopO1-1-dependent ribosylation of PDLP7 in Arabidopsis (Supplemental Figure 6D).

**PDLP7 is required for bacterial immunity**

It was previously reported that the pdlp5 mutant is more susceptible to *P. syringae pv maculicola* (*Psm*) ES4326 (Lee et al., 2011). Because HopO1-1 physically interacts with and destabilizes PDLP7, we speculated that PDLP7 might also play a role in plant immunity. To investigate the role of PDLP7 in plant immunity against bacterial pathogens, we identified and characterized a pdlp7 mutant that carries a transfer DNA (T-DNA) in the first exon of *PDLP7* (Supplemental Figure 7A). An RT-PCR assay showed that the pdlp7 mutant is a knockout as there are no *PDLP7* transcripts in the mutant (Supplemental Figure 7B). The pdlp7 mutant displays normal plant morphology compared to that of the wild type (Supplemental Figure 7C), suggesting that *PDLP7* does not make major contributions to plant growth and development. Consistent with a previous report (Lee et al., 2011), we observed that pdlp5 was more susceptible to *Psm* ES4326 (Figure 7). In addition, pdlp5 showed enhanced disease susceptibility to *Pst* DC3000, but not to the *hrcC* mutant. We observed that the pdlp7 mutant was also more susceptible to both *Psm* ES4326 and *Pst* DC3000, but not to the *hrcC* mutant (Figure 7). A double pdlp5 pdlp7 (pdlp5/7) mutant was generated by crossing. The double mutant displayed similar susceptibility to bacterial infection compared with the single mutants (Supplemental Figure 7D), suggesting that PDLP5 and PDLP7 likely function together in plant immunity. PDLP5 and PDLP7 uniquely contribute to the bacterial immunity among the PDLP family as a pdlp1 pdlp2 pdlp3 (pdlp1/2/3) triple mutant and a pdlp4 single mutant were not compromised in the bacterial defense (Supplemental Figure 7D). We next infected the pdlp5 and pdlp7 mutants with the Δ*hopO1-1* mutant. Both mutants were more susceptible to the Δ*hopO1-1* mutant (Figure 7), suggesting that genetic removal of *PDLP5* and *PDLP7* from the plant is sufficient to substitute for the loss of HopO1-1 in the bacterium.

**HopO1-1 is important for bacterial colonization and invasion of tissues surrounding infection sites**

The finding that *Pst* DC3000 injects HopO1-1 to manipulate PD prompted us to test the role of HopO1-1 in allowing bacteria to colonize surrounding tissues. For this purpose, we locally infected *Pst* DC3000 and the Δ*hopO1-1* mutant on tomato (Castlemart) leaves using a leaf stab assay and counted bacterial numbers in tissues surrounding the infection sites. The Δ*hopO1-1* mutant caused smaller halo spots (Figure 8A) and had
much fewer bacteria in the surrounding tissues (Figure 8B). This result supports the hypothesis that *Pst* DC3000 delivers HopO1-1 to promote bacterial colonization and invade host tissues around infection sites.

**Discussion**

Bacterial effectors have been detected in different cellular compartments within plant cells, including the PM (Shan et al., 2000; Robert-Seilaniantz et al., 2006; Xin et al., 2015), endoplasmic reticulum (Block et al., 2014), trans-Golgi network (TGN)/early endosome (EE) (Nomura et al., 2011), chloroplast (Jelenska et al., 2007; Li et al., 2014), mitochondrion (Block et al., 2009), and nucleocytoplasm (Fu et al., 2007; Giska et al., 2013). Determination of subcellular localization of effectors within plant cells is important for explaining the biologically relevant functions of pathogen effectors. In this study, we found that *Pst* DC3000 HopO1-1 is targeted to PD. This finding strongly suggests that pathogenic bacteria manipulate not only cell-autonomous host functions, but also PD-mediated non-cell-autonomous host processes to spread infection.

Recent findings began to reinforce the notion that PD are important battlegrounds during plant–pathogen interactions (Kankanala et al., 2007; Lee 2014; Cheval and Faulkner, 2017; Sakulkoo et al., 2018; Ganusova and Burch-Smith, 2019). In particular, different members of PDLPs have roles not only in regulating basic PD function, but also in plant immunity. For example, PDLP1 is a receptor for plant viral movement proteins and plays an important role in promoting viral movement (Amari et al., 2010). PDLP1 is also required to resist *Hyaloperonospora arabidopsis*idis (Hpa) infection by depositing callose at haustoria, a feeding structure of the pathogen (Caillaud et al., 2014). We found no evidence that PDLP1 is involved in *Pst* DC3000 infection of Arabidopsis. Instead, HopO1-1 selectively targets PDLP7 and possibly PDLP5, which are involved in bacterial immunity (Figure 5 and Figure 7). We further demonstrated that the short intracellular C-tail of PDLP7 determines the specific interaction between PDLP and HopO1-1 (Figure 5C and D). In fact, changing two amino acids at the C-tail of PDLP7 was sufficient to prevent the protein from being degraded by HopO1-1 (Figure 6E). If many PD-manipulating pathogen effectors target the short C-tails of PDLPs, future efforts to edit the C-tails of PDLPs might provide a broadly applicable novel means of engineering plants with enhanced plasmodesmal immunity against pathogens.

At this point, we favor the hypothesis that HopO1-1 targets immunity-associated PDLP7 for degradation by ribosylating the PDLP protein based on the following observations: HopO1-1 ribosylates a generic substrate (Figure 2), the catalytic activity of HopO1-1 is required for its virulence function (Figure 3B), and the putative ribosylation sites of PDLP7 are required for HopO1-1-dependent protein degradation (Figure 6E). However, we have been unable to detect HopO1-1-dependent ADP-ribosylation of PDLPs or other plant proteins using various methodologies (Supplemental Figure 6). It is possible that the sensitivity of the tested approaches is not high enough to detect the ribosylated signals, although we could robustly detect the ADP-RT activity of HopU1 in all these assays. Alternatively, an unknown biochemical activity of HopO1-1 might regulate the protein
stability of PDLP7 in Arabidopsis. Interestingly, a recent report demonstrated that a putative mono-ADP-RT, *Legionella pneumophila* effector SdeA, functions as a ubiquitin conjugating enzyme in an E1- and E2-independent manner (Qiu et al., 2016). However, HopO1-1 does not share sequence similarity with SdeA. Further study is necessary to elucidate how HopO1-1 precisely destabilizes PDLP7.

Here, we found that expression of HopO1-1 facilitates the movement of YFP molecules between plant cells without drastically changing the apparent PD aperture using the YFP diffusion assays (Figure 4 and Supplemental Figure 3). A recent report showed that PD-dependent trafficking is independent of PD aperture, PD density, or callose deposition at PD (Yan et al., 2019). Yan and colleagues reported that *PHLOEM UNLOADING MODULATOR* (PLM) is required for the formation of the ER–PM contact sites within the cytoplasmic sleeve of PD. PD in the *plm* mutant have no visible cytoplasmic sleeve between the ER and the PM (Nicolas et al., 2017), but exhibit enhanced PD-dependent trafficking. Moreover, in the *plm* mutant apparent PD aperture (as observed by TEM analyses), PD density and PD-associated callose accumulation are comparable to those in wild-type plants (Yan et al., 2019). The findings suggest that, besides apparent PD aperture and density, there are other aspects of PD that might play a crucial role in determining PD function. In this regard, future examination of PD in 35S-HopO1-1 transgenic plants or during *Pst* DC3000 infection may shed light on an aspect of PD regulation that may have evaded discovery so far.

It has long been established that viral pathogens exploit PD to move between plant cells (Benitez-Alfonso et al., 2010; Heinlein 2015; Kumar et al., 2015). Recent studies showed that fungal pathogens also exploit the function of PD to spread in plants (Khang et al., 2010; Cao et al., 2018). Our finding that a bacterial effector protein HopO1-1 targets the host PD suggests that diverse pathogenic microbes have evolved virulence factors to modulate PD-mediated cell-to-cell communication in plants. Understanding how pathogenic microbes modulate PD at the molecular level represents a promising area of research that has potential to substantially advance our understanding of fundamental PD biology and novel disease control strategies in plants.

**Methods**

**Plant Materials, Growth Conditions, Transformation, Plant Selection, and Chemical Treatment**

*Arabidopsis thaliana*, *Nicotiana tabacum* and tomato (Castlemart) plants were grown at 22°C with 50% humidity and irradiated with ~100 μmol m⁻² S⁻² of white light. Arabidopsis T-DNA insertion mutants, *pdlp4* (SALK_028613), *pdlp5* (SAIL_46_E06.v1) and *pdlp7* (SALK_015341), were obtained from ABRC (Columbus, OH). *pdlp1/2/3* (Caillaud et al., 2014) is a gift from Dr. Christine Faulkner’s lab. The presence of the T-DNAs and the homozygosity of mutants were identified by genomic PCR using the following primers: *pdlp4-1* (PDLP4-LP, PDLP4-RP, and LBb1.3), *pdlp5* (PDLP5-LP, PDLP5-RP, and SAIL-LB2) and *pdlp7* (PDLP7-LP, PDLP7-RP, and LBb1.3). The absence of *PDLP4*, *PDLP5*, and *PDLP7* transcripts was determined
by RT-PCR using primers PDLP4-Fwd + PDLP4-Rev, PDLP5-Fwd + PDLP5-Rev and PDLP7-Fwd + PDLP7-Rev, respectively. All primers used in this study are listed in Supplemental Dataset 1. Arabidopsis transgenic plants were generated using the simplified transformation method (https://plantpath.wisc.edu/simplified-arabidopsis-transformation-protocol/). For transgenic plants harboring resistance to kanamycin, T₀ seeds were selected on 0.5 Linsmaier and Skoog (½ LS) medium with 50 µg/ml of kanamycin. For basta resistance transgenic plants, T₀ seeds were germinated on soil and one-week-old seedlings were sprayed with 0.1% Finale Herbicide (Bayer) and 0.05% Silwet L-77 (PhytoTech). The T₂ and T₃ plants were screened on ½ LS medium with 10 µg/ml of Glufosinate-ammonium. For MG132 treatment, Arabidopsis seeds were germinated and grown in ½ LS liquid media with 1% sucrose. Ten-day-old seedlings were treated with 1% DMSO (mock) or 50 µM MG132. Samples were collected 24 hours after the treatment.

Gene Cloning and Plasmid Construction

Plasmid DNAs were constructed using a Gateway cloning system (Invitrogen) or restriction enzyme digest. In this study, we reported the following constructs: 35S-HopO1-1, 35S-HopO1-1DD, 35S-HopO1-1-cMyc, 35S-YFP-HopO1-1, 35S-HopO1-1-YFP, 35S-HopO1-1DD-YFP, 35S-HopO1-1G2A-YFP, 35S-HopO1-1DD-end_YFP, 35S-HopO1-1-40-YFP, 35S-PDLP5-YFP, 35S-PDLP6-YFP, 35S-PDLP7-YFP, 35S-PDLPN6:C7-YFP, 35S-PDLPN7:C6-YFP, 35S-PDLP7R280A/R285A-YFP, 35S-PDLP5-HF, 35S-PDLP6-HF, 35S-PDLP7-HF, 35S-1xYFP, 35S-2xYFP, 35S-3xYFP, 35S-ER-CFP, HopO1-1:NVen210-X:CVen210, HopO1-1:NVen210-PDLP5:CVen210, HopO1-1:NVen210-PDLP6:CVen210, HopO1-1:NVen210-PDLP7:CVen210, HopO1-1-His-MBP, HopO1-1DD-His-MBP, HopU1-His-MBP, and HopU1DD-His-MBP.

The genes of interest were amplified from *Pst* DC3000 genomic DNA or the cDNA synthesized from total RNA of wild-type (Col-0) Arabidopsis seedlings using Phusion High-Fidelity DNA polymerase (New England Biolabs). For Gateway cloning (Invitrogen), the genes of interest were amplified with Gateway-compatible primers. The PCR fragments were cloned into a donor vector (pDONR 207) and different destination vectors using a standard Gateway cloning system (Thermo Fisher). For plasmids cloned by restriction enzyme (RE) digestion, the genes of interest were amplified with gene-specific primers containing the chosen RE recognition sites (see Supplemental Dataset 1 for details). The PCR fragments were digested with the REs and ligated into the RE-digested destination vector using T4 ligase (Thermo Fisher). The vectors used in this study are listed in Supplemental Dataset 2.

To generate the HopO1-1G2A mutation, the mutation site was introduced in the forward primer. To create catalytic mutants (HopO1-1DD and HopU1DD) and chimeric fusion proteins (PDLPN6:C7, and PDLPN7:C6), an overlapping PCR approach (https://gfp.dpb.carnegiescience.edu/protocol/index4.html) was adopted using the overlapping primers. The mutation sites of PDLP7 R280A/R285A-YFP were introduced in the reverse primer used for PCR amplification. For building a single vector BiFC plasmid, *HopO1-1* was amplified with primers...
containing restriction sites Ncol and BamHI on the end of forward and reverse primers, respectively. The amplified fragment was digested with the restriction enzymes and ligated into an enzyme-digested recipient plasmid, pDOE-05 (Gookin and Assmann, 2014), to generate HopO1-1-NVen210. The resulting plasmid HopO1-1:NVen210-X:CVen210 was used as a negative control as well as a vector to introduce PDLPs. PDLP5 was amplified with primers containing AvaII and BspEI cutting sites, whereas PDLP6 and PDLP7 were amplified with SanDI and BspEI cutting sites. The amplified and digested PCR products were ligated into the enzyme-digested recipient plasmid, HopO1-1:NVen210-X:CVen210. ER-CFP and EYFP were amplified using ER-CFP (CD3-953) and ER-YFP (CD3-957), respectively, as templates (Nelson et al., 2007). For cloning 2xYFP, the two fragments of EYFP coding sequences were ligated using EcoRI. 3xYFP was built by ligating the third coding sequence using BamHI at the 3' end of 2xYFP. The amplified products were then cloned into pDnor 207 and pEarley Gate 100 (Earley et al., 2006). To express recombinant proteins in E. coli, HopO1-1 and HopU1 variants were amplified with primers containing restriction sites NdeI and BamHI on the end of forward and reverse primer, respectively. The amplified fragments were digested with the restriction enzymes and ligated into enzyme-digested recipient plasmid, pET17b HMR, to generate His-MPB-fusion protein.

**Transient expression**

For Agrobacterium-mediated transient expression, Agrobacterium strains GV3101 (pMP90) harboring the plasmid of interest (cell density at A_{600} of 0.1) were suspended in ddH_{2}O and infiltrated into the leaves five-week-old N. tabacum or N. benthamiana plants. The infiltrated leaves were subjected to live-cell imaging 2 days after infiltration. A similar transient expression method was used for BiFC assays. The two candidate genes for testing the interaction were cloned into a double ORF expression BiFC system with a XT-Golgi, mTurquoise2 (mTq2) marker (Gookin and Assmann, 2014). The Agrobacterium-infected cells were identified by locating the plant cells with mTq2 expression and the complementation of Venus signals was examined 2 days after infiltration using confocal microscopy.

To examine PD-dependent molecular flux, a microparticle bombardment approach combined with confocal imaging was adopted as previously described (Thomas et al., 2008; Faulkner et al., 2013; Aung et al., 2017). In short, 20 mg of 1.0 μM gold particles (BioRad) were soaked in 70% EtOH for 15 min and rinsed with 1 ml sterile ddH_{2}O three times. The rinsed particles were stored in 333 μl of 50% glycerol. To coat plasmid DNA on the particles, 25 μl of rinsed particles was mixed with 5 μl (1 μg/μl) each of plasmid DNAs (ER-CFP and YFPs), 25 μl of 2.5 M CaCl_{2}, and 5 μl of 0.2 M spermidine. The mixture was vortexed at maximum speed for 3 min, settled for 1 min, and centrifuged at 3,000 xg for 5 sec, and the supernatant was removed. Then, the pellet was resuspended in 100 μl of 100% EtOH. The EtOH-rinsed coated particles were then spun down at 3,000 xg for 5 sec and subjected to two more rounds of EtOH washes. The particles were suspended in 25 μl of 100% EtOH. 8 μl particles were loaded on a macrocarrier disc. The disc was assembled into a Biolistic® PDS-
1000/He Particle Delivery System (BioRad) following the manufacturer’s instructions. The vacuum chamber was set at 27 in. of Hg and the particles were delivered into the abaxial side of 5-week-old Arabidopsis leaves at 1100 psi. The whole procedure was performed at room temperature. The bombarded leaves were kept in the same growth chamber at high humidity for 16–20 hours before imaging. To quantitatively compare the PD-dependent diffusion efficiency of 1xYFP, we collected 518, 402, and 375 images of Col-0, 35S-HopO1-1, and 35S-HopO1-1DD, respectively, from three biological replicates. To determine the PD-dependent trafficking, we first calculated the ratios between transformation events/cells resulting in PD trafficking and total transformation events/cells per experiment. The values from three biological replicates were averaged and standard errors of mean (SEM) calculated. To quantify the number of cells containing YFP, we pooled all images that show PD-dependent movement from three biological replicates. 311, 330, and 221 images from Col-0, 35S-HopO1-1, and 35S-HopO1-1DD, respectively, were used for analysis. The number of cells containing YFP signals was averaged and standard errors of mean (SEM) calculated. A Mann-Whitney U test was used to determine the statistical difference between different genotypes. Mann-Whitney U test results are shown in Supplemental Dataset 3. To determine the PD-dependent trafficking of 2xYFP, we calculated the ratios between transformation events/cells resulting in PD trafficking and total transformation events/cells per experiment. At least 50 images were collected from Col-0 and 35S-HopO1-1 from each biological replicate. Values from three biological replicates were averaged and standard errors of mean (SEM) calculated.

Confocal Imaging and Chemical Staining analyses

A Zeiss Laser Scanning Microscope 510 was used to image fluorescent signals. A small piece (~4 mm²) of leaf tissue was mounted with water on a glass slide with the abaxial side facing upward. Different fluorescent signals are excited with the following laser lines: callose (405 nm), CFP and mTq (458 nm), YFP and Venus (514 nm), and FM 4-64 (595 nm). The signals were then collected using the following settings: callose (BP 420–480 nm), CFP and mTq (BP 460–510 nm), YFP and Venus (BP 530–600 nm), and FM 4-64 (590–630 nm). Callose staining of live tissues was performed as described previously (Guseman et al., 2010). In brief, leaves of 4-week-old Arabidopsis plants were infiltrated with 0.1 mg/ml aniline blue fluorochrome (Biosupplies Australia). The callose signals were collected ~30 min after infiltration for imaging. Cotyledons of 2-week-old Arabidopsis seedlings were stained with 2 μM of FM 4-64 (Life Technologies) for 5 minutes before imaging.

Plasmolysis

Leaves of 2-week-old Arabidopsis transgenic plants expressing 35S-HopO1-1-YFP or 35S-PDLP7-YFP were infiltrated with 1M NaCl and imaged immediately using confocal microscopy as mentioned above.

Generation of P. syringae deletion mutant and complementation strains
A ΔhopO1-1 deletion strain was generated in the Pst DC3000 background as previously described (Kvitko and Collmer 2011) with minor changes. In brief, 1.1 and 1.5-kb genomic DNA fragments flanking hopO1-1 were amplified using the primers listed in Supplemental Dataset 1. The amplified fragments were digested with SalI and ligated with T4 ligase. The ligated 2.6-kb product was gel purified and digested with EcoRI and HindIII and cloned into EcoRI- and HindIII-digested pK18mobsacB using T4 ligase (Invitrogen). The ligated product was then transformed into E. coli RHO5. Both Pst DC3000 and E. coli RHO5 carrying pK18mobsacB plasmid were mixed and spotted on a sterile nitrocellulose filter square on LM medium with 400 μg/ml diaminopimelic acid for conjugation. Transconjugated Pst DC3000 merodiploid were screened with LM media containing rifampicin and kanamycin. Merodiploids were then selected on LM media containing Rifampicin and 10% sucrose to counter-select the integration. The sucrose-resistant and kanamycin-sensitive colonies were then genotyped by PCR using primers listed in Supplemental Dataset 1 to confirm the deletion.

To complement the UNL137 mutant, wild-type hopO1-1 (pLN1622), catalytic mutant hopO1-1DD (pLN4191), and G2A mutant hopO1-1G2A (pLN5543) were fused with their native promoter (schO1pro) and cloned into a pML123 vector. The constructs were then transformed into the UNL137 mutant.

**P. syringae infection assays**

For dip inoculation, Pst DC3000 and ΔhopO1-1 were grown at 30°C overnight in LM media (Kvitko and Collmer 2011). The overnight cultures were then resuspended with water supplemented with 0.02% Silwet L-77 to a final concentration of 2x 10⁸ colony-forming unit (cfu) ml⁻¹. The entire rosette of 5-week-old Arabidopsis plants (Col-0) was dipped into the bacterial suspension with gentle swirling for ~20 seconds. The dipped plants were then placed under a plastic dome to maintain high humidity (~80%). Bacterial multiplication was determined three days after infection by counting cfu per cm² of leaf disc extracts.

For spray inoculation, Pst DC3000 and derivative strains were grown at 30°C overnight on sucrose containing King’s B (KB) (King et al., 1954) agar plates and re-suspended in 10 mM MgCl₂ containing Silwet L-77 (Lehle Seeds, Round Rock, TX) to 5 x 10⁷ cells/ml. The cell suspensions were sprayed onto Arabidopsis plants (Col-0) and the plant leaves were sampled at 0 and 4 days after post-inoculation. For each treatment, 4 leaf discs (0.4 cm²) were crushed in 250 μl of sterilized water and the serial dilutions were plated onto KB agar plates containing rifampicin (100 mg/L). The plates were incubated at 30°C for 2 or 3 days until the bacterial colonies appeared. The following strains were used in the pathogenicity assay: Pst DC3000, UNL137, UNL137 (schO1pro-hopO1-1), UNL137 (schO1pro-hopO1-1DD), and UNL137 (schO1pro-hopO1-1G2A).

For the syringe infiltration assay, bacteria were grown as mentioned above for dip inoculation. 2x 10⁵ cfu/ml of different Pseudomonas strains was infiltrated into the leaves of 5-week-old Arabidopsis plants. The infiltrated plants were dried under low humidity (~20%) for 1 hr to let water evaporate and covered with a plastic dome to
maintain high humidity (~80%). Bacterial multiplication was determined two days after infection by counting cfu per cm² of leaf disc extracts.

For the leaf stab assay, the bacteria were grown at 30°C overnight on LM agar plates. Bacteria were picked and inoculated on tomato leaves using a 30G PrecisionGlide™ needle (BD). The inoculated plants were fully covered with plastic wrap. The distal spreading of bacteria was determined seven days after infection. During the sampling, the infected sites were removed with a 2 mm biopsy punch. The surrounding tissues were then collected using a 4 mm biopsy punch (9 punches for each sample) and counted cfu per cm² of leaf disc extracts.

**Immunoblot Analyses**

Fresh tissues were frozen with liquid nitrogen and homogenized with TissueLyser II (Qiagen). SDS-containing extraction buffer (60 mM Tris-HCL pH 8.8, 2% SDS, 2.5% glycerol, 0.13 mM EDTA pH 8.0, and 1x protease inhibitor cocktail complete from Roche) was added to the homogenized tissues (100 μl/10 mg). The samples were vortexed for 30 s, heated at 70 °C for 10 minutes, and centrifuged at 13,000 g for 5 minutes at room temperature. The supernatants were then transferred to new tubes. For SDS-PAGE analysis, 5 μl of the extract in 1x NuPAGE LDS sample buffer (Life Technologies) was separated on 4–12% NuPage (Life Technologies). The separated proteins were transferred to a PVDF membrane. The membrane was incubated in a blocking buffer (3% BSA, 50 mM Tris-base, 150 mM NaCl, 0.05% Tween 20, pH 8.0) at room temperature for 1 h. Then it was incubated with an antibody prepared in the blocking buffer at 4 °C overnight. The antibodies used are as follows: 1:20,000 α-GFP (abcam catalog no. ab290), 1:20,000 α-Streptavidin-HRP (abcam catalog no. ab7403), 1:10,000 α-cMyc (abcam catalog no. ab9106), 1:100 α-ubiquitin (Sigma catalog no. U5379), 1:10,000 α-Flag-HRP (Sigma catalog no. A8592), and 1:1,000 α-pan-ADP-ribose binding reagent (α-panADPR, EMD Millipore catalog no. MABE1016). The probed membranes were washed three times with 1x TBST (50 mM Tris-base, 150 mM NaCl, 0.05% Tween 20, pH 8.0) for 5 min before being incubated with a secondary antibody at room temperature for 1 h except for α-Streptavidin-HRP and α-Flag-HRP. The secondary antibodies used were: 1:20,000 goat anti-rabbit IgG (ThermoFisher catalog no. 31460). Finally, the membranes were washed four times with 1x TBST for 10 min before the signals were visualized with SuperSignal® West Dura Extended Duration Substrate (Pierce Biotechnology).

**Co-IP assays**

PDLP-YFP and HopO1-1-cMyc proteins were transiently expressed in *N. tabacum* and co-IP assays were performed as previous described (Aung and Hu, 2011) with minor modifications. 1 g fresh weight of infiltrated leaf was collected 2 days after infiltration. The tissues were ground in 3 ml of RIPA buffer (Thermo) with 1x complete protease inhibitor cocktail (Roche) and lysed on a rotator at 4°C for 1 h. The samples were centrifuged at 13,000 g for 10 mins at 4 °C to remove cell debris. 20 μl of the supernatants (total proteins) served as the
input controls. The remaining supernatants were then incubated with 20 μl of GFP-Trap®_A (ChromoTek) on a rotator for 1 h to pull down the YFP-fusion proteins. The agarose beads were then spun down at 3,000 g for 15 sec and washed four times with RIPA buffer. Proteins associated with the YFP-fusion protein were eluted by adding 50 μl of 1x NuPAGE LDS sample buffer (Invitrogen) and heating at 70°C for 10 min. The eluted proteins were analyzed by immunoblot assay as mentioned above.

**Expression and Purification of Recombinant Proteins in E. coli**

The plasmid containing HopO1-1 or HopU1 variants were transformed into *E. coli* Rosetta. The transformants were inoculated in 2 ml of LB with 100 mg/ml of Ampicillin and 10% glucose and incubated in a 37°C shaking incubator. The overnight culture were refreshed in LB media (1:20 ratio) containing 10% glucose at 37°C for another two hours. Expression of the proteins was induced by adding 300 μM IPTG and incubated for 3 hours at 28 °C. Soluble recombinant proteins were purified using Ni-NTA resin as recommended by the manufacturer (Qiagen). In short, bacterial pellet from 200 ml of induced culture was pelleted and suspended with 40 ml of Native Binding buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). The suspension was sonicated and centrifuged at 13,000 g at 4 °C for 10 mins to remove cell debris. The supernatant was filtered through a 0.22 μm sterile filter and incubated with 2 ml of Ni-NTA agarose at 4°C for 1 hour. The agarose beads were then rinsed with Native Wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) and eluted with Elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purity and enrichment of the fusion proteins were determined by separating the proteins with SDS-PAGE and staining with SimplyBlue™ SafeStain (Invitrogen).

**monoADP-Ribosyltransferase (mADP-RT) Activity Assays**

Poly-L-Arginine-ADP-RT assay was performed as previously described (Fu et al., 2007). In brief, 2 mM of the purified proteins (HopO1-1-His-MBP, HopO1-1DD-His-MBP, HopU1-His-MBP, HopU1DD-His-MBP, and His-MBP) were incubated with 0.5 mg of a generic substrate, poly-L-arginine (80 μl of 10 mg/ml in 0.1 M dimethyl glutaric acid buffer pH 7.0), and 0.25 mM of [³²P]-NAD (radiolabelled on the ADP-ribose moiety) at room temperature for 1 hour. Recombinant protein His-MBP was used as a negative control. The reaction was stopped by adding 1 ml of 0.1 M phosphate buffer. The substrate was centrifuged at 3,000 g at room temperature and rinsed with 1 ml of PBS three times. The substrate was then resuspended with 250 μl of 0.1 M HCl and 500 μl of 0.1 M dimethyl glutaric acid buffer pH 7.0. Specific incorporated radioactivity was quantified using liquid scintillation (Beckman LS 5000TD).

To test for mADP-RT activity using plant extracts as substrates, 10 mg F.W. of Arabidopsis or *N. tabacum* was isolated using 100 μl of protein extraction buffer (20 mM Tris-HCl pH7.4, 200 mM NaCl, 1 mM EDTA, and 1 mM DTT). Total proteins were centrifuged at 700 xg for 10 minutes at 4 °C to remove tissue debris. 10 μl of the
total proteins was mixed with 1.25 mM of Biotinalated-NAD+ and 2 μg of recombinant proteins purified from *E. coli*. The reaction was incubated at room temperature for 1 hour and stopped by adding NuPAGE LDS sample buffer (Life Technologies). To use proteins transiently expressed in *N. tabacum* as enzymes, the proteins were pulled-down using 20 μl of GFP-Trap® A (ChromoTek) as mentioned above. The beads were then incubated with 1.25 mM of Biotinalated-NAD+ and total proteins extracted from Arabidopsis or *N. tabacum*. The ribosylated proteins were detected using α-Streptavidin-HRP as mentioned above.

**Detection of ADP-ribosylated proteins in planta**

To detect ADP-ribosylated proteins *in planta*, YFP-fusion proteins were enriched from Arabidopsis transgenic plants expressing 35S-PDLP5-YFP, 35S-PDLP6-YFP, or 35S-PDLP7-YFP in wild-type Col-0 or 35S-HopO1-1 background using 20 μl of GFP-Trap® A (ChromoTek) as mentioned above. Total proteins of wild-type Col-0 and DEX-HopU1 (4-hours post 30 μM Dex treatment) were isolated as described above and served as a negative and positive control, respectively. Ribosylated proteins were detected using an anti-pan-ADP-ribose binding reagent.

**RNA extraction and RT-PCR analyses**

Total RNA from leaves of 2- or 5-week-old Arabidopsis plants was purified as previously described (Chen et al., 2013). 0.32 μg of total RNA was used to make cDNA using SuperScript® VILO™ Master Mix (Life). For RT-PCR, gene-specific primers for *hopO1-1*, *UBQ10*, *PDLP5*, *PDLP6*, and *PDLP7* were used to amplify the target genes (Supplemental Dataset 1). *UBQ10* was served as an internal control and amplified for 25 cycles. The rest of the genes were amplified for 35 cycles. PCR products were separated on a 1% agarose gel.

**Experimental repeats and data analyses**

At least three independent experimental repeats were performed for all experiments. The statistical method and sample size for each experiment were listed in the relevant figures and figure legends.

**Accession Numbers**

The Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this articles are as follows: *PDLP1* (At5g43980), *PDLP2* (At1g04520), *PDLP3* (At2g33330), *PDLP4* (At3g04370), *PDLP5* (At1g70690), *PDLP6* (At2g01660), *PDLP7* (At5g37660), and *UBQ10* (At4g05320). Germplasm identification numbers mentioned in this wrok are as follow: *pdlp4* (SALK_028613), *pdlp5* (SAIL_46_E06.v1), and *pdlp7* (SALK_015341).

**Supplemental Data**

**Supplemental Figure 1.** Expression of HopO1-1 variants in Arabidopsis.
**Supplemental Figure 2.** Subcellular localization of HopO1-1 and PDLP7 in Arabidopsis.

**Supplemental Figure 3.** HopO1-1 promotes PD permeability in Arabidopsis.

**Supplemental Figure 4.** HopO1-1 affects PDLP protein stability in Arabidopsis.

**Supplemental Figure 5.** Expression of PDLP transcripts in Arabidopsis.

**Supplemental Figure 6.** HopO1-1 does not ribosylate plant proteins.

**Supplemental Figure 7.** Characterization of pdlp mutants.

**Supplemental Data Set 1.** Primers used in this study.

**Supplemental Data Set 2.** Vectors used in this study.

**Supplemental Data Set 3.** Summary of statistical tests.

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**Author contributions**

KA and SYH designed the research. KA performed most experiments. PK, AJ, and JRA designed and performed disease assay shown in Figure 3B. BK helped generating the ΔhopO1-1 mutant. ZPL performed immuno blot analyses shown in Figure 6D. KA and SYH analyzed data and wrote the manuscript with input from all authors.

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**References**


Figure 1. HopO1-1 is targeted to the plasma membrane and plasmodesmata in Arabidopsis.

(A) Confocal images of leaf epidermal cells of Arabidopsis transgenic plants expressing fluorescent fusion protein of HopO1-1 variants. Scale = 10 µm.

(B) Confocal images show co-localization between HopO1-1-YFP and FM6-64-stained plasma membrane in Arabidopsis leaf epidermal cells. Scale = 10 µm.

(C) Confocal images show co-localization between HopO1-1-YFP and aniline blue fluorochrome-stained callose (plasmodesmata) in Arabidopsis leaf epidermal cells. Scale = 10 µm.

(D) Confocal images show co-localization between HopO1-1-YFP and PDLP5-CFP. The fusion proteins were transiently expressed in N. benthamiana leaf epidermal cells. Scale = 5 µm.

(E) Confocal images of leaf epidermal cells of Arabidopsis transgenic plants expressing 35S-HopO1-1-YFP. Images were captured right after plasmolysis. Asterisks (*) indicate retracted plasma membrane. Arrowheads show HopO1-1-YFP retained on the cell wall. Scale = 5 µm.

(F) Subcellular localization of N-myristoylation site mutant (HopO1-1G2A-YFP) and two truncated forms (HopO1-141-end-YFP and HopO1-11-40-YFP) of HopO1-1 stably expressed in Arabidopsis leaf epidermal cells. Scale = 10 µm.
Figure 2. HopO1-1 exhibits mono-ADP-ribosyltransferase activity.

(A) SDS-PAGE analysis. Recombinant proteins of wild types (HopO1-1 and HopU1), catalytic mutants (HopO1-1\textsuperscript{DD} and HopU1\textsuperscript{DD}) and His-MBP were purified with Ni-NTA resin. 2 mM of the purified proteins were separated on SDS-PAGE and stained with SimplyBlue\textsuperscript{TM} Safestain. Minuses (–) indicate His-MBP only. Numbers on the left indicate molecular mass in kilodaltons (kDa).

(B) \textit{In vitro} ADP-ribosylation assay. Recombinant proteins were incubated with poly-L-arginine to examine their ribosyltransferase activity. His-MBP served as a negative control. Error bars represent standard error of mean (SEM) from 4 reactions. Statistical differences between different recombinant proteins and His-MBP were analyzed with a two-tailed t-test (\*, \(P<5\times10^{-2}\); **, \(P<5\times10^{-3}\)). Minus (–) indicates His-MBP only.
Figure 3. HopO1-1 is required for full virulence of *Pseudomonas syringae*.

(A) *P. syringae* infection assay. Leaves of Col-0 were dip-inoculated with *P. syringae* pv. *tomato* DC3000 (DC3000) wild type and ΔhopO1-1 mutant strains at 2x 10^8 colony-forming unit (cfu) ml^-1. Bacterial multiplication was determined three days after infection by counting bacterial numbers (cfu/cm² of leaf area). Six replicates were analyzed. Error bars represent standard errors of mean (SEM). Statistical differences between DC3000 and the ΔhopO1-1 mutant were analyzed with a two-tailed *t*-test (***, P<5x10^-5).

(B) Sequence motifs associated with membrane targeting and catalytic activity of HopO1-1 contribute to full virulence of *P. syringae*. Arabidopsis plants (Col-0) were spray-inoculated with 5 x 10^7 cfu/ml of the following DC3000 strains: Wild-type DC3000, UNL137, UNL137 (schO1 pro-hopO1-1), UNL137 (schO1 pro-hopO1-1<sup>DD</sup>), and UNL137 (schO1 pro-hopO1-1<sup>G2A</sup>). Bacterial population was measured at 0 and 4 days post-inoculation. Statistical differences between DC3000 and the mutants are analyzed with a two-tailed *t*-test (*, P<5x10^-2; **, P<5x10^-3).
Figure 4. HopO1-1 promotes PD permeability in Arabidopsis.

(A) Confocal images show the diffusion of YFP. Images were taken of leaf epidermal cells of Col-0 and transgenic plants expressing HopO1-1 variants. YFP and ER-localized CFP (ER-CFP) were co-bombarded into the leaves of wild-type Col-0, 35S-HopO1-1, and 35S-HopO1-1DD. PD-dependent diffusion of YFP molecules was examined. Asterisks indicate the bombarded sites expressing both YFP and ER-CFP. Scale = 50 µm.

(B) Quantitative data show the percentage of transformation events resulting in PD trafficking of YFP in wild-type Col-0, 35S-HopO1-1, and 35S-HopO1-1DD. The degree of trafficking is scored by counting the number of transformation events yielding the diffusion of YFP to surrounding cells vs. the total transformation events per experiment. Error bars represent standard errors of mean (SEM) from three biological replicates. Statistical differences between wild-type Col-0 and the transgenic plants were analyzed with a two-tailed t-test (**, P<0.005).

(C) Quantitative data show the average number of cells containing YFP in wild-type Col-0, 35S-HopO1-1, and 35S-HopO1-1DD. Transformation events resulting in PD trafficking from all three independent experiments are combined for the analysis. Error bars represent standard errors of mean (SEM). Statistical differences among wild-type Col-0 and the transgenic plants were analyzed with a Mann-Whitney U test (**, P<0.0001; ND, no statistical difference).
Figure 5. HopO1-1 physically associates with plasmodesmata-located receptor-like proteins (PDLPs).

(A) Co-immunoprecipitation (co-IP) analysis of the interaction between HopO1-1 and PDLPs. Various combinations of HopO1-1-cMyc and PDLP-YFP fusion proteins as indicated were transiently expressed in \textit{N. tabacum} leaves followed by IP using GFP-Trap\textsuperscript{®}_A. A GFP or cMyc antibody was used to detect the fusion proteins. Three biological replicates were performed for each sample.

(B) Bimolecular fluorescence complementation assay of the interaction between HopO1-1 and PDLPs. Various combinations of HopO1-1:NVen210 and PDLPs:CVen210 were transiently expressed in \textit{N. tabacum} leaves. The infiltrated leaves were subjected for confocal imaging 2-days post infiltration. At least 10 images were captured from randomly chosen regions of infiltrated leaves for each experiment. Three biological replicates were performed for each sample. N, NVen210; C, CVen210; EV, empty vector; XT-Golgi-mTq2, an integrated mTurquoise2 marker labelling Golgi; Scale = 10 µm.

(C) Schematic representations of the sequences of wild-type PDLPs and chimeric PDLPs. TMD: transmembrane domain.

(D) Co-IP analysis of the interaction between HopO1-1 the C-terminal tail of PDLP7. Various combinations of HopO1-1-cMyc and different variants of PDLP-YFP fusion proteins as indicated were transiently expressed in \textit{N. tabacum} leaves followed by IP using GFP-Trap\textsuperscript{®}_A. A GFP or cMyc antibody was used to detect the protein.
Figure 6. HopO1-1 affects PDLP protein stability in Arabidopsis.

(A) Confocal images of 2-week-old Arabidopsis transgenic plants expressing 35S-PDLPs-YFP in wild-type Col-0 or 35S-HopO1-1. Scale = 20 µm.

(B) Immunoblot analysis shows the expression of 35S-PDLP-YFP fusion proteins. The transgenic plants were generated in wild-type Col-0 or 35S-HopO1-1 background. A GFP antibody was used to detect YFP fusion proteins. Rubisco was used as an internal control.

(C) Immunoblot analysis shows that PDLP5 and PDLP7 are degraded through a proteasome-dependent pathway. Arabidopsis transgenic plants expressing 35S-PDLP-YFP fusion proteins were grown in 0.5 Linsmaier and Skoog liquid medium. Ten-day-old seedlings were treated with mock (–, 1% DMSO) and 50 µM MG132 (+). The samples were collected 24 hours after the treatment and subjected to immunoblot analyses. A GFP antibody was used to detect YFP fusion proteins and an ubiquitin antibody was used to detect ubiquitinated proteins. Rubisco was used as an internal control.

(D) Immunoblot analysis shows the expression of 35S-PDLPs-HF upon bacterial infection. Five-week-old Arabidopsis transgenic plants were infiltrated with 2x 10⁸ cfu/ml of Pst DC3000, ΔhopO1-1, or hrcC-. The infected leaves were collected at different time points as indicated (hpi, hours post infection). Expression of the PDLPs was detected using a Flag antibody. Rubisco served as loading control.

(E) Immunoblot analysis detects the expression of 35S-PDLP-YFP fusion proteins in transgenic plants in the wild-type Col-0 or 35S-HopO1-1 background. A GFP antibody was used to detect YFP fusion proteins. Rubisco served as loading control.
Figure 7. PDLP5 and PDLP7 are involved in bacterial immunity.

Leaves of Col-0 and pdlp mutants were syringe-infiltrated with Pst DC3000, the ΔhopO1-1 mutant, the hrcC mutant, or P. syringae pv. maculicola (Psm) ES4326 at 2x 10^5 cfu/ml. Bacterial multiplication was determined 2 days after infection by counting bacterial number (cfu/cm^2 of leaf area). Error bars represent standard errors of mean (SEM) from six biological replicates. Statistical differences between wild-type Col-0 and mutants were analyzed with a two-tailed t-test (*, P<0.05; **, P<0.005).
Figure 8. HopO1-1 is crucial for colonization of *P. syringae* surrounding infection sites.

(A) Disease phenotype of tomato leaves after local infection with *Pst* DC3000 and the ΔhopO1-1 mutant using a needle. The images were taken 7 days after infection.

(B) Bacterial multiplication was determined 7 days after infection by counting bacterial number (cfu/cm² of leaf area). The needle infection sites were removed using a biopsy punch (2 mm radius) and the distal tissues were collected to determine bacterial growth. Error bars represent standard errors of mean (SEM) from six samples. Statistical differences between DC3000 and ΔhopO1-1 are analyzed with a two-tailed t-test (**, P<0.005).