Pseudomonas syringae pv. syringae Uses Proteasome Inhibitor Syringolin A to Colonize from Wound Infection Sites

Johana C. Misas-Villamil
Max Planck Institute for Plant Breeding Research

Izabella Kolodziejek
Max Planck Institute for Plant Breeding Research

Emerson Crabill
University of Nebraska-Lincoln

Farnusch Kaschani
University Duisburg-Essen

Sherry Niessen
The Scripps Research Institute

See next page for additional authors

Follow this and additional works at: http://digitalcommons.unl.edu/plantscifacpub
Inhibitor Syringolin A to Colonize from Wound Infection Sites

Johana C. Misas-Villamil1, Izabela Kolodziejek1, Emerson Crabill2,3, Farnusch Kaschani1,4, Sherry Niessen5, Takayuki Shindo1, Markus Kaiser4, James R. Alfano2,6, Renier A. L. van der Hoorn1*

1 Plant Chemetics Lab, Max Planck Institute for Plant Breeding Research, Cologne, Germany, 2 Center for Plant Science Innovation, University of Nebraska, Lincoln, Nebraska, United States of America, 3 School of Biological Sciences, University of Nebraska, Lincoln, Nebraska, United States of America, 4 Chemical Biology Group, Department of Biology, University Duisburg-Essen, Essen, Germany, 5 Department of Chemical Physiology, The Scripps Research Institute, La Jolla, California, United States of America, 6 Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska, United States of America

Abstract

Infection of plants by bacterial leaf pathogens at wound sites is common in nature. Plants defend wound sites to prevent pathogen invasion, but several pathogens can overcome spatial restriction and enter leaf tissues. The molecular mechanisms used by pathogens to suppress containment at wound infection sites are poorly understood. Here, we studied Pseudomonas syringae strains causing brown spot on bean and blossom blight on pear. These strains exist as epiphytes that can cause disease upon wounding caused by hail, sand storms and frost. We demonstrate that these strains overcome spatial restriction at wound sites by producing syringolin A (SylA), a small molecule proteasome inhibitor. Consequently, SylA-producing strains are able to escape from primary infection sites and colonize adjacent tissues along the vasculature. We found that SylA diffuses from the primary infection site and suppresses acquired resistance in adjacent tissues by blocking signaling by the stress hormone salicylic acid (SA). Thus, SylA diffusion creates a zone of SA-insensitive tissue that is prepared for subsequent colonization. In addition, SylA promotes bacterial motility and suppresses immune responses at the primary infection site. These local immune responses do not affect bacterial growth and were weak compared to effector-triggered immunity. Thus, SylA facilitates colonization from wounding sites by increasing bacterial motility and suppressing SA signaling in adjacent tissues.

Introduction

Wounding of plants by hard wind, hail, heavy rain, sand storms, and frost is common in nature. Many epiphytic leaf pathogens take advantage of this opportunity to infect plants [1]. Although plants have evolved effective immune responses to protect wound sites, many pathogens are able to enter leaf tissues and cause disease [1]. The molecular mechanisms underlying the suppression of plant-mediated restriction of pathogen spreading from wound sites are poorly understood.

Pseudomonas syringae pv. syringae (Psy) causes brown spot on bean plants and blossom blight in pear trees [1], which are serious diseases responsible for significant yield losses in agricultural industries in the US, Africa, and Australia. Psy can grow epiphytically on leaf surfaces and enters the leaf intercellular space (apoplast) through stomata and wounds [1–3]. Upon entering the leaf apoplast, Psy initially propagates biotrophically, keeping the host cells alive, and later causes necrotic lesions [4]. Although Psy is a common leaf epiphyte, disease outbreaks are often seasonal and conditional [5]. For example, the onset of epidemics is associated with heavy rain storms and is related to raindrop momentum rather than an increase in humidity [6]. In addition, heavy wind without significant precipitation, causing damage by hail and blowing sand, has caused a brown spot outbreak causing 55% yield loss [7]. Likewise, although Psy strains are a common and dominant component of the microflora on pear trees, blossom blight disease only occurs after frost injury, which explains the strong seasonal variation of disease outbreaks [8]. These data illustrate that P. syringae takes advantage of natural wound sites to enter host tissue and cause disease. Thus, it is important to understand the molecular mechanisms underlying host entry at wound sites in order to prevent disease outbreaks. However, to date, these mechanisms have been poorly investigated.

We recently discovered that green fluorescent protein (GFP)-expressing P. syringae can escape from wound infection sites and colonize adjacent tissues in the wild tobacco plant Nicotiana benthamiana [9], which has become an important model plant for P. syringae infections [3,10–16]. Colonies appeared up to 1 cm from the primary infection site within a few days. Although these infections are not systemic (throughout the whole plant), these distances, from the perspective of bacteria, are significant and...
increase the area of infection by several orders of magnitude. Using bacterial count assays with controlled inoculation populations, we have shown that bacterial populations can grow nearly 100-fold more if the bacteria colonize adjacent tissue compared to when they remain contained at the primary infection site [9]. The colonization from wound sites follows the vasculature, and electron microscopy experiments indicated that the bacteria move through xylem vessels [9]. The ability to colonize tissues along the vasculature involves four steps: first, the bacteria overcome local containment at the primary infection site; second, they transport themselves over several millimeters through the xylem; third, they escape from the xylem vessel into the apoplast; and finally, SylA promotes bacterial motility and suppresses immune responses at the primary infection site. Consequently, SylA-producing bacteria are more motile and able to spread from the primary infection site through xylem vessels and colonize adjacent, immuno-compromised tissues along the vasculature.

**Author Summary**

Bacterial plant pathogens are usually contained at wound infection sites by an effective immune response. The ways in which bacteria can overcome this spatial restriction is poorly understood. Here, we studied two *Pseudomonas syringae* pv. *syringae* strains that cause brown spot on bean and blossom blight on pea and are known to exist as epiphytes that cause disease upon wounding by hail, sand storms or frost. In this study, we demonstrated that these two strains suppress spatial confinement by producing a low molecular weight proteasome inhibitor, syringolin A (SylA), which acts in two ways. On one hand, SylA diffuses into tissue surrounding the primary infection site to make the tissue insensitive to immune signaling. On the other hand, SylA promotes bacterial motility and suppresses immune responses at the primary infection site. Consequently, SylA-producing bacteria are more motile and able to spread from the primary infection site through xylem vessels and colonize adjacent, immuno-compromised tissues along the vasculature.

Results

The ΔsylC Mutant of PsyB728a Is Unable to Cause Spreading Lesions

Pathovar *syringae* B728a is one of the strains that is able to enter wound sites and colonize adjacent tissues in *N. benthamiana* [9]. At 5 d after wound inoculation (5 dpi) with this strain, fluorescent colonies appeared along the vasculature at regular intervals (Figure 1A), which were absent in the untransformed control not expressing GFP (Figure 1B). To investigate the molecular mechanism underlying wound entry by PsyB728a of *N. benthamiana*, we tested various PsyB728a mutants for their ability to cause spreading lesions following toothpick inoculation. These assays revealed that wound entry by the GFP-expressing ΔsylC mutant of PsyB728a was strongly reduced, even though fluorescence was detected at the inoculation site (Figure 1C). This fluorescence at the inoculation site was caused by bacteria expressing GFP, since the untransformed ΔsylC control did not show this fluorescent signal (Figure 1D). Colonies of ΔsylC in adjacent tissues appeared in less than 20% of the wound inoculations, whereas WT PsyB728a showed colonization from wounding sites (wound entry) in over 80% of the inoculations (Figure 1E).

To demonstrate the relevance of spreading for bacterial growth, we inoculated wound sites with a controlled inoculum (1 µL containing 10⁴ bacteria) and determined the colony-forming units at 5 dpi in leaf discs containing the wound site and adjacent tissue. Under these conditions, WT bacterial populations grew from 10⁴ to over 10⁶ bacteria per infection site, whereas ΔsylC bacteria grew from the same inoculum population to about 3×10⁴ bacteria per inoculation site (Figure 1F). Thus, under these conditions, the ability to spread gives WT bacteria an advantage of 63-fold increased population growth compared to the ΔsylC mutant.

However, strong GFP fluorescence at the inoculation site (Figure 1D) suggested that ΔsylC bacteria were able to grow locally. Indeed, bacterial growth assays on infiltrated tissues did not show significant growth differences between WT and ΔsylC strains (Figure 1G). Furthermore, indistinguishable bacterial growth between WT and ΔsylC bacteria upon infiltration was also observed with different inoculation densities and at different relative humidity levels (Supplemental Figure S1). Although the ΔsylC strain sometimes grew less compared to the WT strain, the differences were only rarely statistically significant (3/16 comparisons with P<0.05 in Figure S1). The absence of a statistically significant growth phenotype upon infiltration is in contrast but not in conflict with earlier reports that the ΔsylC mutant causes less symptoms and less bacterial growth compared to WT bacteria upon spray inoculation [18–19], because SylA suppresses preinvasive immunity by reopening stomata [19]. Our data indicate that, once inside the leaf, WT and ΔsylC bacteria grow equally well at primary infection sites but ΔsylC bacteria are unable to colonize adjacent tissue.

SylA Biosynthesis Is Necessary and Sufficient for Wound Entry by PsyB728a and PsyB301D

To demonstrate that SylA itself is sufficient to promote wound entry, we wound-inoculated the GFP-expressing ΔsylC strain into

PLOS Pathogens | www.plospathogens.org 2 March 2013 | Volume 9 | Issue 3 | e1003281
tissue preinfiltrated with 50 µM SylA or a mock control. We used 50 µM SylA because PsyB301D is able to produce 40–100 µM SylA in cultures [17], and 50 µM SylA inhibits the host proteasome (see below). Exogenous SylA restored wound entry of the ΔsylC strain to a similar frequency as the WT strain (Figure 2A). In contrast, no wound entry by the ΔsylC strain was observed by infiltrating buffer without SylA (Figure 2A). These data demonstrate that SylA is essential and sufficient to promote wound entry by the bean brown spot pathogen PsyB728a.

To determine whether SylA biosynthesis is necessary for wound entry by PsyB301D, we tested SylA-deficient mutants of PsyB301D that lack different SylA biosynthesis enzymes (ΔsylC and ΔsylD mutants, [22]) or the entire SylA biosynthesis cluster (ΔsylA-E, [23]). Importantly, none of these mutant strains was able to colonize adjacent tissue (Figure 2B). The loss of wound entry could be complemented with exogenous SylA (Figure 2C), demonstrating that SylA is also essential and sufficient to promote wound entry by the pear blossom blight pathogen PsyB301D.

To test whether loss of the wound entry phenotype could be complemented by restoring SylA biosynthesis, we transformed the markerless PsyB301D ΔsylA-E mutant with the pPL3syl cosmid [23], which carries the entire SylA biosynthesis cluster. Transformation restored the capability to colonize adjacent tissues (Figure 2D), confirming that SylA biosynthesis genes are required to facilitate wound entry. The ΔsylC and ΔsylD mutants of PsyB301D and PsyB728a could not be transformed with the pPL3syl cosmid as these strains already contain the antibiotic selection marker that was used for the cosmid selection. Taken together, these chemical and genetic complementation assays demonstrate that SylA is required and sufficient to facilitate wound entry by PsyB728a and PsyB301D.

SylA Targets the Proteasome of N. benthamiana

Next, we used proteasome activity profiling [20,24] to investigate whether SylA inhibits the proteasome of N. benthamiana. Labeling of N. benthamiana leaf extracts with an activity-based probe for the proteasome (MVB072, Supplemental Figure S2) revealed a single 24 kDa band on protein gels (Figure 3A). Analysis of the purified labeled proteins by tandem mass spectrometry showed that this signal contained the β1, β2, and β5 proteins, the three catalytic subunits of the proteasome (Figure 3B and Supplemental Figure S3). Preincubation of N. benthamiana leaf extracts with SylA or the selective proteasome inhibitor epoxomicin blocked MVB072 labeling, indicating that SylA inhibited the proteasome of N. benthamiana (Figure 3C). Incubation of N. benthamiana leaf extracts with rhodamine-tagged SylA (RhSylA, [23], Supplemental Figure S2) revealed a single 24 kDa signal on protein gels, similar to MVB072 labeling (Figure 3C). Preincubation with SylA or epoxomicin blocked RhSylA labeling of these proteins, confirming that this signal represented the proteasome (Figure 3C). In contrast to SylA, no proteasome inhibition was detected upon preincubation with SylAsat (Figures 3D and 3E), a synthetic SylA derivative that lacks the double bond required for covalent proteasome inhibition [20]. Inhibition assays at various SylA concentrations showed that proteasome inhibition was detectable at concentrations greater
than 6.3 μM and that inhibition was always incomplete (Figures 3E and 3F). These data are consistent with the observation that SylA preferentially inhibits two of the three catalytic subunits of the Arabidopsis proteasome, suggesting that the remaining signal was caused by the β1 catalytic subunit [20].

**SylA Blocks SA Signaling and SA-mediated Immunity in N. benthamiana**

Since wound entry might be associated with the suppression of acquired resistance in adjacent tissues, we tested whether SylA could inhibit SA signaling, a key regulator of acquired resistance in tobacco [26]. Treatment of *N. benthamiana* plants with the SA analog benzothiadiazole (BTH, 300 μM [27]) resulted in the transcriptional activation of the SA-responsive *PR1a* gene within 6 h ([Figure 4A](#fig4a){refig}). However, this transcriptional activation of *PR1a* was blocked when tissues were preinfiltrated with 50 μM SylA, but not SylAsat ([Figure 4A](#fig4a){refig}), indicating that SylA blocked SA signaling by proteasome inhibition. A SylA dilution series showed that suppression of SA signaling occurred at 3.1 μM SylA, but complete inhibition of SA signaling required concentrations greater than 12.5 μM SylA ([Figure 4B](#fig4b){refig}).

To determine if SylA could also block immunity mediated by SA signaling, we infected BTH- and water-treated plants with ΔsyIC bacteria in the absence or presence of SylA. In the absence of SylA, BTH treatment caused reduced bacterial growth of the ΔsyIC strain compared to the control ([Figure 4C](#fig4c){refig}). However, SylA treatment increased growth of ΔsyIC bacteria in BTH-treated tissue to the same level as the control ([Figure 4C](#fig4c){refig}). These data demonstrate that exogenous SylA can complement bacterial growth of the ΔsyIC strain by suppressing SA-mediated immunity.

The above experiments indicate that SylA-producing WT bacteria will grow better during SA signaling than SylA-deficient ΔsyIC bacteria. To test this hypothesis, we inoculated BTH-treated plants with WT and ΔsyIC bacteria and measured bacterial growth. These assays demonstrated a statistically significant growth reduction of the ΔsyIC mutant bacteria compared to WT bacteria ([Figure 4D](#fig4d){refig}). Thus, during SA signaling, SylA-producing WT bacteria grow better than SylA-deficient ΔsyIC bacteria, in contrast to naive plants where both strains grow equally well ([Figure 1G](#fig1g){refig}).

To test whether SA signaling suppresses wound entry, we inoculated plants with WT-GFP and ΔsyIC-GFP 1 d after spraying with water or BTH. BTH treatment significantly reduced the frequency of wound entry by WT bacteria ([Figure 4E](#fig4e){refig}). Thus, activated SA signaling for 1 d was sufficient to suppress wound entry, even of SylA-producing WT bacteria. However, addition of
exogenous SylA before BTH treatment promoted wound entry of both WT-GFP and \( \Delta \text{yC} \)-GFP bacteria in BTH-treated tissue (Figure 4E). Thus, SylA can promote wound entry by inhibiting SA signalling downstream of SA.

SylA Deficiency Triggers Early Host Cell Death and Immune Responses

While performing the infiltration assays, we noticed that \( \Delta \text{yC} \) bacteria triggered early host cell death upon infiltration (Figure 5A). Cell death upon \( \Delta \text{yC} \) inoculation occurred at 2 dpi, whereas WT bacteria caused host cell death usually after 5 dpi (Figure 5A). At later time points, late cell death developing upon infiltration of the WT strain often spread into tissues surrounding the primary infection site, whereas early cell death induced by the \( \Delta \text{yC} \) strain remained confined to the infiltrated region (Figure 5B).

To test whether \( \Delta \text{yC} \)-induced early host cell death is a form of programmed cell death reminiscent of the hypersensitive response (HR), we co-inoculated leaves with lanthanum chloride and sodium vanadate, two chemicals that can prevent programmed cell death by blocking transport of calcium ions and ATPase activity, respectively [28]. Importantly, \( \Delta \text{yC} \)-induced cell death was blocked both by lanthanum chloride and sodium vanadate (Figure 5C), indicating that early host cell death is a program that can be blocked.

Early host cell death by \( \Delta \text{yC} \) bacteria was preceded at 1 dpi by callose deposition (Figure 5D), increased SA levels (Figure 5E), and transcriptional activation of the HR-marker \( \text{Hin1} \) (Figure 5F). These data demonstrate that the \( \Delta \text{yC} \) strain triggered early cell death associated with typical HR-like responses. This is remarkable, since the growth of \( \Delta \text{yC} \) bacteria was indistinguishable from that of WT bacteria during these assays (Figure 1G).

\( \Delta \text{yC} \)-induced Local Responses Are Weak when Compared to ETI/NHR Responses

To further investigate why immune responses induced by the \( \Delta \text{yC} \) strain did not affect bacterial growth, we transformed WT and \( \Delta \text{yC} \) strains with \textit{hopQ1-1} of \textit{PtoDC3000}, a type III effector.
that triggers nonhost resistance (NHR) in *N. benthamiana* [29]. Immune responses triggered by strains carrying hopQ1-1 were compared to strains containing the empty vector (EV). Importantly, the presence of the HopQ1-1 effector increased callose deposition ([Figure 6A](#fig6a){ref#}) and reactive oxygen species in the ΔsylC strain ([Figure 6B](#fig6b){ref#}), indicating that SylA deficiency of the ΔsylC strain induces immune responses that are similar but much weaker compared to the ETI/NHR responses induced by HopQ1-1 [21]. Interestingly, HopQ1-1 also induced strong immune responses in the WT strain ([Figures 6A](#fig6a){ref#} and 6B), indicating that SylA does not suppress HopQ1-1-induced responses.

Importantly, the presence of the HopQ1-1 effector in WT and ΔsylC strains was associated with strongly reduced bacterial growth upon infiltration, as expected for a strong ETI response ([Figure 6C](#fig6c){ref#}). Leaf tissue infiltrated with HopQ1-1-expressing WT and ΔsylC bacteria did not exhibit disease symptoms at low cell densities ([Figure 6D](#fig6d){ref#}), in agreement with the reduced bacterial growth. In contrast, early host cell death, induced by ΔsylC(EV), occurred shortly after infection but stayed confined to the infiltrated area. Cell death induced by WT(EV) occurred later and expanded beyond the infiltrated area ([Figure 6D](#fig6d){ref#}). However, when infiltrated at high densities, HopQ1-1-expressing WT and ΔsylC bacteria triggered cell death that could be blocked by

---

**Figure 4. SylA blocks SA-mediated immunity.** (A) SA signaling is blocked by SylA but not SylAsat. *N. benthamiana* leaves were infiltrated with 50 μM SylA or SylAsat and sprayed with 300 μM BTH. RNA was isolated from infiltrated tissues 6 h after BTH treatment and used as a template for semi-quantitative RT-PCR with gene-specific primers for PR1a and Actin. (B) Dose-dependent inhibition of BTH-induced NbPR1a expression by SylA. Leaves were infiltrated with various concentrations of SylA and sprayed 6 h later with 300 μM BTH. RNA was isolated from infiltrated tissues 6 h after BTH treatment and used as a template for semi-quantitative RT-PCR with gene-specific primers for PR1a (30 cycles) and Actin (24 cycles). (C) SylA blocks BTH-induced acquired resistance. *N. benthamiana* leaves were infiltrated with or without SylA and immediately sprayed with BTH. The ΔsylC mutant bacteria were infiltrated with 2 × 10^8^ bacteria/mL 6 h after SylA/BTH treatment, and bacterial populations were determined at 0 and 3 d after inoculation. Error bars represent SEM of four independent bacterial counts. This experiment was repeated three times with similar results. (D) SylA-producing bacteria grow better in BTH-treated tissues than ΔsylC bacteria. WT and ΔsylC bacteria were infiltrated into *N. benthamiana* leaves at 2 × 10^8^ bacteria/mL, and plants were sprayed with 300 μM BTH 6 h later. Bacterial populations were determined at 0, 3, and 7 dpi. Error bars represent SEM of four independent bacterial counts. This experiment was repeated two times with similar results. (E) SylA promotes wound entry in BTH-treated tissue. Leaves of *N. benthamiana* plants were infiltrated with 50 μM SylA or 0.025% DMSO. After 1 h, the infiltrated area was wound-inoculated with GFP-expressing WT or ΔsylC bacteria and sprayed with 300 μM BTH or water. Wound entry was scored at 5 dpi by fluorescence microscopy. Error bars represent SEM of four independent experiments. P-values determined using the Student’s t-test are indicated.

doi:10.1371/journal.ppat.1003281.g004
lanthanum chloride and sodium vanadate (Figure 6E). Taken together, these data indicate that SylA-deficiency leads to an ETI-like immune response that is too weak to suppress bacterial growth.

SylA Diffuses and Inhibits the Proteasome in Adjacent Tissue

Since SylA is a small molecule that facilitates wound entry, we tested whether SylA could act at a distance from the primary inoculation site. Therefore, we inoculated RhsylA and monitored rhodamine-based fluorescence by fluorescence microscopy. These studies demonstrated that RhsylA moved through the vasculature up to 1 cm from the inoculation site within 2 h (Figure 7A). To test whether RhSylA also targeted the proteasome in adjacent tissues, we extracted proteins from adjacent tissues and analyzed labeling by protein gel electrophoresis. These experiments demonstrated that RhSylA labeled the proteasome in adjacent tissue (Figure 7B). Co-inoculation of RhSylA with unlabeled SylA suppressed labeling, indicating that SylA itself could also move to adjacent tissue (Figure 7B). To confirm that SylA itself could inhibit the proteasome in adjacent tissues, we inoculated SylA locally and monitored proteasome activity in extracts of adjacent tissue using MVB072 labeling. The suppression of MVB072 labeling upon inoculation with SylA (Figure 7C) demonstrate that SylA inhibits the proteasome in adjacent tissues.

SylA Promotes Wound Entry by Suppressing SA-mediated Immunity at a Distance

To investigate whether SylA also promotes colonization at adjacent sites, we infiltrated 50 μM SylA or a mock control into N. benthamiana plants and analyzed cell death and SA accumulation at 24 hpi (Figure 5F). SylA-deficientDsylC bacteria induced early host cell death that was preceded by callose deposition (Figure 5D) and SA accumulation (Figure 5E). Early host cell death induced by SylA-deficientDsylC bacteria was preceded by SA accumulation (Figure 5E). The calcium transport inhibitor lanthanum chloride and the ATPase inhibitor sodium vanadate blocked cell death induced by SylA-deficientDsylC bacteria (Figure 6C). These results demonstrate that SylA-deficiency triggers immune responses at primary infection sites and promotes colonization at adjacent sites by suppressing SA-mediated immunity at a distance.

Figure 5. SylA-deficiency triggers immune responses at primary infection sites. (A) Time course of cell death induced by WT and ΔsylC strains. Leaves were stained with trypan blue at various days after infiltration. Scale bar, 10 mm. (B) Cell death spreads from zones infected with WT but not ΔsylC bacteria. Leaves were infiltrated and stained with trypan blue at 7 dpi. (C) Cell death induced by ΔsylC is blocked by the calcium transport inhibitor lanthanum chloride and the ATPase inhibitor sodium vanadate. Leaves were co-infiltrated with 1×10⁸ bacteria/mL with 50 μM lanthanum chloride or 1 μM sodium vanadate, and pictures were taken at 24 hpi. (D) Early host cell death induced by SylA-deficient ΔsylC bacteria is preceded by callose deposition. Leaves were stained for callose at 24 hpi, examined by fluorescence microscopy, and depicted with equal settings. The callose spots per 0.56 mm² were quantified and printed below the picture with the SEM (n = 3). Scale bar, 0.1 mm. (E) Early host cell death induced by SylA-deficient ΔsylC bacteria is preceded by SA accumulation. N. benthamiana plants were infiltrated with 10⁵ bacteria/mL of WT and ΔsylC bacteria, and SA concentrations were measured at 3 and 24 hpi. Error bars represent SEM of three technical replicates. Student’s t-test: P = 0.21 (3 hpi) and P = 0.096 (24 hpi). The experiment was repeated twice with similar results. (F) Early host cell death induced by SylA-deficient ΔsylC bacteria is preceded by upregulated transcript levels of the hypersensitive cell death marker Hin1. Semi-quantitative RT-PCR was performed on mRNA isolated at 24 hpi. (A–F) Bacteria were infiltrated with 2×10⁶ bacteria/mL into mature leaves of N. benthamiana and analyzed at 24 hpi unless stated otherwise. doi:10.1371/journal.ppat.1003281.g005

Figure 6. SylA-deficiency triggers immune responses at primary infection sites. (A) Time course of cell death induced by WT and ΔsylC strains. Leaves were stained with trypan blue at various days after infiltration. Scale bar, 10 mm. (B) Cell death spreads from zones infected with WT but not ΔsylC bacteria. Leaves were infiltrated and stained with trypan blue at 7 dpi. (C) Cell death induced by ΔsylC is blocked by the calcium transport inhibitor lanthanum chloride and the ATPase inhibitor sodium vanadate. Leaves were co-infiltrated with 1×10⁸ bacteria/mL with 50 μM lanthanum chloride or 1 μM sodium vanadate, and pictures were taken at 24 hpi. (D) Early host cell death induced by SylA-deficient ΔsylC bacteria is preceded by callose deposition. Leaves were stained for callose at 24 hpi, examined by fluorescence microscopy, and depicted with equal settings. The callose spots per 0.56 mm² were quantified and printed below the picture with the SEM (n = 3). Scale bar, 0.1 mm. (E) Early host cell death induced by SylA-deficient ΔsylC bacteria is preceded by SA accumulation. N. benthamiana plants were infiltrated with 10⁵ bacteria/mL of WT and ΔsylC bacteria, and SA concentrations were measured at 3 and 24 hpi. Error bars represent SEM of three technical replicates. Student’s t-test: P = 0.21 (3 hpi) and P = 0.096 (24 hpi). The experiment was repeated twice with similar results. (F) Early host cell death induced by SylA-deficient ΔsylC bacteria is preceded by upregulated transcript levels of the hypersensitive cell death marker Hin1. Semi-quantitative RT-PCR was performed on mRNA isolated at 24 hpi. (A–F) Bacteria were infiltrated with 2×10⁶ bacteria/mL into mature leaves of N. benthamiana and analyzed at 24 hpi unless stated otherwise. doi:10.1371/journal.ppat.1003281.g005
benthamiana leaves and inoculated ΔylC-GFP bacteria at 5 mm outside the infiltrated region (Figure 7D). The ΔylC-GFP bacteria colonized adjacent tissue significantly more frequently in tissue next to SyLA-infiltrated zones compared to zones infiltrated with the mock control (Figure 7E), demonstrating that SyLA promotes wound entry in adjacent tissues. To investigate whether SyLA-producing WT bacteria also promoted wound entry by ΔylC bacteria in adjacent tissues, we infiltrated WT bacteria and inoculated ΔylC-GFP bacteria at 5 mm from the infiltrated area 1 d after infiltration. The presence of a nearby WT-infiltrated region significantly enhanced wound entry of the ΔylC-GFP strain, compared to a mock-infiltrated region (Figure 7F).

To determine whether WT bacteria spread outside the infiltrated area, we repeated the assay by infiltrating WT-GFP bacteria followed by wound inoculation of ΔylC-GFP bacteria in adjacent tissue. The empty space between the marked infiltration zone and the bacterial colonies originating from the wound inoculation site distinguished the WT-GFP from the ΔylC-GFP bacteria (Figure 7G). Colonization of adjacent tissues by infiltrated WT-GFP bacteria did not occur at 6 dpi, whereas the ΔylC-GFP bacteria were already colonizing adjacent tissues from wounding sites (Figure 7G). Thus, WT bacteria promote wound entry of ΔylC-GFP bacteria at adjacent sites, presumably by producing diffusing molecules, rather than being present themselves.

To demonstrate that SyLA suppresses SA signaling in adjacent tissues, we (1) infiltrated leaves with SyLA or mock control; (2) wound inoculated WT-GFP next to the infiltrated area; and (3) sprayed plants with BTH to induce SA signaling (Figure 7H). BTH treatment resulted in a strong suppression of wound entry by WT-GFP bacteria compared to the water-treated control (Figure 7I), confirming that SA signaling suppresses wound entry even of WT bacteria (Figure 4E). Importantly, exogenous SyLA infiltrated at a distance from the inoculated area significantly increased the frequency of wound entry by WT-GFP bacteria in BTH-treated tissue (Figure 7I). To test whether SyLA-producing WT bacteria also suppressed SA-mediated immunity in the vasculature of adjacent tissues, we wound inoculated WT-GFP next to a zone infiltrated with WT bacteria 1 d after infiltration and sprayed the plants with BTH. BTH treatment suppressed wound entry of WT-GFP bacteria, but the presence of a nearby WT-infiltrated zone significantly enhanced wound entry of WT-GFP bacteria in BTH-treated tissues (Figure 7J). These data
demonstrate that SylA-producing bacteria suppress SA responses in the vasculature of adjacent tissues.

A Second Layer of Immunity: NahG Blocks Immunity in Adjacent Tissues but Not Escape from Primary Infection Sites

The accumulation of SA during infection with the ΔsylC strain suggested that adjacent tissues might have acquired resistance. To test whether tissues surrounding ΔsylC-infected areas also mounted immunity, we inoculated WT bacteria next to regions that were preinfiltrated with WT or ΔsylC bacteria (Figure 8A, left). When inoculated next to ΔsylC-infected regions, WT bacteria were unable to spread (Figure 8A), indicating that ΔsylC-infected regions triggered acquired resistance in adjacent tissue. We performed the same experiment on NahG-transgenic N. benthamiana plants which cannot accumulate SA because NahG expresses a bacterial salicylate hydroxylase that converts SA into catechol [30]. When WT bacteria were inoculated next to ΔsylC-infected
regions in NahG-transgenic plants, wound entry was observed with the same frequency as when inoculated next to regions infiltrated with WT bacteria (Figure 8A). These data show that NahG blocks immunity in adjacent tissues triggered by ΔsylC mutant bacteria.

To test whether blocking immune responses in adjacent tissues was sufficient for wound entry by SylA-deficient bacteria, ΔsylC-GFP bacteria were inoculated into NahG-transgenic plants. Importantly, we observed more frequent wound entry by ΔsylC bacteria from inoculation sites on NahG plants compared to WT plants (Figure 8B). However, the frequency of wound entry by ΔsylC bacteria was still less than that of WT bacteria. This difference was also evident from the colonization pattern upon wound entry: the number of veins along which ΔsylC-GFP bacteria colonized was significantly less compared to WT-GFP in NahG plants (Figure 8C). These data indicate that colonization from wound sites is suppressed by two mechanisms: an immune response in adjacent tissues, which is absent in NahG plants, and immune responses at the primary infection site that are not suppressed by NahG.

Local Control of Wound Entry Is Associated with Reduced Motility

Our data indicate that, in addition to SA-dependent immune responses in adjacent tissues, local immune responses also suppress the escape of SylA-deficient bacteria from primary infection sites. To determine whether ΔsylC-GFP bacteria have reduced motility at the primary infection site, WT-GFP and ΔsylC-GFP bacteria were monitored by confocal microscopy at different time points during the wound infection assays. The bacterial motility was indistinguishable between WT-GFP and ΔsylC-GFP bacteria when grown in NYG liquid medium (Figure 9A and Movies S1 and S2) and 6 h after infiltration (Figure 9B and Movie S3 and S4). At later stages, colonies developed in the leaf for both WT-GFP and ΔsylC-GFP bacteria. Bacteria inside these colonies were not motile (Figure 9B and Movies S5 and S6). However, bacteria at the edge of WT-GFP colonies were motile (Figure 9B and Movie S5), but bacteria at the edge of ΔsylC-GFP colonies were not (Figure 9B and Movie S6). Furthermore, WT-GFP bacteria in exudates that leaked from a cut through an infected leaf were motile (Figure 9C and Movie S7), but ΔsylC/bacteria only gained motility at the edge of the exudate and seemed to be embedded in a glue-like matrix (Figure 9C and Movie S8). The reduced motility of ΔsylC bacteria could be complemented chemically by co-infiltrating exogenous 50 μM SylA but not the mock control (Figure 9D and Movies S9 and S10), confirming that the reduced motility of the ΔsylC mutant is caused by the absence of SylA. In conclusion, bacterial motility was identical during the initial stages of infection and was reduced for ΔsylC-GFP bacteria during the later stages of infection.

Discussion

This work uncovered a key role for SylA in facilitating wound entry by suppressing immune responses, both locally and in adjacent tissue. A model for the dual role of SylA is summarized in Figure 10 and is discussed below. Our data indicate that the role of SylA as a virulence factor depends on the assay. SylA is a virulence factor in the classical sense, since the SylA-deficient mutant causes less symptoms on bean plants upon spray...
response triggered by the SylA was not the scope of this study but is important to classify the role of the factor(s) that trigger immune responses in the absence of PTI by flagellin and other elicitors [13]. The identification of bacteria (SylA-deficient bacteria grew equally well locally compared to WT by the presence of HopQ1-1 (Figure 4C)), associated with a 63-fold increased bacterial population level (Figure 1F). We believe that this phenotype is important for the biology of PsyB728a and PsyB301D, since both strains are known to infect bean leaves and pear blossoms via wounds caused by wind or frost, respectively [7,8].

1. SylA Deficiency Triggers Local Immune Responses without Affecting Bacterial Populations

SylA-deficient PsyB728a strains triggered local immune responses, including early host cell death and the accumulation of SA, callose, and HIN1 transcripts (Figure 5). The ΔsylC-induced early host cell death could be prevented with calcium channel or ATPase inhibitors (Figure 5C), indicating that this is a form of programmed cell death. The ΔsylC-induced immune responses were relatively weak compared to NHR/ETI responses induced by the presence of HopQ1-1 (Figure 6), which explains why SylA-deficient bacteria grew equally well locally compared to WT bacteria (Figure 1G). PsyB728a carries several type III effectors that can trigger HR and other ETI responses, resulting in reduced bacterial growth in N. benthamiana [14]. PsyB728a is also likely to trigger PTI by flagellin and other elicitors [13]. The identification of the factor(s) that trigger immune responses in the absence of SylA was not the scope of this study but is important to classify the response triggered by the ΔsylC strain.

The observation that the SylA-deficient ΔsylC mutant grew similarly to WT bacteria upon infiltration (Figures 1G and S1), demonstrated the striking ability of PsyB728a to survive in dead host tissue, despite the widely accepted view that the HR and immune responses suppress pathogen survival. The survival of P. syringae in tissue undergoing HR and immune responses is not unprecedented. Unaffected bacterial populations in dying host tissue have also been observed in tobacco plants infiltrated with avirulent P. syringae pv. maculicola (Pma) M2 and pv. tabaci (Pta) [31]. The absence of cell death in SGT1-silenced N. benthamiana plants did not affect bacterial growth of PsyB728a and only moderately affected growth of PtoDC3000, Pma, and Pta [32]. High-throughput silencing in N. benthamiana has revealed many genes that suppress AvrPto/Pto-mediated HR when silenced, but only a few genes have an effect on bacterial growth [33]. These data indicate that P. syringae is able to survive and even grow in the presence of dead host tissue, despite the likely presence of toxic components generated by immune responses of the dying host. These data are consistent with the notorious survival of PsyB728a as an epiphyte, even during dry periods [3,34].

2. SylA Deficiency Triggers SA Accumulation and Acquired Resistance in Surrounding Tissues

Inoculation of WT-GFP bacteria next to regions preinfiltrated with ΔsylC bacteria demonstrated that ΔsylC triggered immune responses in surrounding tissues that suppressed wound entry (Figure 8A). The ΔsylC bacteria triggered the accumulation of SA (Figure 5E), and SA is known to cause acquired resistance in tobacco [26]. These data demonstrated that ΔsylC bacteria triggered SA-dependent acquired resistance in adjacent tissue (ILR, induced local resistance) (Figure 10A). ILR is similar to the well-described systemic acquired resistance (SAR), which occurs in the entire plant and is also dependent on SA signaling [26]. The
experiments with NahG-transgenic plants demonstrated that the suppression of wound entry occurred at two levels: an acquired response in surrounding tissues, absent in the NahG line (Figure 8A); and a local response confining the bacteria at the primary infection site, still mostly present in the NahG line (Figure 8BC). The suppression of acquired resistance in adjacent tissue in the NahG line might be due to degradation of SA or due to the accumulation of catechol, which can affect bacterial growth [35–36].

3. SylA Production Suppresses Local Immune Responses

In contrast to ΔsylC bacteria, WT bacteria did not trigger early cell death and prevented the early accumulation of callose, Hul1 transcripts, and SA (Figure 5). Thus, SylA production suppressed local immune responses. The finding that local immune responses could be suppressed by inhibiting the proteasome is consistent with previous reports. Hatsugai and colleagues found that silencing of the β1 proteasome subunit suppressed AvrRpm1-induced HR [37]. Furthermore, proteasome inhibitors also have been shown to inhibit early cell death induced by P. syringae pv. phaseolicola [38] and pv. tabaci [31] in tobacco. Taken together, these studies implicate proteasome involvement in cell death and immunity triggered by P. syringae. Interestingly, the HopQ1-1-induced HR was not suppressed in SylA-producing WT bacteria (Figure 6E). These results either mean that SylA could not block all ETI responses or that the timing and/or concentration of SylA production was insufficient to prevent HopQ1-1-induced ETI.

The principle that proteasome inhibition can suppress early cell death might be counterintuitive, since proteasome inhibitors generally are assumed to cause cell death. However, proteasome inhibition can also promote cell survival or cell death, depending on the concentrations and potencies of the proteasome inhibitors used [39]. During PsyB728a infection, SylA intercepted a pro-death program, implying that SylA production was carefully timed and targeted by PsyB728a during infection to provide a sublethal dose. Proteasome inhibition also seems counterintuitive, since many effectors like AvrPtoB, HopM1, and coronatine depend on proteasome activity to execute degradation of their host targets [40–42]. The PsyB728a genome lacks biosynthesis genes for coronatine, but it contains AvrPtoB and HopM1 homologs [43–44]. Therefore, the SylA concentration and timing may be essential parameters that act in concert with the expression of other effectors. These data suggest that ETI suppression by SylA can be overruled if ETI is triggered stronger and/or faster, e.g. in the case of HopQ1-1.

4. SylA Diffuses and Creates a Zone of SA-insensitive Vasculature Tissue

Our data indicate that SylA diffuses through the vasculature and inhibits the host proteasome in adjacent tissues. Although analytical tools to detect SylA in plant tissues are not yet available, we showed that SylA suppressed proteasome activity in adjacent tissues (Figure 7C) and found that rhodamine-tagged SylA quickly moves through the vasculature (Figure 7AB). At this stage, it is unclear whether the SylA movement is apoplastic.
(through the xylem) or symplasmic (through the phloem) and whether the transport is active or passive. Although SylA is produced by bacteria residing in the apoplast, SylA is also quickly taken up by host cells and may enter the symplastic transportation route [20].

Since SylA acts along the vasculature, we used vasculature-specific immunity assays to detect the suppression of SA signaling. For these assays, we used wound inoculations, which provide bacteria with direct access to the vasculature. Importantly, the presence of SylA, when supplemented at a distance, complemented wound entry by ΔpIC-GFP bacteria (Figure 7E). Also, SylA-producing WT bacteria promoted wound entry of ΔpIC-GFP bacteria in adjacent tissues (Figure 7FG), indicating that sufficient SylA was produced during infection to promote colonization from wounding sites.

SylA directly blocks SA signaling in N. benthamiana, which was demonstrated by blocking BTH-induced PR1α expression using SylA (Figure 4A). These data are consistent with the observation that SylA blocks SA signaling in Arabidopsis [19]. One possible molecular mechanism is that SylA prevents the degradation of phosphorylated NPR1 in the nucleus, which needs to be removed by the nuclear proteasome to allow continued transcriptional activation of NPR1-responsive genes [45]. The inhibition of SA signaling in the nucleus is consistent with the observation that SylA targets the nuclear compartment [20]. The subcellular targeting of SylA also explains why SylA blocks SA signaling at concentrations that are less than required for full proteasome inhibition in the total extracts. Thus, new assays are required to detect tissue-specific and subcellular proteasome inhibition during infection.

Importantly, we showed that SylA and SylA-producing bacteria blocked SA signaling in adjacent tissues, since BTH-mediated suppression of wound entry by WT-GFP bacteria was restricted when SylA or WT bacteria were preinfiltrated next to the infection sites (Figure 7J). Vasculature-specific infection assays were required, since SylA acted along the vasculature at a low dose. These experiments indicate that SylA-producing bacteria use effector diffusion to create a zone of SA-insensitive vasculature tissue around the infection site.

**Remote Control by Effector Diffusion**

Our data indicate that SylA moves and inhibits the nuclear proteasome in host cells along the vasculature to create SA-insensitive tissue that was ready for subsequent colonization. Effector diffusion is a common strategy used by pathogens to control immune responses in adjacent tissues. Several fungal effectors that translocate to the host cytoplasm move to adjacent plant cells and are thought to prepare adjacent cells for infection [46–47]. These effectors typically move up to four adjacent cells (less than 0.1 mm). In contrast, small molecule effectors, such as SylA, can move over greater distances (several millimeters) and can reach a larger area surrounding the primary infection site. Although the SylA biosynthesis gene cluster so far only has been found in phylogroup II P. syringae strains, other strains produce other small molecule effectors that can interfere with SA signaling in adjacent tissues. A well-studied example is coronatine, which is produced by several P. syringae strains and also suppresses SA signaling, in this case by activating the jasmonate signaling cascade that results in the downregulation of the SA biosynthetic enzymes and upregulation of SA-converting enzymes [48–49]. P. syringae strains producing coronatine increase the susceptibility of non-inoculated leaves, a phenomenon called systemic induced susceptibility (SIS) [50]. Coronatine is required and sufficient to induce SIS, and SIS is suppressed in NahG-transgenic plants. However, this increased susceptibility is only moderate (3-fold increased growth), and the biological relevance is unclear as it benefits other pathogens as well as the coronatine-producing strain. We focused on adjacent tissues because they are relevant for the wound entry phenotype. We found that SylA production is beneficial for SylA-producing strains as these strains can escape primary infection sites, and increase the bacterial population. These experiments indicate that control of adjacent tissue by effector diffusion may be a common strategy for P. syringae and that different strains use different toxins, in different hosts, and through different molecular mechanisms.

**Both Adjacent and Local Immune Responses Suppress Wound Entry**

The absence of WT-like wound entry by ΔpIC-GFP bacteria in ILR-deficient NahG plants (Figure 8) indicated that also local responses prevent colonization from wound sites. The escape from local confinement in the ILR-deficient NahG plants occurred sporadically, resulting in only a few infected vasculatures where wound entry occurred (Figure 8C). Notably, time-resolved confocal microscopy demonstrated that ΔpIC-GFP bacteria were embedded in a rigid extracellular matrix (ECM) that seemed to immobilize the bacteria at the primary infection site (Figure 9). This rigid ECM was probably similar to the fibrillar ECM described in the 1980s for incompatible interactions with P. syringae bacteria [51–52]. The rigid ECM might have been derived from the bacteria or created by vacuolar content release during the HR. The proteasome is essential for fusion of the vacuolar membrane with the plasma membrane during the HR [37]. Therefore, inhibition by SylA could prevent vacuolar content release into the apoplast. However, a rigid ECM might not be the only possible mechanism of local confinement. Immune responses might also change the motility of the bacteria by affecting their metabolic state. The nature of the matrix, the metabolic status of the embedded bacteria, and how SylA prevents these events remain topics for future studies.

**Conclusion**

In conclusion, our studies revealed that SylA facilitated wound entry of PsyB28a and PsyB301D by blocking immune responses in both local and adjacent tissues. Local immune responses did not affect bacterial population levels but contained the bacteria to the primary infection site. Immune responses in adjacent tissues were SA-dependent and suppressed colonization by bacteria that escaped confinement at the primary infection site. SylA suppressed this two-layered immune response by blocking immune responses in both local and adjacent tissues. Our data indicate that SylA diffuses through the vasculature and blocks SA signaling in adjacent tissues, creating an SA-insensitive zone of vasculature tissue that makes the tissue ready for subsequent colonization.

**Methods**

**Generation of Transgenic Pseudomonas Strains**

All plasmids and strains are summarized in Supplemental Table S1. The ORF encoding GFP was amplified from DNA isolated from GFP-transgenic Pf/DC3000 [42] using primers 5′-tccctaggggtaaggaggaag-3′ and 5′-cccccacagtgaactctctcctgac-3′ and cloned into pGEM-T (Promega), resulting in pFK69. The GFP ORF was amplified from pFK69 using primers 5′-atacgagcagtgaactctctcctgac-3′ (introducing a ribosome binding site, RBS) and 5′-tagagcagctctgtcagatcc-3′ and cloned into pBlueScript using HindIII and XbaI restriction sites, resulting in pFK74. The RBS-GFP cassette of pFK74 was cloned into pML123 [53] using
BamHI and XbaI restriction sites, resulting in pFK78. *Pseudomonas syringae* strains were transformed with pFK78 by electroporation and selected on gentamycin (10 μg/mL). Several fluorescent colonies were used for infection and were found to behave similarly. *PaW718-GFP* was transformed with cosmid pPL3syl carrying *sylA-E* genes of *P. syringae* *Pv0301D-R* [25] by tripolar mating using a helper *E. coli* strain carrying pRK600 and selected with tetracyclin (10 μg/mL), gentamycin (10 μg/mL) and rifampicin (25 μg/mL) [25].

### Plant Growth and Infection Assays

*Nicotiana benthamiana* plants were grown under a 12 h light regime at 22°C and 60% relative humidity and used at 3–5 weeks old, before flowering, unless indicated otherwise. For infection by infiltration, bacteria were grown overnight in 10 mL NYG medium (5 g/L peptone, 5 g/L yeast extract, 2% glycerol), centrifuged and resuspended into 10 mM MgCl2. The OD600 was measured and bacteria were diluted to an OD600 of 0.0002 in 10 mM MgCl2. Bacteria were infiltrated into *N. benthamiana* leaves with a 1 mL syringe without needle. Leaves were examined at various time points. GFP fluorescence was detected by a LeicaMZ16FA using the GFP filter. All experiments were done with similar acquisition settings.

For wound inoculation, the three youngest expanded leaves of 3–5 week-old, nonflowering *N. benthamiana* plant were selected. Bacteria were taken from fresh plates with a sterile toothpick and punched through the leaf using a new toothpick for every infection. In case of infection with controlled inoculum (Figure 1F), wound sites were immediately inoculated with 1 μL of a 107 bacteria/mL. Usually two wounds were compared in opposite leaf halves. Plants were incubated at 22°C under cover and pictures were taken at 5 dpi using stereo fluorescence microscopy. Pictures of every infection site were taken for blind scoring and verification. An example experiment is shown as Figure S3. The percentage of wound entry was calculated for at least 12 toothpicks by dividing the number of times that colonies appear in adjacent tissues by the number of times that colonization occurred at the toothpick inoculation site itself. Inoculation sites that did not show GFP fluorescence were rare and were not included in the count. The average and standard error of the mean (SEM) was calculated from four independent experiments.

Endophytic bacterial populations were determined by colony count assays from opposite leaf halves as described previously [54]. Briefly, leaves were surface-sterilized in 15% H2O2 for 5 min on a rotary shaker at 200 rpm and washed with sterile water. Alternatively, leaves were sterilized by 70% ethanol for 5 min (Supplemental Figure S1). Leaf discs (13 mm diameter, unless otherwise indicated) from two infiltrated leaves were combined and ground in 1 mL 10 mM MgCl2 using metal beads. 20 μL droplets of a 107 bacteria/mL suspension were put on selective agar medium (25 g/mL) [23]. Serial 10-fold dilution series were put on selective agar medium (25 g/mL) containing 160 pmol 2-hydroxybenzoic-3,4,5,6-d4 acid (SA-d4; Campro Scientific, http://www.campro.eu/) as internal standard. After shaking for 10 min at 37°C, samples were centrifuged and re-extracted with 0.5 μL chloroform/methanol (1:2). After phase separation through the addition of 0.5 μL H2O, the polar extract was dried. Samples were acidified with 30 μL 10% trifluoroacetic acid (TFA) and extracted twice with 0.6 mL ethyl acetate/hexane (3:1). Following evaporation of organic solvents, analytes were derivatized with 80 μL pyridine/N-methyl-N-(trimethylsilyl)trifluoroacetamide (1:1) (Sigma) and 1 μL was injected into a gas chromatograph coupled to a mass spectrometer (GC-MS; Agilent, http://www.agilent.com/). Masses of SA-d4 (m/z 271) and SA (m/z 267) were detected by selected ion monitoring and quantified using the Chemstation software from Agilent.

#### Callose Deposition Assays

Callose deposits were measured in leaves of *N. benthamiana* after infiltration with 2 × 107 bacteria/mL. Leaf discs were excised 24 h after infiltration and incubated in 95% ethanol alcohol at 37°C for 2 h and labelled proteins were detected from protein gels using the Typhoon 8600 scanner (Molecular Dynamics) with excitation and emission at 532 and 580 nm, respectively.

#### Measurements of SA

SA concentrations were measured as described in Straus et al. [56]. Briefly, SA was extracted from 100 mg plant material in 1 mL chloroform/methanol/water (1:2:0.3) containing 160 pmol 2-hydroxybenzoic-3,4,5,6-d4 acid (SA-d4; Campro Scientific, http://www.campro.eu/) as internal standard. After shaking for 10 min at 37°C, samples were centrifuged and re-extracted with 0.5 mL chloroform/methanol (1:2). After phase separation through the addition of 0.5 μL H2O the polar extract was dried. Samples were acidified with 30 μL 10% trifluoroacetic acid (TFA) and extracted twice with 0.6 mL ethyl acetate/hexane (3:1). Following evaporation of organic solvents, analytes were derivatized with 80 μL pyridine/N-methyl-N-(trimethylsilyl)trifluoroacetamide (1:1) (Sigma) and 1 μL was injected into a gas chromatograph coupled to a mass spectrometer (GC-MS; Agilent, http://www.agilent.com/). Masses of SA-d4 (m/z 271) and SA (m/z 267) were detected by selected ion monitoring and quantified using the Chemstation software from Agilent.

### Reactive Oxygen Species (ROS) Assays

*N. benthamiana* leaves were infiltrated with 2 × 108 bacteria/mL. Leaf discs were excised 24 h after infiltration and incubated in 95% ethanol alcohol at 37°C for 2 h and labelled proteins were detected from protein gels using the Typhoon 8600 scanner (Molecular Dynamics) with excitation and emission at 532 and 580 nm, respectively.

### Supporting Information

#### Figure S1  
**Bacterial growth of WT and ΔsylC mutant *PsyB728a* upon infiltration.** GFP-expressing (A–C, G, H) or non-transgenic bacteria (D, E, F and I) were infiltrated with 2 × 107 (A–G and I) or 2 × 106 (H) bacteria/mL, and infected plants were kept at 60%–90% relative humidity (RH) (A–F), 60% RH (F–H) or transferred to 2 dpi from high RH to 60% RH (I). Bacterial populations were determined at different days-post-infiltration.

#### Figure S2  
**Callose Deposition Assays.** Callose deposits were measured in leaves of *N. benthamiana* after infiltration with 2 × 107 bacteria/mL. Leaf discs were excised 24 h after infiltration and incubated in 95% ethanol alcohol at 37°C for 2 h and labelled proteins were detected from protein gels using the Typhoon 8600 scanner (Molecular Dynamics) with excitation and emission at 532 and 580 nm, respectively.
inoculation (dpi). Leaves were surface-sterilized with hydrogen peroxide (A–C, G–I) or ethanol (D–E) before leaf extracts were generated, diluted and plated. Experiments were performed in Cologne (A–E, G–I) or Nebraska (F). (A–I) Independent leaves were taken for n independent counts, indicated at the bottom. All error bars represent SEM. Pairwise comparisons between WT and ΔsylC growth was calculated using the Student t-test. NA, not analyzed. (JPG)

**Figure S2** Structures of chemicals used in this study. Reactive groups (red), biotin (blue) and fluorescent reporter (yellow). (JPG)

**Figure S3** Representative wound entry assay experiment. WT-GFP and ΔsylC-GFP bacteria were toothpick-inoculated from a fresh plate into different leaves of different plants of *N. benthamiana*. Pictures were made at 5 dpi using stereo fluorescence microscopy using identical settings. The frequency of host entry at each wound inoculation sites was counted over 12 toothpick sites, as shown at the bottom. Scale bar, 1 mm. (JPG)

**Movie S1** Bacterial motility of WT-GFP in hanging droplet assay. *WT-GFP* bacteria were grown in NYG liquid medium and their motility was monitored by confocal microscopy by hanging droplet assay. Shown is the edge of the droplet for 200 seconds. The size of the movie frame corresponds to 512 × 512 μm. (AVI)

**Movie S2** Bacterial motility of ΔsylC-GFP in hanging droplet assay. ΔsylC-GFP bacteria were grown in NYG liquid medium and their motility was monitored by confocal microscopy by hanging droplet assay. Shown is the edge of the droplet for 200 seconds. The size of the movie frame corresponds to 512 × 512 μm. (AVI)

**Movie S3** Bacterial motility of WT-GFP at 6 h after infiltration. *WT-GFP* bacteria were infiltrated at 10^8* bacteria/mL and their motility was monitored six hours later by confocal microscopy. The length of the movie is 200 seconds. The size of the movie frame corresponds to 512 × 512 μm. (AVI)

**Movie S4** Bacterial motility of ΔsylC-GFP at 6 h after infiltration. ΔsylC-GFP bacteria were infiltrated at 10^8* bacteria/mL and their motility was monitored six hours later by confocal microscopy. The length of the movie is 200 seconds. The size of the movie frame corresponds to 512 × 512 μm. (AVI)

**Movie S5** Bacterial motility of WT-GFP in colonies at 24 hpi. Leaves were infiltrated with 10^7 *WT-GFP* bacteria/mL and subjected to confocal microscopy at 24 hpi. Host cells are intact (middle and bottom) and carry normal-looking chloroplasts (red). Bacteria (green) are static in the colony (bottom), but are motile along the edge of the colony (top). The length of the movie is 200 seconds. The size of the movie frame corresponds to 512 × 512 μm. (AVI)

**Movie S6** Bacterial motility of ΔsylC-GFP in colonies at 24 hpi. Leaves were infiltrated with 10^7 ΔsylC-GFP bacteria/mL and subjected to confocal microscopy at 24 hpi. Bacteria (green) are not motile and have occupied a collapsed host cell containing degenerating chloroplasts (red). The length of the movie is 200 seconds. The size of the movie frame corresponds to 512 × 512 μm. (AVI)

**Movie S7** Bacterial motility of WT-GFP in colony exudates at 24 hpi. Leaves were infiltrated with 10^7 *WT-GFP* bacteria/mL, cut, and subjected to confocal microscopy at 24 hpi. GFP-expressing bacteria in the exudate that leaks from the leaf cut through infected tissue. The length of the movie is 100 seconds. The size of the movie frame corresponds to 512 × 512 μm. (AVI)

**Movie S8** Bacterial motility of ΔsylC-GFP in colony exudates at 24 hpi. Leaves were infiltrated with 10^7 ΔsylC-GFP bacteria/mL, cut, and subjected to confocal microscopy at 24 hpi. GFP-expressing bacteria in the exudate that leaks from the leaf cut through infected tissue. The length of the movie is 100 seconds. The size of the movie frame corresponds to 512 × 512 μm. (AVI)

**Movie S9** Bacterial motility at 24 hpi in sylC-GFP colonies with 50 μM SyIA. Leaves were infiltrated with 10^7 ΔsylC-GFP bacteria/mL containing 50 μM SyIA and bacterial colonies were imaged at 24 hpi by confocal microscopy. The length of the movie is 100 seconds. The size of the movie frame corresponds to 512 × 512 μm. (AVI)

**Movie S10** Bacterial motility at 24 hpi in sylC-GFP colonies with 0.25% DMSO. Leaves were infiltrated with 10^7 ΔsylC-GFP bacteria/mL containing 0.25% DMSO and bacterial colonies were imaged at 24 hpi by confocal microscopy. The length of the movie is 100 seconds. The size of the movie frame corresponds to 512 × 512 μm. (AVI)

**Table S1** Identification of MVB072-labeled proteins from *N. benthamiana*. (PDF)

**Table S2** Plasmids and strains used in this study. (PDF)

**Acknowledgments**

We would like to thank Elmon Schmelzer and Rainer Franzen for their help with scanning electron microscopy; Arjen van Doorn and Johannes Stuttmann for measuring SA concentrations; and Robert Dudler and Silke Robatzek for providing other materials. We also like to thank Paul Schulze-Lefert, Jane Parker, Imre Somssich, Re´ka To´th, Matthias Joosten and John Rathjen for providing other materials. We also like to thank Paul Schulze-Lefert, Jane Parker, Imre Somssich, Re´ka To´th, Britta Maedge, Haibin Lu and Silke Robatzek for suggestions and comments on the manuscript.

**Author Contributions**

Conceived and designed the experiments: JCMV IK EC JRA RALvdH. Performed the experiments: JCMV IK EC FK TS RALvdH. Analyzed the data: JCMV IK EC JRA RALvdH. Contributed reagents/materials/analysis tools: MK. Wrote the paper: RALvdH.
