BREAKTHROUGH REPORT

Direct and Indirect Visualization of Bacterial Effector Delivery into Diverse Plant Cell Types During Infection

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Short title: Visualizing effector delivery in plants

One-sentence summary: The GFP strand system enables spatial and temporal visualization of bacterial effector delivery during infection.

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ABSTRACT

To cause disease, diverse pathogens deliver effector proteins into host cells. Pathogen effectors can inhibit defense responses, alter host physiology, and represent important cellular probes to investigate plant biology. However, effector function and localization have primarily been investigated after overexpression in planta. Visualizing effector delivery during infection is challenging due to the plant cell wall, autofluorescence, and low effector abundance. Here, we utilized a GFP strand system to directly visualize bacterial effectors delivered into plant cells through the Type III secretion system. GFP is a beta barrel that can be divided into 11 strands. We generated transgenic Arabidopsis thaliana plants expressing GFP1-10 (strands 1 to 10). Multiple bacterial effectors tagged with the complementary strand 11 epitope retained their biological function in Arabidopsis and tomato (Solanum lycopersicum). Infection of plants expressing GFP1-10 with bacteria delivering GFP11-tagged effectors enabled direct effector detection in planta. We investigated the temporal and spatial delivery of GFP11-tagged effectors during infection with the foliar pathogen Pseudomonas syringae and the vascular pathogen Ralstonia solanacearum. Thus, the GFP strand system can be broadly used to investigate effector biology in planta.

INTRODUCTION
Plants can be infected by all classes of pathogens and rely on their innate immune system to recognize and respond to invading organisms (Henry et al., 2013). An important aspect of pathogenicity is the delivery of pathogen proteins, termed effectors, into host cells (Toruno et al., 2016). Effectors can modulate host metabolism, shut down host defense signaling, and suppress cell death (Toruno et al., 2016). Gram-negative bacterial pathogens such as the foliar pathogen *Pseudomonas syringae* and the vascular pathogen *Ralstonia solanacearum* use the Type III secretion system (TTSS), a proteinaceous needle-like structure, to directly deliver effectors inside host cells (Chang et al., 2014). Mutations in core components of the TTSS, such as the homopolymeric ring hrcC, block the ability to cause disease (Deng et al., 1998; Vasse et al., 2000). Plants have evolved intracellular immune receptors with nucleotide binding leucine-rich repeat (NLR) domain architecture that specifically recognize pathogen effectors leading to effector-triggered immunity (ETI) (Chiang and Coaker, 2015). A hallmark of ETI is the hypersensitive response (HR), a specialized form of programmed cell death. Much of our understanding of the plant innate immune system has been gained through investigation of the model plant *Arabidopsis thaliana*, which can be infected by pathogens with diverse tissue preferences, including *P. syringae* and *R. solanacearum*.

Intracellular delivery of GFP-tagged effectors from the fungal pathogen, *Magnaporthe oryzae*, has been successfully visualized inside plant cells (Khang et al., 2010). However, this approach has not been successful for other fungal pathogens, possibly due to the GFP tag, which can interfere with effector delivery or function, or due to low-level effector expression (Tanaka et al., 2015). Despite the importance of bacterial effectors in the modulation of host-microbe interactions, direct TTSS effector delivery has not been visualized in whole organisms. Recently, delivery of the virulence protein VirE2 by *Agrobacterium tumefaciens* through the Type IV secretion system was successfully visualized using the GFP strand system in yeast, Arabidopsis and tobacco (Li et al., 2014; Li et al., 2017; Yang et al., 2017). The Type IV secretion system is responsible for delivery and uptake of proteins and DNA, with a conduit diameter of about 18.5 nm (Wallden et al., 2010). Size constraints of the inner needle (2-3 nm conduit) of the TTSS require proteins to be unfolded prior to secretion and fusion with a full-length fluorophore would likely block delivery (Akeda and Galan, 2005; Chang et al., 2014). Bacterial effector delivery by the TTSS has been studied indirectly in plant genotypes during ETI, via cell viability staining.
during the HR (Torres et al., 2002; Greenberg and Yao, 2004). However, due to the spread of defense signaling by apoplastic reactive oxygen species (ROS) and potentially other small molecules that can move through plasmodesmata (including some pathogen effectors), it is impossible to determine which cells are direct recipients of bacterial effectors, or which host cells are capable of directly recognizing effectors (Allan and Fluhr, 1997; Torres et al., 2002; Greenberg and Yao, 2004; Khang et al., 2010). Additionally, effector detection in the host has relied primarily on overexpression in *Nicotiana benthamiana* or Arabidopsis, but these approaches may not reflect their subcellular localization/targeting and accumulation under natural infection. Thus, multiple questions remain regarding which cells are targeted for effector delivery and where effectors localize within the host cell during infection.

Here, we investigated cell-specific immune responses and used the GFP strand system to directly visualize the delivery of bacterial effectors *in planta*. This approach allowed direct visualization of the *P. syringae* effectors AvrPto, AvrPtoB, and AvrB delivered into diverse leaf cell types during natural infection and visualization of the *R. solanacearum* effector PopP2. The GFP strand system enables the investigation of effector biology during natural infection in intact plants and our findings provide insight to the overlap of cell-type-specific immune responses and patterns of effector delivery.

**RESULTS**

**Diverse cell types in Arabidopsis leaves recognize and respond to the bacterial effector AvrB**

To investigate the capacity of various leaf cell types to recognize and respond to pathogenic bacteria, cell death was used as a proxy for effector recognition. The HR was visualized using trypan blue, a vital stain that selectively accumulates in dead cells turning them blue (van Wees, 2008). In Arabidopsis, the RPM1 NLR immune receptor recognizes phosphorylation of the plant protein RIN4 induced in the presence of the AvrB and AvrRpm1 effectors (Chung et al., 2011; Liu et al., 2011). The RPS2 NLR immune receptor recognizes cleavage and elimination of RIN4 by the AvrRpt2 effector protease (Axtell and Staskawicz, 2003; Mackey et al., 2003). Dip inoculation of Arabidopsis Col-0 with virulent *P. syringae* pv. *tomato* (*Pst*) DC3000 carrying an empty vector (EV) did not result in trypan blue staining of leaf cells 14 hours post-inoculation (hpi) (Figure 1A). In contrast, Col-0 infection with *Pst* carrying
the AvrB effector resulted in activation of RPM1-mediated resistance and accumulation of the trypan blue stain in the epidermal pavement and guard cells as well as internal mesophyll cells (Figure 1A).

To probe the ability of specific cell types to elicit ETI responses, we complemented the Arabidopsis double mutant *rps2-101C/rin4* (*r2r4*) with genomic T7-tagged *RIN4* driven by previously published cell-specific promoters (CSPs) for guard cells (*pGC1*), epidermal cells (*pCER6*), and mesophyll cells (*pCAB3*) (Yang et al., 2008; Ranjan et al., 2011) (Figure 1B). Expression of *RIN4* in the CSP lines as compared to Col-0 was determined by anti-*RIN4* immunoblotting (Supplemental Fig. 1). In order to enable detection of *RIN4* in transgenic lines using different CSPs, protein loading for the Col-0 control was diluted by half to avoid overexposure. *RIN4* expression in the cell-specific transgenic lines was still less than the diluted Col-0 sample. Lines were dip inoculated with *Pst* DC3000 (*avrB*) and assessed for the ability to elicit a microscopic HR by trypan blue staining (Figure 1B). In the *r2r4* background, RPM1 is not functional due to the absence of *RIN4* (Mackey et al., 2002). As expected, the *r2r4* line did not elicit an HR and was not stained by trypan blue, whereas the *rps2-101C* single mutant (*r2*) used as a positive control displayed an HR in all cell types (Figure 1C). When the *rin4* mutation is complemented in a cell-specific manner, single-cell HR is also detected in *RIN4* expressing cell types using trypan blue staining (Figure 1C).

In order to visualize macroscopic HR, we infiltrated the same *Arabidopsis* genotypes with a high dose of *Pst* DC3000 (EV) or (*avrB*) (Figure 1D). After *Pst* DC3000 (*avrB*) inoculation we could visualize macroscopic HR spread throughout the infiltrated area in all lines containing *RIN4* (Figure 1D). Infiltration with *Pst* DC3000 (EV) did not induce an HR in any lines. To quantify macroscopic HR in the CSP lines, we measured ion leakage, a proxy for cell death (Henry et al., 2015). Consistent with the limited expression of *RIN4* in different cell types, we found the ion leakage in the CSP lines was reduced with respect to the positive controls Col-0 and *r2* after infiltration with *Pst* DC3000 (*avrB*) (Figure 1E). Together, these results indicate that discrete cell types within the leaf tissue are capable of responding to recognized effectors and may propagate cell death signals across tissues either through plasmodesmata linkages or by apoplastic ROS signaling. Alternatively, there may be a small amount of *RIN4* expressed in other cells in CSP lines that is sufficient to induce cell death after high density inoculation.
The GFP strand system functions in *Nicotiana benthamiana* and effector function is retained when fused with GFP strand 11

Although diverse cell types are able to recognize AvrB, this does not demonstrate direct bacterial effector delivery into these cells (Figure 1). To visualize bacterial effector delivery *in planta* we adopted the GFP strand system based on spontaneous assembly of two complementary GFP fragments (Cabantous et al., 2005). GFP is a beta barrel protein and can be divided into 11 strands (Cabantous et al., 2005). The success of the GFP strand system for detection of type three secreted proteins was previously demonstrated for the *Salmonella* effectors PpB2 and SteA into HeLa cell culture, indicating that the strand 11 tag does not interfere with bacterial effector delivery in *Salmonella* (Van Engelenburg and Palmer, 2010). We used a superfolder GFP variant, which exhibits enhanced solubility, folding, and fluorescence (Cabantous et al., 2005; Pedelacq et al., 2006). The small 16 amino acid eleventh strand (RDHMVLHEYVNAAGIT) was fused via a flexible linker (GDGGSGGGGS) on the C-terminus of the *P. syringae* effectors AvrB, AvrPto, and AvrPtoB (Figure 2A). When the effector-GFP₁₁ fusion is co-expressed with GFP₁₋₁₀, the two complementary GFP fragments should spontaneously associate/self-assemble to form a functional GFP molecule (Figure 2A). First, we demonstrated proof-of-concept for the GFP strand system using *Agrobacterium*-mediated transient plant expression system in *N. benthamiana*. GFP₁₋₁₀ and the individual effector-GFP₁₁ constructs were transiently expressed in *N. benthamiana* cells (Figure 2B). Expression of GFP₁₋₁₀ alone did not result in fluorescence, whereas co-expression with effector-GFP₁₁ resulted in clear GFP fluorescence (Figure 2B). Expression of full-length GFP₁₋₁₁ also resulted in clear fluorescence. Plasmolysis revealed that effector subcellular localization was consistent with previous reports: AvrB-GFP₁₁ and AvrPto-GFP₁₁ were detected at the plasma membrane with visible Hechtian strands after plasmolysis and AvrPtoB-GFP₁₁ exhibited cytosolic localization (Nimchuk et al., 2000; de Vries et al., 2006) (Figure 2B).

*Pst* DC3000 infects both tomato and Arabidopsis, causing bacterial speck disease. In resistant tomato genotypes such as the cultivar Rio Grande (RG) 76R, AvrPto and AvrPtoB both interact with tomato protein kinase Pto, which is guarded by the tomato NLR Prf (Martin et al., 1993; Salmeron et al., 1996). In the susceptible tomato genotype RG *prf3*, AvrPto and AvrPtoB promote bacterial virulence (Martin et al., 1993; Lin and Martin, 2005). In order to verify that effector fusion to GFP strand 11 does not impact effector delivery and function, inoculations
were performed in tomato using *Pst* expressing AvrPto- GFP<sub>11</sub> and AvrPtoB- GFP<sub>11</sub>. Effectors were cloned into the pBBR1 plasmid under the control of their native promoters and expressed with a C-terminal fusion to GFP strand 11 as described above (Kovach et al., 1995). The resulting plasmids were transformed into *Pst* DC3000. Since AvrPto and AvrPtoB are functionally redundant with respect to the host ETI response, they were singly complemented into a *Pst* double deletion mutant for both effectors (DC3000 ΔavrPto/ΔavrPtoB) (Lin and Martin, 2005). *Pst* DC3000 ΔavrPto/ΔavrPtoB caused disease on both RG and RG prf3 although symptoms were attenuated compared to wild-type *Pst* DC3000 (Figure 3A, B). Complementation of DC3000 ΔavrPto/ΔavrPtoB with either AvrPto-GFP<sub>11</sub> or AvrPtoB-GFP<sub>11</sub> resulted in recovery of recognition and resistance in the RG background (Figure 3A, B). Bacterial titers were assessed at four days post-dip inoculation. Complementation of *Pst* DC3000 ΔavrPto/ΔavrPtoB with AvrPto-GFP<sub>11</sub> leads to a complete recovery of virulence and bacterial titers in RG prf3 (Figure 3A-B). Complementation of *Pst* DC3000 ΔavrPto/ΔavrPtoB with AvrPtoB-GFP<sub>11</sub> enhanced disease symptoms, but did not result in a significant increase in bacterial virulence in RG prf3 (Figure 3A-B). Previously, AvrPtoB was demonstrated to enhance DC3000 disease symptoms but not bacterial titers at four days post-inoculation (Lin and Martin, 2005). These data demonstrate that both AvrPto and AvrPtoB effectors are able to be delivered into plant cells via the TTSS and can be recognized by the plant innate immune system when fused to GFP strand 11.

In order to verify that fusion to GFP strand 11 does not impact AvrB delivery and function, inoculations were performed in Arabidopsis using *Pst* DC3000 expressing AvrB-GFP<sub>11</sub>. AvrB is recognized by the RPM1 NLR in the Arabidopsis Col-0 ecotype (Boyes et al., 1998). The ability of AvrB-GFP<sub>11</sub> to elicit RPM1-mediated responses was assessed using trypan blue staining as well as bacterial growth assays. To assess macroscopic HR, leaves of Col-0 plants were syringe infiltrated with a bacterial suspension of either *Pst* DC3000 (EV) or *Pst* DC3000 (avrB-GFP<sub>11</sub>). Infection with *Pst* DC3000 (avrB-GFP<sub>11</sub>) elicited a robust macroscopic HR, visualized by trypan blue at 24hpi (Figure 3C). Bacterial growth assays demonstrate that AvrB-GFP<sub>11</sub> is delivered and recognized by RPM1, as the bacterial growth of *Pst* DC3000 (avrB-GFP<sub>11</sub>) was attenuated at 4 days post-inoculation compared to DC3000 EV on Col-0 but not the rpm1-3 mutant line (Figure 3D-E). Taken together, these results indicate that effector-
GFP fusions, when delivered by the TTSS of Pst DC3000, are recognized by host NLR immune receptors and can promote bacterial virulence in susceptible genetic backgrounds.

**Effectors are delivered into multiple cell types in Arabidopsis leaves**

To visualize effector delivery during natural infection, we infiltrated Pst DC3000 strains carrying the complementary GFP strand 11 tagged effectors into homozygous transgenic Arabidopsis lines expressing 35S:GFP (Supplemental Figure 2). To avoid the cell death elicited by RPM1-mediated recognition of AvrB in wild-type Col-0, rpm1-3/rps2-101C mutant plants (r1r2) lacking both RPM1 and RPS2 NLR receptors expressing GFP1-10 were used to analyze AvrB-GFP delivery. The following Pst genotypes were used to detect bacterial effector delivery in the Col-0 background: DC3000 ΔavrPto/ΔavrPtoB + avrPto-GFP11, DC3000 ΔavrPto/avrPtoB-GFP11, DC3000 ΔavrPto/avrPtoB (negative control), DC3000 ΔhrcC + avrPtoB-GFP11 (negative control), and DC3000 ΔhrcC + avrPto-GFP11 (negative control). The following Pst genotypes were used to detect bacterial effector delivery in the r1r2 background: DC3000 + avrB-GFP11 and DC3000 EV (negative control). Confocal micrographs were acquired with the Zeiss LSM710 confocal microscope. We analyzed four different Arabidopsis plants for each bacterial strain. Two inoculation methods were assessed: syringe (Figure 4) and surface inoculation (Figure 5).

Effector delivery was first assessed after syringe inoculation of four-week-old Arabidopsis leaves with Pst DC3000 carrying each of the three GFP fusions tagged effectors. We examined and quantified confocal micrographs taken from four biological replicates to record the temporal and spatial delivery of AvrB-GFP11, AvrPto-GFP11 and AvrPtoB-GFP11 at 24 and 48hpi. At 24hpi, effector delivery events were detected in mesophyll cells for AvrB-GFP11 and AvrPto-GFP11 (Supplemental Figure 3, Figure 4D). Delivery events of AvrB-GFP11 and AvrPto-GFP11 effectors at this time point were visualized as small foci at the cell periphery in both mesophyll and epidermal pavement cells (Supplemental Figure 3). At 24 hpi, AvrPtoB-GFP11 was not detected in mesophyll cells but a small amount of fluorescence was observed in the epidermal pavement cells and the GFP signal had a more diffuse localization around the cell periphery than the other two effectors (Supplemental Figure 3, Figure 4D). By 48hpi, we observed robust delivery of all three effectors in both mesophyll and epidermal pavement cells (Figure 4A, B). The number of cells positive for GFP fluorescence was greater for the effectors...
AvrB-GFP<sub>11</sub> and AvrPto-GFP<sub>11</sub> than for AvrPtoB-GFP<sub>11</sub>, suggesting enhanced effector delivery or stability (Figure 4D). At 48 hpi, fluorescent stretches of effectors were observed, indicating effector accumulation over time (Figure 4A-B). Surprisingly, the highest frequency of delivery events was not in the mesophyll, but the epidermal pavement cells (Figure 4D). GFP fluorescence localized to the cell periphery was observed at both 24 and 48hpi (Supplemental Figure 3 and Figure 4B). The AvrB-GFP<sub>11</sub> signal was strongest of the three effectors investigated, and was frequently detected at junctions between pavement cells. Delivery into adjacent pavement cells could be detected as parallel stretches of GFP fluorescence (Figure 4B inset). To clearly demonstrate separation of the GFP signal and chlorophyll autofluorescence, we used the Zeiss ZEN lite software to create 3D projections of representative Z-stacks for each effector (Figure 4C). These projections further establish a predominance of GFP signal in the epidermal layer. This may reflect a preference for effector delivery at the leaf surface or it may be a consequence of signal reduction when attempting to move the focal plane more deeply into the leaf interior. Expression of GFP<sub>1-10</sub> under the control of 35S promoter within individual cell types may vary and contribute to an observational bias for effector delivery in cells with higher expression of the transgene. We did not detect GFP fluorescence after inoculation with the control Pst strains DC3000 EV, DC3000 ΔhrcC carrying effector-GFP<sub>11</sub> constructs, or DC3000 ΔavrPto/ΔavrPtoB, indicating that the fluorescence signal was specifically detecting effector delivery (Figure 4A, B).

Syringe inoculation delivers bacteria directly to the apoplast. Surface inoculation (dip or spray inoculation) more closely mimics natural infection conditions, enables epiphytic growth on the surface of leaves, and facilitates the detection of early invasion events into the leaf interior (Katagiri et al., 2002). Due to the robust delivery of AvrB-GFP<sub>11</sub> and AvrPto-GFP<sub>11</sub> and their plasma membrane localization (effectively concentrating the fluorescent signal), we chose to focus on these two effectors for surface inoculation experiments. In surface-inoculated leaves, effector delivery events were primarily detected in epidermal pavement and guard cells (Figure 5A-C). Guard cells can exhibit autofluorescence in their inner walls flanking the stomatal pore. Therefore, guard cells only exhibiting fluorescence in their inner walls were not included in the quantification of effector delivery. Effector delivery was detected when fluorescence occurred at the guard cell outer edge, which is adjacent to the surrounding epidermal pavement cells. Effector delivery into pavement cells gave characteristic stretches of GFP fluorescence at the cell
periphery, while delivery events into guard cells appeared as more discretely localized in puncta (Figures 5B). It is possible that effector delivery is initially concentrated as foci at the membrane near the tip of the TTSS needle before spreading along the plasma membrane, as previously described in vitro (Jin and He, 2001). Although we were able to visualize AvrB-GFP delivered into mesophyll cells at 24hpi, AvrPto-GFP could not be detected in mesophyll cells after surface inoculation at 24 or 48hpi (Supplemental Figure 4, Figure 5A and C). Regardless of the inoculation method, we observed that epidermal pavement cells exhibited the highest number of cells positive for effector delivery (Figure 4C, 5C-D). These data indicate that pavement cells are sites for effector delivery by Pseudomonas. These findings are supported by previously published work, which demonstrated that transcription of avrPto in Pseudomonas was highest on the leaf surface when bacteria were in contact with pavement cells (Lee et al., 2012).

Epidermal pavement cells in Arabidopsis display a wide size distribution with dimensions ranging 10 µm to 200 µm, correlated with endopolyploidy of the cell (Melaragno et al., 1993). In contrast, the average size of an epiphytically colonizing Pseudomonad is 1.2 µm (Monier and Lindow, 2003a). The size disparity between bacterial and host cells at the leaf surface could enable multiple bacterial cells to attach and deliver effectors into the same host cell from discrete locations. In order to investigate the ability of Pst DC3000 to deliver effectors at multiple sites within each cell, we quantified the number of distinct fluorescent foci within an individual cell from confocal micrographs. The number of fluorescent foci per cell differed depending on the effector and ranged from 1-25 (Supplemental Figure 5). Compared to other effectors, AvrPto-GFP exhibited a significantly higher number fluorescent foci in pavement cells at 48 hpi, but a significantly lower number of foci in mesophyll cells at 24 hpi (Supplemental Figure 5). At 24 hpi, AvrB-GFP was not only delivered into more mesophyll cells than AvrPto-GFP, but the number of distinct foci in a single mesophyll cell was also significantly higher (Supplemental Figure 5C-D). Taken together, these data demonstrate that the GFP strand system allows analysis of native promoter driven effector delivery during natural infection and the positive detection of cells targeted for effector delivery.

The GFP strand system allows visualization of effector delivery by the xylem colonizing pathogen Ralstonia solanacearum
To demonstrate the utility of the GFP strand system across diverse bacterial pathogens, we applied this technology to detect effector delivery from *Ralstonia solanacearum*. This soil-borne Gram-negative bacterial pathogen colonizes the xylem of infected plants, causing devastating bacterial wilt disease in over 200 plant species (Schell, 2000). In contrast to *P. syringae*, *R. solanacearum* gains entry through the root apex or secondary root emergence sites. After invading the root xylem vessels, *R. solanacearum* disseminates into the stem, where it multiplies and induces wilting through excessive production of exopolysaccharides (Schell, 2000). Mutation of core *R. solanacearum* TTSS components renders the bacteria nonpathogenic (Arlat et al., 1992). PopP2 is a well-characterized effector from the *R. solanacearum* strain GM1000. PopP2 contains a nuclear localization signal and is targeted to the nucleus where it inactivates defensive plant transcription factors to dampen basal immunity (Deslandes et al., 2003). In the Arabidopsis Ws-0 ecotype, PopP2 is recognized by the NLR receptors RPS4 and RRS1-R that cooperate molecularly to trigger resistance (Deslandes et al., 2003; Tasset et al., 2010; Le Roux et al., 2015; Sarris et al., 2015).

Using the GFP strand system, we investigated PopP2 delivery during natural infection with *R. solanacearum* GM1000. PopP2-GFP$_{11}$ was cloned into the integrative plasmid pRCT under its native promoter and transformed into *R. solanacearum* GM1000 ∆*popP2* (Monteiro et al., 2012). In order to determine if PopP2-GFP$_{11}$ was functional when delivered from *R. solanacearum*, susceptible Arabidopsis Col-0 and resistant Ws-0 plants were root-inoculated (Figure 6A). Compared to the GM1000 ∆*popP2*+*popP2*, ∆*popP2* complemented with PopP2-GFP$_{11}$ had similar levels of disease development in Col-0 and the Col-0 GFP$_{1-10}$ transgenic lines (Figure 6A). Similarly, both strains were recognized in the Ws-0 ecotype. Bacterial virulence of PopP2-containing strains was recovered in the absence of NLR recognition in *rrs1*-1 and *rps4*-21/*rrs1*-1 mutant lines (Figure 6A). PopP2 is a member of the YopJ-like family of acetyltransferases and the PopP2-C321A catalytic mutation abolishes effector recognition in Ws-0 (Tasset et al., 2010). The *R. solanacearum* ∆*popP2*+*popP2*-C321A catalytic mutant had a slight reduction in disease severity in susceptible Col-0 and Col-0 35S:GFP$_{1-10}$ lines compared to PopP2 and PopP2-GFP$_{11}$, using a previously described disease scale (Figure 6A) (Tasset et al., 2010). These results demonstrate that the PopP2-GFP$_{11}$ is functionally similar to the wild-type PopP2 in both susceptible and resistant Arabidopsis ecotypes.
To investigate delivery of PopP2-GFP<sub>11</sub>, four-week-old Col-0 $GFP_{1-10}$ plants were root-inoculated with \textit{R. solanacearum} GMI1000 $\Delta popP2+popP2-GFP_{11}$ as previously described (Deslandes et al., 2003) and confocal micrographs were taken 7 days post-inoculation. \textit{R. solanacearum} PopP2-GFP<sub>11</sub> was observed accumulating in nuclei of cells surrounding the sites of lateral root emergence (Figure 6B-D). Roots inoculated with \textit{R. solanacearum} GMI1000 $\Delta popP2+popP2$ did not show fluorescence accumulation in the nuclei (Figure 6E-F).

Interestingly, PopP2-GFP<sub>11</sub> nuclear accumulation was also observed in cells surrounding vasculature in the shoot petiole (Figure 6G). These results demonstrate the functionality and translatability of the GFP strand system for detecting effector delivery across pathogens colonizing diverse host tissues. Effectors delivered to the cytoplasm, plasma membrane, and nucleus can be visualized with the 35S:$GFP_{1-10}$ system. However, effector visualization is dependent on the subcellular distribution of $GFP_{1-10}$ and it is likely that specific organelle-targeted variants of $GFP_{1-10}$ will be necessary to visualize effector in other cellular organelles.

**DISCUSSION**

Over 30 effectors are delivered by \textit{P. syringae} DC3000 and over 72 by \textit{R. solanacearum} GMI1000 (Alfano and Collmer, 2004; Coll and Valls, 2013). Pathogen effectors are virulence factors that suppress diverse aspects of plant immunity and thus can be excellent cellular probes for investigating plant innate immune responses (Toruno et al., 2016). Here, we have developed the GFP strand system to facilitate investigation of effector delivery and function during natural infection. We have demonstrated that four effector-GFP<sub>11</sub> fusions are functional, delivered by the TTSS, and can be visualized \textit{in planta} using the GFP strand system. Thus, the GFP strand system provides a powerful tool to examine effector biology, including temporal and spatial differences in effector delivery.

\textit{P. syringae} is a hemibiotrophic pathogen and it is currently unknown how the switch from biotrophy to necrotrophy is regulated \textit{in planta} (Xin and He, 2013). Filamentous pathogens demonstrate spatial and temporal regulation of effector expression (Wang et al., 2011; Kleemann et al., 2012). Effector transcription is temporally regulated in filamentous pathogens corresponding to the switch between biotrophic and necrotrophic life stages. In filamentous pathogens, cell death-suppressing effectors are expressed early and cell death-promoting effectors are expressed later during infection (Wang et al., 2011; Kleemann et al., 2012; Jupe et
P. syringae effectors share a common hrp box in their promoter sequences which is recognized by the alternative sigma factor HrpL (Xin and He, 2013). The presence of a conserved hrp box indicates that the transcriptional regulation of individual bacterial effectors may not differ. Using the GFP strand system, we detected differences in the quantity of *Pseudomonas* effector delivery over time. For example, AvrB-GFP$_{11}$ was most easily detected in *planta*. Robust AvrB delivery is consistent with the robust activation of RPM1-mediated resistance. RPM1 can trigger a macroscopic HR at 5-6 hpi, compared to other NLRs which trigger an HR around 10-12 hpi (Boyes et al., 1998). The differences we detected in effector abundance and number of delivery events per cell may reflect kinetics of effector maturation or stability, or a difference in substrate preference of the TTSS for different effectors. Additionally, variation in plasmid copy number or replication may have contributed to observed effector delivery differences, although all three *Pseudomonas* effectors were expressed from the same freely replicating plasmid, pBBR1, which should limit such variation. Future research will focus on investigating effector delivery after integrating the GFP$_{11}$ tag into endogenous sites at the C-termini of effectors in the genome. Our data indicate that variation in effector delivery may exist for *P. syringae* and paves the way for future detailed investigations into the hierarchy of effector delivery using the GFP strand system.

Effector proteins play an important role in epiphytic leaf colonization. Previous studies using *P. syringae* pv. *syringae* B728a detected bacterial effector expression at 24 and 48hpi on the leaf surface, and bacterial strains with mutations in the TTSS exhibited reduced epiphytic growth (Lee et al., 2012). In a natural infection, *P. syringae* initially colonizes the leaf surface, with aggregates forming at cell-cell junctions between pavement cells (Monier and Lindow, 2003b; Lee et al., 2012). We observed effector delivery at the pavement cell junctions (Figure 4B), supporting cell junctions as important environmental niches for *P. syringae* colonization and initiation points for effector dissemination. Effector delivery into pavement cells occurred after syringe or surface inoculation indicating *P. syringae* can directly deliver diverse effectors into pavement cells on the cell surface or once inside the leaf from the apoplast. Pavement cells have been shown to actively sense pathogen associated microbial patterns (PAMPs), triggering immune responses such as actin filament rearrangements required for delivery of antimicrobial compounds to infection sites, callose deposition or mobilization of immune response machinery via the secretory pathway (Henty-Ridilla et al., 2013). Likewise, *Pseudomonas* effectors HopW1
and HopG1 have been shown to disrupt actin dynamics to suppress these host cell immune
responses (Kang et al., 2014; Shimono et al., 2016). Thus, targeting effector delivery into
pavement cells may be an attempt to inhibit or delay plant perception.

Stomatal pores on the leaf surface serve as ports of entry into the leaf interior for multiple
pathogens, including *P. syringae* (McLachlan et al., 2014). Several effectors (AvrB, HopX1,
HopZ1a, HopF2) have been shown to interfere with PAMP-induced stomatal closure and
promote pathogen entry to the leaf apoplast (Jiang et al., 2013; Gimenez-Ibanez et al., 2014;
Hurley et al., 2014; Zhou et al., 2015). Here, we demonstrated that AvrB and AvrPtoB can be
directly delivered into guard cells, which flank stomatal pores, upon surface inoculation using
the GFP strand system. Thus, guard cells can be directly targeted by pathogen effectors to
promote colonization. Consistent with this finding, AvrB has been demonstrated to promote
bacterial growth and stomatal opening upon surface inoculation (Zhou et al., 2015). Collectively,
these data indicate that effectors can be directly delivered into cells of the leaf surface in order to
enhance entry into the leaf interior and suppress defense responses.

Relatively little is known about the delivery of effectors by vascular pathogens such as *R.
solanacearum*. Xylem vessels are non-living water conduits within the plant, and are thus poor
reservoirs of nutrients to support a pathogenic microbial population (Yadeta and BP, 2013).
Effectors from xylem-limited pathogens are thought to be delivered into surrounding live tissues.
Multiple NLR immune receptors have been identified recognizing effectors from xylem-
colonizing pathogens, including ZAR1 that recognizes AvrAC from *Xanthomonas campestris*,
and the RRS1-R/RPS4 pair recognizing PopP2 from *R. solanacearum* (Le Roux et al., 2015;
Sarris et al., 2015; Wang et al., 2015). Furthermore, the TTSS is absolutely required for ability of
*R. solanacearum* to cause bacterial wilt disease, highlighting the importance of effector delivery
(Schell, 2000).

We were able to visualize the *R. solanacearum* effector PopP2-GFP\textsuperscript{11} at lateral root sites,
suggesting effector delivery is important for early colonization. *R. solanacearum* invades plant
roots through wounds or cracks, primarily those caused by lateral root emergence (Vasse et al.,
1995). Effector delivery at these sites of invasion could be important to suppress early defense
responses until *R. solanacearum* can gain entry into the xylem vessels. Once established in the
xylem vessels, the bacteria are able to enter the intercellular spaces of the parenchyma cells in
the cortex and pith in various areas of the plant (Deslandes et al., 1998). Consistent with this, we
were able to detect PopP2-GFP\textsubscript{11} delivery and nuclear localization in cells surrounding vasculature. Delivery of effectors from the xylem to surrounding live tissue cells may also metabolically reprogram host cells to support pathogen proliferation.

The GFP strand system is versatile and we have demonstrated its capability to enable visualization of effector delivery in a foliar, epiphytic bacterial pathogen in addition to a root-associated, xylem-colonizing vascular pathogen. This system could be used to investigate effector delivery across kingdoms, and may work well for fungal, oomycete and viral protein delivery. The short GFP strand 11 did not interfere with effector function, as opposed to a full-length fluorophore which would not be delivered by the TTSS and can also interfere with effector function \textit{in planta} (Akeda and Galan, 2005; Van Engelenburg and Palmer, 2010; Radics et al., 2014). Natural infection allows visualization of effector localization within host cells. For example, we observed discrete fluorescent puncta or short fluorescent stretches at the cell periphery for AvrB-GFP\textsubscript{11} and AvrPto-GFP\textsubscript{11} in inoculated leaves, indicating effectors are delivered in non-homogenous microenvironments within a cell (Figures 4, 5, Supplemental Figures 3, 4). This is in contrast to robust fluorescence observed around the entire cell periphery in our transient overexpression validation assays for the GFP strand system in \textit{N. benthamiana} (Figure 2). Creation of microenvironments within a host cell by delivering effectors into discrete subcellular locations may play a key role in effector function during natural infection. Taken together, the GFP strand system provides effector biologists in with a valuable tool to advance the understanding plant-pathogen interactions.

\section*{METHODS}

\textbf{Plant Materials and Growth Conditions}

\textit{Arabidopsis thaliana} plants were grown in a controlled environment chamber at 23°C, 75% relative humidity, and a 10h/14h light/dark photoperiod with light intensity of 100 \textmu E.m\textsuperscript{-2}.s\textsuperscript{-1} using T12 high output bulbs. The \texttt{rps2-101c/rpm1-3} and \texttt{rps2-101c/rpm1-3/rin4} genotypes were previously described (Mindrinos et al., 1994; Boyes et al., 1998; Mackey et al., 2002). Line \texttt{r2} refers to \texttt{rps2-101c}, \texttt{r1} refers to \texttt{rpm1-3}, and \texttt{r4} refers to \texttt{rin4} (Mindrinos et al., 1994; Boyes et al., 1998). Seedlings used in detecting GFP\textsubscript{1-10} expression in roots vs. shoots were grown on MS plates for two weeks before harvesting. \textit{Nicotiana benthamiana} plants were grown in a controlled environmental chamber at 25°C, 85% relative humidity, 16/8-hr light/dark photoperiod
with light intensity of $180\mu E.m^{-2}.S^{-1}$. *Solanum lycopersicum* cv. Rio-Grande (RG) 76R lines [RG (Pto/Pto, Prf/Prf), RG prf3 (Pto/Pto, prf3/prf3)] were grown as previously described (Thapa et al., 2015).

**Molecular Cloning**

All primers used for cloning are described in Supplemental Table 1. The cell-specific promoters pGC1 ((Yang et al., 2008), 1730bp), pCER6 ((Ranjan et al., 2011), 1230bp) and pCAB3 ((Ranjan et al., 2011), 1550bp) were PCR amplified and cloned into pENTR (Invitrogen). *RIN4*’s genomic DNA with an N-terminal fusion to the T7 epitope was cloned into pENTR as previously described (Lee et al., 2015). Cell-specific promoter T7-gRIN4 constructs were moved into the binary vector pGWB1 using gateway technology (Invitrogen, (Nakagawa et al., 2007)). The Arabidopsis *rps2-101c/rin4* mutant was used as the background genotype for all cell-specific promoter transformations. All transgenic plants were generated using the floral-dip method and homozygous T3 lines were used for all assays (Clough and Bent, 1998). For effector delivery assays, Arabidopsis Col-0 and *rpm1-3/rps2-101C (r1r2)* backgrounds were transformed with pZP222 carrying *35S:GFP*$_{1-10}$ by floral-dip method (Clough and Bent, 1998) to generate transgenic plants with constitutive expression of GFP strands 1-10.

Bacterial effectors with C-terminal fusions to GFP strand 11 (GFP$_{11}$) were cloned into binary vectors for expression in *N. benthamiana* as well as broad host range vectors for expression in *Pseudomonas syringae* pv. *tomato* (Pst) strain DC3000. In-fusion cloning (Clontech) was used to seamlessly clone effector-GFP$_{11}$ constructs into pGWB514 (Nakagawa et al., 2007), followed by electroporation into *Agrobacterium tumefaciens* strain GV3101 for transient co-expression with *35S:GFP*$_{1-10}$. The broad host range vector pBBR1 MCS5 (Kovach et al., 1995) was linearized with XhoI and the gateway cassette B with C-terminal GFP$_{11}$ was ligated back in, creating a new vector named pBBR1$_{GW}$-GFP$_{11}$. The *avrPtoB* promoter and coding sequence was PCR amplified from *Pst*, cloned into pENTR, and moved into pBBR1$_{GW}$-GFP$_{11}$. In-fusion cloning (Clontech) was used to seamlessly clone *npro:avrB-GFP*$_{11}$ and *npro:avrPto-GFP*$_{11}$ into pBBR1-MCS5 without the gateway footprint (*npro:avrB* in pBBR1$_{GW}$-GFP$_{11}$ was non-functional *in planta*). The pBBR1 *avrPto-GFP*$_{11}$ plasmid and pBBR1$_{GW}$ *avrPtoB-GFP*$_{11}$ plasmid were conjugated into Pto DC3000 ΔavrPto/ΔavrPtoB using tri-parental mating (Lin and Martin, 2005). pBBR1$_{GW}$ *avrPtoB-GFP*$_{11}$ was electroporated into *Pst* DC3000
ΔhrcC as a negative control for type three secretion of GFP11 tagged effector. The avrB promoter and coding sequence was PCR amplified from a previously described pENTR plasmid (Lee et al., 2015). The pBBR1 avrB-GFP11 plasmid was electroporated into Pst DC3000. Native promoters for each effector were defined as follows: 85bp upstream ATG for AvrPtoB, 256bp upstream ATG for AvrB and 116bp upstream of ATG for AvrPto. Promoters were designed to include Hrp box and intergenic regions.

The PopP2-GFP11 sequence was generated by two-step PCR using PrimeStar HS DNA polymerase from Takara Bio Inc. (Otsu, Japan). Primers used are listed in Supplementary Table 1: fragment 1 was generated using primers PopP2-Fw and PopP2-GFP11-rev, fragment 2 used primers PopP2-GFP11-Fw and GFP11-Rev. PCR fragment 3, generated by mixing fragments 1 and 2 in presence of primers AttB1-PopP2 and AttB2-GFP11 was recombined into pDONR207 (Invitrogen) to generate the pENTR-PopP2-GFP11 entry clone. The PopP2-GFP11 insert was recombined into the pRCT-GWY destination vector allowing the expression of PopP2 under the control of its native promoter (383 pb upstream ATG). Integrative pRCT-PopP2-GFP11 plasmid allowing expression of the PopP2-GFP11 coding sequence under the control of native popP2 promoter was introduced in Ralstonia solanacearum GMI1000 ΔpopP2 strain by natural transformation.

Immunoblotting

SDS-PAGE and subsequent immunoblotting were performed according to standard procedures (Harlow and Lane, 1988). RIN4 immunoblots were performed with Anti-RIN4 rabbit polyclonal antibody at a concentration of 1:3,000 (Lee et al., 2015). GFP immunoblots were performed with Anti-GFP (ab290, Abcam) rabbit polyclonal antibody at a concentration of 1:8,000. Secondary goat anti-rabbit IgG-HRP conjugate (Biorad) was used at a concentration of 1:3,000 for detection via enhanced chemiluminescence (Pierce).

Pathogen Assays

Microscopic cell death assays were performed 12 hpi with Pst DC3000 (avrB). Four-week-old Col-0, pGC1 and pCER6 promoter lines were dip inoculated with 1×10⁹ CFU/mL, whereas pCAB3 lines were syringe inoculated with 5×10⁵ CFU/ml Pst DC3000 (avrB). Dead cells were visualized using trypan blue staining (9.3mL Phenol (liquid), 10mL Lactic Acid,
10mL Glycerol, 10mL water, 10mg trypan blue). Leaves were covered with trypan blue stain (Alfa Aesar) and incubated in a boiling water bath for 5-10min. Samples were allowed to cool at room temp for 45-60 min before removing the trypan blue stain and washing three times with water to remove excess stain. Tissue was cleared in chloral hydrate overnight (2.5g chloral hydrate/mL of water), then transferred to 60% glycerol for storage and microscopy.

For macroscopic cell death assays, Pst DC3000 carrying empty pBBR1 and Pst DC3000 (avrB-GFP in pBBR1) were syringe infiltrated into four-week-old Arabidopsis Col-0 leaves at a concentration of 4×10^7 CFU/mL. After infiltration, plants were placed under a light bank and macroscopic cell death (HR) was observed using trypan blue staining at 12hpi as described above. For the ion leakage assay, leaf disks of infiltrated leaves were harvested with a cork borer #4 and incubated in water for 30 min. Water was replaced and conductivity was measured 5 h later using an Orion 3 Star conductivity meter (Thermo Electron Corporation, Beverly, MA).

Bacterial growth assays in Arabidopsis were performed using syringe inoculation, whereby four-week-old Col-0 plants were syringe infiltrated with 2×10^5 CFU/ml bacteria in 10 mM MgCl_2 as described by Kim and colleagues (Kim et al., 2005). Experiments were repeated at least twice, with a minimum of six biological replicates (six individual plants) per time point.

Bacterial growth assays were performed on three-week-old tomato plants. The S. lycopersicum genotypes Rio Grande 76R (Pto/Pto, Prf/Prf), RG prf3 (Pto/Pto, prf3/prf3) were dip inoculated with 5×10^7 CFU/ml bacteria in 10mM MgCl_2 with 0.01% Silwet L-77 as previously described (Thapa et al., 2015). For inoculations with R. solanacerarum GMI1000 ∆popP2 strains, four week-old plants were root inoculated as described before and disease symptoms were scored 7-8 dpi (Deslandes et al., 1998).

Microscopy

All microscopy for single cell HR trypan blue staining was performed using a Leica DM 5000B epifluorescent microscope under brightfield conditions. All confocal microscopy with P. syringae infections were performed using a Zeiss LSM710 confocal microscope equipped with a LDC-apochromat 40×/1.1W Korr M27 water-immersion objective (NA 1.1). GFP was excited at 488nm, emission gathered at 500-550nm. Chloroplast autofluorescence emission was gathered at 650-750nm. Imaging of transient co-expression of effector-GFP and GFP in Nicotiana benthamiana was performed at 36 hpi. Agrobacterium tumefaciens strains (described above)
carrying 35S::GFP<sub>1-10</sub> or avrPto-GFP<sub>11</sub>, avrPtoB-GFP<sub>11</sub> and avrB-GFP<sub>11</sub> were induced with 100 µM acetosyringone and co-infiltrated at an OD600 = 0.6 into N. benthamiana. Plasmolysis was performed using 1M NaCl.

For effector delivery experiments where surface inoculation was used, two-week-old seedlings of Arabidopsis Col-0, Col-0 GFP<sub>1-10</sub> or rlr2 GFP<sub>1-10</sub> were painted with 1×10<sup>9</sup> CFU/mL bacteria re-suspended in 10 mM MgCl<sub>2</sub>. Effector delivery assays using syringe inoculation were performed on four-week-old plants using bacterial suspensions of 2×10<sup>6</sup> CFU/mL. The following bacterial strains were used: Pst DC3000, Pst DC3000 (∆avrPto/∆avrPtoB), Pst DC3000 (∆avrPto/∆avrPtoB +avrPtoB-GFP<sub>11</sub>), Pst DC3000 (∆avrPto/∆avrPtoB +avrPtoB-GFP<sub>11</sub>), Pst DC3000 (+avrB-GFP<sub>11</sub>), Pst DC3000 ΔhrcC (+avrPto-GFP<sub>11</sub>), and Pst DC3000 ΔhrcC (+avrPto-GFP<sub>11</sub>). Plant leaves were scanned for the presence of GFP fluorescence to detect effector delivery. Confocal micrographs were collected 24hpi and 48hpi. Micrographs taken from four independent plants per treatment were analyzed for delivery into specific cells and discrete delivery events ranging from puncta to larger plasma membrane or cytosolic sheets. A total of 107 micrographs were analyzed, with 63 from the three combined effector treatments. Of the 63 micrographs, 26 were of Pst DC3000 (+avrB-GFP<sub>11</sub>), 27 of Pst DC3000 (∆avrPto/∆avrPtoB +avrPtoB-GFP<sub>11</sub>) and 10 were from Pst DC3000 (∆avrPto/∆avrPtoB +avrPtoB-GFP<sub>11</sub>) inoculated leaves. Pst DC3000 (∆avrPto/∆avrPtoB +avrPtoB-GFP<sub>11</sub>) had fewer total micrographs because it was only tested using syringe inoculation.

Detection of PopP2-GFP<sub>11</sub> in root or petiole cells was performed seven days after inoculation of four-week-old Arabidopsis 35S::GFP<sub>1-10</sub> plants with R. solanacearum GMI1000 ∆popP2 expressing PopP2-GFP<sub>11</sub> or PopP2 (negative control). Entire roots were washed with distilled water, mounted on a glass slide and covered with a coverslip. Plant petioles and roots were scanned for the presence of GFP fluorescence to detect effector delivery. Images were acquired with a confocal microscope (Leica SP2 AOBS, Mannheim) using a 40x water immersion lens (N.A. 0.8). For excitation, a 405 nm ray line of a diode laser and the 488 nm ray line of an argon laser were used and the emitted fluorescence collected in the blue range between 410 nm and 470 nm and in the green range between 500 nm and 530 nm. Maximal projections of 20-25 confocal planes were acquired in z-dimension. From the z-stack of confocal images, the maximal projections of the two color channels were then computed and overlaid.
Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: GC1 (At1g22690), CER6 (At1g68530), CAB3 (At1g29910), RIN4 (At3g25070), RPM1 (At3g07040), RPS2 (At4g26090), PTO (101268866), PRF (101263413), AvrPto (1185679), AvrPtoB (1184744), AvrB (3366713), and PopP2 (16105295).

Supplemental Data

Supplemental Figure 1. RIN4 expression in Arabidopsis tissue-specific lines (Supports Figure 1).

Supplemental Figure 2. GFP₁₋₁₀ is detected by immunoblot using anti-GFP after expression in Arabidopsis and Nicotiana benthamiana (Supports Figure 2).

Supplemental Figure 3. Visualization of Pseudomonas syringae DC3000 effector delivery in Arabidopsis 24 hours post-syringe inoculation (Supports Figure 4).

Supplemental Figure 4. Visualization of Pseudomonas syringae DC3000 effector delivery in Arabidopsis 24 hours post-surface inoculation (Supports Figure 5).

Supplemental Figure 5. Bacterial effectors are delivered at multiple foci per cell (Supports Figures 4 and 5).

Supplemental Table 1. Primers used in experiments listed 5'-3'.

Supplemental Table 2. Analysis of variance of conductivity in Arabidopsis cell-specific promoter lines (Supports Figure 1E).

Supplemental Table 3. Analysis of variance of bacterial growth in tomato (Supports Figure 3B).

Supplemental Table 4. Analysis of variance of bacterial growth in Arabidopsis (Supports Figure 3E).

AUTHOR CONTRIBUTIONS

EH, TYT, LD, and GC designed experiments. EH, TYT, AJ performed experiments. GC and LD supervised the study. EH, TYT, and GC wrote the manuscript with input from AJ and LD.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1. Cell-specific immune responses in plant leaves.**

A) All leaf cell types are able to elicit cell death upon effector recognition. Four-week-old Arabidopsis Col-0 plants were dip inoculated with *Pseudomonas syringae* pv. *tomato* DC3000 expressing empty vector (EV) or the bacterial effector *avrB*. Twelve hours post inoculation (hpi), microscopic cell death was visualized using trypan blue staining. Circles indicate representative trypan blue staining in leaf mesophyll, epidermal, and guard cells elicited by recognition of *P. syringae avrB* but not by inoculation with *P. syringae* EV.

B) Complementation of specific leaf cell types with the immune regulator *RIN4* to assess cell-specific immune responses. Transgenic lines were generated expressing *RIN4* under cell specific promoters in the rps2-101c/rin4 knockout genetic background. $r2 = rps2-101c$, $r4 = rin4$.

C) Four-week-old Arabidopsis plants expressing *RIN4* in a cell specific manner were dip inoculated with *P. syringae* (EV) and (*avrB*). Twelve hpi, trypan blue staining was used to visualize cell death in $r2r4$, $r2$, and cell specific promoter lines. All cell types were capable of eliciting cell death after inoculation with *P. syringae* (*avrB*). Bar = 100µm.

D) Four-week-old Arabidopsis plants of the indicated genotypes were subjected to half-leaf syringe infiltration with *P. syringae* DC3000 (*avrB*) or (EV). Eight plants per genotype were infiltrated with each bacterial strain and macroscopic cell death was recorded 16hpi.

E) Four-week-old Arabidopsis plants of the indicated genotypes were syringe infiltrated as described in (D) and ion leakage (µS/cm) was measured with a conductivity meter 5 hpi. Bars represent means, $n = 3$ individual plants, error bars represent standard deviation. Statistical differences were conducted by ANOVA followed by LSD mean separation, alpha = 0.05. Experiments were repeated three times with similar results.

**Figure 2. The GFP strand system is able to detect effector localization in Nicotiana benthamiana.**

A) Overview of the GFP strand system for effector detection *in planta*. Superfold GFP is a beta barrel protein consisting of eleven strands. GFP can be split into strands 1-10, which are transformed into plant cells and constitutively expressed. GFP strand 11 is fused to the C-terminus of a bacterial effector via a flexible linker. Here, the AvrB effector is C-terminally tagged with GFP$_{11}$. When AvrB-GFP$_{11}$ is expressed *in planta*, GFP$_{1-10}$ and AvrB-GFP$_{11}$ spontaneously recombine to give a functional fluorescent GFP molecule. A protein model of
superfolder GFP and AvrB was generated from existing crystal structures (PDB: 2B3P and 1NH1, respectively). **B) Validation of the GFP strand system in* N. benthamiana *using Agrobacterium-mediated transient expression of GFP**1-10** and the *P. syringae* effectors AvrB-GFP**11**, AvrPto-GFP**11** and AvrPtoB-GFP**11**. GFP fluorescence was visualized by confocal microscopy 48 h post-infiltration. Right panel: plant leaves were subjected to plasmolysis with 1 M NaCl for 5 minutes. Plasmolysis demonstrates that the subcellular localization of AvrB and AvrPto effectors are at the plasma membrane and the AvrPtoB effector is cytosolic. GFP**1-10** alone was used as a negative control and full-length GFP was used as a positive control for plasmolysis. Bar = 20 µm.

**Figure 3. Effectors fused to GFP**11** retain their biological activity.** Functional validation of strand 11 tagged effectors delivered by *Pseudomonas syringae pv. tomato* DC3000 in tomato and Arabidopsis. **A) Disease symptoms on indicated tomato genotypes four days post-inoculation.** Four-week-old tomato plants were dip inoculated with the indicated strains. Effectors were expressed from their native promoters in the pBBR1 broad host range vector. Tomato Rio Grande (RG) 76R recognizes the AvrPto and AvrPtoB effectors and is resistant to *Pst* DC3000, but not DC3000 ΔavrPtoΔavrPtoB (ΔΔ). The tomato line RG 76R prf3 is unable to recognize the AvrPto and AvrPtoB effectors, and is susceptible to *Pst* DC3000. RG 76R prf3 exhibits characteristic necrotic lesions when inoculated with DC3000 (+avrPto/+avrPtoB), DC3000 ΔΔ (+avrPto-GFP**11**) and DC3000 ΔΔ (avrPto-GFP**11**). **B) Quantification of bacterial growth in tomato.** Bacterial inoculations were conducted as described in (A) and bacterial titeres were determined four days post-inoculation. RG 76R is able to recognize DC3000 ΔavrPtoΔavrPtoB is complemented with either AvrPto-GFP**11** or AvrPtoB-GFP**11**. The susceptible cultivar 76R prf3 cannot mount a response to AvrPto-GFP**11** or AvrPtoB-GFP**11**. Bacterial titeres are represented as Log colony forming units per cm² (Log CFU/cm²) of leaf tissue. Bars represent means, n =6 individual plants, error bars indicate standard deviation. Statistical differences were conducted with by ANOVA followed by LSD mean separation, alpha = 0.05. The experiment was repeated 3 times, with similar results. **C) The AvrB effector is recognized by the RPM1 immune receptor in Arabidopsis Col-0.** DC3000 carrying *avrB-GFP**11** or *avrB-3xFLAG* is recognized in Arabidopsis Col-0 and elicits cell death, as visualized by trypan blue staining. The DC3000 empty vector (EV) control does not elicit cell death. Four-week-old plants were syringe
infiltrated in one leaf half with the indicated bacterial genotypes and leaves harvested for trypan blue staining 12 h post-inoculation. D) Disease symptoms of Arabidopsis Col-0 four days post-syringe infiltration with DC3000 (EV), (avrB-GFP), or (avrB-3XFLAG). E) Quantification of bacterial growth from leaves infiltrated as described in (D). Bacterial titers demonstrate significantly reduced growth of DC3000 (avrB-GFP) compared to DC3000 (EV) on Col-0 but not rpm1-3. Bars represent means, n = 4 individual plants at day 0 and n = 8 individual plants at day 4. Error bars indicate standard deviation from the mean. Statistical differences were conducted with by ANOVA followed by LSD mean separation, alpha = 0.05. The experiment was repeated twice with similar results.

Figure 4. The GFP strand system enables visualization of type III delivered effectors into diverse cell types. Four-week-old Arabidopsis plants expressing 35S:GFP were syringe infiltrated with Pseudomonas syringae pv. tomato DC3000 expressing GFP tagged effectors and effector delivery was visualized by confocal microscopy at 24h and 48h post-inoculation. A) Effector delivery into mesophyll cells 48 hours post-syringe inoculation. AvrB-GFP was visualized after inoculation onto rpm1-3/rps2-101c (r1r2) plants expressing GFP. AvrPto-GFP and AvrPtoB-GFP were visualized after inoculation onto Col-0 expressing GFP. Effectors were expressed from their native promoters in the broad host range vector pBBR1. EV = pBBR1 empty vector, ∆∆= DC3000 ∆avrPto/∆avrPtoB. Bar = 10 µm. B) Effector delivery into the epidermal pavement cells of Col-0 and r1r2 expressing GFP 48h post-syringe inoculation. Inset and circle highlight plasma membrane GFP localization in two adjacent epidermal cells for the AvrB-GFP effector. Bar = 20 µm. C) Cross sectional views of syringe inoculated leaves indicate effector delivery predominately localizes to the epidermal cell layers. Each effector cross section represents the time of maximal delivery for that effector, AvrB-GFP cross section is from 24 hpi and AvrPto-GFP and AvrPtoB-GFP cross sections are from 48 hpi. Cross sections were generated from 3D projections of Z-stacks ranging from 15-27µm in thickness. Magenta indicates chlorophyll autofluorescence. Bar = 20 µm. D) Graph indicates the overall temporal differences in delivery of three effectors across cell types in both surface and syringe inoculations, n = 4 individual plants per treatment. Numbers reflect the sum of all positive cells over four individual plant replicates at 24 and 48h post-inoculation after both syringe and surface inoculation.
Figure 5. Visualization of DC3000 type III delivered effectors in Arabidopsis after surface inoculation. Two-week-old Arabidopsis plants expressing 35S:GFP\textsubscript{1-10} were painted with *Pseudomonas syringae* pv. *tomato* expressing empty vector (EV), AvrPto-GFP\textsubscript{11} and AvrB-GFP\textsubscript{11}. Effector delivery was visualized by confocal microscopy 24h and 48h post-inoculation. AvrB-GFP\textsubscript{11} was visualized after inoculation onto *rpm1-3/rps2-101c* (*r1r2*) plants expressing GFP\textsubscript{1-10}. AvrPto-GFP\textsubscript{11} was visualized after inoculation onto Col-0 expressing GFP\textsubscript{1-10}. Effectors were expressed from their native promoters in the broad host range vector pBBR1. A) Effector delivery into epidermal pavement cells 48h post-surface inoculation. EV = pBBR1 empty vector, \( \Delta \Delta = \) DC3000 \( \Delta \)avrPto/\( \Delta \)avrPtoB. Bar = 20 \( \mu \)m. B) Effector delivery into guard cells 48 h post-surface inoculation. Both AvrB-GFP\textsubscript{11} and AvrPto-GFP\textsubscript{11} were delivered into epidermal guard cells. Bar = 5 \( \mu \)m. C) Temporal distribution of effector delivery into mesophyll, epidermal pavement and guard cells differs for each effector. Graph indicates the total number of cells where effector delivery was detected after surface inoculation, \( n = 4 \) individual plants per treatment and time point. Numbers reflect the sum of all positive cells at 24 or 48 h post-inoculation. D) Graph indicates cell-type specific distribution of effector delivery, contrasting syringe and surface inoculations \( n = 4 \) individual plants per treatment and time point. Numbers reflect the sum of all positive cells at both 24 and 48 h post-inoculation.

Figure 6. The GFP strand system enables visualization of the *Ralstonia solanacearum* PopP2 effector in the nuclei of root and petiole cells. A) Roots of four-week-old plants were inoculated with the indicated *R. solanacearum* genotypes and disease symptoms were scored eight days post-inoculation (dpi). The inoculated plant genotypes included: Col-0 (does not recognize PopP2), transgenic Col-0 expressing GFP\textsubscript{1-10}, Ws-0 (recognizes PopP2), the *rrs1-1* mutant, and the *rrs4-21/rrs1-1* double mutant. Plants were inoculated with *R. solanacearum* GMI1000 \( \Delta \)popP2 expressing wild-type PopP2, PopP2-C321A or PopP2-GFP\textsubscript{11}. The disease index was scored based on the following scale: 0 = no wilting, 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100% of wilted leaves. Mean and SD values were calculated from scores of >7 plants. B-D) Confocal micrographs showing the presence of PopP2-GFP\textsubscript{11} effector in nuclei of root cells, seven dpi of four-week-old transgenic Col-0 35S:GFP\textsubscript{1-10} plants with *R. solanacearum* GMI1000
ΔpopP2 expressing PopP2-GFP<sub>11</sub>. E-F) Confocal micrographs of root cells of transgenic Col-0 35S:GFP<sub>1-10</sub> plants seven dpi with <i>R. solanacearum</i> GMI1000 ΔpopP2 expressing PopP2 (negative control). Note no nuclear signal is observed. B-C and E-F are an overlay of maximal projections from 20-25 confocal planes acquired in z dimension (z-stacks 4 µm in thickness). D is an overlay of a confocal plane. G) Confocal images of Col-0 35S:GFP<sub>1-10</sub> petioles inoculated with <i>R. solanacearum</i> GMI1000 ΔpopP2 expressing PopP2-GFP<sub>11</sub>. Scale bars = 30 µm.
Figure 1. Cell-specific immune responses in plant leaves. A) All leaf cell types are able to elicit cell death upon effector recognition. Four-week-old Arabidopsis Col-0 plants were dip inoculated with Pseudomonas syringae pv. tomato DC3000 expressing empty vector (EV) or the bacterial effector avrB. Twelve hours post inoculation (hpi), microscopic cell death was visualized using trypan blue staining. Circles indicate representative trypan blue staining in leaf mesophyll, epidermal, and guard cells elicited by recognition of P. syringae avrB but not by inoculation with P. syringae EV. B) Complementation of specific leaf cell types with the immune regulator RIN4 to assess cell-specific immune responses. Transgenic lines were generated expressing RIN4 under cell specific promoters in the rps2-101c/rin4 knockout genetic background. r2 = rps2-101c, r4 = rin4. C) Four-week-old Arabidopsis plants expressing RIN4 in a cell specific manner were dip inoculated with P. syringae (EV) and (avrB). Twelve hpi, trypan blue staining was used to visualize cell death in r2r4, r2, and cell specific promoter lines. All cell types were capable of eliciting cell death after inoculation with P. syringae (avrB). Bar = 100µm. D) Four-week-old Arabidopsis plants of the indicated genotypes were subjected to half-leaf syringe infiltration with P. syringae DC3000 (avrB) or (EV). Eight plants per genotype were infiltrated with each bacterial strain and macroscopic cell death was recorded 16hpi. E) Four-week-old Arabidopsis plants of the indicated genotypes were syringe infiltrated as described in (D) and ion leakage (µS/cm) was measured with a conductivity meter 5 hpi. Bars represent means, n = 3 individual plants, error bars represent standard deviation. Statistical differences were conducted by ANOVA followed by LSD mean separation, alpha = 0.05. Experiments were repeated three times with similar results.
Figure 2. The GFP strand system is able to detect effector localization in *Nicotiana benthamiana*. A) Overview of the GFP strand system for effector detection *in planta*. Superfold GFP is a beta barrel protein consisting of eleven strands. GFP can be split into strands 1-10, which are transformed into plant cells and constitutively expressed. GFP strand 11 is fused to the C-terminus of a bacterial effector via a flexible linker. Here, the AvrB effector is C-terminally tagged with GFP<sub>11</sub>. When AvrB-GFP<sub>11</sub> is expressed *in planta*, GFP<sub>1-10</sub> and AvrB-GFP<sub>11</sub> spontaneously recombine to give a functional fluorescent GFP molecule. A protein model of superfolder GFP and AvrB was generated from existing crystal structures (PDB: 2B3P and 1NH1, respectively). B) Validation of the GFP strand system in *N. benthamiana* using *Agrobacterium*-mediated transient expression of GFP<sub>1-10</sub> and the *P. syringae* effectors AvrB-GFP<sub>11</sub>, AvrPto-GFP<sub>11</sub>, and AvrPtoB-GFP<sub>11</sub>. GFP fluorescence was visualized by confocal microscopy 48 h post-infiltration. Right panel: plant leaves were subjected to plasmolysis with 1 M NaCl for 5 minutes. Plasmolysis demonstrates that the subcellular localization of AvrB and AvrPto effectors are at the plasma membrane and the AvrPtoB effector is cytosolic. GFP<sub>1-10</sub> alone was used as a negative control and full-length GFP was used as a positive control for plasmolysis. Bar = 20 μm.
Figure 3. Effectors fused to GFP11 retain their biological activity. Functional validation of strand 11 tagged effectors delivered by Pseudomonas syringae pv. tomato DC3000 in tomato and Arabidopsis. A) Disease symptoms on indicated tomato genotypes four days post-inoculation. Four-week-old tomato plants were dip inoculated with the indicated strains. Effectors were expressed from their native promoters in the pBBR1 broad host range vector. Tomato Rio Grande (RG) 76R recognizes the AvrPto and AvrPtoB effectors and is resistant to Pst DC3000, but not DC3000 ΔavrPtoΔavrPtoB (ΔΔ). The tomato line RG 76R prf3 is unable to recognize the AvrPto and AvrPtoB effectors, and is susceptible to Pst DC3000. RG 76R prf3 exhibits characteristic necrotic lesions when inoculated with DC3000 (+avrPto/+avrPtoB), DC3000 ΔΔ (+avrPto-GFP11) and DC3000 ΔΔ (+avrPto-GFP11). B) Quantification of bacterial growth in tomato. Bacterial inoculations were conducted as described in (A) and bacterial titers were determined four days post-inoculation. RG 76R is able to recognize DC3000 ΔavrPtoΔavrPtoB is complemented with either AvrPto-GFP11 or AvrPtoB-GFP11. The susceptible cultivar 76R prf3 cannot mount a response to AvrPto-GFP11, or AvrPtoB-GFP11. Bacterial titers are represented as Log colony forming units per cm² (Log CFU/cm²) of leaf tissue. Bars represent means, n = 6 individual plants, error bars indicate standard deviation. Statistical differences were conducted with by ANOVA followed by LSD mean separation, alpha = 0.05. The experiment was repeated 3 times, with similar results. C) The AvrB effector is recognized by the RPM1 immune receptor in Arabidopsis Col-0. DC3000 carrying avrB-GFP11, or avrB-3xFLAG is recognized in Arabidopsis Col-0 and elicits cell death, as visualized by trypan blue staining. The DC3000 empty vector (EV) control does not elicit cell death. Four-week-old plants were syringe infiltrated in one leaf half with the indicated bacterial genotypes and leaves harvested for trypan blue staining 12 h post-inoculation. D) Disease symptoms of Arabidopsis Col-0 four days post-syringe infiltration with DC3000 (EV), (avrB-GFP11), or (avrB-3xFLAG). E) Quantification of bacterial growth from leaves infiltrated as described in (D). Bacterial titers demonstrate significantly reduced growth of DC3000 (avrB-GFP11) compared to DC3000 (EV) on Col-0 but not rpm1-3. Bars represent means, n = 4 individual plants at day 0 and n = 8 individual plants at day 4. Error bars indicate standard deviation from the mean. Statistical differences were conducted with by ANOVA followed by LSD mean separation, alpha = 0.05. The experiment was repeated twice with similar results.
Figure 4. The GFP strand system enables visualization of type III delivered effectors into diverse cell types. Four-week-old Arabidopsis plants expressing 35S::GFP\textsubscript{11-10} were syringe infiltrated with Pseudomonas syringae pv. tomato DC3000 expressing GFP\textsubscript{11}, tagged effectors and effector delivery was visualized by confocal microscopy at 24h and 48h post-inoculation. A) Effector delivery into mesophyll cells 48 hours post-syringe inoculation. AvrB-GFP\textsubscript{11} was visualized after inoculation onto rpm1-3/rps2-101c (r1r2) plants expressing GFP\textsubscript{11-10}. AvrPto-GFP\textsubscript{11} and AvrPtoB-GFP\textsubscript{11} were visualized after inoculation onto Col-0 expressing GFP\textsubscript{11-10}. Effectors were expressed from their native promoters in the broad host range vector pBBR1. EV = pBBR1 empty vector. ΔΔ = DC3000 ΔavrPto/ΔavrPtoB. Bar = 10 μm. B) Effector delivery into the epidermal pavement cells of Col-0 and r1r2 expressing GFP\textsubscript{11-10} 48h post-syringe inoculation. Inset and circle highlight plasma membrane GFP localization in two adjacent epidermal cells for the AvrB-GFP\textsubscript{11} effector. Bar = 20 μm. C) Cross sectional views of syringe inoculated leaves indicate effector delivery predominately localizes to the epidermal cell layers. Each effector cross section represents the time of maximal delivery for that effector, AvrB-GFP\textsubscript{11} cross sections is from 24 hpi and AvrPto-GFP\textsubscript{11} and AvrPtoB-GFP\textsubscript{11} cross sections are from 48 hpi. Cross sections were generated from 3D projections of Z-stacks ranging from 15-27μm in thickness. Magenta indicates chlorophyll autofluorescence. Bar = 20 μm. D) Graph indicates the overall temporal differences in delivery of three effectors across cell types in both surface and syringe inoculations, n = 4 individual plants per treatment. Numbers reflect the sum of all positive cells over four individual plant replicates at 24 and 48h post-inoculation after both syringe and surface inoculation.
Figure 5. Visualization of DC3000 type III delivered effectors in Arabidopsis after surface inoculation.
Two-week-old Arabidopsis plants expressing 35S:GFP 1-10 were painted with Pseudomonas syringae pv. tomato expressing empty vector (EV), AvrPto-GFP 11, and AvrB-GFP 11. Effector delivery was visualized by confocal microscopy 24h and 48h post-inoculation. AvrB-GFP 11 was visualized after inoculation onto rpm1-3/rps2-101c (r1r2) plants expressing GFP 1-10. AvrPto-GFP 11 was visualized after inoculation onto Col-O expressing GFP 1-10. Effectors were expressed from their native promoters in the broad host range vector pBBR1. A) Effector delivery into epidermal pavement cells 48h post-surface inoculation. EV = pBBR1 empty vector, ΔΔ = DC3000 ΔavrPto/Δ avrPtoB. Bar = 20 μm. B) Effector delivery into guard cells 48 h post-surface inoculation. Both AvrB-GFP 11 and AvrPto-GFP 11 were delivered into epidermal guard cells. Bar = 5 μm. C) Temporal distribution of effector delivery into mesophyll, epidermal pavement and guard cells differs for each effector. Graph indicates the total number of cells where effector delivery was detected after surface inoculation, n = 4 individual plants per treatment and time point. Numbers reflect the sum of all positive cells at 24 or 48 h post-inoculation. D) Graph indicates cell-type specific distribution of effector delivery, contrasting syringe and surface inoculations n = 4 individual plants per treatment and time point. Numbers reflect the sum of all positive cells at both 24 and 48 h post-inoculation.
Figure 6. The GFP strand system enables visualization of the *Ralstonia solanacearum* PopP2 effector in the nuclei of root and petiole cells. A) Roots of four-week-old plants were inoculated with the indicated *R. solanacearum* genotypes and disease symptoms were scored eight days post-inoculation (dpi). The inoculated plant genotypes included: Col-0 (does not recognize PopP2), transgenic Col-0 expressing GFP$_{1-10}$, Ws-0 (recognizes PopP2), the *rrs1-1* mutant, and the *rps4-21/rrs1-1* double mutant. Plants were inoculated with *R. solanacearum* GMI1000 ΔpopP2 expressing wild-type PopP2, PopP2-C321A or PopP2-GFP$_{11}$. The disease index was scored based on the following scale: 0 = no wilting, 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100% of wilted leaves. Mean and SD values were calculated from scores of >7 plants. B-D) Confocal micrographs showing the presence of PopP2-GFP$_{11}$ effector in nuclei of root cells, seven dpi of four-week-old transgenic Col-0 35S:GFP$_{1-10}$ plants with *R. solanacearum* GMI1000 ΔpopP2 expressing PopP2-GFP$_{11}$. E-F) Confocal micrographs of root cells of transgenic Col-0 35S:GFP$_{1-10}$ plants seven dpi with *R. solanacearum* GMI1000 ΔpopP2 expressing PopP2 (negative control). Note no nuclear signal is observed. B-C and E-F are an overlay of maximal projections from 20-25 confocal planes acquired in z dimension (z-stacks 4 μm in thickness). D is an overlay of a confocal plane. G) Confocal images of Col-0 35S:GFP$_{1-10}$ petioles inoculated with *R. solanacearum* GMI1000 ΔpopP2 expressing PopP2-GFP$_{11}$. Scale bars = 30 μm.


