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A human pathogenic bacterium *Shigella* proliferates in the plant through the adoption of type III effectors for Shigellosis

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Abstract:

Human intestinal pathogenic bacteria has been reported to be transmitted by fresh vegitables. *Shigella*, which infects primates, is reportedly transmitted by fresh vegetables; however, its molecular interactions with plants have not been ellucidated. Here, we show that four *Shigella* strains, *S. boydii* (*S. b*), *S. sonnei* (*S. s*), *S. flexneri* 2a (*S. f* 2a), and *S. flexneri* 5a (*S. f* 5a), proliferated at different levels in *Arabidopsis thaliana*. Microscopic studies revealed that these bacteria were present inside leaves and damaged plant cells. GFP-labeled *S. b*, *S. f* 2a, and *S. f* 5a entered plants via guard cells, and *S. f* 2a infiltrated root tissues and colonized roots. Using mutants lacking type III secretion systems (T3SS), we found that T3SS of *Shigella* that regulate the pathogenesis of shigellosis in humans also play a central role in attachment and multiplication in *Arabidopsis.* Furthermore, the immunosuppressive activity of two T3S effectors, OspF and OspG, were needed for the proliferation of *Shigella* in *Arabidopsis*. Of note, delivery of OspF or OspG effectors inside of plant cells upon *Shigella* inoculation was confirmed by using a split GFP system, respectively. These findings demonstrate that the human pathogen *Shigella* can proliferate in plants by adoption of immunosuppressive machinery for its original host human.

Keywords: Alternative host, Arabidopsis plants, Enteropathogenic bacteria, PAMP-triggered immunity, Proliferation, *Shigella* spp., Split GFP, Trans-kingdom pathogenesis, Type III secretion system (T3SS), T3S effectors

Introduction

Human hosts are primarily infected by the ingestion of water or food contaminated with bacteria from feces (the fecal-oral route), including ingestion of undercooked contaminated meats and improperly washed contaminated vegetables (Mead et al., 1999; Weir, 2002; Gupta et al., 2004). Fresh fruits and vegetables, such as lettuce, tomatoes, and green peppers, are responsible for the widespread transmission of food-borne infections by *Salmonella* or *Shigella* (Guchi and Ashenafi, 2010; Semenov et al., 2010; Gu et al., 2013). These observations suggest that human pathogenic bacteria use plants as alternative hosts as a stable environmental niche. Several human pathogenic bacteria, including *Salmonella enterica* serovar Typhimurium, *Escherichia coli* O157:H7, and *Pseudomonas aeruginosa*, are known to use plants as alternative hosts (Plotnikova et al., 2000; Semenov et al., 2010). These bacteria can attach to the plant surface, and actively invade and proliferate in plant tissues (Cevallos-Cevallos et al., 2012; Martínez-Vaz et al., 2014). In particular, several enteropathogenic bacteria, including *E. coli* O157:H7 and *Salmonella*, spread within plants through vascular tissues after infection via contaminated water (Solomon et al., 2002).

Shigella is a human-adapted pathogen that infects the host via multiple transmission routes. It is a non-motile, rod-shaped, facultative intracellular and invasive pathogen, very closely related to *Escherichia coli*. Based on the carbohydrate composition of the O-antigen, i.e., the polysaccharide component of the lipopolysaccharide molecule that is the major bacterial surface antigen, *Shigella* is classified into four serogroups. These have been given species designations, namely, *S. dysenteriae* 1 (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D) (Lindberg et al., 1991; Schroeder and Hilbi, 2008). *Shigella* spp. are important epidemic pathogens and serious public health concern in developed and developing countries. About 164,300 deaths in all age groups, and 54,900 deaths in children younger than 5 years, were reported globally in 2015 (Mortality and Causes of Death, 2016). However, the actual number of infections might be higher because mild symptoms are not reported (Mortality and Causes of Death, 2016), suggesting that the microorganism may employ a variety of survival strategies not only for human intestinal infection but also for survival in a non-adapted host. Although *Shigella* contamination has

also been reported in plants (Naimi et al., 2003; Ohadi et al., 2013), it is not yet known whether the bacterium actively invades and/or proliferates inside the plant.

Unlike animals, plants have no adaptive immune system; instead, each cell possesses an innate immune system. The innate immune system in plants and animals recognizes and suppresses pathogens and has common features that are preserved throughout evolution. Plant pattern recognition receptors recognize conserved microbial or pathogen-associated molecular patterns; the pattern-triggered immunity (PTI) is activated via the mitogen-activated protein kinase (MAPK) cascades (Jones and Dangl, 2006). To suppress PTI, bacteria inject effector proteins into plant cells using type III secretion systems (T3SS). To counteract this PTI evasion response, the plant nucleotide binding-leucine rich repeat proteins recognize the pathogen effectors; effector-triggered immunity is then activated to accompany the hypersensitive response (Jones and Dangl, 2006). For human or animal intestinal bacteria to infect plants, the PTI must first be disabled. *S. enterica* serovar Typhimurium, similar to its activity in the mammalian host, uses T3SS to suppress plant immune responses (Schikora et al., 2011; Schikora et al., 2012). In particular, one of the T3S effector proteins of *S. enterica,* SpvC targets the MAPK signaling system as in animals to suppress the host PTI, when expressed in plants under the control of plant binary vector (Neumann et al., 2014).

Here, we examined the ability of four *Shigella* strains (*S. s* (Holt et al., 2012), *S. b* and *S. f* 2a (Wei et al., 2003), and *S. f* 5a (Onodera et al., 2012)) to proliferate in *Arabidopsis* plants. We found that the four strains invaded and proliferated differently in plant tissues. A Proliferation of mutants lacking the T3S effectors, i.e., noninvasive human strains, was reduced *in planta*. Reverse genetics and molecular biology experiments demonstrated that the immunosuppressive function of *Shigella* T3S effectors OspF and OspG was essential for *Shigella* proliferation in plants. Notably, we first observed the delivery of *Shigella* Type III effector proteins inside of plant cells by *Shigella* inoculation using the split GFP technology. These observations indicate that *Arabidopsis* may be useful as a model host for studying the pathogenesis of *Shigella*.

Materials and Methods

Plant materials and growth

Arabidopsis thaliana accession Columbia (*Col-0*) was used for *Shigella* infection. Briefly, *Arabidopsis* seeds were surface-sterilized in 70% (v/v) ethanol for 2 min followed by an incubation in 50% household bleach for 10 min. Seeds were washed extensively with sterile deionized water before placed on 1/2 Murashige and Skoog (MS) medium (Duchefa Biochemie, Haarlem, Netherlands) supplemented with 1% sucrose and solidified with 0.6% (w/v) agar (Murashige and Skoog, 1962). *Nicotiana benthamiana* plants were grown at 22 ± 3°C under a 16 h light/8 h dark cycle in plastic pots containing steam-sterilized mixed soil (2:1:1, v/v/v, soil/vermiculite/perlite) (Moon et al., 2016). To measure the plant immune response in terms of MAPK activity, 1 μM flg22 peptide (Cat No: FLG22-P-1; Alpha Diagnostics, Inc., San Antonio, TX, USA) was used as a positive control (Bethke et al., 2009).

Bacterial strains, growth conditions, and plasmids

The bacterial strains and plasmids used in the study are described in Table S1. *Shigella* and *Pseudomonas* strains harboring a plasmid pDSK-GFPuv were generated by electroporation, as described previously (Wang et al., 2007; Hong et al., 2016).

Shigella spp. were grown at 37°C in Luria-Bertani (LB) medium or tryptic soy agar containing 0.003% (w/v) Congo red dye (Sigma-Aldrich, St. Louis, MO, USA) (Runyen-Janecky and Payne, 2002). *Pseudomonas syringae* strains were grown at 28°C (with shaking at 200 rpm) in King's B liquid medium (Sigma-Aldrich) containing appropriate antibiotics (King et al., 1954). As a non-pathogenic control, *Escherichia coli* DH5α was grown in LB medium at 37°C with shaking (Kennedy, 1971). *Agrobacterium tumefaciens* GV2260 was grown at 28°C in LB broth with shaking at 200 rpm (Shamloul et al., 2014).

The coding region of *ospF* or *ospG* was PCR-amplified using *attB*-containing PCR primers (Table S2). The PCR fragments were cloned into the pDONR™207 vector by BP recombination using the Gateway® BP Clonase™ II kit (Invitrogen, Carlsbad, CA, USA). The products were then transferred to pBAV178 (for AvrRpt2 fusion) or pBAV179 (for HA fusion) vectors by LR recombination (Gateway® LR Clonase™ II, Invitrogen). pBAV178, pBAV179, and pME6012 (empty vector control) were kindly provided

by Dr. Jean T. Greenberg (University of Chicago) (Vinatzer et al., 2005).

Bacterial inoculation assay *in planta*

Arabidopsis seedlings (2 weeks old) grown in 1/2 MS medium were used for flood-inoculation (Ishiga et al., 2011). Briefly, 10 *Arabidopsis* seedlings in one container were incubated for 3 min with 35 ml of each bacterial strain suspension (5×10^6 or 5×10^5 cfu/ml) containing 0.02% Silwet L-77 (Lehle Seeds, Round Rock, TX, USA) or buffer. After the bacterial suspensions were removed by decantation, plates containing inoculated plants were incubated in a growth room (23 ± 2°C, 16 h light/8 h dark). Bacterial cell counts from inoculated plants were monitored as described previously (Ishiga et al., 2011). Three inoculated seedlings in one container were harvested by cutting the hypocotyls, and total fresh weight was measured. The cfu were normalized to cfu/mg using sample weight. The cfu of seedlings in three separate containers (as biological replicates) were measured. In addition, the bacterial population was evaluated in more than three independent experiments conducted successively under the same conditions.

To assess root invasion, 10-d-old *Arabidopsis* seedlings grown vertically in 1/2 MS medium were inoculated by dropping 2.0 μl of bacterial suspension (5×10^7 cfu/ml) onto the root tips. Symptoms were observed under white light, and bacterial proliferation was monitored at 5 dpi by observation of GFP-expressing bacteria under UV light. Three biological replicates were generated in separate plates, and three independent experiments were conducted under the same conditions.

To analyze the effector secretion, pBAV178 was used to transform *P. syringae* pv. *tomato* DC3000 (*Pst*) by electroporation (Cadoret et al., 2014). Bacterial suspensions (2.5×10^8 cfu/ml) were used to syringe-infiltrate *Arabidopsis* leaves; 2 d after the infiltration, the hypersensitive response was assessed by trypan blue staining (Koch and Slusarenko, 1990).

To test the virulence of *Shigella* effectors via *Pst,* plasmid pBAV179 was used to transform *Pst*; 5×10^7 cfu/ml bacterial suspensions containing 0.02% (w/v) Silwet L-77 were used to spray-infect *Arabidopsis* leaves. After infection, the plants were covered with a clear lid to maintain humidity and transferred to a

growth room (22 ± 3 ºC, 16 h light/8 h dark). The symptoms and bacterial proliferation were assessed at 4 dpi.

Shigella infection to visualize of effector secretion into plant cells

Transgenic *Arabidopsis* expressing Nucleus-targeted Nu-sfGFP1-10^{OPT} were flood inoculated (5×10^5 cfu/ml) with *S. f* 5a, *S. f 5a*::OspF-sfGFP11 or *S. f 5a*::OspG-sfGFP11. To produce the sfGFP11-fused *Shigella* effectors, *OspF* or *OspG* was transferred to pEP119T by LR recombination. At specific time points, *Arabidopsis* leaf discs were observed under a confocal microscope (Park et al., 2017). Each experiment included at least three independent plants.

Microscopy

For SEM, flood-inoculated *Arabidopsis* leaves were fixed in 4% (w/v) paraformaldehyde and dehydrated in an ethanol series (30%, 50%, 70%, 96%, and 100%). The fixed leaves were then dried, coated with gold-palladium, and visualized using a scanning electron microscope (LEO 1455VP, Oberkochen, Germany) (Plotnikova et al., 2000). For TEM, flood-inoculated *Arabidopsis* leaves were cut off, fixed overnight in 2.5% (w/v) glutaraldehyde, post-fixed in 2% (w/v) osmium tetroxide, dehydrated in ethanol, and embedded in the resin. After staining in 2% (w/v) uranyl acetate and lead citrate, samples were observed under an electron microscope (Bio-TEM; Tecnai G2 Spirit Twin; FEI, USA) (Chae and An, 2016).

For fluorescence confocal microscopy, expression of GFP-labeled bacteria or GFP-tagged *Shigella* effector proteins in plants was observed under a Nikon laser scanning confocal microscope C2 (Nikon, Tokyo, Japan) using filter sets for GFP (λ_{ex}, 488 nm; λ_{em}, 505–530 nm) or RFP (λ_{ex}, 561 nm; λ_{em}, 570–620 nm). For each microscopic method, three leaves were used per treatment and at least three microscopic fields were observed for each leaf, including the control.

Expression of _Shigella_ virulence genes in _Arabidopsis_ plants

Total RNA was extracted from *Shigella*-infected leaves (from three plants) using RNAiso plus (Cat

No: 9108; TaKaRa, Otsu, Japan), according to the manufacturer's protocol. RT-PCR was performed using M-MLV reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Quantitative RT-PCR was carried out in a CFX Connect™ Real Time System (BioRad, Hercules, CA, USA) using iQ™ SYBR® Green Supermix (BioRad) and primers specific for target genes (*ipaB*, *ipaC*, *icsA*, *icsB*, *virB*, and *virF*; Table S1 (Bando et al., 2010)). The qRT-PCR results were normalized to the expression of 16s rRNA.

Immunoblotting

Total protein was extracted from *Shigella*- or *Agrobacterium*-infected leaves (from three plants) using denaturing extraction buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% Triton-X, 1× protease inhibitor cocktail (Roche, Basel, Switzerland), 0.4 M DTT, 1 M NaF, and 1 M Na_3VO_3]. The extracted proteins were separated on 12% SDS-PAGE gels and transferred to a PVDF membrane (Pierce, Rockford, IL, USA). Antibodies specific for phospho-p44/p42 MAPK ERK1/2 (Cat No: 4377; Cell Signaling Technology, Danvers, MA, USA) and ERK1 (Cat No: sc-94; Santa Cruz, Dallas, TX, USA) were used for immunoblot analyses. Target proteins were detected using ECL plus reagent (GE Healthcare, Wauwatosa, WI, USA) and visualized using an Alliance 9.7 chemiluminescence imaging system (UVITEC, Cambridge, UK).

Statistical analysis

All data are expressed as the mean ± SD. The statistical significance of bacterial cell growth in infected plants was examined using Student's t-test (Microsoft Office Excel) and ANOVA (SPSS v.18; IBM, Armonk, NY, USA) (Moon et al., 2016). Asterisks and letters indicate significant differences between samples ($P < 0.05$).

Results

Four *Shigella* spp. strains interact differently with *Arabidopsis*

To observe the behavior of the human pathogen *Shigella* in plants, we investigated the interaction of four *Shigella* spp. strains representing three serogroups (*S. b*, *S. s*, *S. f* 2a, and *S. f* 5a), with the model plant *A. thaliana*. *Shigella* is a water-borne pathogen, with infection spreading via contaminated water (Pandey et al., 2014). Hence, we chose to use a flood-inoculation approach (Ishiga et al., 2011), which is thought to mimic natural inoculation. Infection of the phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) was observed as a positive control and *Pst* Δ*hrcC*, a mutant lacking the T3SS of *Pst*, and the non-pathogenic bacterium *E. coli* DH5α were used as negative controls for infection. When 2-week-old *Arabidopsis* seedlings were inoculated with *S. s* or *S. f* 2a, clear infections were observed, such as yellowing and necrosis of the leaves, while mild symptoms were detected after inoculation with *S. b* and *S. f* 5a (Figure 1A) (Liu et al., 2015).

In addition to observing the symptoms, the bacterial growth *in planta* was also evaluated to detect initial plant adherence and proliferation. Early attachment of all *Shigella* strains and DH5α was more than 10 times lower than that of *Pst* and *Pst* Δ*hrcC* (Figure 1B; Day 0, grey bars). At 3 days post-inoculation (dpi), the cell number of all strains significantly increased than initial bacterial cells, although the extent of cell amplification differed depending on the *Shigella* strain (Figure 1B; black bars). Notably, *S. s* cell numbers increased over 10^5 times, comparable to the proliferation of *Pst*. By contrast, the interaction between *S. f* 5a and *Arabidopsis* was similar to that between the plant and non-pathogenic DH5α.

Previous studies demonstrate that several human pathogenic bacteria, including *E. coli* O157:H7 and *Enterococcus faecalis*, invade the leaves and roots of *A. thaliana* (Jha et al., 2005; Deering et al., 2012). Therefore, to determine whether *Shigella* strains invade through *A. thaliana* roots, we dropped bacterial solutions onto root tips and observed symptoms in *Arabidopsis* plants for 14 d. Interestingly, all the strains inhibited Arabidopsis root growth (Figure 1C). Interestingly, *S. b* strain caused severe root growth inhibition unlike the mild disease symptom of the leaves after the infection of this strain (compare Figure 1A, 1C). Inoculation of *S. f 5a* caused slight inhibition of root growth but caused much less damage to the plant than other strains. Taken together, these results indicated that, although the proliferative capacity of the *Shigella* strains differs, the cells can invade and colonize the apoplast of plants, thereby causing structural damage to

the host.

Penetration and subsequent internalization of *Shigella* spp. into *Arabidopsis*

Since we observed that *Shigella* proliferate and induce disease-like symptoms in *Arabidopsis*, we examined whether the bacterium multiplies on the leaf surface and in the intercellular space (apoplast) by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), respectively. *Pst*, known to infects plants via open stomata (Panchal et al., 2016), colonized guard cells at 24 h post-infection (Figure 2A). Similarly, all tested *Shigella* strains clustered around guard cells and the surface of epidermal cells. *S. s* and *S. b* formed relatively broad clusters in the surrounding areas, including guard cells (Figure 2A). In particular, *S. b* and *S. f* 2a intensely colonized guard cells (Figure 2A), leading us to speculate that they enter plants via open stomata, similar to *Pst*.

Most plant pathogenic bacteria infect and colonize the apoplast (Sattelmacher, 2009; Gao et al., 2016). Therefore, we investigated whether *Shigella* is capable of intercellular colonization and causing damage to plant cells. Indeed, the TEM images revealed colonization of *Shigella* in the intercellular spaces and attachment to the host cell walls (Figure 2B). The presence of the microbes in the intercellular space resulted in the alteration of the host organelle structure, such as the separation of the plasma membrane from the cell wall, the liberation of cell organelles, and disruption of chloroplasts (Figure 2B (Gao et al., 2016)). This effect was most pronounced in plant cells inoculated with *S. b* and *S. s*; further, *S. b* and *S. s* were more commonly found in the intercellular spaces than *S. f* 2a and *S. f* 5a.

To observe internalization of bacterial cells in the plant via root cells, we attempted to label the four *Shigella* strains with a green fluorescent protein (GFP) and obtained *S. b*, *S. f* 2a, and *S. f* 5a strains labeled with GFP successfully. GFP labeling did not affect the bacterial growth in plants (Figure S2). Bacterial suspensions were dropped onto the root tips of vertically grown *Arabidopsis* plants. Five days later, whole plants and root tissues were photographed under ultraviolet (UV) light to observe the distribution of fluorescently labeled bacteria (Figure 3A). In accordance with the disease phenotypes observed, *Pst* (which is a foliar pathogen) exhibited strong fluorescence in the leaves, despite the fact that it was applied to root

tips (Figure 3A). To observe *Pst* in root tissues, inoculated roots were disinfected with hypochlorite solution and observed under a fluorescence microscope. GFP fluorescence was observed only in epidermal cells after application of *Pst* to root tips (Figure 3B). Consistantly, this finding indicates that *Pst* infects throughthe leaf tissues, not by invading root tissues or colonizing the roots. Interestingly, when GFP-labeled *S. f 2a* was applied to the root tip, strong green fluorescence was observed in both roots and leaves, while other GFP-labeled *Shigella* inoculated plants showed only weak fluorescence in leaves (Figure 3A). In root tissues, GFP fluorescence was observed in root endodermal cells only in *S. f* 2a-treated plants (Figure 3B). Taken together, these results indicate that *Shigella* invades plant leaves and roots in a strain-dependent manner, and then moves along the surface and through the internal vascular tissues of the plant.

Shigella* T3S effectors are necessary for attachment and multiplication in *Arabidopsis

T3SS is the crutial determinant for the virulence of many Gram-negative bacteria, including animal and plant pathogens (He, 1998; Ogawa et al., 2008; Buttner and He, 2009). Thus, we investigated if pathogenic proteins for animal host infections are required for invasion and multiplication of *Shigella* in plants. To study the biological role of the T3SS in the interaction between *Shigella* and plants, we used noninvasive variants of *S. f* 2a and *S. f* 5a (strains Δvp and BS176, respectively) (Sansonetti et al., 1982; Wenneras et al., 2000; Shim et al., 2007). To facilitate observation of bacterial invasion in living plants, the noninvasive variants of the *Shigella* strains were also labeled with GFP, and bacterial growth and expression of effector proteins were verified (Figure S1-S3). Bacterial proliferation in plants after inoculation with Δvp or BS176 strains was 10 times lower than that after inoculation by parental *Shigella* strains; similar results were observed for GFP-labeled *Shigella* strains and mutants (Figure 4A). To examine the involvement of the T3SS in bacterial invasion of the plant surface, leaves were flood-inoculated with GFP-labeled bacteria, and leaf surfaces were observed 24 h later (Figure 4B). Examination under UV light revealed that the levels of GFP-labeled *S. f* 2a and *S. f* 5a on leaf surfaces were higher than those of Δvp and BS176, especially in open stomata (Figure 4B). We also generated a non-pathogenic mutant of *S.s* and have tested if the severe infection of *S.s* strain is T3SS dependent (Figure S4). When plants were inoculated with this T3SS-deficient

S. s strain, disease phenotypes and bacterial growth were significantly reduced (Figure S4), as observed with Δvp and BS176. Altogether, these results suggested that the T3SS of *Shigella* that operate during infection in human is also required for the colonization in plants.

To further investigate involvement of *Shigella* T3S effectors in plant interactions, we measured expression of critical virulence effectors related to human pathogenesis, including *ipaB*, *ipaC*, *icsA*, *icsB*, *virB*, and *virF*, in *S. f* 2a-, *S. f* 5a-, Δvp-, and BS176-infected *Arabidopsis* plants (Bando et al., 2010). These effectors play a role in mammalian cell lysis (*ipaB*, *ipaC*), intracellular spread (*icsA*, *icsB*), and regulation of virulence factor (*virF*, *virB*) (Ogawa et al., 2008). Total RNAs were isolated from *Arabidopsis* leaves at 6 and 12 h after *Shigella* inoculation, and changes in expression of virulence genes were confirmed by quantitative RT-PCR (Figure 4C). Expression of all *Shigella* virulence genes examined in WT *S. f* 2a- or *S. f* 5a-inoculated *Arabidopsis* leaves increased. Interestingly, induction was higher and faster in *S. f* 2a-treated plants than in *S. f* 5a-treated plants, which is in agreement with the earlier results showing that *S. f* 2a was more pathogenic to plants than *S. f* 5a. Expression of virulence genes was not detected in plants inoculated with non-pathogenic mutants Δvp or BS176, similar to buffer-treated control plants (Figure 4C), suggesting that common virulence factors regulate interactions with both plants and human intestinal cells.

To investigate the plant innate immune responses to *Shigella* inoculation, MAPK phosphorylation was evaluated. The activation of MAPK by phosphorylation is a conserved response of the earliest microbe-triggered immune signaling in both plants and animals (Zipfel, 2009). The flg22 peptide is a representative microbe-associated molecular pattern in plants (Bethke et al., 2009). In plants treated with flg22, pronounced MAPK phosphorylation was apparent within 5 min of treatment and this response lasted up to 30 min (Figure 4D). On the other hand, MAPK phosphorylation in plants treated with *S. f* 2a or *S. f* 5a was reduced; from 15 min on, it was strongly suppressed and almost completely disappearing after ca. 30 min (Figure 4D). Meanwhile, in plants treated with the virulence plasmid-deficient mutants, Δvp or BS176, MAPK activation was recovered, in contrast to *S. f* 2a- or *S. f* 5a-treated plants, although the degree and duration of the phosphorylation were lower than those elicited by the flg22 treatment (Figure 4D). These observations indicated that *Shigella* suppresses the innate immunity of *Arabidopsis* via its T3SS.

Suppression of immune signaling in *Arabidopsis* plants by *Shigella* T3S effectors OspF and OspG

Shigella OspF has been reported to inhibit MAPK signaling, which is conserved in plants and animals (Arbibe et al., 2007; Li et al., 2007). OspG is an essential immunosuppressive effector protein secreted at the later stages of infection; this protein interferes with activation of the NF-κB pathway, which is absent from plants (Kim et al., 2005). Therefore, we examined whether OspF and OspG have virulence activity in plants by introducing them heterologously into a phytopathogen, *Pst*, and monitoring its pathogenicity. First, we used an AvrRpt2-derived T3SS reporter system (Figure S5A; (Mudgett, 2005). AvrRpt2$^{101\text{-}255}$ is sufficient to induce cell death but lack in delivery to the plant cells. When *Arabidopsis* leaves were syringe-infiltrated with various *Pst* strains which express effectors fused to AvrRpt2$^{101–255}$, we observed that *Pst* producing OspF:AvrRpt2$^{101–255}$ or OspG:AvrRpt2$^{101–255}$ successfully induced cell death at 1 day after delivery implying that both OspF and OspG are successfully delivered into *Arabidopsis* cells via *Pst* T3SS (Figure 5A). Next, we examined whether the virulence of *Pst* was increased by expression of *Shigella* OspF or OspG. Production of OspF:HA and OspG:HA by *Pst* was confirmed by immunoblotting with an anti-HA antibody (Figure S5B, S5C). Plants infected with *Pst* cells producing OspF: HA or OspG: HA showed more severe symptoms than plants infected with the empty vector control (Figure 5B). In addition, the number of bacterial cells was 10 times higher than in the plants infected with the empty vector control (Figure 5C). Taken together, these results indicate that OspF and OspG preserve their function as virulence proteins in plant hosts.

On the other hand, *in vitro* studies demonstrated that OspF exerts a phosphothreonine lyase activity and irreversibly removes phosphate groups from MAPK (Arbibe et al., 2007; Li et al., 2007). To investigate whether the virulence associated with *Shigella* OspF in plants was linked to the same mechanism of action as in animals, we used a phenotypic screening system involving a MEK2 (a tobacco MAP kinase kinase2) mutant, MEK2^{DD} (Yang et al., 2001; Kim and Zhang, 2004). MEK2^{DD}, a constitutively active mutant of MEK2, induces cell death when overproduced in plant leaves In this experiment, an HA:MEK2^{DD} clone fused to an HA epitope tag at the N-terminus was used to monitor expression of MEK2^{DD}. As expected, co-

production of HA:MEK2^{DD} and GFP (control) resulted in pronounced cell death (Figure S6A). Co-production of OspF:GFP, but not OspG:GFP, and MEK2^{DD} completely inhibited the MEK2^{DD}-induced cell death (Figure S6A). The production of the two effectors fused with GFP was then evaluated in the MEK2^{DD}-producing plant leaves; the production of OspF:GFP was apparent, but that of OspG:GFP was not (Figure S6B), even though both proteins were stably produced in the absence of MEK2^{DD} (Figure S6B). The degradation of the OspG protein might be associated with the activation of MAPK. Indeed, as assessed by immunoblotting with specific anti-phosphorylated MAPK antibodies, MAPK phosphorylation was very weak in the OspF:GFP-producing plant samples in comparison with GFP- or OspG:GFP-producing plant samples (Figure S6B). The production of MEK2^{DD} protein was confirmed in all samples using anti-HA antibodies (Figure S6B). These observations strongly suggest that the *Shigella* effector OspF inhibits plant immune responses by inhibiting activation of MAPK (as in humans), and that OspG induces immunosuppression in plants by targeting distinct MAPK pathways.

OspF or OspG affects *Shigella* proliferation in plants

To confirm the role of the OspF or OspG proteins in the interaction between *Shigella* and plants (as in human cells), we inoculated plants with *S. f* 5a mutants lacking the OspF or OspG proteins and examined their growth. The growth of mutants lacking *ospF* or *ospG* was as deficient as that of the virulence plasmid-deficient mutant BS176 (Figure 6A). Reduced growth of *S. f* 5a *ΔospF* or *S. f* 5a *ΔospG* mutants was entirely restored by complementation of the mutation, indicating that these two effector proteins are indeed important for bacterial growth in plants (Figure 6A). Next, we monitored the activation of MAPKs to determine whether plant immune suppression was affected by the deletion of *ospF* or *ospG*. As shown in Figure 6B, *S. f.* 5a Δ*ospF* induced stronger phosphorylation of MAPKs than wild-type *S. f* 5a; this was offset by complementation with OspF. By contrast, phosphorylation of MAPK in *S. f* 5a *ΔospG*-inoculated plants was no different from that in plants inoculated with wild-type *S. f* 5a (Figure 6B). These results are consistent with the inhibition of MAPK-induced immune responses by OspF, but not by OspG, using the plant expression binary vector described above (Figure S6). Taken together, these results indicate that

Shigella effectors OspF or OspG play an important role in increasing bacterial proliferation in both plant and animal hosts.

Delivery of OspF and OspG into plant cells during *Shigella* inoculation

To visualize direct delivery of type III effectors to plant cells by *Shigella*, we took advantage of a newly developed split superfolder green fluorescent protein (sfGFP^{OPT}) system (Park et al., 2017). In this system, the non-fluorescent GFP protein (GFP1-10) with β –strand 1 to 10 is expressed in the host cell and the bacterial effector is linked to the 11th β-strand (GFP11). Only when the effector fused with GFP11 is introduced into plant cells via bacterial T3SS, fluorescent signals are induced through GFP reconstitution (Park et al., 2017; Figure 7A). First, in order to select a host plant expressing GFP1-10 capable of exploring the delivery of OspF and OspG, we observed the subcellular localization of OspF:GFP and OspG:GFP by expressing them in *Nicotiana benthamiana* leaves using the *Agrobacterium* system (Figure S7). The fluorescence signal for both proteins was strong in the plant cell nucleus; OspF:GFP fluorescence was also observed along the cytoplasmic membrane, and punctate OspG:GFP fluorescence was observed in the cytosol (Figure S7C). However, immunoblot of OspG:GFP expression in *Nicotiana benthamiana* detected OspG:GFP as well as free GFP (Figure S7B), suggesting that overexpression of free GFP might be responsible for the cytosolic GFP expression. Based on these results, we choose *Arabidopsis* plants expressing sfGFP1-10 (sfGFP1-10^{OPT}) in nucleus or cytosol and the OspF and OspG were fused with 11th β-strand of sfGFP, respectively (Figure S8A). GFP signals were observed at nucleus in both *S. f 5a*::OspF-sfGFP11 and *S. f 5a*::OspG-sfGFP11 flood-inoculated *Arabidopsis* leaves at 3 hpi, respectively, while control *S. f* 5a WT did not (Figure 7B). In contrast, when *Arabidopsis* seedlings expressing cytosolic sfGFP1-10^{OPT} were flood inoculated with *S. f* 5a producing OspF-sfGFP11 or OspG-sfGFP11, we detected no reconstituted GFP signals in all tested bacteria (Figure S8B). This suggests that *Shigella* effectors OspF and OspG were successfully secreted into *Arabidopsis* cells through the *Shigella* T3SS. In the host human cell, OspF localization is nuclear (Zurawski et al., 2006; Arbibe et al., 2007) and that of OspG is nuclear and cytoplasmic (Kim et al., 2005; Zhou et al., 2013; de Jong and Alto, 2014), confirming that the subcellular

localization in plant cells is similar to that of animal cells. Taken together, the *Shigella* effectors OspF and OspG can be delivered into plant cells through T3SS.

Discussion

In the current study, we investigated the interaction of the human pathogenic bacterium, *Shigella*, with an alternative host, the *Arabidopsis* plant. We demonstrated that four *Shigella* strains, *S. b*, *S. s*, *S. f* 2a, and *S. f* 5a, invade and colonize *Arabidopsis* to different extents. Different symptoms and bacterial proliferation rates for different *Shigella* strain suggested that the variability of the plant interaction mechanisms among the strains, e.g., the adherence and multiplication, might contribute to the differences. Differences in the nutritional requirements of bacterial strains may constitute another reason for the differences in the growth rates within the *Arabidopsis* host.

It has been demonstrated that, in human, *Shigella* initially enters the epithelial layer via the M cells through transcytosis, leading to the invasion of the basolateral surfaces of the intestinal epithelial cells. A subsequent gut inflammation leads to the activation of the innate immune response (Phalipon and Sansonetti, 2007). We demonstrated, in addition to the human host, *Shigella* invades *Arabidopsis* through vascular tissues and leaf stomata pores (Figure 2-4). In particular, *S. s* and *S. b* formed relatively wide clusters in the surrounding areas, including the guard cells (Figure 2), similar to *Pseudomonas syringae* which has built a host-pathogen relationship with Arabidopsis (Panchal et al., 2016). Interestingly, we found that the four studied-strains of *Shigella* associate with the plant cells and induce different plant responses. The bacterial loads of *S. s in planta* were relatively higher than those of the other strains. By contrast, inoculation of *S. f* 5a was associated with lower bacterial proliferation and less severe symptoms than observed for other strains (Figure 1-3). *S. f* 2a and *S. f* 5a, which belong to the same serogroup (Lindberg et al., 1991), elicited distinctly different plant responses with respect to disease symptoms, suggesting that the virulent effectors may play a relatively more important role in *Shigella*-plant interactions than PAMPs. These observations also indicate that specific plant immune systems may be useful in the search for novel virulence factors expressed by different *Shigella* strains.

405 Many Gram-negative bacterial pathogens utilize common infection strategies to colonize and invade
406 plant and animal cells, and pathogenicity appears to depend on highly conserved T3SSs, which deliver the
407 effector proteins to host cells (Buttner and Bonas, 2003). By using avirulent mutant strains, we were able to
408 show that effectors that regulate the pathogenesis of shigellosis in humans also play a central role in
409 regulating interactions with *Arabidopsis*. We showed that secretion of T3S effectors is required for the
410 *Shigella* proliferation and attachment in plants (Figure 4, Figure S4). Furthermore, the effector proteins
411 impacted MAPK-dependent/independent immune responses in *Arabidopsis* (Figure 4, 6). These
412 observations further support the suggestion that the function of the main effector proteins of *Shigella*
413 appears to be conserved in plant and animal hosts, and that this contributes to bacterial intracellular survival
414 or suppression of the host defense against the pathogens. Reduced colonization of T3SS-deficient
415 pathogenic *E. coli* in plants was previously reported, they suggested that *E. coli* uses the T3SS apparatus for
416 attachment to leaves, rather than for bacterial growth inside plants (Shaw et al., 2008). The relevance of
417 T3SS for multiplication of *Salmonella* in plants remains unclear due to the different effects of T3SS function
418 on *Salmonella*-plant interactions (Iniguez et al., 2005; Schikora et al., 2011; Shirron and Yaron, 2011).
419 In general, animal bacterial pathogens with short needle lengths have been thought to be difficult to
420 secrete T3S effectors on plant cells due to their cell walls and have been regarded as a major cause of not
421 infecting plants. Recently, however, several studies have reported that the length and width of bacterial
422 T3SS depend on the type of host cell, the environment, and the infection cycle of the bacteria (Deane et al.,
423 2010). In this paper, we showed direct evidence that the effector proteins of *Shigella* were delivered into
424 plant cells through T3SS utilizing recently developed the split GFP technology (Figure 7). Further study,
425 identifying plant factors that trigger and control *Shigella*'s T3SS assembly will be a great help in developing
426 a strategy to prevent the spread of *Shigella* through plants.
427 Expression of the T3SS of *Shigella* is regulated at the transcriptional level and is activated at a
428 permissive temperature (≥ 32°C) (Tobe et al., 1991; Campbell-Valois and Pontier, 2016). We were able to
429 observe the expression of the T3SS genes of *Shigella* under temperatures at which plants grow (22 ± 3°C)
430 (Figure 5A). A recent study showed that elevation of the temperature increases T3SS-mediated virulence of

the phytopathogen *Pst* in plants, which is in contrast with the negative effect of high temperature on expression of the T3SS of *Pst in vitro* (Huot et al., 2017). Regardless of the temperature of host cells, it will be interesting to determine whether *Shigella* regulates T3SS gene expression *in vivo* and to identify factors that influence T3SS gene expression other than plant temperature.

Salmonella strains capable of proliferating on plant leaves and actively entering plant tissues, root hairs, or trichomes were recently shown to exhibit virulence in animals (Barak et al., 2011; Golberg et al., 2011; Schikora et al., 2011). We demonstrated that *Shigella* strains actively colonize the surface of and inside *Arabidopsis* leaves and root tissues (Figure 1–3) and that bacteria recovered from plants maintain expression of pathogenic proteins (Figure S3). Collectively, these findings suggest that, similar to *Salmonella*, *Shigella*-inoculated plants are a serious risk to food safety and that contamination of plants is another route underlying infection of *Shigella*, an important human pathogenic bacterium. In this study, we only observed plants artificially inoculated with *Shigella* in a laboratory environment. Therefore, to confirm the food safety concern surrounding *Shigella*-inoculated plants, the ability of *Shigella* to infect a variety of crops grown in the field should be tested. The pathogenicity of plant-isolated *Shigella* in animals should also be investigated.

The current study provides new insights into host invasion mechanisms utilized by *Shigella* to interact with an alternative host, the plant *Arabidopsis*. Studying trans-kingdom pathogenesis involving human-adapted pathogens, such as *Shigella*, may uncover novel pathogenic mechanisms uniquely activated in response to specific hosts. When we isolated the two *Shigella* effectors OspF and OspG, and produced them in plant cells, their localization coincided with that in the animal cells (Figure 7 and S6C), and it was apparent that the production of both proteins increased the virulence of plant pathogens (Figure 6). In addition, we confirmed that OspF inhibits the innate immune response of plants via the same enzymatic activity as in animals (Figure 5 and S6). In animals, OspG inhibits the host immune response by inhibiting the activity of NF-κB by blocking degradation of IκB (Ashida et al., 2015). Plants possess an IκB-like protein called NIM1 (Ryals et al., 1997); however, no other published studies have investigated whether an NF-κB–induced immune response exists in plants. In the current study, we demonstrated the ability of OspG

to increase the phytopathogenicity of non-*Shigella* bacteria (Figure 5), and also observed degradation of OspG:GFP upon constitutive activation of MAPK signaling (Figure S6B). The existence of a plant immune signaling pathway similar to that of animal NF-κB, which would also be the target of OspG, may hence be assumed. Characterization of the previously unrecognized stress-activated mediators of the innate immunity in plants upon infection with food-borne pathogens, would help us to define the defensive functions of plants. Finally, the characterization of plants as an alternative host for food-borne pathogens will be critical in developing effective means to prevent their transmission and disease.

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Author contributions

JMP, SHJ, and JL conceived and designed the study. SHJ, JL, and DHL carried out the experiments. JMP, SHJ, JL, DWK, and EP analyzed the data. JMP, SHJ, JL, and EP wrote the manuscript. JMP, SHJ, EP, CMR and DC edited and discussed the manuscript and all authors agreed with the final version.

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657 virulence. Infection and immunity **74,** 5964-5976.
658

Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 The growth of *Shigella* strains producing GFP or virulence-deficient mutants is not affected *in vitro* growth.

Fig. S2 Comparison of Bacterial proliferation in *Arabidopsis* inoculated with *Shigella* and GFP-labeled *Shigella* by flooding

Fig. S3 Expression of effector proteins of *Shigella* recovered from inoculated plants.

Fig. S4 Spontaneous type III secretion system-deficient mutant of *S. sonnei* is reduced growth and symptoms in *Arabidopsis.*

Fig. S5 Expression of OspF or OspG in *Pst* for virulence assay.

Fig. S6 Co-expression of OspF:GFP suppresses tobacco $MEK2^{DD}$-triggered cell death.

Fig. S7 Expression GFP fused OspF or OspG in plant.

Figure S8. Delivery of OspF or OspG fused with GFP11 through T3SS

Table S1 Bacterial strains and plasmids used in the study.

Table S2 Sequences of PCR primers used for Gateway cloning.

Table. S3. PCR primer list and sequences used for qRT-PCR.

Figure legends

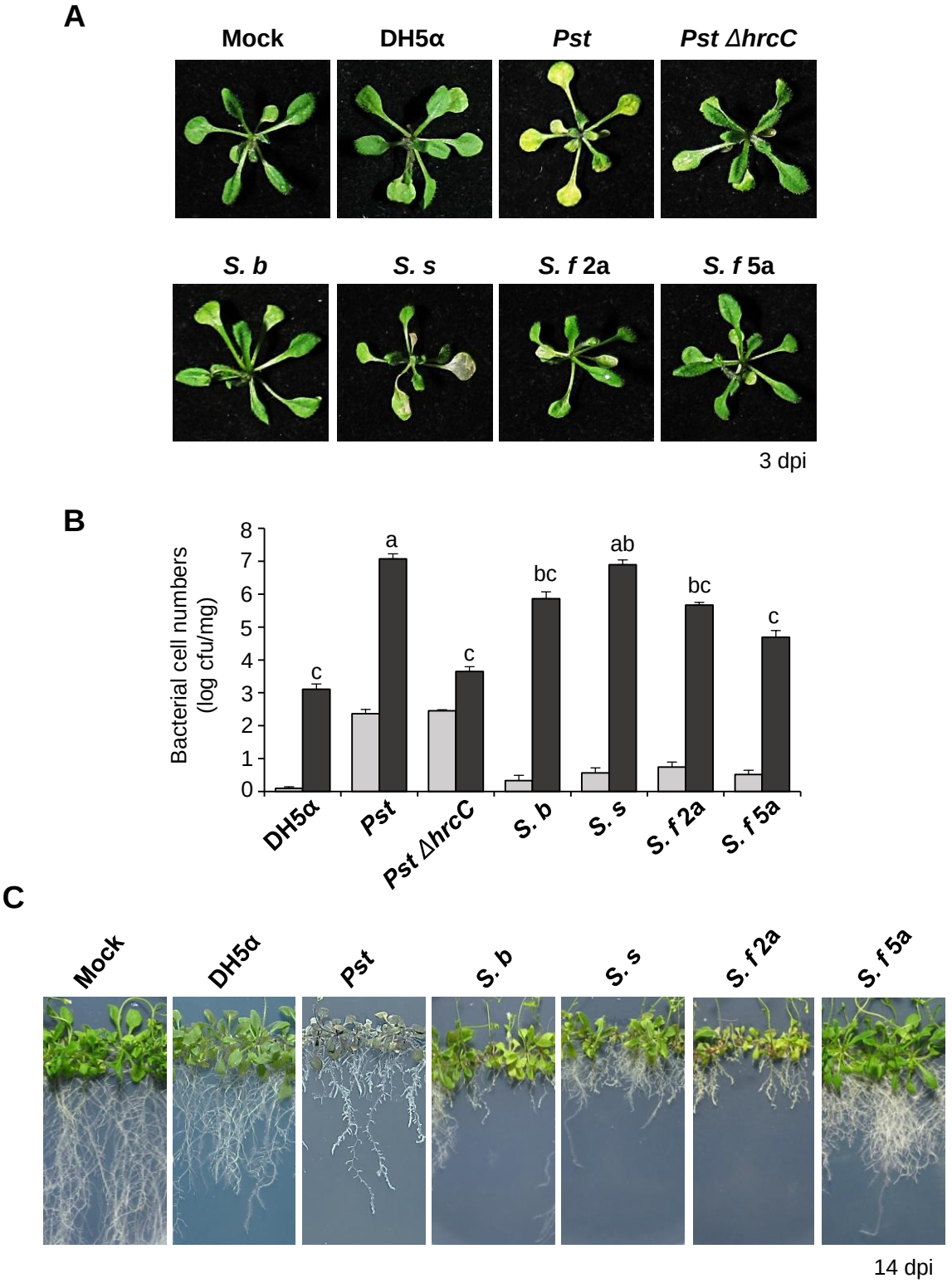
Figure 1. *Shigella* proliferates and induces disease symptoms in *Arabidopsis* plants. (A, B) *Arabidopsis* seedlings in 1/2 MS medium were flood-inoculated with distilled H_2O containing 0.025% Silwet L-77 (Mock) or bacterial suspensions (5×10^5 cfu/ml). (A) The symptoms of *Arabidopsis* inoculated with *Shigella*

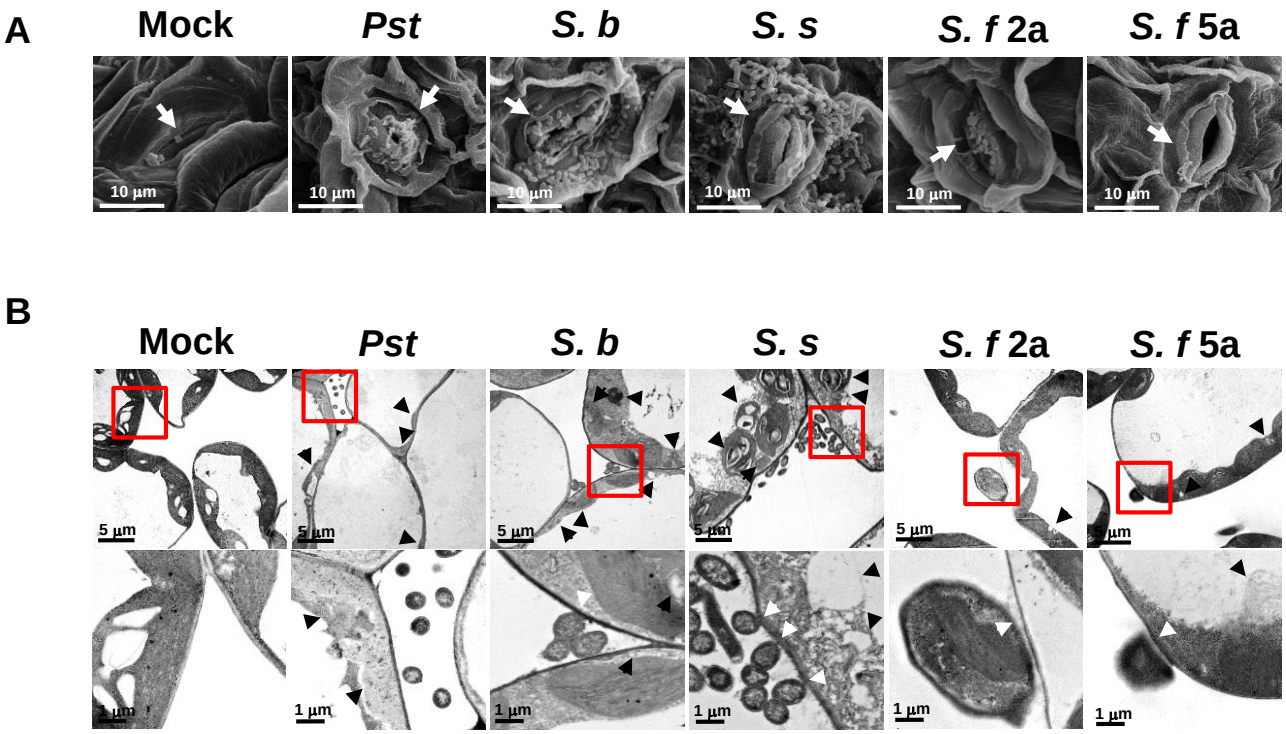
691 spp. by flood-inoculation. Photographs of representative symptoms were taken at 3 dpi. (B) Bacterial cell
692 numbers were evaluated on Days 0 (gray bar) and 3 (black bar) after flood-inoculation. Plants were surface-
693 sterilized with 5% H_2O_2 and washed several times with sterile distilled water to remove unattached bacteria
694 from their surface, before evaluating attached and proliferated bacterial population. The bars represent the
695 mean ± SD of three replicates and the different letters indicate significant differences between samples ($P <$
696 0.05, one-way ANOVA). (C) *Arabidopsis* seedlings in 1/2 MS medium were infected with 2 μl of buffer or
697 bacterial suspensions (5×10^7 cfu/ml) by dropping onto the root tips. The photographs were taken 14 dpi and
698 are representative of three independent experiment. All experiments were repeated three times
699 independently and representative results are shown.
700
701 **Figure 2.** *Shigella* clusters around guard cells and localizes in the apoplast affecting plant cell structures. (A,
702 B) *Arabidopsis* seedlings were flood-inoculated with a buffer or bacterial suspensions (5×10^6 cfu/ml). (A)
703 After 24 h, the leaves were fixed and analyzed under a SEM. The *Pst* cells were observed over the stomata.
704 *Shigella* strains were associated with the stomata. Bar, 10 μm. Representative image showing bacteria
705 around guard cells (indicated by white arrows). (B) After 24 h, the leaves were evaluated under a TEM. The
706 *Shigella* strains colonized the intercellular spaces. Red boxed regions in the micrographs at the upper panel
707 were maginified at the bottom panel. Bar, 5 μm (upper panels). Bar, 1 μm (bottom panels). TEM images
708 revealed the *Shigella* (white arrowheads in the bottom panel) attached to the cell wall in the intercellular
709 spaces and altered mesophyll cells. Black arrowheads at the upper panel indicate separation of the plasma
710 membrane from the cell wall, the abnormal organelles and disruption of chloroplasts. The results are
711 representative of two independent experiments.
712
713 **Figure 3.** *Shigella S. f 2a* invades and colonizes plant roots. (A, B) Root tips of *Arabidopsis* seedlings were
714 drop-inoculated with GFP-labeled bacterial suspensions (5×10^7 cfu/ml). (A) Bacterial colonization of
715 inoculated plants was photographed under white and UV light at 5 dpi. (B) GFP-labeled *Shigella* are

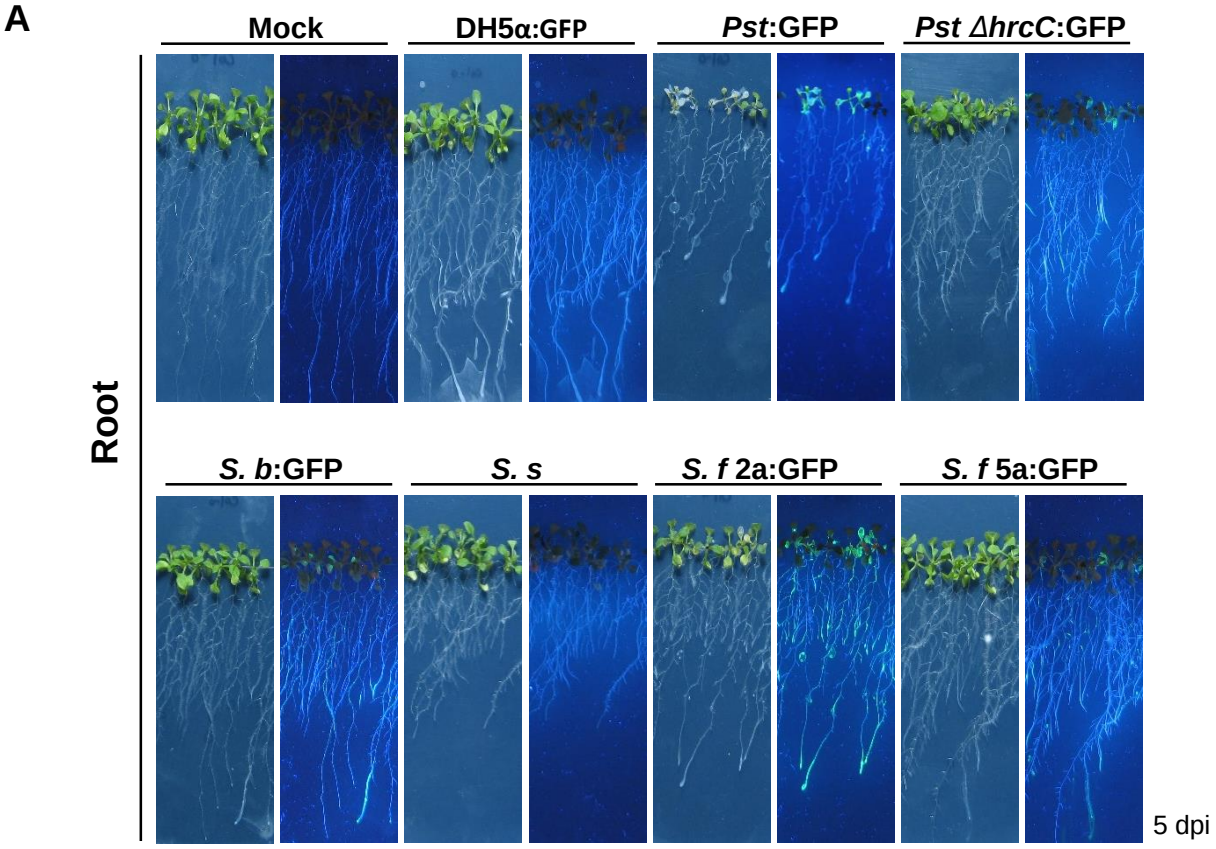
716 localized in the epidermal or endodermal cells of *Arabidopsis* roots. GFP images were taken using a
717 confocal microscope. Higher magnification micrographs and 3D Raman confocal volume images show
718 internalization of *S. f* 2a in *Arabidopsis* roots. All experiments were repeated at least three times, and
719 representative results are shown.
720
721 **Figure 4.** The virulence plasmid-deficient mutant of *Shigella* is impaired in the *Arabidopsis* proliferation.
722 (A, B) *Arabidopsis* seedlings in 1/2 MS medium were flood-inoculated with *S. f* 2a, Δvp, *S. f* 5a, or BS176,
723 and their GFP-labeled variants (5×10^5 cfu/ml). (A) The bacterial cell numbers *in planta* were determined at
724 0 and 3 dpi. Bars represent the mean ± SD of three replicates and the different letters indicate significant
725 differences between samples ($P < 0.05$, one-way ANOVA). (B) Images of GFP-labeled *Shigella* in leaf
726 epidermal cells of *Arabidopsis* acquired at 24 hpi under a fluorescence confocal microscope. Bar, 10 μm.
727 The blue represents auto-fluorescence of chlorophyll. All experiments were repeated at least three times, and
728 representative results are shown. (C, D) *Arabidopsis* leaves were syringe-infiltrated with buffer, 1 μM flg22,
729 or bacterial suspension (5×10^6 cfu/ml), and samples were collected at the indicated times. (C) Transcription
730 of *Shigella* effectors (*ipaB*, *ipaC*, *icsA*, *icsB*, *virB*, and *virF*) in inoculated *Arabidopsis* leaves was analyzed
731 by qRT-PCR. qRT-PCR results were normalized to expression of 16s rRNA. Expression of effectors by
732 plasmid-deficient mutant strains was compared with that in the WT. Data are expressed as the mean ± SD of
733 three replicates. (D) Immunoblotting was performed using either anti-phospho-p44/42 (ERK1/2, upper
734 panels) or anti-ERK1 (lower panels) antibodies. All experiments were repeated at least three times, each
735 with similar results.
736
737 **Figure 5.** Increased virulence of phytopathogen *Pst* in the presence of *Shigella* effectors OspF or OspG. (A)
738 *Pst* strains expressing the *Shigella* effector proteins were used in *Arabidopsis* for effector secretion assay.
739 *Arabidopsis* leaves were syringe-infiltrated with *Pst* cells (5×10^7 cfu/ml) producing AvrRpt2, carrying the
740 empty vector (pME6012), and producing OspF:AvrRpt2$^{101–255}$ or OspG:AvrRpt2$^{101–255}$. The hypersensitive

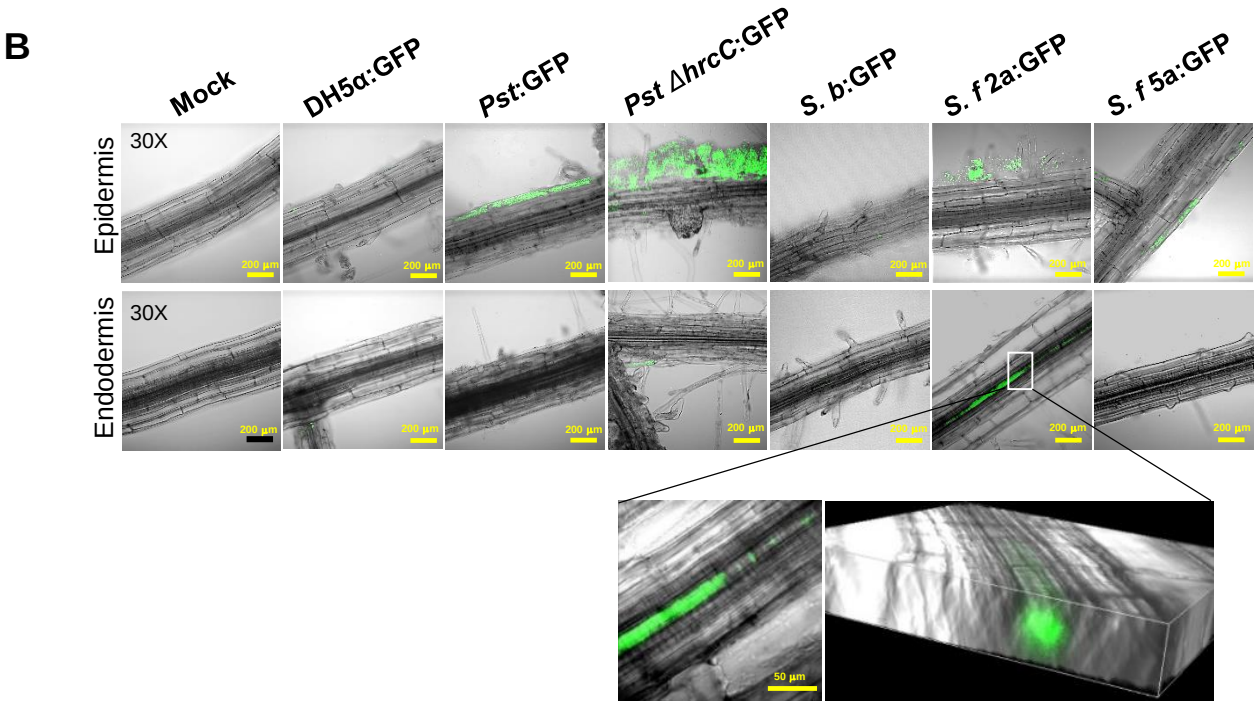
741 response in the infiltrated leaves was monitored using the trypan blue staining and photographed at 1 dpi.
742 (B, C) *Arabidopsis* plants were sprayed with *Pst* cells carrying an empty vector (pME6012), or with cells
743 producing OspF:HA or OspG:HA (5×10^7 cfu/ml). (B) Disease symptoms were monitored for 4 d after
744 spraying. (C) Proliferation of *Pst* cells producing *Shigella* effector proteins in *Arabidopsis* at 0 and 4 d after
745 spray-inoculation. Bars represent the mean ± SD of six replicates, and different letters indicate significant
746 differences between samples ($P < 0.05$, two-way ANOVA). All experiments were repeated at least three
747 times.
748
749 **Figure 6.** The *Shigella* effector proteins OspF and OspG underpin the bacterial virulence in plants. (A) The
750 virulence of the OspF- or OspG-deficient *Shigella* mutants is impaired in the *Arabidopsis* model.
751 *Arabidopsis* seedlings in 1/2 MS medium were flood-inoculated with *S. f* 5a, Δ*ospF*, Δ*ospF*+p*ospF*, Δ*ospG*,
752 or Δ*ospG*+p*ospG* (5×10^5 cfu/ml). Photographs of representative disease symptoms were taken, and
753 bacterial cell numbers *in planta* were determined, at 3 dpi. The bars represent the mean ± SD of three
754 replicates. The different letters indicate significant differences between samples ($P < 0.05$, one-way
755 ANOVA). (B) MAPK activation in *Arabidopsis* plants in response to infection by ospF or ospG deletion
756 mutants. *Arabidopsis* leaves were infiltrated with each bacterial strain as described previously and then
757 collected at the indicated times. Anti-phospho-p44/42 (ERK1/2, upper panels) or anti-ERK1 (lower panels)
758 antibodies were used for immunoblotting. All experiments were repeated at least three times, with similar
759 results.
760
761 **Figure 7.** *Shigella* T3S effectors OspF and OspG with sfGFP11 are delivered through *S. flexneri* 5a into
762 *Arabidopsis.* (A) Illustration of a split $sfGFP^{OPT}$ system for monitoring the transfer of bacterial effectors by
763 T3SS to the nucleus of host cells. (B) Transgenic *Arabidopsis* seedlings expressing $sfGFP1\text{-}10^{OPT}$ in 1/2 MS
764 medium were flood-inoculated with *S. f* 5a containing OspF-sfGFP11 or OspG-sfGFP11(5×10^5 cfu/ml). 3
765 hours after inoculation reconstituted sfGFP signals in the nucleus of *Arabidopsis* leaf epidermal cells were

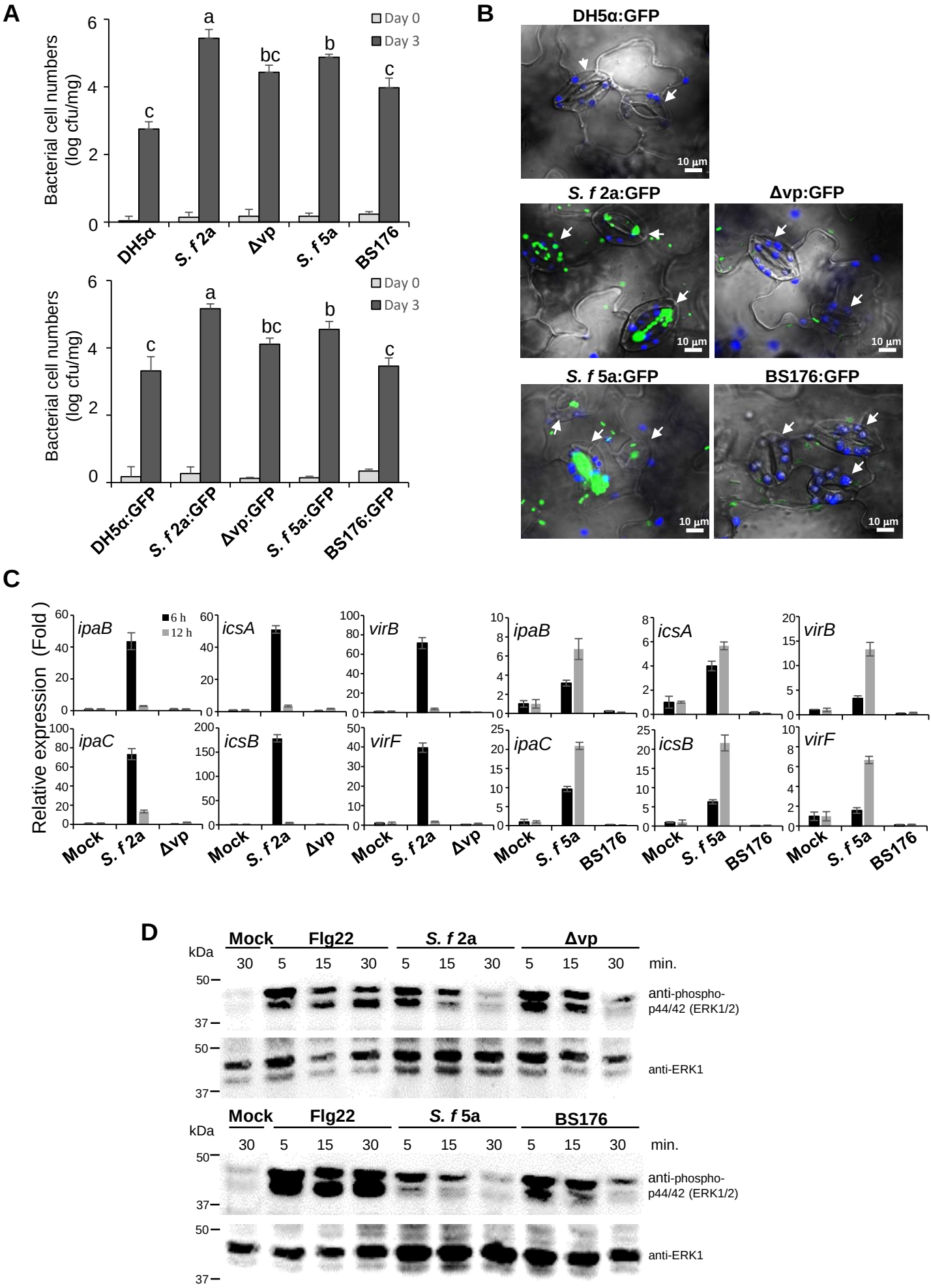
766 observed under a fluorescence confocal microscope. Yellow arrows indicate the complemented sfGFP
767 signals in nuclei. Yellow perporated-boxed regions were magnified at the right column. The magenta
768 represents auto-fluorescence of chlorophyll. Bar, 10 μm and 20 μm.
769


A
Mock
DH5α
Pst
Pst ΔhrcC
S. b
S. s
S. f 2a
S. f 5a
3 dpi
B
Bacterial cell numbers
(log cfu/mg)
0
1
2
3
4
5
6
7
8
c
a
c
bc
ab
bc
c
DH5α
Pst
Pst ΔhrcC
S. b
S. s
S. f 2a
S. f 5a
C
Mock
DH5α
Pst
S. b
S. s
S. f 2a
S. f 5a
14 dpi

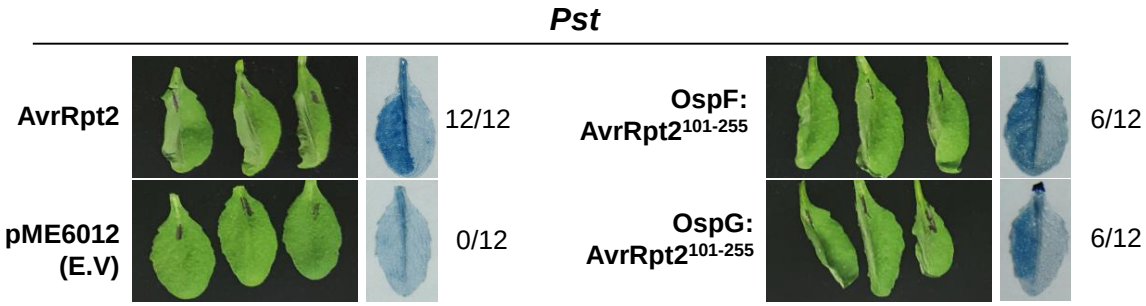

A
Mock
Pst
S. b
S. s
S. f 2a
S. f 5a
10 µm
B
Mock
Pst
S. b
S. s
S. f 2a
S. f 5a
5 µm
1 µm


A
Mock
DH5α:GFP
Pst:GFP
Pst ΔhrcC:GFP
Root
S. b:GFP
S. s
S. f 2a:GFP
S. f 5a:GFP
5 dpi

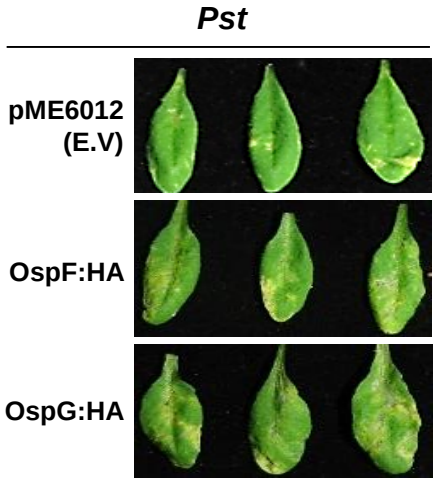

B
Mock
DH5α:GFP
Pst:GFP
Pst ΔhrcC:GFP
S. b:GFP
S. f 2a:GFP
S. f 5a:GFP
Epidermis
Endodermis
30X
30X
200 μm
50 μm


A
Bacterial cell numbers (log cfu/mg)
Day 0
Day 3
a
bc
b
c
c
DH5α
S. f 2a
Δvp
S. f 5a
BS176
DH5α:GFP
S. f 2a:GFP
Δvp:GFP
S. f 5a:GFP
BS176:GFP
B
DH5α:GFP
S. f 2a:GFP
Δvp:GFP
S. f 5a:GFP
BS176:GFP
10 μm
C
Relative expression (Fold)
6 h
12 h
ipaB
icsA
virB
ipaC
icsB
virF
Mock
S. f 2a
Δvp
S. f 5a
BS176
D
Mock
Flg22
S. f 2a
Δvp
S. f 5a
BS176
kDa
min.
anti-phospho-p44/42 (ERK1/2)
anti-ERK1

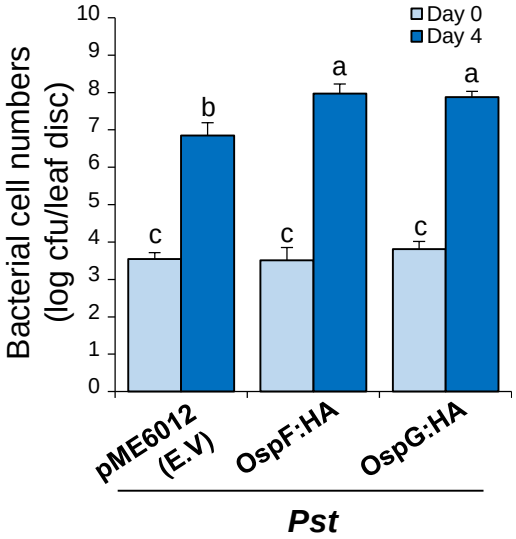
A

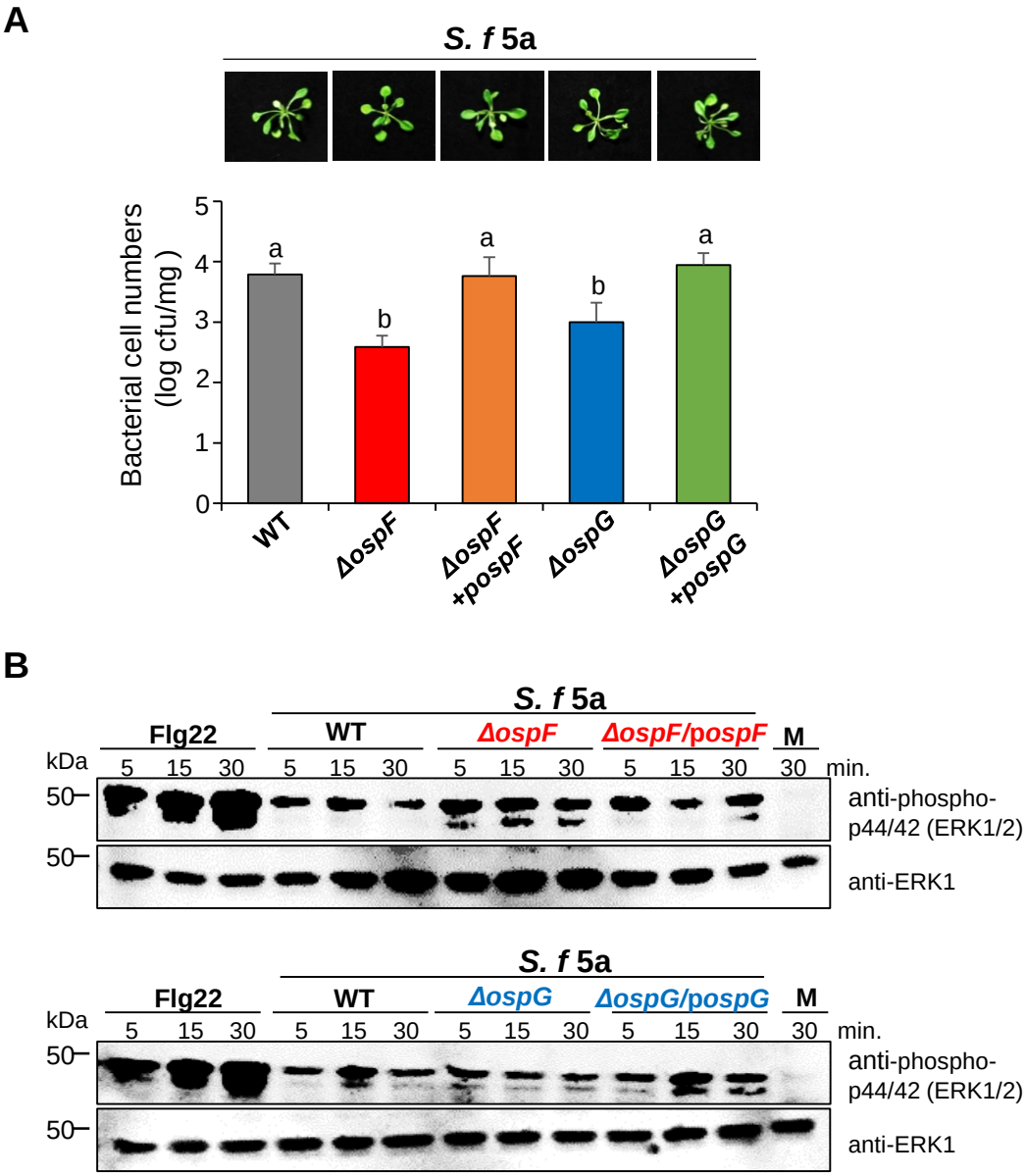

Pst
AvrRpt2
12/12
pME6012 (E.V)
0/12
OspF: AvrRpt2101-255
6/12
OspG: AvrRpt2101-255
6/12

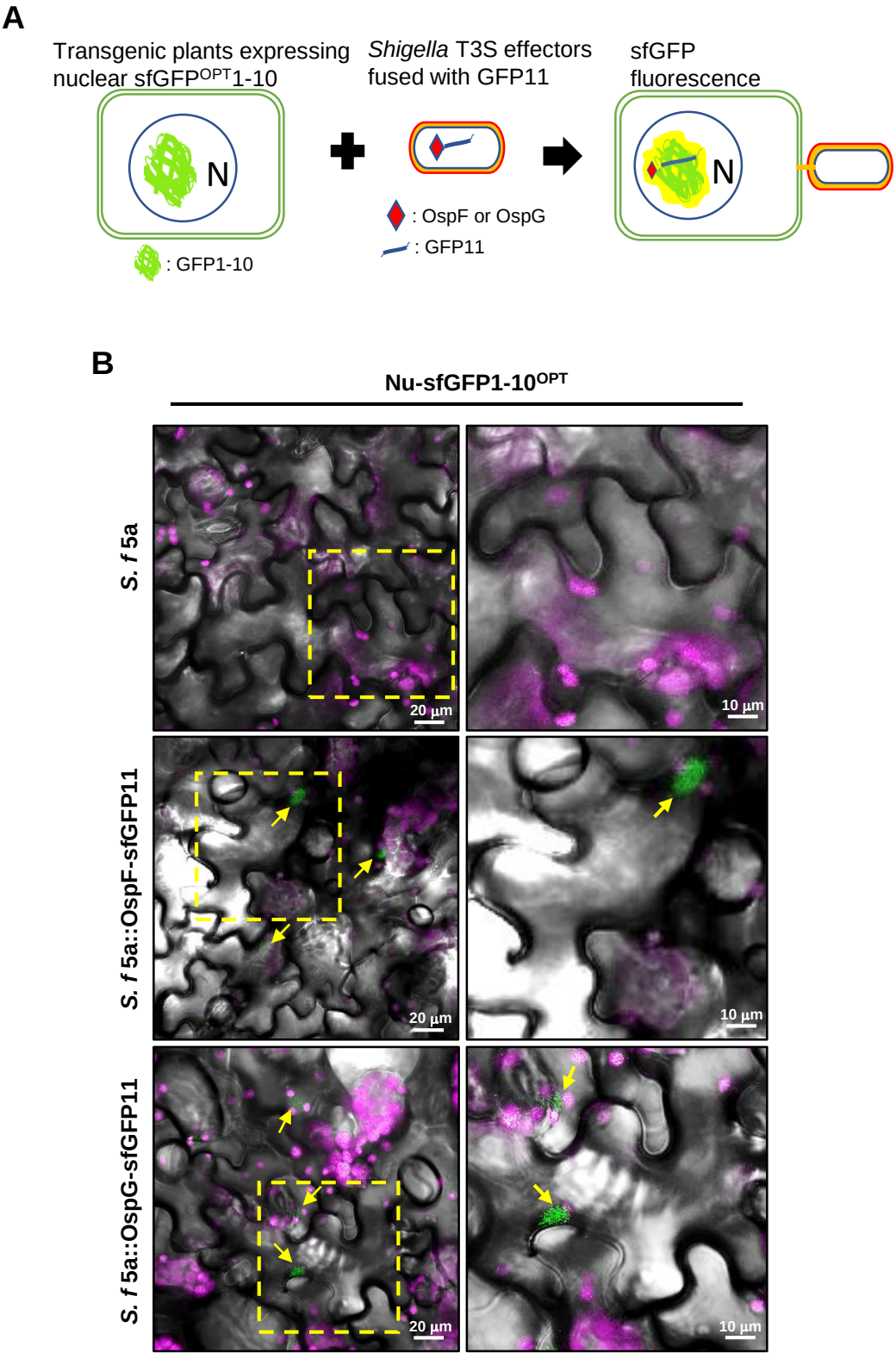
B


Pst
pME6012 (E.V)
OspF:HA
OspG:HA

C


Bacterial cell numbers (log cfu/leaf disc)
0
1
2
3
4
5
6
7
8
9
10
Day 0
Day 4
c
b
c
a
c
a
pME6012 (E.V)
OspF:HA
OspG:HA
Pst


A
S. f 5a
Bacterial cell numbers
(log cfu/mg)
0
1
2
3
4
5
a
b
a
b
a
WT
ΔospF
ΔospF
+pospF
ΔospG
ΔospG
+pospG
B
S. f 5a
Flg22
WT
ΔospF
ΔospF/pospF
M
kDa
5 15 30 5 15 30 5 15 30 5 15 30 30 min.
50
anti-phospho-
p44/42 (ERK1/2)
50
anti-ERK1
S. f 5a
Flg22
WT
ΔospG
ΔospG/pospG
M
kDa
5 15 30 5 15 30 5 15 30 5 15 30 30 min.
50
anti-phospho-
p44/42 (ERK1/2)
50
anti-ERK1


A
Transgenic plants expressing nuclear sfGFP^OPT1-10
Shigella T3S effectors fused with GFP11
sfGFP fluorescence
N
N
: OspF or OspG
: GFP11
: GFP1-10
B
Nu-sfGFP1-10^OPT
S. f 5a
S. f 5a::OspF-sfGFP11
S. f 5a::OspG-sfGFP11
20 µm
10 µm
20 µm
10 µm
20 µm
10 µm