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A human pathogenic bacterium *Shigella* proliferates in plants through adoption of type III effectors for shigellosis

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Abstract

Shigella, which infects primates, can be transmitted via fresh vegetables; however, its molecular interactions with plants have not been elucidated. Here, we show that four *Shigella* strains, *Shigella boydii*, *Shigella sonnei*, *Shigella flexneri* 2a, and *S. flexneri* 5a, proliferate at different levels in *Arabidopsis thaliana*. Microscopic studies revealed that these bacteria were present inside leaves and damaged plant cells. Green fluorescent protein (GFP)-tagged *S. boydii* and *S. flexneri* 5a colonized leaves only, whereas *S. flexneri* 2a colonized both leaves and roots. Using *Shigella* mutants lacking type III secretion systems (T3SSs), we found that T3SSs that regulate the pathogenesis of shigellosis in humans also play a central role in bacterial proliferation in *Arabidopsis*. Strikingly, the immunosuppressive activity of two T3S effectors, OspF and OspG, was required for proliferation of *Shigella* in *Arabidopsis*. Of note, delivery of OspF or OspG effectors inside plant cells upon *Shigella* inoculation was confirmed using a split GFP system. These findings demonstrate that the human pathogen *Shigella* can proliferate in plants by adapting immunosuppressive machinery used in the original host human.

KEYWORDS

alternative host, *Arabidopsis* plants, enteropathogenic bacteria, PAMP-triggered immunity, proliferation, *Shigella* spp., split GFP, T3S effectors, trans-kingdom pathogenesis, type III secretion system (T3SS)

1 | INTRODUCTION

Human may be infected by enteric pathogens via the ingestion of water or food contaminated with enteropathogenic bacteria from feces (the fecal-oral route; Gupta, Polyak, Bishop, Sobel, & Mintz, 2004; Mead et al., 1999; Weir, 2002). Fresh fruits and vegetables, including lettuce, tomatoes, and green peppers, are responsible for widespread transmission of food-borne pathogens such as *Salmonella* and *Shigella* (Gu, Cevallos-Cevallos, & van Bruggen, 2013; Guchi & Ashenafi, 2010; Semenov, Kuprianov, & van Bruggen, 2010). These observations suggest that human pathogenic bacteria use plants as alternative hosts because they provide a stable environmental niche.

Indeed, several human pathogenic bacteria, including *Salmonella enterica* serovar Typhimurium, *Escherichia coli* O157:H7, and *Pseudomonas aeruginosa*, use plants as alternative hosts (Deering, Mauer, & Pruitt, 2012; Plotnikova, Rahme, & Ausubel, 2000; Semenov et al., 2010). These bacteria attach to the plant surface and actively invade and proliferate in plant tissues (Cevallos-Cevallos, Gu, Danyluk, & van Bruggen, 2012; Martínez-Vaz, Fink, Diez-Gonzalez, & Sadowsky, 2014). In particular, several enteropathogenic bacteria such as *E. coli* O157:H7 and *Salmonella* have been found to internalize in both root and shoots in various plant species, but the mechanism on how they spread from initial infection site to other areas of the plant is uncertain (Bernstein, Sela, Pinto, & Ioffe, 2007; Cooley, Miller, & Mandrell, 2003; Warriner, Spaniolas, Dickinson, Wright, & Waites, 2003).

Shigella is a human-adapted pathogen that infects the host via multiple transmission routes. It is a nonmotile, rod-shaped, facultative intracellular and invasive pathogen that is very closely related to *E. coli*. Based on the carbohydrate composition of the O-antigen, that is, 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, *Shigella dysenteriae* 1 (serogroup A), *Shigella flexneri* (serogroup B), *Shigella boydii* (serogroup C), and *Shigella sonnei* (serogroup D; Lindberg, Karnell, & Weintraub, 1991; Schroeder & Hilbi, 2008). *Shigella* spp. are important epidemic pathogens and a serious risk to public health 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 & Causes of Death, 2016). However, the actual number of infections might be higher because mild symptoms are not reported (Mortality & Causes of Death, 2016); this suggests that the microorganism may employ a variety of survival strategies, not only for human intestinal infection but also for survival in a non-human host. Although *Shigella* contamination has been reported in plants (Naimi et al., 2003; Ohadi, Rasouli, Darzi-Eslam, Jafari, & Ehsani, 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; this pattern-triggered immunity (PTI) is activated via mitogen-activated protein kinase (MAPK) cascades (Jones & Dangl, 2006). To suppress PTI, bacteria inject effector proteins into plant cells using type III secretion systems (T3SSs). To counteract this, plant nucleotide-binding leucine-rich repeat proteins recognize the pathogen effectors; effector-triggered immunity is then activated to accompany the hypersensitivity response (Jones & Dangl, 2006). For human or animal intestinal bacteria to infect plants, the PTI must first be disabled. Similar to its activity in mammalian hosts, *S. enterica* serovar Typhimurium uses the T3SS to suppress plant immune responses (Schikora et al., 2011; Schikora, Garcia, & Hirt, 2012). In particular, when expressed in plants under the control of a plant binary vector, one of the T3S effector proteins of *S. enterica*, SpvC, targets the MAPK signalling system to suppress the host PTI (Neumann et al., 2014).

Here, we examined the ability of four *Shigella* strains (*S. sonnei*, Holt et al., 2012; *S. boydii* and *S. flexneri* 2a, Wei et al., 2003; and *S. flexneri* 5a, Onodera et al., 2012) to proliferate in *Arabidopsis* plants. We found that the four strains invaded and proliferated differently in plant tissues. Proliferation of mutants lacking the T3S effectors, that is, non-invasive 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 used split green fluorescent protein (GFP) technology to observe delivery of *Shigella* type III effector proteins inside plant cells after *Shigella* inoculation. These observations indicate that *Arabidopsis* may be a useful model host for studying the pathogenesis of *Shigella*.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth

Arabidopsis thaliana accession Columbia (Col-0; obtained from the Arabidopsis Biological Resource Center) was used for *Shigella* infection. Briefly, *Arabidopsis* seeds were surface sterilized for 2 min in 70% (v/v) ethanol, followed by incubation for 10 min in 50% household bleach. Seeds were washed extensively with sterile deionized water before placed on half-strength Murashige and Skoog (1/2 MS) medium (Duchefa Biochemie, Haarlem, Netherlands) supplemented with 1% sucrose and solidified with 0.6% (w/v) agar (Murashige & Skoog, 1962). 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).

2.2 | Bacterial strains, growth conditions, and plasmids

The bacterial strains and plasmids used in the study are described in Table S1. *Shigella* and *Pseudomonas* strains harbouring plasmid pDSK-GFPuv were generated by electroporation, as described previously (Hong, Kwon, & Park, 2016; Wang, Kang, Anand, Lazarovits, & Mysore, 2007).

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 & 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, Ward, & Raney, 1954). As a nonpathogenic control, *E. coli* DH5α was grown at 37°C in LB medium with shaking (Kennedy, 1971). *Agrobacterium tumefaciens* GV2260 was grown at 28°C in LB broth with shaking at 200 rpm (Shamloul, Trusa, Mett, & Yusibov, 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, Jelenska, & Greenberg, 2005).

2.3 | Bacterial inoculation assay *in planta*

Arabidopsis seedlings (2 weeks old) grown in half-strength MS medium were used for flood inoculation (Ishiga, Ishiga, Uppalapati, & Mysore, 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 colony forming unit [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 hr light/8 hr 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 samples were sterilized for 2 min with 5% H_2O_2 and then washed three times with sterile distilled water. The number of cfu was normalized to cfu/mg using sample weight. The number of cfu of seedlings in three separate containers (as biological replicates) was 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-day-old *Arabidopsis* seedlings grown vertically in half-strength MS medium (Mullen et al., 1998) 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 ultraviolet (UV) light. Three biological replicates were generated in separate plates, and three independent experiments were conducted under the same conditions.

To analyse effector secretion, pBAV178 was used to transform *P. syringae* pv. *tomato* DC3000 (*Pst*) by electroporation (Cadoret, Soscia, & Voulhoux, 2014). Bacterial suspensions (2.5×10^8 cfu/ml) were used to syringe infiltrate *Arabidopsis* leaves; 1 days after infiltration, the hypersensitivity response was assessed by trypan blue staining (Koch & Slusarenko, 1990).

To test the virulence of *Shigella* effectors, *Pst* was transformed with plasmid pBAV179; 5×10^7 cfu/ml bacterial suspension containing 0.02% (w/v) Silwet L-77 was 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°C, 16 hr light/8 hr dark). The symptoms and bacterial proliferation were assessed at 4 dpi.

2.4 | *Shigella* infection to visualize effector secretion into plant cells

Transgenic *Arabidopsis* expressing nucleus-targeted Nu-sfGFP1-10^{OPT} were flood inoculated (5×10^5 cfu/ml) with *S. flexneri* 5a, *S. flexneri* 5a::OspF-sfGFP11, or *S. flexneri* 5a::OspG-sfGFP11. To generate sfGFP11-fused *Shigella* effectors, *OspF* or *OspG* was transferred to pEP119T by LR recombination. At specific time points, *Arabidopsis* leaf discs were observed using a confocal microscope (Park, Lee, Woo, Choi, & Dinesh-Kumar, 2017). Each experiment included at least three independent plants.

2.5 | Microscopy

For scanning electron microscopy, 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 under a scanning electron microscope (LEO 1455VP, Oberkochen, Germany; Plotnikova et al., 2000). For transmission electron microscopy (TEM), flood-inoculated *Arabidopsis* leaves were cut off, fixed overnight in 2.5% (w/v) glutaraldehyde, postfixed in 2% (w/v) osmium tetroxide, dehydrated in ethanol, and embedded in resin. After staining with 2% (w/v) uranyl acetate and lead citrate, samples were observed using an electron microscope (Bio-TEM; Tecnai G2 Spirit Twin; FEI, USA; Chae & An, 2016).

For fluorescence confocal microscopy, expression of GFP-labelled bacteria or GFP-tagged *Shigella* effector proteins in plants was observed using the Nikon C2 confocal microscope (Nikon, Tokyo, Japan) at 30× or 60× magnification and filter sets for GFP (λ_{ex}, 488 nm; λ_{em}, 505–530 nm) or red fluorescent protein (λ_{ex}, 561 nm; λ_{em}, 570–620 nm). For each microscopic method, three leaves from three different plants were used per treatment, and at least three microscopic fields were observed for each leaf, including the control.

2.6 | 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 (q)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 S3; Bando et al., 2010). The qRT-PCR results were normalized to expression of 16s rRNA.

2.7 | 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% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred to a polyvinylidene fluoride 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).

2.8 | 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 analysis of variance (SPSS v.18; IBM, Armonk, NY, USA; Moon, Lee, Oh, Kang, & Park, 2016). Asterisks and letters indicate significant differences between samples ($P < .05$).

3 | RESULTS

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

To observe the behaviour of the human pathogen *Shigella* in plants, we investigated the interaction between four *Shigella* spp. strains representing three serogroups (*S. boydii*, *S. sonnei*, *S. flexneri* 2a, and *S. flexneri* 5a) and the model plant *A. thaliana*. *Shigella* is a water-borne pathogen that spreads via contaminated water (Pandey, Kass, Soupir, Biswas, & Singh, 2014). Hence, we chose to use a flood-inoculation approach (Ishiga et al., 2011), which is thought to mimic natural inoculation. Infection of *Pst* was observed as a positive control, and *Pst* Δ*hrcC* (a mutant lacking the T3SS of *Pst*) and the nonpathogenic bacterium *E. coli* DH5α were used as negative controls for infection. When 2-week-old *Arabidopsis* seedlings were inoculated with *S. sonnei* or *S. flexneri* 2a, clear symptoms were observed, such as yellowing and necrosis of the leaves, whereas mild symptoms such as wilting were detected after inoculation with *S. boydii* and *S. flexneri* 5a (Figure 1a; Liu et al., 2015).

In addition to observing symptoms, bacterial growth *in planta* was also evaluated to detect initial adherence to plants and subsequent 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 postinoculation (dpi), the cell number of all strains significantly increased than that measured at Day 0, although the extent of cell number amplification differed depending on the *Shigella* strain (Figure 1b; black bars). Notably, *S. sonnei* cell numbers increased by more than 10^5 times, which was comparable with proliferation of *Pst*. By contrast, the interaction between *S. flexneri* 5a and *Arabidopsis* was similar to that between the plant and nonpathogenic 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* (Deering et al., 2012; Jha, Bais, & Vivanco, 2005). Therefore, to determine whether *Shigella* strains invade through *A. thaliana* roots, we dropped bacterial solutions onto root tips and observed *Arabidopsis* plants for 14 days to detect symptoms. Interestingly, all strains inhibited *Arabidopsis* root growth (Figure 1c). *S. boydii* strain caused severe root growth inhibition, unlike the mild symptoms observed in leaves infected with this strain (cf. Figure 1a,c). Inoculation of *S. flexneri* 5a caused slight inhibition of root growth but caused much less damage to the plant than other strains. Taken together, these results suggest that *Shigella* actively interact with *Arabidopsis* plants and have various interaction mechanisms depending on the strains.

3.2 | Penetration and subsequent internalization of *Shigella* spp. in *Arabidopsis*

Because we observed that *Shigella* proliferate and induce disease-like symptoms in *Arabidopsis*, we used scanning electron microscopy and TEM to examine whether the bacterium multiplies on the leaf surface and in the intercellular space (apoplast), respectively. *Pst*, which infects plants via open stomata (Panchal et al., 2016), colonized guard cells at 24 hr postinfection (Figure 2a). Similarly, all tested *Shigella* strains clustered around guard cells and the surface of epidermal cells. *S. sonnei* and *S. boydii* formed relatively broad clusters in the surrounding areas, including guard cells (Figure 2a). In particular, *S. boydii* and *S. flexneri* 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 (Gao et al., 2016; Sattelmacher, 2009). Therefore, we investigated whether *Shigella* is capable of intercellular colonization and causing damage to plant cells. Indeed, TEM images revealed colonization of *Shigella* in the intercellular spaces and apparent attachment to host cell walls (Figure 2b). The presence of the microbes in the intercellular space was associated with 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. boydii* and *S. sonnei*; furthermore, *S. boydii* and *S. sonnei* were more commonly found in intercellular spaces than *S. flexneri* 2a and *S. flexneri* 5a.

To observe internalization of bacterial cells in plant roots, we labelled *S. boydii*, *S. flexneri* 2a, and *S. flexneri* 5a strains with GFP; *S. sonnei* did not label with GFP. GFP labelling did not affect 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 UV light to observe distribution of fluorescently labelled bacteria

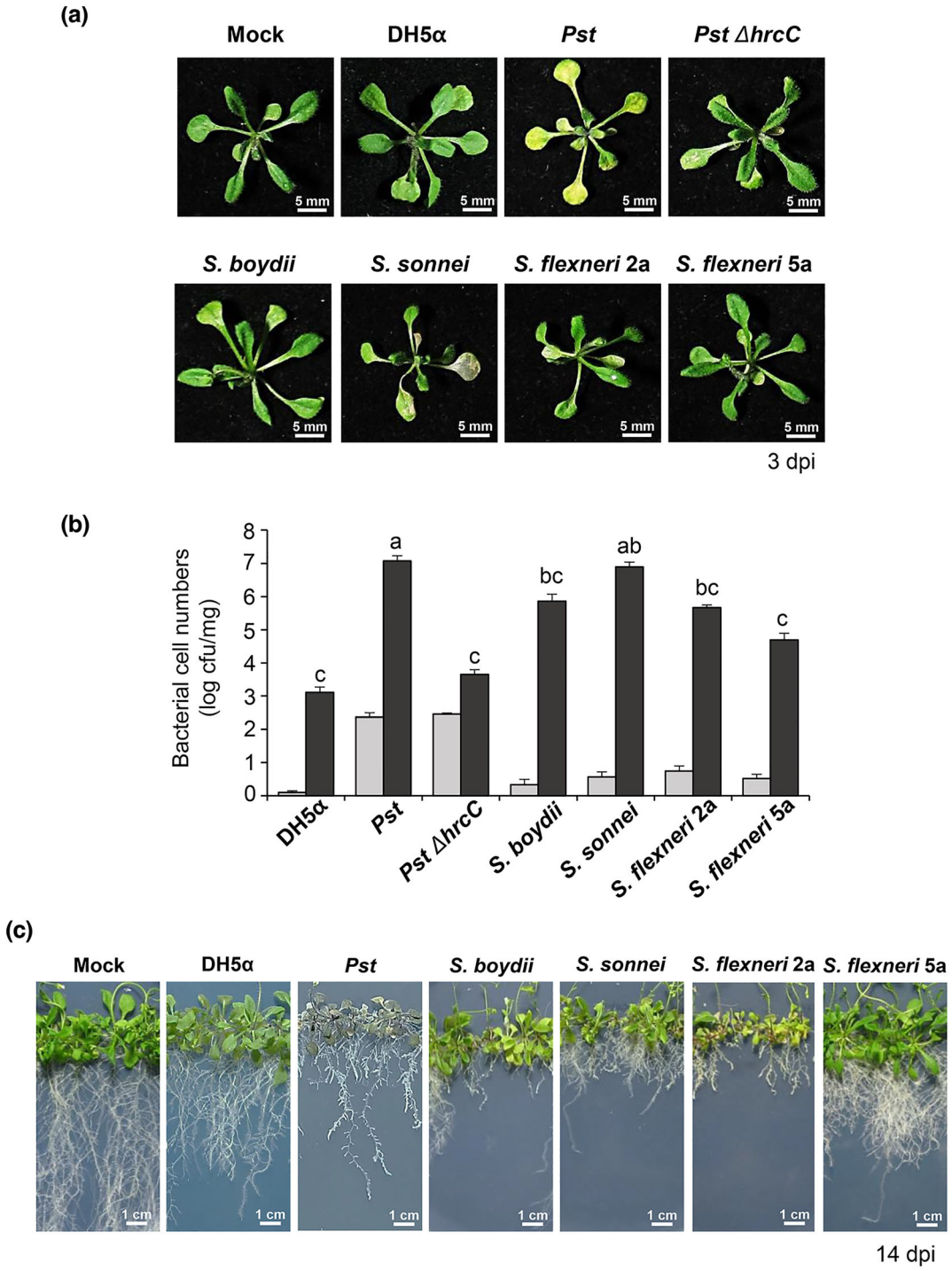

FIGURE 1 *Shigella* proliferates and induces disease symptoms in *Arabidopsis* plants. (a, b) *Arabidopsis* seedlings grown in half-strength MS medium were flood inoculated with distilled water containing 0.025% Silwet L-77 (Mock) or bacterial suspensions (5×10^5 cfu/ml). (a) The symptoms of *Arabidopsis* flood inoculated with *Shigella* spp. Photographs of representative symptoms were taken at 3 dpi. (b) Bacterial cell numbers were evaluated on Days 0 (grey bar) and 3 (black bar) after flood inoculation. Plants were surface sterilized with 5% H_2O_2 and then washed several times with sterile distilled water to remove unattached bacteria from the surface before evaluating the attached and proliferating bacterial populations. The bars represent the mean ± *SD* of three replicates, and the different letters indicate significant differences between samples ($P < .05$, one-way analysis of variance). (c) *Arabidopsis* seedlings grown in half-strength MS medium were infected with 2 μl of buffer or bacterial suspension (5×10^7 cfu/ml) by dropping onto the root tips. Photographs were taken at 14 dpi and are representative of three independent experiments. All experiments were repeated three times independently, and representative results are shown

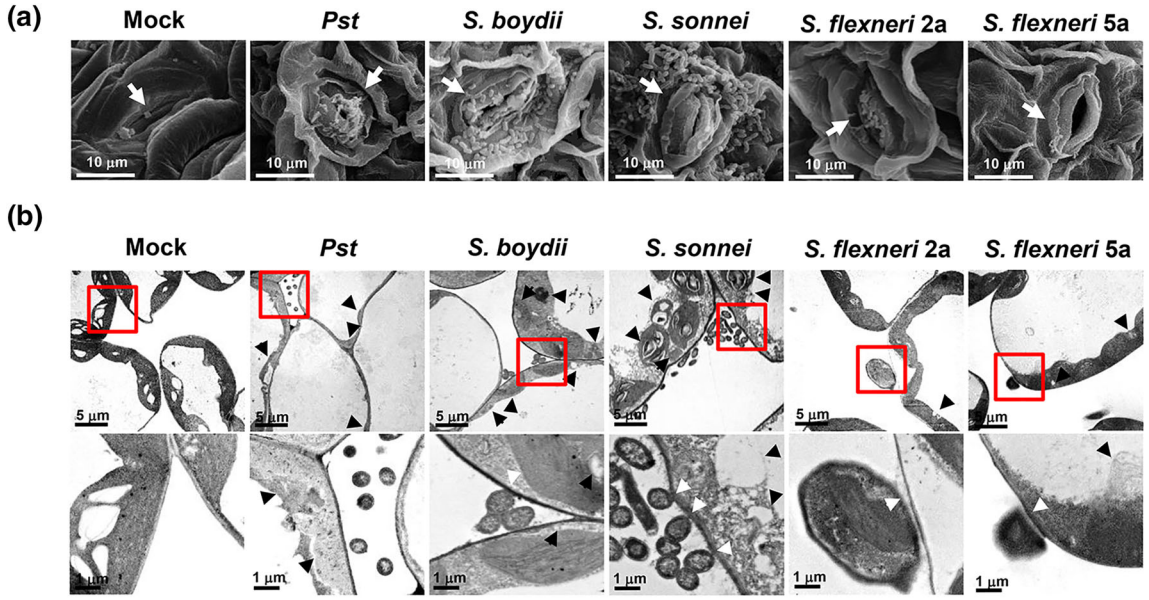

FIGURE 2 *Shigella* clusters around guard cells and localizes in the apoplast. (a, b) *Arabidopsis* seedlings were flood inoculated with buffer or bacterial suspension (5×10^6 cfu/ml). (a) After 24 hr, the leaves were fixed and analysed by scanning electron microscopy. *Pst* cells were observed over the stomata. *Shigella* strains were associated with the stomata. Bar, 10 μm. Representative image showing bacteria around guard cells (indicated by white arrows). (b) After 24 hr, leaves were evaluated by transmission electron microscopy. *Shigella* strains colonized the intercellular spaces. The red boxes shown in the micrographs in the upper panel are magnified in the bottom panel. Bar, 5 μm (upper panels); bar, 1 μm (bottom panels). Transmission electron microscopy images revealed *Shigella* (white arrowheads in the bottom panel) presented in the intercellular spaces, associated with abnormal mesophyll cells. Black arrowheads in the upper panel indicate separation of the plasma membrane from the cell wall, abnormal organelles, and disrupted chloroplasts. The results are representative of two independent experiments

(Figure 3a). In accordance with the disease phenotypes observed, *Pst* (which is a foliar pathogen) exhibited strong fluorescence in 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 at the surface of epidermal cells after application of *Pst* to root tips (Figure 3b). This finding indicates that *Pst* infects and colonizes leaf tissues. Interestingly, when GFP-labelled *S. flexneri* 2a was applied to the root tip, strong green fluorescence was observed in both roots and leaves, whereas other GFP-labelled *Shigella*-inoculated plants showed only weak fluorescence in roots (Figure 3a). In root tissues of *S. flexneri* 2a:GFP-treated plants, GFP fluorescence was observed in the inner vessel tissues only (Figure 3b). Taken together, these results indicate that *Shigella* colonizes plant leaves and roots in a strain-dependent manner.

3.3 | *Shigella* T3S effectors are necessary for attachment and proliferation in *Arabidopsis*

T3SS is the crucial determinant for the virulence of many Gram-negative bacteria, including animal and plant pathogens (Buttner & He, 2009; He, 1998; Ogawa, Handa, Ashida, Suzuki, & Sasakawa, 2008). Thus, we investigated whether pathogenic proteins required to infect animal hosts are also required for invasion and proliferation of *Shigella* in plants. To study the biological role of the T3SS during the interaction between *Shigella* and plants, we used non-invasive variants of *S. flexneri* 2a and *S. flexneri* 5a (strains Δvp and BS176, respectively; Sansonetti, Kopecko, & Formal, 1982; Shim et al., 2007; Wenneras et al., 2000). To facilitate observation of bacterial invasion in living plants, non-invasive variants of *Shigella* strains were also labelled with GFP; we then observed bacterial growth and expression of effector proteins (Figures 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-labelled *Shigella* strains and mutants (Figure 4a). To examine involvement of the T3SS in bacterial invasion of the plant surface, leaves were flood inoculated with GFP-labelled bacteria, and leaf surfaces were observed 24 hr later (Figure 4b). Examination under UV light revealed that the levels of GFP-labelled *S. flexneri* 2a and *S. flexneri* 5a on leaf surfaces were frequently observed than those of Δvp and BS176, especially in open stomata (Figure 4b). We also generated a nonpathogenic mutant of *S. sonnei* and tested whether severe infection by *S. sonnei* strain is T3SS dependent (Figure S4). When plants were inoculated with this T3SS-deficient *S. sonnei* strain, disease phenotypes and bacterial growth were reduced significantly (Figure S4), as observed with Δvp and BS176. Altogether, these results suggest that the T3SS of *Shigella* that operate during infection of humans is also required for colonization of plants.

To further investigate involvement of *Shigella* T3S effectors in plant interactions, we measured expression of critical virulence effectors

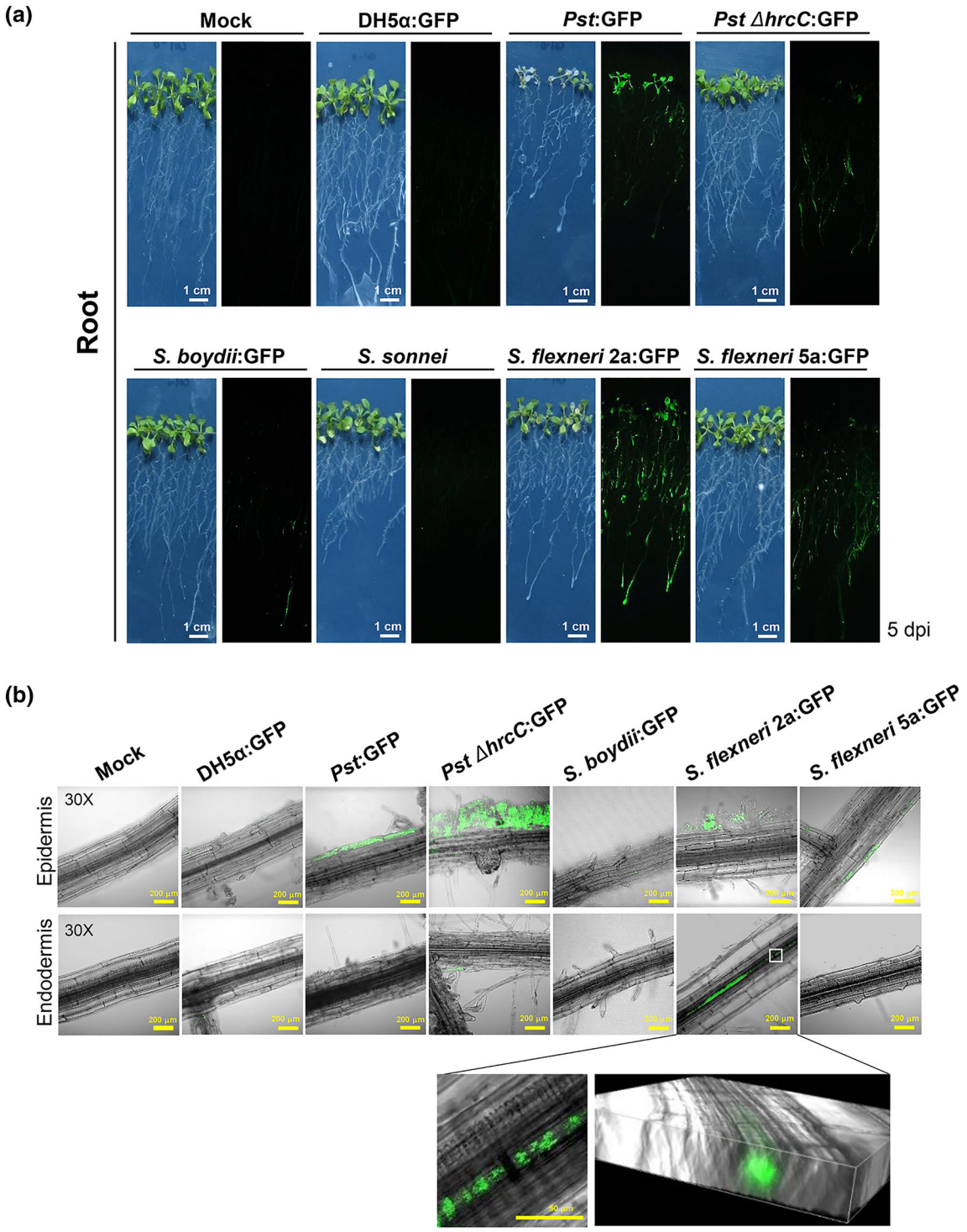

FIGURE 3 *Shigella flexneri* 2a invades and colonizes plant roots. (a, b) Root tips of *Arabidopsis* seedlings were drop inoculated with GFP-labelled bacterial suspensions (5×10^7 cfu/ml). (a) Bacterial colonization of inoculated plants was photographed under white and ultraviolet light at 5 dpi. (b) GFP-labelled *Shigella* localized in the epidermal or endodermal cells of *Arabidopsis* roots. GFP images were taken using a confocal microscope. Higher magnification micrographs and 3D Raman confocal volume images show internalization of *S. flexneri* 2a in *Arabidopsis* roots. All experiments were repeated at least three times, and representative results are shown

related to human pathogenesis, including *ipaB*, *ipaC*, *icsA*, *icsB*, *virB*, and *virF*, in *S. flexneri* 2a-, *S. flexneri* 5a-, Δvp-, and BS176-infected *Arabidopsis* plants (Bando et al., 2010). These effectors play a role in mammalian cell lysis (*ipaB* and *ipaC*), intracellular spread (*icsA* and *icsB*), and regulation of virulence factors (*virF* and *virB*; Ogawa et al., 2008). Total RNAs were isolated from *Arabidopsis* leaves at 6 and 12 hr after *Shigella* inoculation, and changes in expression of virulence genes were confirmed by qRT-PCR (Figure 4c). Expression of all *Shigella* virulence genes examined in wild-type (WT) *S. flexneri* 2a- or *S. flexneri* 5a-inoculated *Arabidopsis* leaves increased. Interestingly, induction

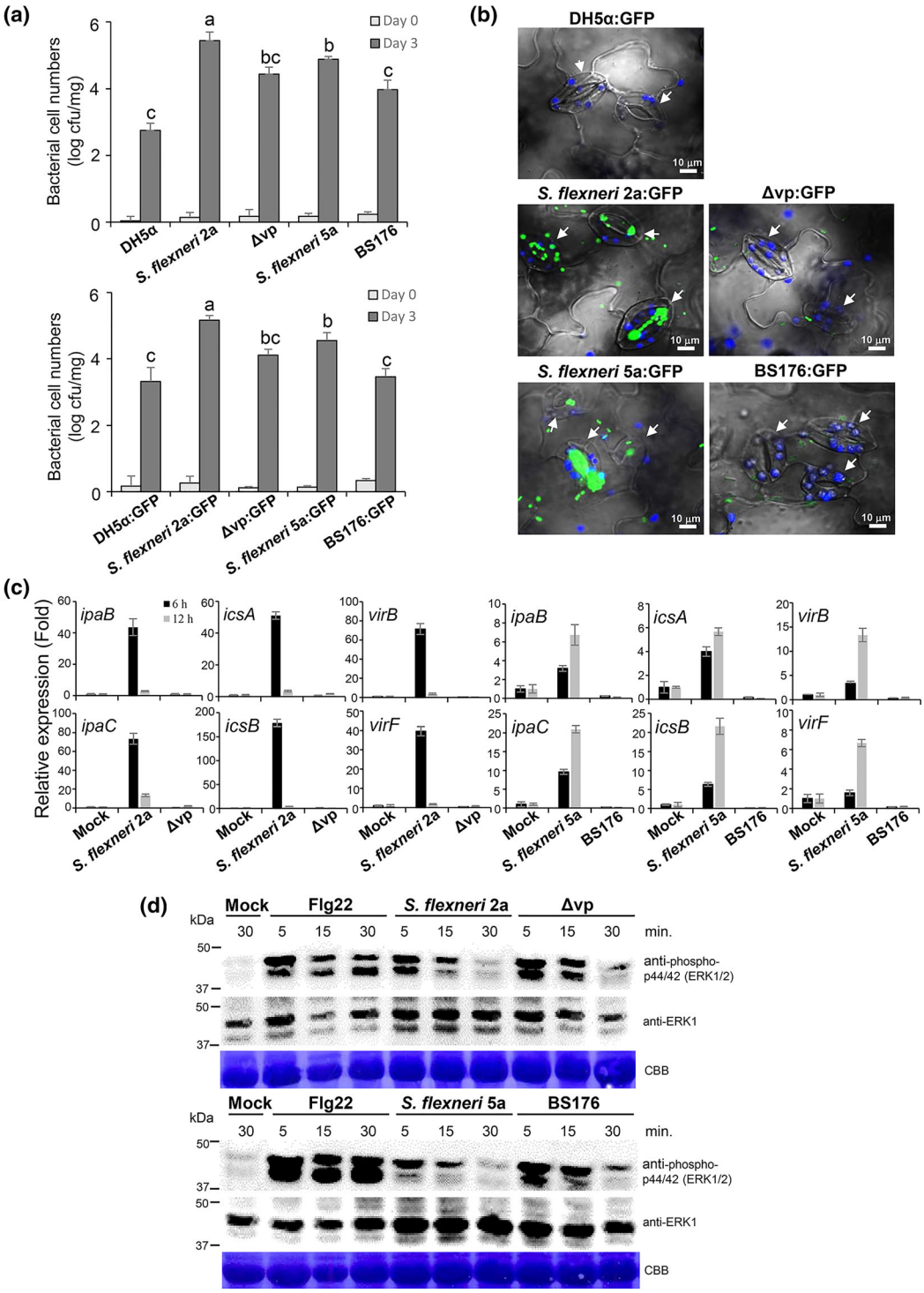

FIGURE 4 A virulence plasmid-deficient mutant of *Shigella* shows impaired proliferation in *Arabidopsis*. (a, b) *Arabidopsis* seedlings grown in half-strength MS medium were flood inoculated with *Shigella flexneri* 2a, Δvp, *S. flexneri* 5a, or BS176 and their GFP-labelled variants (5×10^5 cfu/ml). (a) The number of bacteria cells *in planta* was determined at 0 and 3 dpi. Bars represent the mean ± *SD* of three replicates, and different letters indicate significant differences between samples ($P < .05$, one-way analysis of variance). (b) Images of GFP-labelled *Shigella* in leaf epidermal cells of *Arabidopsis* acquired at 24 hpi under a fluorescence confocal microscope. Bar, 10 μm. Blue represents auto-fluorescence of chlorophyll. All experiments were repeated at least three times, and representative results are shown. (c, d) *Arabidopsis* leaves were syringe infiltrated with buffer, 1-μM flg22, or bacterial suspension (5×10^6 cfu/ml), and samples were collected at the indicated times. (c) Transcription of *Shigella* effectors (*ipaB*, *ipaC*, *icsA*, *icsB*, *virB*, and *virF*) in inoculated *Arabidopsis* leaves was analysed by qRT-PCR. qRT-PCR results were normalized to expression of 16s rRNA. Expression of effectors by plasmid-deficient mutant strains was compared with that by wild-type cells. Data are expressed as the mean ± *SD* of three replicates. (d) Immunoblotting was performed using either antiphospho-p44/42 (ERK1/2, upper panels) or anti-ERK1 (lower panels) antibodies. All experiments were repeated at least three times, each with similar results

was higher and faster in *S. flexneri* 2a-treated plants than in *S. flexneri* 5a-treated plants, which is in agreement with the earlier results showing that *S. flexneri* 2a was more pathogenic to plants than *S. flexneri* 5a. Expression of virulence genes was not detected in plants inoculated with nonpathogenic 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 plant innate immune responses to *Shigella* inoculation, we evaluated MAPK phosphorylation. Activation of MAPK by phosphorylation is a conserved response 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). By contrast, MAPK phosphorylation in plants treated with *S. flexneri* 2a or *S. flexneri* 5a was reduced; from 15 min onward, it was strongly suppressed, and it almost completely disappeared after ~30 min (Figure 4d). Meanwhile, plants treated with the virulence plasmid-deficient mutants Δvp or BS176 showed recovery of MAPK activation, which was in contrast to *S. flexneri* 2a- or *S. flexneri* 5a-treated plants; however, the degree and duration of phosphorylation were lower than those elicited by flg22 treatment (Figure 4d). These observations indicate that *Shigella* T3SS suppresses innate immune responses in *Arabidopsis*.

3.4 | Suppression of immune signalling in *Arabidopsis* plants by *Shigella* T3S effectors OspF and OspG

Shigella OspF inhibits MAPK signalling, 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). Expression of the two effector genes in *S. flexneri* 5a-inoculated plants was observed by qRT-PCR (Figure S5). Therefore, we examined whether OspF and OspG have virulence activity in plants by introducing them heterologously into a phytopathogen, *Pst*, and then monitoring its pathogenicity. First, we used an AvrRpt2-derived T3SS reporter system (Figure S6A; Mudgett, 2005). AvrRpt2$^{101-255}$ lacks the N-terminal region for secretion into plant cells and induces RPS2-dependent hypersensitivity responses only when a translocation signal is added (Vinatzer et al., 2005). When *Arabidopsis* leaves were syringe infiltrated with various *Pst* strains expressing effectors fused to AvrRpt2$^{101-255}$, we observed that *Pst* producing OspF:AvrRpt2$^{101-255}$ or OspG:AvrRpt2$^{101-255}$ successfully induced cell death 1 day after delivery, implying that both OspF and OspG were delivered successfully into *Arabidopsis* cells via the *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 S6B,C). 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 that in 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.

By contrast, in vitro studies demonstrated that OspF exerts phosphothreonine lyase activity and removes phosphate groups from MAPK irreversibly (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} (Kim & Zhang, 2004; Yang, Liu, & Zhang, 2001). 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 marked cell death (Figure S7A). Co-production of OspF:GFP, but not OspG:GFP, and MEK2^{DD} completely inhibited MEK2^{DD}-induced cell death (Figure S7A). Production of the two effectors fused with GFP was then evaluated in MEK2^{DD}-producing plant leaves; production of OspF:GFP was apparent, but that of OspG:GFP was not (Figure S7B), even though both proteins were stably produced in the absence of MEK2^{DD} (Figure S8B). Degradation of the OspG protein might be associated with activation of MAPK. Indeed, as assessed by immunoblotting with specific antiphosphorylated 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 S7B). Production of MEK2^{DD} protein in all samples was confirmed using anti-HA antibodies (Figure S7B). 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.

3.5 | 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. flexneri* 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. flexneri* 5a Δ*ospF* or *S. flexneri* 5a Δ*ospG* 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 activation of MAPKs to determine whether plant immunosuppression was affected by deletion of *ospF* or *ospG*. As shown in Figure 6b, *S. flexneri* 5a Δ*ospF* induced stronger phosphorylation of MAPKs than WT *S. flexneri* 5a; this was offset by complementation with OspF. By contrast, phosphorylation of MAPK in *S. flexneri* 5a Δ*ospG*-inoculated plants was no different from that

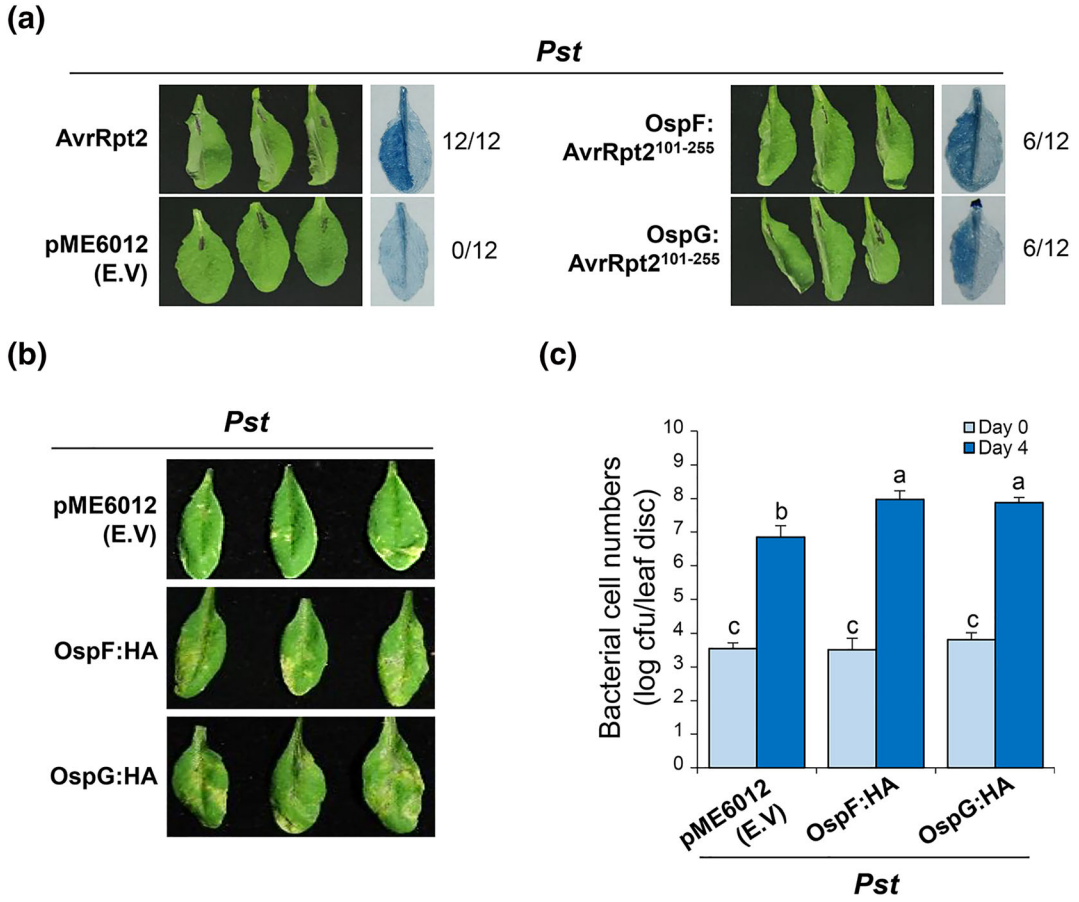

FIGURE 5 Increased virulence of the phytopathogen *Pst* in the presence of *Shigella* effector OspF or OspG. (a) *Pst* strains expressing the *Shigella* effector proteins were used for effector secretion assay in *Arabidopsis*. *Arabidopsis* leaves were syringe infiltrated with *Pst* cells (5×10^7 cfu/ml) producing AvrRpt2, cells carrying the empty vector (pME6012), or cells producing OspF:AvrRpt2$^{101-255}$ or OspG:AvrRpt2$^{101-255}$. The hypersensitivity response in infiltrated leaves was monitored using trypan blue staining and photographed at 1 dpi. (b, c) *Arabidopsis* plants were sprayed with *Pst* cells carrying an empty vector (pME6012) or with cells producing OspF:HA or OspG:HA (5×10^7 cfu/ml). (b) Disease symptoms were monitored for 4 days after spraying. (c) Proliferation of *Pst* cells producing *Shigella* effector proteins in *Arabidopsis* at 0 and 4 days after spray inoculation. Bars represent the mean ± *SD* of six replicates, and different letters indicate significant differences between samples ($P < .05$, two-way analysis of variance). All experiments were repeated at least three times

in plants inoculated with WT *S. flexneri* 5a (Figure 6b). These results are consistent with inhibition of MAPK-induced immune responses by OspF, but not by OspG, using the plant expression binary vector described above (Figure S7). Taken together, the results indicate that *Shigella* effectors OspF and OspG play an important role in increasing bacterial proliferation in both plant and animal hosts.

3.6 | Delivery of OspF and OspG into plant cells after *Shigella* inoculation

To visualize direct delivery of type III effectors to plant cells by *Shigella*, we took advantage of a newly developed split superfolder GFP (sfGFP^{OPT}) system (Park et al., 2017). In this system, the nonfluorescent GFP protein (GFP1–10) harbouring β-strands 1–10 is expressed in the host cell, and the bacterial effector is linked to the 11th β-strand (GFP11). Only when the effector fused to GFP11 is introduced into plant cells via bacterial T3SS is a fluorescent signal induced through reconstitution of GFP (Park et al., 2017; Figure 7a). First, to select a host plant expressing GFP1–10 capable of exploring the delivery of OspF and OspG, we observed subcellular localization of OspF:GFP and OspG:GFP by expressing them in *Nicotiana benthamiana* leaves using the *Agrobacterium* system (Figure S8). 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 S8C). However, immunoblot analysis of OspG:GFP expression in *N. benthamiana* revealed both OspG:GFP and free GFP (Figure S8B), suggesting that overexpression of free GFP might be responsible for cytosolic GFP expression. On the basis of these results, we chose *Arabidopsis* plants expressing sfGFP1–10 (sfGFP1–10^{OPT}) in the nucleus or cytosol and OspF and OspG fused to the 11th β-strand of sfGFP, respectively (Figure S9A). GFP signals were observed in the nucleus of both *S. flexneri* 5a::OspF-sfGFP11

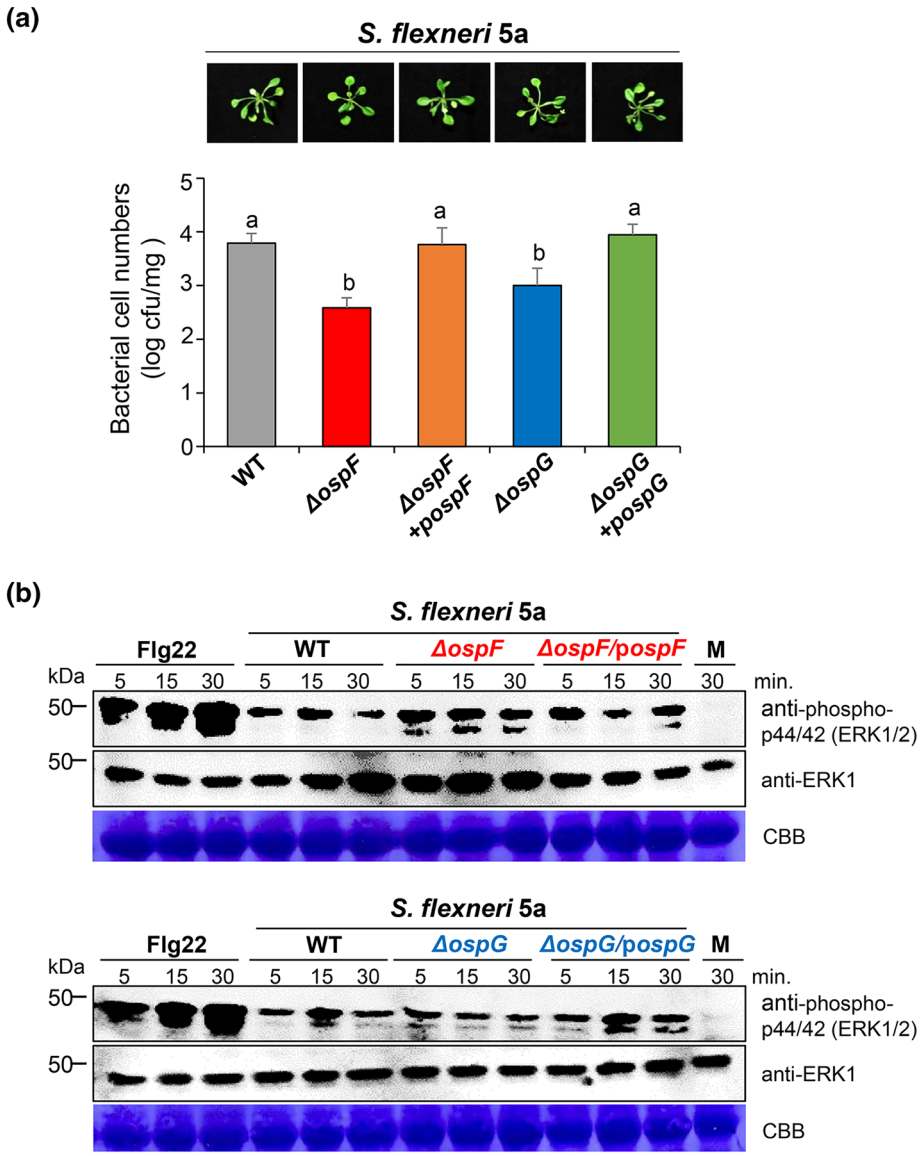

FIGURE 6 The *Shigella* effector proteins OspF and OspG underpin bacterial virulence in plants. (a) The virulence of the OspF- or OspG-deficient *Shigella* mutants in the *Arabidopsis* model is impaired. *Arabidopsis* seedlings grown in half-strength MS medium were flood inoculated with *Shigella flexneri* 5a, Δ*ospF*, Δ*ospF* + p*ospF*, Δ*ospG*, or Δ*ospG* + p*ospG* (5×10^5 cfu/ml). Photographs of representative disease symptoms were taken, and the number of bacteria *in planta* was determined at 3 dpi. The bars represent the mean ± *SD* of three replicates. Different letters indicate significant differences between samples ($P < .05$, one-way analysis of variance). (b) MAPK activation in *Arabidopsis* plants in response to infection by ospF or ospG deletion mutants. *Arabidopsis* leaves were infiltrated with each bacterial strain, as described previously, and then collected at the indicated times. Antiphospho-p44/42 (ERK1/2, upper panels) or anti-ERK1 (lower panels) antibodies were used for immunoblotting. All experiments were repeated at least three times, each with similar results

and *S. flexneri* 5a::OspG-sfGFP11 flood-inoculated *Arabidopsis* leaves at 3 hpi; by contrast, control *S. flexneri* 5a WT did not show a GFP signal (Figure 7b). Furthermore, when *Arabidopsis* seedlings expressing cytosolic sfGFP1-10^{OPT} were flood inoculated with *S. flexneri* 5a producing OspF-sfGFP11 or OspG-sfGFP11, we detected no reconstituted GFP signals in any of the tested bacteria (Figure S9B). This suggests that *Shigella* effectors OspF and OspG were secreted successfully into *Arabidopsis* cells through the *Shigella* T3SS. In the host human cell, OspF localization is nuclear (Arbibe et al., 2007; Zurawski, Mitsuhata, Mumy, McCormick, & Maurelli, 2006) whereas that of OspG is both nuclear and cytoplasmic (de Jong & Alto, 2014; Kim et al., 2005; Zhou, Dong, Hu, & Shao, 2013); this confirms that subcellular localization in plant cells is similar to that in animal cells. Taken together, the data suggest that *Shigella* effectors OspF and OspG can be delivered into plant cells through the T3SS.

4 | DISCUSSION

Here, we investigated the interaction between the human pathogenic bacterium *Shigella* and an alternative host, the *Arabidopsis* plant. We demonstrated that four *Shigella* strains, *S. boydii*, *S. sonnei*, *S. flexneri* 2a, and *S. flexneri* 5a, invade and proliferate in *Arabidopsis*, albeit to

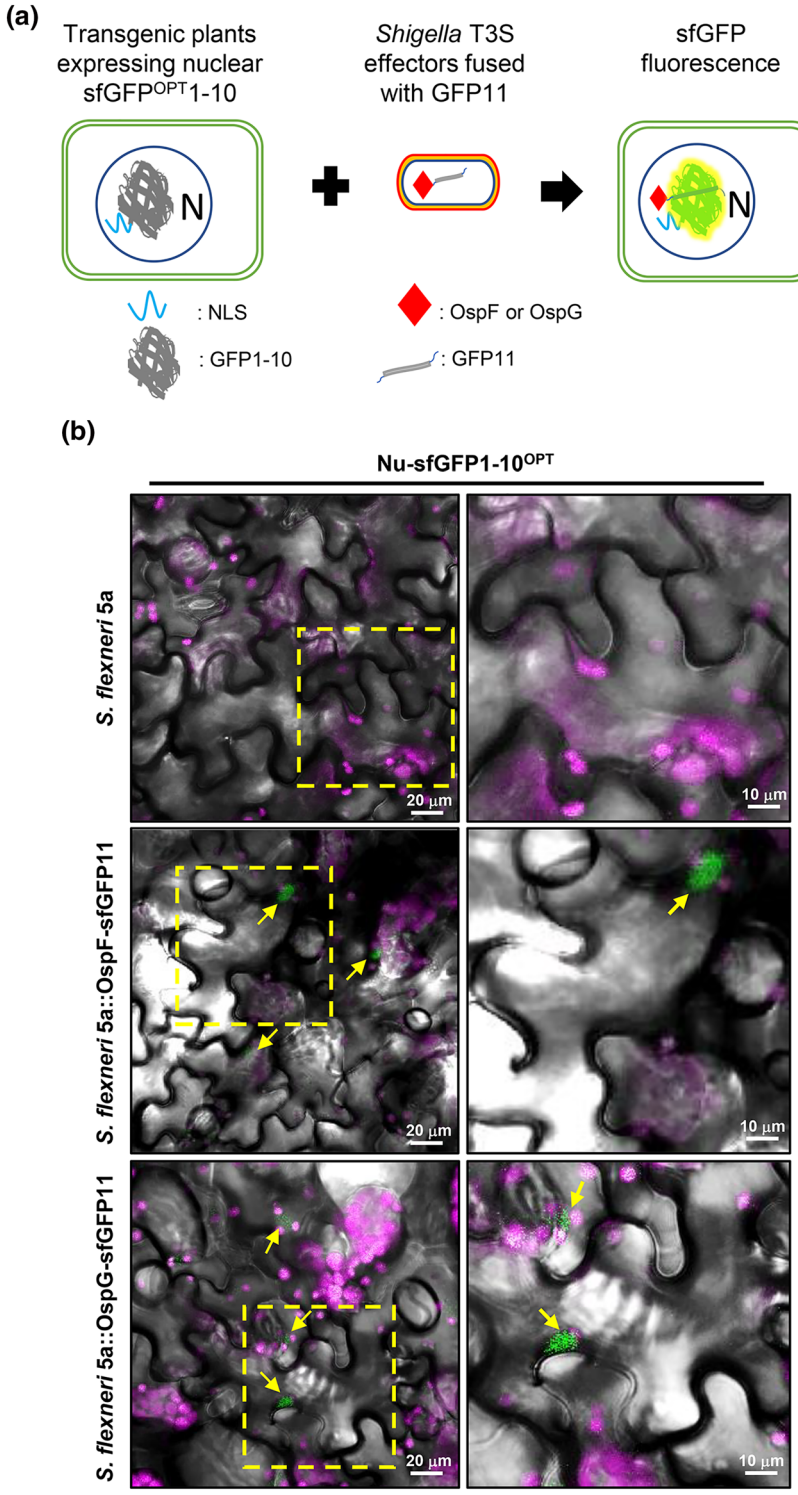

FIGURE 7 *Shigella* T3S effectors OspF and OspG plus sfGFP11 are delivered into *Arabidopsis* via *Shigella flexneri* 5a. (a) Illustration of a split sfGFP^{OPT} system used to monitor T3SS-mediated transfer of bacterial effectors to the nucleus of host cells. NLS, nuclear localization signal. (b) Transgenic *Arabidopsis* seedlings expressing sfGFP1-10^{OPT} grown in half-strength MS medium were flood inoculated with *S. flexneri* 5a containing OspF-sfGFP11 or OspG-sfGFP11 (5×10^5 cfu/ml). At 3 hr postinoculation, reconstituted sfGFP signals in the nucleus of *Arabidopsis* leaf epidermal cells were observed under a fluorescence confocal microscope. Yellow arrows indicate complemented sfGFP signals in the nuclei. Yellow perforated-boxed regions are magnified in the right column. Magenta represents auto-fluorescence of chlorophyll. Bar, 10 and 20 μm

different extents. Variability of plant interaction mechanisms among strains, for example, adherence and proliferation, might be a major contributor to the different symptoms and bacterial proliferation rates observed for the four *Shigella* strains. Differences in the nutritional requirements of bacterial strains may be another reason for differences in the growth rates within the *Arabidopsis* host.

In humans, *Shigella* initially enters the epithelial layer via M cells (through transcytosis), leading to the invasion of the basolateral surfaces of intestinal epithelial cells. Subsequent gut inflammation activates the innate immune response (Phalipon & Sansonetti, 2007). Here, we demonstrated that *Shigella* invades *Arabidopsis* through vascular tissues and leaf stomata (Figures 2–4). In particular, *S. sonnei* and *S. boydii* formed relatively wide clusters in the surrounding areas, including guard cells (Figure 2); this is similar to *Pst*, which has a host–pathogen relationship with *Arabidopsis* (Panchal et al., 2016). Interestingly, we found that the four strains of *Shigella* studied herein associate with plant cells and induce different plant responses. The bacterial loads of *S. sonnei in planta* were higher than those of the other strains. By contrast, inoculation of *S. flexneri* 5a was associated with lower bacterial proliferation and less severe symptoms than observed for other strains (Figures 1–3). *S. flexneri* 2a and *S. flexneri* 5a, which belong to the same serogroup (Lindberg et al., 1991), elicited distinctly different plant responses with respect to disease symptoms, suggesting that virulence 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.

Many Gram-negative bacterial pathogens utilize common infection strategies to colonize and invade plant and animal cells, and pathogenicity appears to depend on highly conserved T3SSs, which deliver the effector proteins to host cells (Buttner & Bonas, 2003). By using avirulent mutant strains, we were able to show that effectors that regulate pathogenesis of shigellosis in humans also play a central role in regulating interactions with *Arabidopsis*. We showed that secretion of T3S effectors is required for proliferation and attachment of *Shigella* in plants (Figures 4 and S4). Furthermore, the effector proteins affected MAPK-dependent/independent immune responses in *Arabidopsis* (Figures 4 and 6). These observations further support the suggestion that the function of the main effector proteins in *Shigella* appears to be conserved in plant and animal hosts and that this contributes to intracellular survival of bacteria or to suppression of host defence against the pathogen. Reduced colonization of plants by T3SS-deficient pathogenic *E. coli* was reported previously; indeed, reports suggest that *E. coli* uses the T3SS apparatus to attach to leaves rather than for growth inside plants (Shaw et al., 2008). The relevance of the T3SS to multiplication of *Salmonella* in plants remains unclear due to the different effects of T3SS function on *Salmonella*–plant interactions (Iniguez et al., 2005; Schikora et al., 2011; Shirron & Yaron, 2011).

In general, animal bacterial pathogens with short needle lengths are thought to have difficulty secreting T3S effectors into plant cells. Recently, however, several studies reported that the length and width of bacterial T3SS depends on the type of host cell, the environment, and the infection cycle of the bacterium (Deane, Abrusci, Johnson, & Lea, 2010). Here, we used the recently developed split GFP technology to provide direct evidence that the effector proteins of *Shigella* are delivered to plant cells through the T3SS (Figure 7). Further studies that identify plant factors that trigger and control assembly of the *Shigella* T3SS will be of great help when developing a strategy to prevent the spread of *Shigella* via plants.

Expression of the *Shigella* T3SS is regulated at the transcriptional level and is activated at a permissive temperature (≥32°C; Campbell-Valois & Pontier, 2016; Tobe et al., 1991). We were able to observe expression of the T3SS genes of *Shigella* at temperatures below which plants grow (22 ± 3°C; Figure 4c). A recent study showed that an increase in temperature increases T3SS-mediated virulence of the phytopathogen *Pst* in plants, which is in contrast to 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 other than plant temperature that influence T3SS gene expression.

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, Kramer, & Hao, 2011; Golberg, Kroupitski, Belausov, Pinto, & Sela, 2011; Schikora et al., 2011). We demonstrated that *Shigella* strains actively colonize both the surface and inside of *Arabidopsis* leaves and root tissues (Figures 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 by *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 concerns 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 insight 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 animal cells (Figures 7 and S8C), and it was apparent that production of both proteins increased the virulence of plant pathogens (Figure 5). In addition, we confirmed that OspF inhibits the innate immune response of plants via the same enzymatic activity observed in animals (Figures 6 and S7). In animals, OspG inhibits the host immune response by suppressing activity of NF-κB by blocking degradation of IκB (Ashida, Mimuro, & Sasakawa, 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 that OspG increases the phytopathogenicity of non-*Shigella* bacteria (Figure 5), along with degradation of OspG:GFP upon constitutive activation of MAPK signalling (Figure S7B). Hence, the existence of a plant immune signalling pathway similar to NF-κB in animals, which would also be the target of OspG, may

be assumed. Characterization of previously unrecognized stress-activated mediators of innate immunity in plants upon infection with food-borne pathogens would help us to define the defensive functions of plants. Finally, characterization of plants as an alternative host for food-borne pathogens will be critical for developing effective means of preventing their transmission.

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AUTHOR CONTRIBUTIONS

J. M. P., S. H. J., and J. L. conceived and designed the study. S. H. J., J. L., and D. H. L. carried out the experiments. J. M. P., S. H. J., J. L., D. W. K., and E. P. analysed the data. J. M. P., S. H. J., J. L., and E. P. wrote the manuscript. J. M. P., S. H. J., E. P., C. M. R., and D. C. edited and discussed the manuscript. All authors approved the final version.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Bacterial strains and plasmids used in this study.

Table S2. Sequences of PCR primer used for Gateway cloning.

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

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

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

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

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

Figure S5. Expression of o*spF* and o*spG* in *Arabidopsis* plants inoculated with *S. flexneri* 5a WT or BS176, respectively.

Figure S6. Expression of OspF or OspG in *Pst* for virulence assay.

Figure S7. Co-expression of OspF:GFP suppresses tobacco MEK2DD-triggered cell death.

Figure S8. Expression GFP fused OspF or OspG in plant.

Figure S9. Delivery of OspF or OspG fused with GFP11 through T3SS

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