Human pathogenic bacterium *Shigella* infects *Arabidopsis* plants using type-III effectors that suppress conserved MAP kinase signaling

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Abstract

Although human enteropathogenic bacteria can survive both on the surface of and inside plants, little is known about the molecular interactions between bacteria and plants. Here, we examined the interaction between the human pathogenic bacterium *Shigella* and the plant *Arabidopsis*. Four *Shigella* strains actively proliferated in, and showed virulence against, *Arabidopsis*. Using mutants lacking type-III secretion systems (T3SS), we showed that T3SS that regulate the pathogenesis of shigellosis in humans also play a central role in pathogenesis in *Arabidopsis* by inhibiting mitogen-activated protein kinase (MAPK)-mediated plant immune responses. Using *Shigella* mutants deficient for a single T3S effector, we showed that the virulence activity of T3S effectors (OspF or OspG) was needed for pathogenicity of *Shigella* in *Arabidopsis*. Production of OspF in plant cells suppressed a constitutively active form of tobacco mitogen-activated protein kinase kinase 2 ($MEK2^{DD}$)-triggered cell death by inhibiting phosphorylation of MAPK, indicating that suppression of MAPK signaling activation by OspF is conserved in both human and plants. These findings demonstrate for the first time that determinants of *Shigella*-mediated virulence and pathogenic symptoms are expressed in both plant and mammalian models.

Keywords: *Arabidopsis thaliana*, Effectors, Enteropathogenic bacteria, MAPK signaling, *Shigella*, Type-III secretion system

Introduction

Shigella species are Gram-negative bacteria from the *Enterobacteriaceae* family. They are non-motile, rod-shaped, facultative intracellular and invasive pathogens, 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 & Hilbi, 2008). *Shigella* spp. are important epidemic pathogens and a serious public health concern in developed and developing countries. Although about 164,300 deaths in all age groups, and 54,900 deaths in children younger than 5 years, were reported globally in 2015, the actual number of infections might be higher because mild symptoms are not reported (Mortality & Causes of Death, 2016). This means that the microorganism may employ a variety of survival strategies not only for infection of animals but also for survival in a restricted host such as a plant. In recent years, the morbidity or mortality of the infection caused by the emergence of *Shigella* that produces β-lactamase, an enzyme that underpins the resistance to the third-generation antibiotics cephalosporins, has been increasing (Zhang *et al.*, 2011).

Shigella is a human-adapted pathogen that infects the host via multiple transmission routes. 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 (Franz *et al.*, 2008; Ferens & Hovde, 2011). 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 & Ashenafi, 2010; Semenov *et al.*, 2010; Gu *et al.*, 2013b). 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; Cevallos-Cevallos *et al.*, 2012; Gu *et al.*, 2013a). Although *Shigella* contamination has also

been reported in plants, 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 & 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 & 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, SpvC, one of the T3S effector proteins of *S. enterica*, targets the MAPK signaling system in both plant and animal hosts to suppress the host PTI (Neumann *et al.*, 2014).

Here, we examined the ability of four *Shigella* strains [*S. sonnei* (*S. s*) (Holt *et al.*, 2012), *S. boydii* (*S. b*), *S. flexneri* 2a (*S. f* 2a) (Wei *et al.*, 2003), and *S. flexneri* 5a (*S. f* 5a) (Onodera *et al.*, 2012)] to proliferate in *Arabidopsis* plants. We found that the bacteria actively invaded and proliferated in plant root and leaf tissues. Proliferation of mutants lacking the T3S effectors, i.e., noninvasive human strains, was reduced both *in vitro* and *in planta*. Reverse genetics and molecular biology experiments demonstrated that the *Shigella* T3S effectors OspF and OspG were delivered to plant cells and functioned as pathogenic proteins. 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 for 2 min in 70% (v/v) ethanol, incubated in 50% household bleach for 10 min, washed extensively with sterile deionized water, and planted 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 & Skoog, 1962). *Nicotiana benthamiana* plants were germinated and 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 (#FLG22-P-1; Alpha Diagnostics, Inc., San Antonio, TX, USA) was used as a positive control in the experiments (Bethke *et al.*, 2009).

Bacterial strains, growth conditions, and plasmids

The bacterial strains and plasmids used in the study are described in Supporting Table S1. *Shigella and Pseudomonas* strains harboring plasmid pDSK-GFPuv were generated by electroporation as previously described (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 & 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). *Escherichia coli* DH5α was grown in LB medium at 37°C with shaking (Kennedy, 1971) and used as a non-pathogenic control. *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 (Supporting Table S2), and the PCR fragments were cloned into the pDONR™207 vector using the pENTR Directional TOPO Cloning kit (Invitrogen, Carlsbad, CA, USA). The products were then transferred to the 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). The products were also transferred to pK7FWG2 (obtained from Ghent University, Belgium) by LR recombination to produce the green fluorescent protein (GFP)-fused *Shigella* effectors OspF:GFP and OspG:GFP (Karimi *et al.*, 2002).

Bacterial inoculation assay *in planta*

Leaves (4 weeks old) from *Arabidopsis* plants grown in soil were infected with each strain of bacteria by syringe infiltration (5×10^7 or 5×10^5 cfu/ml) or spray-inoculation (5×10^7 cfu/ml) (Katagiri *et al.*, 2002). After infection, plants were covered with a clear lid to

maintain humidity and transferred to a growth room (22 ± 3°C, 16 h light/8 h dark). The bacterial populations were measured via serial dilution assays (Katagiri *et al.*, 2002). Colony forming units (cfu) were normalized to cfu/mg using the total weight of inoculated leaves. Bacterial populations were evaluated on three plants per treatment (as biological replicates) and in three independent experiments conducted successively.

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 (5×10^5 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 pathogenicity, 10-d-old *Arabidopsis* seedlings grown vertically in 1/2 MS medium were infected by dropping 2.0 μl of bacterial suspension (5×10^7 cfu/ml) onto the root tips. Disease symptoms were observed under white light (WL) at 7 or 14 dpi, and bacterial proliferation was monitored at 7 dpi by observation of GFP-expressing bacteria under ultra violet (UV) light. Three biological replicates were generated in separate plates, and three independent experiments were conducted under the same conditions.

H_2O_2 and cell death staining

Bacterial suspensions (5×10^7 cfu/ml) were injected into one half of each *Arabidopsis* leaf using a syringe infiltration method. Accumulation of H_2O_2 in infected leaves was monitored using a 3,3′-diaminobenzidine (DAB) staining method (Thordal-Christensen *et al.*, 1997). At 12 h post-infiltration, the leaves were cut and incubated with 1 mg/ml DAB (Sigma-Aldrich) solution for 12 h as previously described (Thordal-Christensen *et al.*, 1997). Cell death was observed by trypan blue staining as described previously (Koch & Slusarenko, 1990).

Microscopy

For scanning electron microscopy (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 transmission electron microscopy (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 & 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 (#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*) (Bando *et al.*, 2010).

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 (#4377; Cell Signaling Technology, Danvers, MA, USA), ERK1 (#sc-94; Santa Cruz, Dallas, TX, USA), hemagglutinin (HA) (#S2930; Clontech Laboratories, Mountain View, CA, USA), or GFP (#sc-9996; Santa Cruz) 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).

Agrobacterium-mediated transient gene expression

A. tumefaciens strain GV2260 harboring the *GFP*, *OspF:GFP*, or *OspG:GFP* genes driven by the 35S promoter was prepared as described previously (Lee *et al.*, 2013). The inoculum (OD_{600} = 0.4) was infiltrated into 4-week-old *N. benthamiana* leaves using a 1 ml needleless syringe. To observe $MEK2^{DD}$-triggered cell death suppression by *Shigella* effectors, *Agrobacterium* (OD_{600} = 0.4) expressing HA:$MEK2^{DD}$ was mixed with *Agrobacterium* containing *GFP*, *OspF:GFP*, or *OspG:GFP* at a 1:1 ratio and infiltrated into the leaves of *N. benthamiana*. Each experiment was repeated using at least three leaves from the plant, and each experiment included at least three different plants.

Statistical analysis

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

Results

Disease severity and symptomatic development in *A. thaliana* according to the infection method and *Shigella* infection load

To test whether the human pathogen *Shigella* is also a phytopathogen, we investigated the interaction of four *Shigella* strains representing three serogroups (*S. b*, *S. s*, *S. f* 2a, and *S. f* 5a) with the model plant *A. thaliana*. Inoculation of *Arabidopsis* plant leaves with high-density suspensions of *Pseudomonas syringae* pv. *tomato* DC3000 cells enables observation of the plant immune response (e.g., production of reactive oxygen species) (Chinchilla *et al.*, 2007; Smith & Heese, 2014) and necrosis (Katagiri *et al.*, 2002). Hence, we first inoculated *Arabidopsis* leaves with high-density bacterial cell suspensions (≅ 5×10^7

cfu/ml) using syringe-pressure infiltration. This method is an artificial method of injecting pathogens directly into the intercellular space of the plant cells; it is widely used to reduce variation in plant cell immune responses among infiltrated plants (Liu *et al.*, 2015). *Pst hrcC*$^-$, a mutant lacking the T3SS of *Pst*, and the non-pathogenic bacterium *E. coli* DH5α were used as negative controls. *Arabidopsis* leaves infected with *Pst* showed an apparent accumulation of H_2O_2 [at 1 d post-infection (dpi)] and necrosis (at 3 dpi), whereas no such responses were observed in leaves treated with the buffer or either of the two non-pathogenic bacteria (Fig. 1a). In the majority of leaves that had been syringe-infiltrated with *S. s* and *S. f* 2a, cell death, i.e., wilting and obvious accumulation of H_2O_2, was observed (Fig. 1a). On the other hand, infection with *S. b* and *S. f* 5a resulted in a slight production of H_2O_2 compared with the buffer treatment or the non-pathogenic bacterium treatments; unlike the other *Shigella* strains investigated, no apparent cell death was observed (Fig. 1a). These observations indicated that the bacterium can elicit an immune response in plants, and that the extent of the response is strain-dependent.

Shigella is commonly known as a water-borne pathogen, with infection spread through contaminated water (Pandey *et al.*, 2014). Hence, we chose to use a flood-inoculation approach (Ishiga *et al.*, 2011), which is thought to closely mimic natural infection. Similarly to *Pst*, when 2-week-old *A. thaliana* seedlings were inoculated with *S. s* or *S. f* 2a, clear disease phenotypes were observed, such as yellowing and necrosis of the leaves (Fig. 1b). On the other hand, obvious symptoms of disease were not apparent with *S. b* and *S. f* 5a, such as after syringe-pressure inoculation (Fig. 1b). 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 was more than 10 times lower than that of *Pst* and *Pst hrcC*$^-$ (Fig. 1c). However, 3 d after the inoculation, the extent of cell amplification differed depending on the *Shigella* strain; nevertheless, the cell numbers of all strains increased over 1 000 times. Notably, *S. s* cell number increased over 10^5 times. This level of proliferation was comparable to that of plant pathogens.

Previously published studies demonstrated that several human pathogenic bacteria, including *E. coli* O157:H7 and *Enterococcus faecalis*, can infect the leaves and roots of *A. thaliana* (Jha *et al.*, 2005; Deering *et al.*, 2012). To determine whether the selected *Shigella* strains could be transmitted through *A. thaliana* roots, we investigated if these strains existed within the *Arabidopsis* roots, and if the plants could be infected via the roots. The tissues of the flood-infected *Arabidopsis* plants were split into the aerial and root sections, and the

propagation of *Shigella* was assessed in each tissue type; a large number of bacteria were detected in the former and also latter tissue types (Fig. S1). In addition, dropping the bacterial solutions onto the root tips elicited similar disease symptoms to those caused by the *Shigella* strains flooding, supporting these observations (Fig. S2).

Apparently different disease phenotypes and bacterial proliferation rates were observed for different *Shigella* strains. This suggested that a variability of the infection mechanisms among the strains, e.g., the adherence and invasion efficiency, might contribute to the differences in pathogenesis. Differences in the nutritional requirements of bacterial strains may constitute another reason for the differences in the growth rates within the *Arabidopsis* host.

Plant cell entrance and internalization of *Shigella*

Since we observed that *Shigella* proliferate in *Arabidopsis*, we next directly examined the plant cell entry and intercellular presence of the bacterium using SEM and TEM. As anticipated, *Pst*, which infects plants via open stomata (Panchal *et al.*, 2016), colonized the guard cells (Fig. 2). All *Shigella* strains invaded the plant through the stomata (Fig. 2). Most plant pathogenic bacteria *in planta* infect and colonize the intercellular spaces (Abramovitch *et al.*, 2006; Gao *et al.*, 2016). To investigate whether *Shigella* is capable of intercellular colonization and induces plant responses, e.g., alteration of host cell structures, we analyzed *A. thaliana* leaves infected with *Shigella* by TEM (Fig. 3). Indeed, the TEM images revealed *Shigella* colonization of the intercellular spaces and bacterial attachment to the host cell walls. 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 (Fig. 3). This effect was most pronounced in plant cells infected with *S. b* and *S. s*; further, *S. s* and *S. b* were more commonly found in the intercellular spaces than *S. f* 2a and *S. f* 5a.

In a previous study, *Salmonella* was labeled with GFP and its presence in plant cytoplasm was reported (Schikora *et al.*, 2008). We also labeled four *Shigella* strains with GFP and then observed them in live plant tissues to see whether plant penetration by *Shigella* occurs via the guard cells. *S. b*, *S. f* 2a and *S. f* 5a strains were successfully labeled with GFP (the *S. s* strain was not) and then inoculated into the plant using the flood-inoculation method. The leaf surface was then observed under a confocal microscope (Fig. S3). Similar to the previous SEM results, GFP-labeled *Shigella* were observed in the guard cells, and strain *S. b*

was more abundant than strains *S. f* 2a and *S. f* 5a.

Taken together, these results indicated that, although the proliferative capacities of the *Shigella* strains differ, the cells can infect the plants through the stomata, proliferate in the intercellular spaces, and cause structural damage to the host during an infection.

T3S *Shigella* effectors are necessary for both epiphytic and endophytic growth in *Arabidopsis*

GFP was used to label the *Shigella* strains to facilitate the observation of bacterial migration in living plants. In addition, to study the role of *Shigella* T3SS in plant infection, 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). First, we verified that the GFP labeling did not visibly affect *Shigella* growth and pathogenicity (Figs 4a, S1, S4). Strains *S. f* 2a, *S. f* 5a and their GFP-labeled derivatives were used to flood-inoculate *Arabidopsis* seedlings, and their growth *in planta* was compared. Bacterial proliferation after infection with Δvp or BS176 strains was 10 times lower than that after infection by parental *Shigella* strains; similar results were confirmed when we observed growth of GFP-labeled *Shigella* strains and mutants (Fig. 4a). To more readily observe bacterial growth, taking advantage of the fluorescent tagging, the bacteria were dropped onto transparent root tissues and their proliferation was compared (Fig. 4b). Under UV light, we observed that the GFP-producing *S. f* 2a and *S. f* 5a strains colonized and multiplied along the whole root system 7 d after the inoculation (Fig. 4b). Notably, *S. f* 2a:GFP cells formed biofilm-like communities on the root surface to a greater extent than *S. f* 5a:GFP (Fig. 4b). Unlike *S. f* 2a:GFP and *S. f* 5a:GFP, strong fluorescence was not observed in the root tissues of plants treated with Δvp:GFP or BS176:GFP, suggesting that T3S effectors of *Shigella* indeed play an important role in the bacteria epiphytic growth in plants (Fig. 4b). We also confirmed that none of the *Shigella* strains grew in the plant culture medium (Fig. S5).

To determine if these microorganisms were also present inside the root cells *per se*, the roots of the infected plants were sampled and their surface was disinfected, and they were observed under a fluorescence microscope (Fig. 4c). *S. f* 2a:GFP and *S. f* 5a:GFP were both found in the proximal vascular tissue; the former also appeared to form communities in the root epidermal tissue. On the other hand, communities inside the root cells formed by the mutant strains were not readily apparent.

Altogether, these results suggested that the T3S effectors of *Shigella* that operate

during an infection in human are also required for the bacterial pathogenicity in plants. Similar observations were also made for the plant pathogen *Pst* and its T3SS deletion mutant *Pst hrcC*$^-$ (Fig. S6a, b). However, *Pst*:GFP cells proliferated along the root surface but no growth was observed in the vascular tissue inside the root. The *Pst hrcC*$^-$:GFP cells were rarely observed on either the root surface or inside the root.

Expression of *Shigella* T3S effector genes and activation of *Arabidopsis* MAPK signaling during *Shigella* infection in *Arabidopsis* plants

To further investigate T3S effectors involved in plant pathogenesis, we measured expression of key 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. These effectors play a role in mammalian cell lysis (*ipaB*, *ipaC*) and intracellular spread (*icsA*, *icsB*), and regulation of virulence factor (*virF* and *virB*) expression (Ogawa *et al.*, 2008). Total RNAs were isolated from *A. thaliana* leaves at 6 and 12 h after *Shigella* inoculation and changes in expression of virulence genes were confirmed by quantitative RT-PCR (Fig. 5a). Expression of all *Shigella* virulence genes examined in WT *S. f* 2a- or *S. f* 5a-infected *Arabidopsis* leaves increased. In particular, induction was higher and faster in *S. f* 2a-infected plants than in *S. f* 5a-infected 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 infected with non-pathogenic mutants Δvp or BS176, similar to buffer-treated control plants. These results indicate that common virulence factors regulate pathogenesis in both plants and human intestinal cells.

To investigate the plant innate immune responses to *Shigella* infection, MAPK phosphorylation was evaluated. The activation of MAPK by phosphorylation is a conserved response of the earliest pathogen-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 (Fig. 5b). 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 (Fig. 5b). 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 (Fig. 5b). These observations indicated that *Shigella* suppresses the innate immunity of *Arabidopsis* via its T3S virulence effectors.

Two *S. f* 5a effectors, OspF and OspG, function as virulence factors in *Arabidopsis*

To see if the *Shigella* effector protein really has pathogenic functions in plants as in human cells, we next investigated its effector proteins OspF and OspG. These two proteins interfere with the human innate immune response during infection (Kim *et al.*, 2005; Arbibe *et al.*, 2007; Ashida & Sasakawa, 2014; Pruneda *et al.*, 2014). To test whether OspF and OspG are required for plant infection by *Shigella*, we tested single *S. f.* 5a Δ*ospF* and *S. f 5a* Δ*ospG* deletion mutants. We observed a clear reduction in bacterial proliferation in plants infected with *S. f.* 5a Δ*ospF* or *S. f* 5a Δ*ospG* when compared with wild-type *S. f* 5a (Fig. 6a). Pathogenicity was fully restored by complementation of the mutations, indicating that these effector proteins indeed play an important role in *Shigella* pathogenesis in plants (Fig. 6a). We then monitored activation of MAPKs to determine whether plant immune suppression was altered by single mutation of *OspF* and *OspG*. As shown in Figure 6B, *S. f.* 5a Δ*ospF* induced stronger phosphorylation of MAPKs than wild-type *S. f* 5a; this induction was offset by complementation with OspF. On the other hand, *S. f 5a*-deficient OspG showed little change in the inhibition of MAPK phosphorylation compared to wild-type *S. f* 5a, but rather a slight increase in MAPK activation due to overexpression of OspG (Fig. 6b). These results suggest that OspG - induced plant immunosuppression may be present in the lower part of the MAPK signaling or may be involved in the plant immune response other than inhibition of MAPK activation. Taken together, these results indicate that *Shigella* effectors OspF or OspG play an important role in increasing bacterial invasion and proliferation in plants as well as in animal hosts.

To understand how the *Shigella* effectors OspF and OspG affect the pathogenesis of plant pathogens, each gene was introduced into *Pst*. First, we used an AvrRpt2-derived T3SS reporter system to determine whether the *Shigella* effector was delivered into plant cells via *Pst* T3SS (Mudgett, 2005). In this system, AvrRpt2 (a T3S effector protein from *Pseudomonas syringae* pv. tomato) interacts with the RPS2 protein of *Arabidopsis* plants to induce plant cell death (Mudgett, 2005). We introduced the *ospF* and *ospG* genes into the pBAV178 vector encoding the AvrRpt$^{101–255}$ fragment (Fig. S7a). When *Arabidopsis* leaves were syringe-infiltrated with *Pst* producing various AvrRpt$^{101–255}$ constructs, *Pst* producing

404 OspF$_{S.f5a}$:AvrRpt2$^{101–255}$ or OspG$_{S.f5a}$:AvrRpt2$^{101–255}$ induced a strong cell death response at 1
405 dpi, while control *Pst* cells containing the empty vector did not, indicating that the *Shigella*
406 effectors OspF and OspG are successfully delivered into *Arabidopsis* cells via *Pst* T3SS (Fig.
407 7a). Full-length AvrRpt2 was used as a positive control for the cell death response.
408 Furthermore, we tested whether the pathogenicity of *Pst* increased in the presence of
409 *Shigella* OspF or OspG. Plants infected with *Pst* cells producing OspF:HA or OspG:HA
410 showed more severe disease symptoms than plants infected with the empty vector control;
411 furthermore, bacterial cell numbers were 10 times higher than those in the control (Fig. 7b, c).
412 Production of OspF:HA and OspG:HA in *Pst* was also confirmed by immunoblotting with an
413 anti-HA antibody (Fig. S7b). Overall, these observations suggest that the *Shigella* effectors
414 OspF and OspG function as virulence factors in plants.
415
416 **OspF completely suppresses the MEK2^{DD}-dependent hypersensitive response**
417 We next attempted to produce the OspF and OspG proteins in the plant cells, to
418 verify whether their known function in the human host is different from that in the plant cell.
419 To do this, subcellular localization of the two proteins fused with GFP was evaluated in the
420 plant cells. The DNA fragments encoding the OspF:GFP and OspG:GFP fusions were
421 inserted into a binary plant expression vector and transiently expressed in *N. benthamiana*
422 leaves using the *Agrobacterium* system (Fig. 8a). The fluorescence signal for both proteins
423 was strong in the plant cell nucleus; OspF:GFP fluorescence was also observed along the
424 cytoplasmic membrane, and punctate OspG:GFP fluorescence was observed in the cytosol
425 (Fig. 8b). In the host human cell, OspF localization is nuclear (Zurawski *et al.*, 2006; Arbibe
426 *et al.*, 2007) and that of OspG is nuclear and cytoplasmic (Kim *et al.*, 2005; Zhou *et al.*, 2013;
427 de Jong & Alto, 2014), i.e., their subcellular localization is similar to that of plant cells.
428 Previously, *in vitro* studies demonstrated that OspF exerts a phosphothreonine lyase
429 activity and irreversibly removes phosphate groups from MAPK (Arbibe *et al.*, 2007; Li *et al.*,
430 2007). We used a phenotypic screening system involving a MEK2 (a tobacco MAP kinase
431 kinase2) mutant, MEK2^{DD}, to investigate whether the virulence associated with *Shigella*
432 OspF in plants was linked to the same mechanism of action as in animals. MEK2^{DD}, a
433 constitutively active mutant of MEK2, induces cell death when overproduced in plant leaves
434 (Yang *et al.*, 2001; Kim & Zhang, 2004). In this experiment, an HA:MEK2^{DD} clone fused to
435 an HA epitope tag at the N-terminus was used to monitor expression of MEK2^{DD}. As
436 expected, co-production of HA:MEK2^{DD} and GFP (control) resulted in a pronounced cell

death (Fig. 8c). Surprisingly, co-production of OspF:GFP, but not OspG:GFP, and MEK2^{DD} completely inhibited the MEK2^{DD}-induced cell death (Fig. 8c). 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 (Fig. 8d), even though both proteins were stably produced in the absence of MEK2^{DD} (Fig. 8a). The degradation of the OspG protein may have been associated with the activated 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 (Fig. 8d). The production of MEK2^{DD} protein was confirmed in all samples using anti-HA antibodies (Fig. 8d). These observations strongly suggested that the *Shigella* effector OspF suppressed plant MAPK activation through its dephosphorylation, as in the human host.

DISCUSSION

In the current study, we investigated the interaction of the human pathogenic bacterium, *Shigella*, with a novel host, the *Arabidopsis* plant. We demonstrated that four *Shigella* strains, *S. b*, *S. s*, *S. f* 2a, and *S. f* 5a, promote proliferation and confer virulence on *Arabidopsis*. It has been clearly 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 & Sansonetti, 2007). We demonstrated in the current study that, in addition to the human host, *Shigella* infects *Arabidopsis*, through the vascular tissues of the root and leaf stomata pores (Figs 2, 4). In particular, *S. s* and *S. b* formed relatively wide clusters in the surrounding areas, including the guard cells (Figs 2, S3). 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 serogroups. By contrast, *S. f* 5a infection was associated with reduced bacterial proliferation and disease symptoms compared with the other strains (Figs 1, S1, S2). *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. The specific plant responses, which were different for bacterial strains within the same serogroup, suggest that virulent effectors may play a relatively more important role in plant pathogenesis than PTI. These observations also indicate that specific plant immune systems may be useful in

the search for novel virulence factors expressed by different *Shigella* serogroups.

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 the pathogenesis of shigellosis in humans also play a central role in regulating pathogenesis in *Arabidopsis*. We showed that secretion of T3S effectors is required to trigger both pathogenic symptoms and expression of the invasive phenotypes in plants (Fig. 4). Furthermore, the effector proteins impacted MAPK-dependent innate immune responses in *Arabidopsis* (Figs 5, 6). Although reduced colonization of T3SS-deficient pathogenic *E. coli* in plants was previously reported, in contrast with the present study, they suggested that *E. coli* uses the T3SS apparatus for attachment to leaves, rather than for delivery of effector proteins (Shaw *et al.*, 2008). The relevance of T3SS for infection of plants with *Salmonella* 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). Until now, the transfer of effector proteins into plant cells by T3SS of animal bacterial pathogens has not been reported. Our results indirectly suggest that *Shigella* effectors can be transferred into plant cells; however, direct evidence, such as visualization of the delivery of effectors using split fluorescent proteins (Mach, 2017), is required.

Expression of the T3SS of *Shigella* is regulated at the transcriptional level and is activated at a permissive temperature (≥ 32°C) (Erickson, 2012). We were able to observe expression of the T3SS genes of *Shigella* under temperatures at which plants grow (22 ± 3°C) (Fig. 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.

The current study provides new insights into host invasion mechanisms utilized by *Shigella* to infect 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 (Fig. 8b), and it was apparent that the production of both proteins increased the virulence of plant pathogens (Fig. 7b, c). In addition, we confirmed that OspF inhibits the innate immune response of plants via the same enzymatic activity as in animals (Fig. 8c, d). OspG inhibits the host immune response by inhibiting the activity of NF-κB by blocking the degradation of IκB in animals (Ashida *et al.*, 2015). Plants possess an IκB-like protein called NIM1 (Ryals *et al.*, 1997), but no other published studies have investigated whether a NF-κB–induced immune response exists in plants. In the current study, we demonstrated the ability of OspG to enhance the phytopathogenicity of non-*Shigella* bacteria (Fig. 7b, c) and also observed the degradation of OspG:GFP upon the constitutive activation of MAPK signaling (Fig. 8c, d). 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 better 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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Authors' contributions

JMP, SHJ, and JL conceived and designed the study and wrote the manuscript. SHJ, JL, and DHL carried out the experiments. JMP, SHJ, JL, DWK, and CMR analyzed and discussed the data.

References

Abramovitch RB, Anderson JC, Martin GB. 2006. Bacterial elicitation and evasion of plant innate immunity. *Nat Rev Mol Cell Biol* **7**(8): 601-611.

Arbibe L, Kim DW, Batsche E, Pedron T, Mateescu B, Muchardt C, Parsot C, Sansonetti PJ. 2007. An injected bacterial effector targets chromatin access for transcription

factor NF-kappaB to alter transcription of host genes involved in immune responses. *Nat Immunol* **8**(1): 47-56.

Ashida H, Mimuro H, Sasakawa C. 2015. Shigella Manipulates Host Immune Responses by Delivering Effector Proteins with Specific Roles. *Frontiers in Immunology* **6**(219).

Ashida H, Sasakawa C. 2014. Shigella hacks host immune responses by reprogramming the host epigenome. *EMBO J* **33**(22): 2598-2600.

Bando SY, Moreno AC, Albuquerque JA, Amhaz JM, Moreira-Filho CA, Martinez MB. 2010. Expression of bacterial virulence factors and cytokines during in vitro macrophage infection by enteroinvasive Escherichia coli and Shigella flexneri: a comparative study. *Mem Inst Oswaldo Cruz* **105**(6): 786-791.

Bethke G, Unthan T, Uhrig JF, Poschl Y, Gust AA, Scheel D, Lee J. 2009. Flg22 regulates the release of an ethylene response factor substrate from MAP kinase 6 in Arabidopsis thaliana via ethylene signaling. *Proc Natl Acad Sci U S A* **106**(19): 8067-8072.

Buttner D, Bonas U. 2003. Common infection strategies of plant and animal pathogenic bacteria. *Curr Opin Plant Biol* **6**(4): 312-319.

Cevallos-Cevallos JM, Gu G, Danyluk MD, van Bruggen AH. 2012. Adhesion and splash dispersal of Salmonella enterica Typhimurium on tomato leaflets: effects of rdar morphotype and trichome density. *Int J Food Microbiol* **160**(1): 58-64.

Chae Y, An YJ. 2016. Toxicity and transfer of polyvinylpyrrolidone-coated silver nanowires in an aquatic food chain consisting of algae, water fleas, and zebrafish. *Aquat Toxicol* **173**: 94-104.

Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nurnberger T, Jones JD, Felix G, Boller T. 2007. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* **448**(7152): 497-500.

de Jong MF, Alto NM. 2014. Thinking outside the Osp(G)--kinase activation by E2-ubiquitin. *EMBO J* **33**(5): 403-404.

Deering AJ, Mauer LJ, Pruitt RE. 2012. Internalization of E. coli O157:H7 and Salmonella spp. in plants: A review. *Food Research International* **45**(2): 567-575.

Erickson MC. 2012. Internalization of Fresh Produce by Foodborne Pathogens. *Annual Review of Food Science and Technology* **3**(1): 283-310.

Ferens WA, Hovde CJ. 2011. Escherichia coli O157:H7: animal reservoir and sources of human infection. *Foodborne Pathog Dis* **8**(4): 465-487.

Franz E, Semenov AV, van Bruggen AH. 2008. Modelling the contamination of lettuce with

Escherichia coli O157:H7 from manure-amended soil and the effect of intervention strategies. *J Appl Microbiol* **105**(5): 1569-1584.

Gao X, Huang Q, Zhao Z, Han Q, Ke X, Qin H, Huang L. 2016. Studies on the Infection, Colonization, and Movement of Pseudomonas syringae pv. actinidiae in Kiwifruit Tissues Using a GFPuv-Labeled Strain. *PLoS One* **11**(3): e0151169.

Gu G, Cevallos-Cevallos JM, Vallad GE, van Bruggen AH. 2013a. Organically managed soils reduce internal colonization of tomato plants by Salmonella enterica serovar Typhimurium. *Phytopathology* **103**(4): 381-388.

Gu G, Cevallos-Cevallos JM, van Bruggen AH. 2013b. Ingress of Salmonella enterica Typhimurium into tomato leaves through hydathodes. *PLoS One* **8**(1): e53470.

Guchi B, Ashenafi M. 2010. Microbial load, prevalence and antibiograms of salmonella and Shigella in lettuce and green peppers. *Ethiop J Health Sci* **20**(1): 41-48.

Holt KE, Baker S, Weill F-X, Holmes EC, Kitchen A, Yu J, Sangal V, Brown DJ, Coia JE, Kim DW, et al. 2012. Shigella sonnei genome sequencing and phylogenetic analysis indicate recent global dissemination from Europe. *Nat Genet* **44**(9): 1056-1059.

Hong CE, Kwon SY, Park JM. 2016. Biocontrol activity of Paenibacillus polymyxa AC-1 against Pseudomonas syringae and its interaction with Arabidopsis thaliana. *Microbiological Research* **185**: 13-21.

Huot B, Castroverde CDM, Velásquez AC, Hubbard E, Pulman JA, Yao J, Childs KL, Tsuda K, Montgomery BL, He SY. 2017. Dual impact of elevated temperature on plant defence and bacterial virulence in Arabidopsis. *Nature Communications* **8**(1): 1808.

Iniguez AL, Dong Y, Carter HD, Ahmer BMM, Stone JM, Triplett EW. 2005. Regulation of Enteric Endophytic Bacterial Colonization by Plant Defenses. *Molecular Plant-Microbe Interactions* **18**(2): 169-178.

Ishiga Y, Ishiga T, Uppalapati SR, Mysore KS. 2011. Arabidopsis seedling flood-inoculation technique: a rapid and reliable assay for studying plant-bacterial interactions. *Plant Methods* **7**: 32.

Jha AK, Bais HP, Vivanco JM. 2005. Enterococcus faecalis mammalian virulence-related factors exhibit potent pathogenicity in the Arabidopsis thaliana plant model. *Infect Immun* **73**(1): 464-475.

Jones JD, Dangl JL. 2006. The plant immune system. *Nature* **444**(7117): 323-329.

Karimi M, Inzé D, Depicker A. 2002. GATEWAY™ vectors for Agrobacterium-mediated plant transformation. *Trends Plant Sci* **7**(5): 193-195.

Katagiri F, Thilmony R, He SY. 2002. The Arabidopsis thaliana-pseudomonas syringae interaction. *Arabidopsis Book* **1**: e0039.

Kennedy CK. 1971. Induction of colicin production by high temperature or inhibition of protein synthesis. *J Bacteriol* **108**(1): 10-19.

Kim CY, Zhang S. 2004. Activation of a mitogen-activated protein kinase cascade induces WRKY family of transcription factors and defense genes in tobacco. *The Plant Journal* **38**(1): 142-151.

Kim DW, Lenzen G, Page AL, Legrain P, Sansonetti PJ, Parsot C. 2005. The Shigella flexneri effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes. *Proc Natl Acad Sci U S A* **102**(39): 14046-14051.

King EO, Ward MK, Raney DE. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. *J Lab Clin Med* **44**(2): 301-307.

Koch E, Slusarenko A. 1990. Arabidopsis is susceptible to infection by a downy mildew fungus. *Plant Cell* **2**(5): 437-445.

Lee JH, Kim YC, Choi D, Park JM. 2013. Identification of novel pepper genes involved in Bax- or INF1-mediated cell death responses by high-throughput virus-induced gene silencing. *Int. J. Mol. Sci.* **14**(11): 22782-22795.

Li H, Xu H, Zhou Y, Zhang J, Long C, Li S, Chen S, Zhou JM, Shao F. 2007. The phosphothreonine lyase activity of a bacterial type III effector family. *Science* **315**(5814): 1000-1003.

Lindberg AA, Karnell A, Weintraub A. 1991. The lipopolysaccharide of Shigella bacteria as a virulence factor. *Rev Infect Dis* **13 Suppl 4**: S279-284.

Liu X, Sun Y, Korner CJ, Du X, Vollmer ME, Pajerowska-Mukhtar KM. 2015. Bacterial Leaf Infiltration Assay for Fine Characterization of Plant Defense Responses using the Arabidopsis thaliana-Pseudomonas syringae Pathosystem. *J Vis Exp*(104).

Mach J. 2017. Tracking the Bacterial Type III Secretion System: Visualization of Effector Delivery Using Split Fluorescent Proteins. *The Plant Cell* **29**(7): 1547-1548.

Martínez-Vaz BM, Fink RC, Diez-Gonzalez F, Sadowsky MJ. 2014. Enteric Pathogen-Plant Interactions: Molecular Connections Leading to Colonization and Growth and Implications for Food Safety. *Microbes and Environments* **29**(2): 123-135.

Moon JY, Lee JH, Oh C-S, Kang H-G, Park JM. 2016. Endoplasmic reticulum stress responses function in the HRT-mediated hypersensitive response in Nicotiana benthamiana. *Molecular Plant Pathology* **17**(9): 1382-1397.

Mortality GBD, Causes of Death C. 2016. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **388**(10053): 1459-1544.

Mudgett MB. 2005. New insights to the function of phytopathogenic bacterial type III effectors in plants. *Annu Rev Plant Biol* **56**: 509-531.

Murashige T, Skoog F. 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum* **15**(3): 473-497.

Neumann C, Fraiture M, Hernandez-Reyes C, Akum FN, Virlogeux-Payant I, Chen Y, Pateyron S, Colcombet J, Kogel KH, Hirt H, et al. 2014. The Salmonella effector protein SpvC, a phosphothreonine lyase is functional in plant cells. *Front Microbiol* **5**: 548.

Ogawa M, Handa Y, Ashida H, Suzuki M, Sasakawa C. 2008. The versatility of Shigella effectors. *Nat Rev Micro* **6**(1): 11-16.

Onodera NT, Ryu J, Durbic T, Nislow C, Archibald JM, Rohde JR. 2012. Genome Sequence of Shigella flexneri Serotype 5a Strain M90T Sm. *Journal of Bacteriology* **194**(11): 3022.

Panchal S, Roy D, Chitrakar R, Price L, Breitbach ZS, Armstrong DW, Melotto M. 2016. Coronatine Facilitates Pseudomonas syringae Infection of Arabidopsis Leaves at Night. *Front Plant Sci* **7**: 880.

Pandey PK, Kass PH, Soupir ML, Biswas S, Singh VP. 2014. Contamination of water resources by pathogenic bacteria. *AMB Express* **4**: 51.

Phalipon A, Sansonetti PJ. 2007. Shigella's ways of manipulating the host intestinal innate and adaptive immune system: a tool box for survival? *Immunol Cell Biol* **85**(2): 119-129.

Plotnikova JM, Rahme LG, Ausubel FM. 2000. Pathogenesis of the human opportunistic pathogen Pseudomonas aeruginosa PA14 in Arabidopsis. *Plant Physiol* **124**(4): 1766-1774.

Pruneda JN, Smith FD, Daurie A, Swaney DL, Villen J, Scott JD, Stadnyk AW, Le Trong I, Stenkamp RE, Klevit RE, et al. 2014. E2~Ub conjugates regulate the kinase activity of Shigella effector OspG during pathogenesis. *EMBO J* **33**(5): 437-449.

Runyen-Janecky LJ, Payne SM. 2002. Identification of chromosomal Shigella flexneri genes induced by the eukaryotic intracellular environment. *Infect Immun* **70**(8): 4379-4388.

Ryals J, Weymann K, Lawton K, Friedrich L, Ellis D, Steiner HY, Johnson J, Delaney TP, Jesse T, Vos P, et al. 1997. The Arabidopsis NIM1 protein shows homology to the mammalian transcription factor inhibitor I kappa B. *Plant Cell* **9**(3): 425-439.

Sansonetti PJ, Kopecko DJ, Formal SB. 1982. Involvement of a plasmid in the invasive ability of Shigella flexneri. *Infect Immun* **35**(3): 852-860.

Schikora A, Carreri A, Charpentier E, Hirt H. 2008. The dark side of the salad: Salmonella typhimurium overcomes the innate immune response of Arabidopsis thaliana and shows an endopathogenic lifestyle. *PLoS One* **3**(5): e2279.

Schikora A, Garcia AV, Hirt H. 2012. Plants as alternative hosts for Salmonella. *Trends Plant Sci* **17**(5): 245-249.

Schikora A, Virlogeux-Payant I, Bueso E, Garcia AV, Nilau T, Charrier A, Pelletier S, Menanteau P, Baccarini M, Velge P, et al. 2011. Conservation of Salmonella infection mechanisms in plants and animals. *PLoS One* **6**(9): e24112.

Schroeder GN, Hilbi H. 2008. Molecular pathogenesis of Shigella spp.: controlling host cell signaling, invasion, and death by type III secretion. *Clin Microbiol Rev* **21**(1): 134-156.

Semenov AM, Kuprianov AA, van Bruggen AH. 2010. Transfer of enteric pathogens to successive habitats as part of microbial cycles. *Microb Ecol* **60**(1): 239-249.

Shamloul M, Trusa J, Mett V, Yusibov V. 2014. Optimization and utilization of Agrobacterium-mediated transient protein production in Nicotiana. *J Vis Exp*(86).

Shaw RK, Berger CN, Feys B, Knutton S, Pallen MJ, Frankel G. 2008. Enterohemorrhagic Escherichia coli Exploits EspA Filaments for Attachment to Salad Leaves. *Applied and Environmental Microbiology* **74**(9): 2908-2914.

Shim DH, Suzuki T, Chang SY, Park SM, Sansonetti PJ, Sasakawa C, Kweon MN. 2007. New animal model of shigellosis in the Guinea pig: its usefulness for protective efficacy studies. *J Immunol* **178**(4): 2476-2482.

Shirron N, Yaron S. 2011. Active suppression of early immune response in tobacco by the human pathogen Salmonella Typhimurium. *PLoS One* **6**(4): e18855.

Smith JM, Heese A. 2014. Rapid bioassay to measure early reactive oxygen species production in Arabidopsis leave tissue in response to living Pseudomonas syringae. *Plant Methods* **10**(1): 6.

Solomon EB, Yaron S, Matthews KR. 2002. Transmission of Escherichia coli O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. *Appl Environ Microbiol* **68**(1): 397-400.

Thordal-Christensen H, Zhang Z, Wei Y, Collinge DB. 1997. Subcellular localization of H2O2 in plants. H2O2 accumulation in papillae and hypersensitive response during the barley—powdery mildew interaction. *The Plant Journal* **11**(6): 1187-1194.

Vinatzer BA, Jelenska J, Greenberg JT. 2005. Bioinformatics correctly identifies many type III secretion substrates in the plant pathogen Pseudomonas syringae and the biocontrol isolate P. fluorescens SBW25. *Mol Plant Microbe Interact* **18**(8): 877-888.

Wang K, Kang L, Anand A, Lazarovits G, Mysore KS. 2007. Monitoring in planta bacterial infection at both cellular and whole-plant levels using the green fluorescent protein variant GFPuv. *New Phytologist* **174**(1): 212-223.

Wei J, Goldberg MB, Burland V, Venkatesan MM, Deng W, Fournier G, Mayhew GF, Plunkett G, Rose DJ, Darling A, et al. 2003. Complete Genome Sequence and Comparative Genomics of Shigella flexneri Serotype 2a Strain 2457T. *Infection and Immunity* **71**(5): 2775-2786.

Wenneras C, Ave P, Huerre M, Arondel J, Ulevitch RJ, Mathison JC, Sansonetti P. 2000. Blockade of CD14 increases Shigella-mediated invasion and tissue destruction. *J Immunol* **164**(6): 3214-3221.

Yang KY, Liu Y, Zhang S. 2001. Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco. *Proc Natl Acad Sci U S A* **98**(2): 741-746.

Zhang R, Zhou HW, Cai JC, Zhang J, Chen GX, Nasu M, Xie XY. 2011. Serotypes and extended-spectrum beta-lactamase types of clinical isolates of Shigella spp. from the Zhejiang province of China. *Diagn Microbiol Infect Dis* **69**(1): 98-104.

Zhou Y, Dong N, Hu L, Shao F. 2013. The Shigella type three secretion system effector OspG directly and specifically binds to host ubiquitin for activation. *PLoS One* **8**(2): e57558.

Zipfel C. 2009. Early molecular events in PAMP-triggered immunity. *Curr Opin Plant Biol* **12**(4): 414-420.

Zurawski DV, Mitsuhata C, Mumy KL, McCormick BA, Maurelli AT. 2006. OspF and OspC1 are Shigella flexneri type III secretion system effectors that are required for postinvasion aspects of virulence. *Infect Immun* **74**(10): 5964-5976.

Supporting Information

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

Fig. S1 Bacterial proliferation in *Arabidopsis* shoots and roots inoculated with *Shigella* by flooding.

Fig. S2 *Shigella*-infected *Arabidopsis* plants show various disease symptoms at a late stage of infection.

Fig. S3 Imaging of the *Arabidopsis* leaf stomata challenged with *Shigella* labeled GFP.

Fig. S4 The growth of *Shigella* strains producing GFP or virulence-deficient mutants is not affected *in vitro*.

Fig. S5 *Shigella* does not grow in the plant MS growth medium.

Fig. S6 An overview of *Arabidopsis* plants infected with DH5α, *Pst hrcC*$^-$, or *Pst*.

Fig. S7 Expression of OspF or OspG for the virulence assay.

Table S1 Bacterial strains and plasmids used in the study.

Table S2 Sequences of PCR primers used for Gateway cloning.

Figure legends

Fig. 1 *Shigella* cells proliferate and induce disease symptoms in *Arabidopsis* plants. (a) *Arabidopsis* leaves were syringe-infiltrated with buffer or bacterial suspensions (5×10^7 cfu/ml). *S. s*, and *S. f* 2a induced wilting of infected leaves. Accumulation of reactive oxygen species was measured by 3,3′-diaminobenzidine staining 1 d after infiltration. The fractions indicate the number of times that the symptoms were observed relative to the number of tests. (b, c) *Arabidopsis* seedlings in 1/2 MS medium were flood-inoculated with distilled H_2O containing 0.025% Silwet L-77 (buffer) or bacterial suspensions (5×10^5 cfu/ml). (b) The

disease phenotypes of *Arabidopsis* infected with *Shigella* spp. by flood-inoculation. Photographs of representative disease symptoms were taken at 3 dpi. (c) Bacterial cell numbers were evaluated on Days 0 and 3 after flood-inoculation. The bars represent the mean ± SD of three replicates and asterisks indicate significant differences between samples (*P < 0.05, *t*-test). All experiments were repeated three times independently and representative results are shown.

Fig. 2 *Shigella* cells adhere to the *Arabidopsis* epidermis and enter the plant via open stomata. *Arabidopsis* seedlings were flood-inoculated with a buffer or bacterial suspensions (5×10^6 cfu/ml). After 24 h, the leaves were fixed and analyzed under a SEM. The *Pst* cells were observed over the stomata. *Shigella* strains were associated with the stomata. Bar, 10 μm. Representative image showing bacteria around open stomata (indicated by white arrows). The results are representative of two independent experiments.

Fig. 3 *Shigella* cells associate with and colonize plant cell structures. *Arabidopsis* seedlings were flood-inoculated with a buffer or bacterial suspensions (5×10^6 cfu/ml). After 24 h, the leaves were evaluated under a TEM. The *Shigella* strains colonized the intercellular spaces. Bar, 5 μm (left panels). Bar, 1 μm (right panels). Each column of micrographs represents a different magnification. TEM images revealed the *Shigella* (white arrows) attached to the cell wall in the intercellular spaces and altered mesophyll cells. Black arrows indicate separation of the plasma membrane from the cell wall and disruption of chloroplasts. The results are representative of two independent experiments.

Fig. 4 The virulence of a plasmid-deficient mutant of *Shigella* is impaired in the *Arabidopsis* model. (a) *Arabidopsis* seedlings in 1/2 MS medium were flood-inoculated with *S. f* 2a, Δvp, *S. f* 5a, or BS176, and their GFP-labeled variants (5×10^5 cfu/ml). The bacterial cell numbers *in planta* were determined at 3 dpi. Bars represent the mean ± SD of three replicates and the different letters indicate significant differences between samples (P < 0.05, one-way ANOVA). (b, c) The root tips of *Arabidopsis* seedlings were drop-inoculated with suspensions of GFP-labeled bacteria (5×10^7 cfu/ml). (b) Biofilm formation and colonization of *Arabidopsis* seedling roots by GFP-labeled *Shigella* was observed under white (WL) and ultra violet (UV) light at 7 dpi. (c) Images of GFP-labeled *Shigella* wild types in the epidermis or vascular tissue of the *Arabidopsis* roots acquired using a fluorescence microscope at 7 dpi. Bar, 100 μm. All experiments were repeated at least three times, and

representative results are shown.

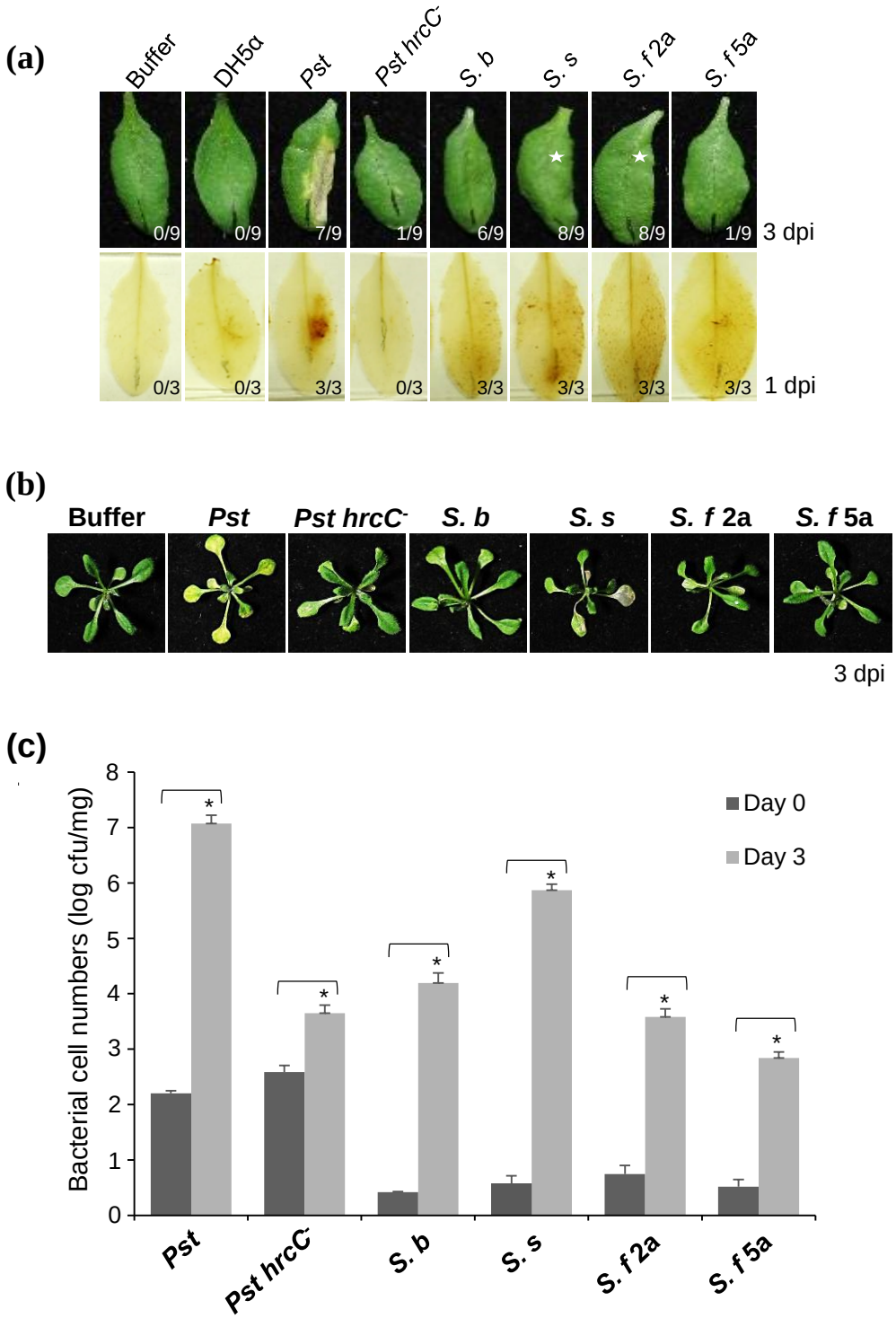
Fig. 5 Expression of *Shigella* effectors and MAPK activation in *Arabidopsis* plants in response to virulence plasmid-deficient *Shigella* mutants. (a, b) *Arabidopsis* leaves were syringe-infiltrated with buffer, 1 μM flg22, or bacterial suspension (5×10^6 cfu/ml), and samples were collected at the indicated times. (a) Transcription of *Shigella* effectors (*ipaB*, *ipaC*, *icsA*, *icsB*, *virB*, and *virF*) in infected *A. thaliana* leaves was analyzed 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 in the WT. Data are expressed as the mean ± SD of three replicates. (b) Immunoblotting was performed using either anti-phospho-p44/42 (ERK1/2, upper panels) or anti-ERK1 (lower panels) antibodies. All experiments were repeated at least three times, each with similar results.

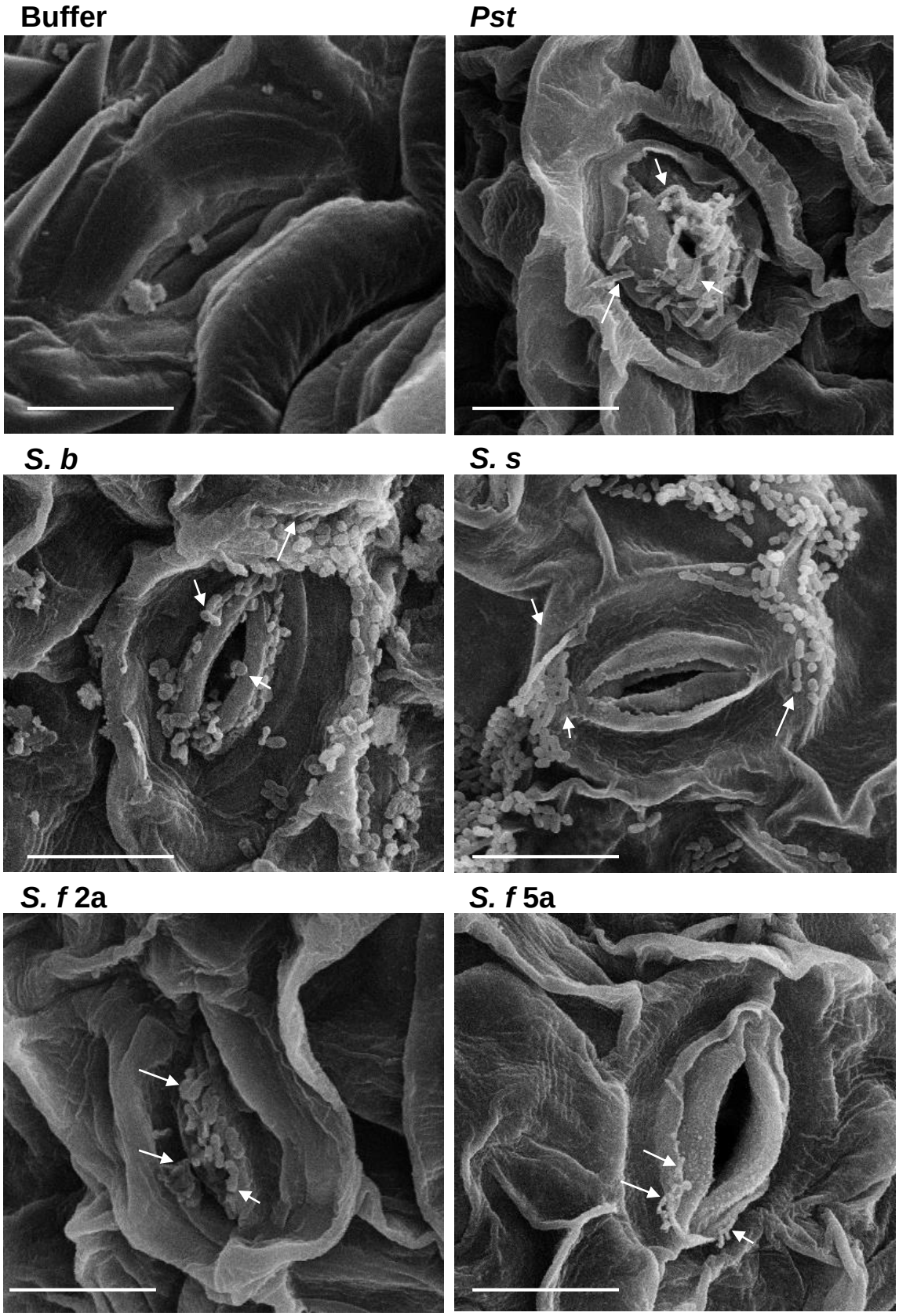
Fig. 6 The *Shigella* effector proteins OspF and OspG underpin the bacterial virulence in plants. (a) The virulence of the OspF- or OspG-deficient *Shigella* mutants is impaired in the *Arabidopsis* model. *Arabidopsis* seedlings in 1/2 MS medium were flood-inoculated with *S. f* 5a, Δ*ospF*, Δ*ospF*+p*ospF*, Δ*ospG*, or Δ*ospG*+p*ospG* (5×10^5 cfu/ml). Photographs of representative disease symptoms were taken, and bacterial cell numbers *in planta* were determined, at 3 dpi. The bars represent the mean ± SD of three replicates. The different letters indicate significant differences between samples ($P < 0.05$, one-way ANOVA). (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. Anti-phospho-p44/42 (ERK1/2, upper panels) or anti-ERK1 (lower panels) antibodies were used for immunoblotting. All experiments were repeated at least three times, with similar results.

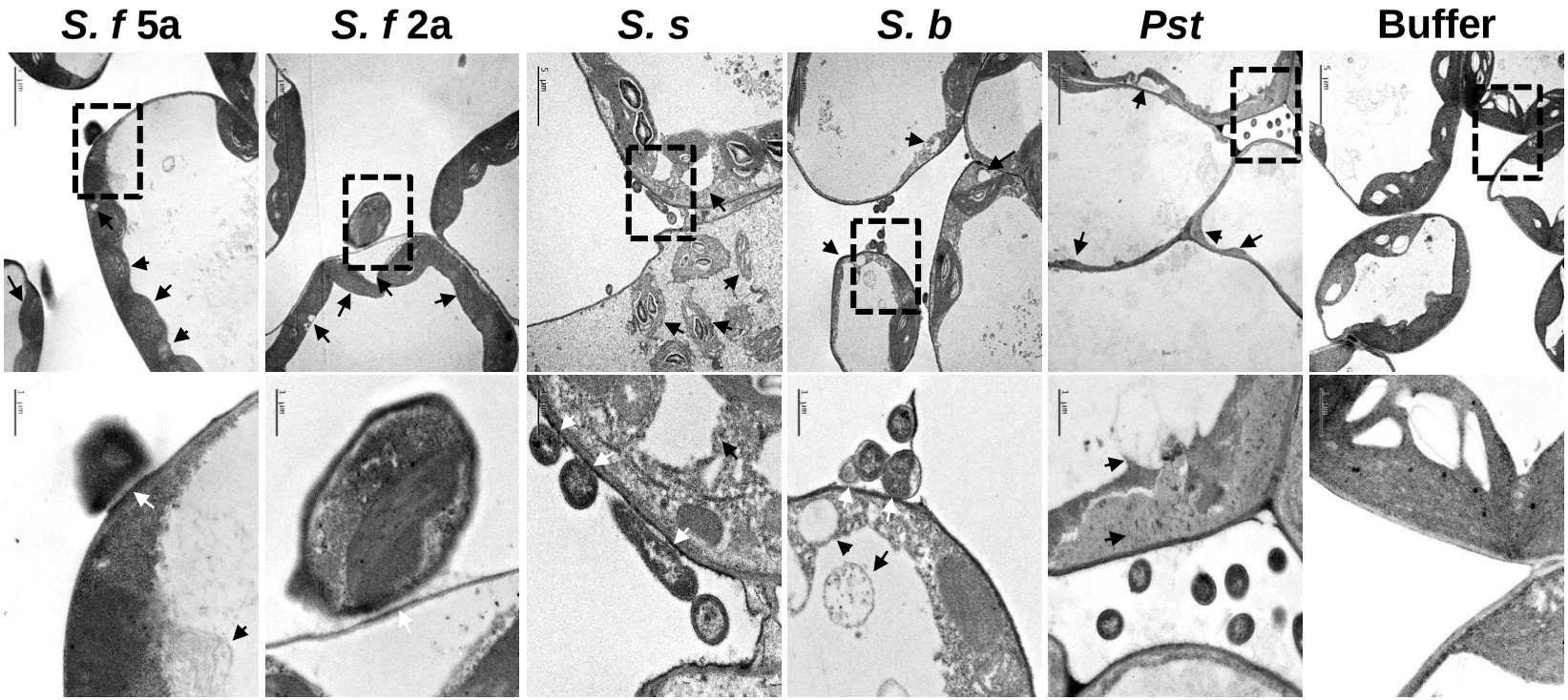
Fig. 7 Increased virulence of phytopathogen *Pst* in the presence of *Shigella* effectors OspF or OspG. (a) *Arabidopsis* leaves were syringe-infiltrated with *Pst* cells (5×10^7 cfu/ml) producing AvrRpt2, carrying the empty vector (pME6012), and producing OspF:AvrRpt2$^{101–255}$ or OspG:AvrRpt2$^{101–255}$. The hypersensitive response in the infiltrated leaves was monitored using the trypan blue staining and photographed at 1 dpi. (b, c) The *Arabidopsis* plants were sprayed with *Pst* cells carrying an empty vector (pME6012), or producing OspF:HA or OspG:HA (5×10^7 cfu/ml). (b) The disease symptoms were monitored 4 d after spraying. (c) The proliferation of *Pst* cells producing the *Shigella* effector proteins in

Arabidopsis, 0 and 4 d after spray-inoculation. The bars represent the mean ± SD of six replicates, and different letters indicate significant differences between samples ($P < 0.05$, two-way ANOVA). All experiments were repeated at least three times.

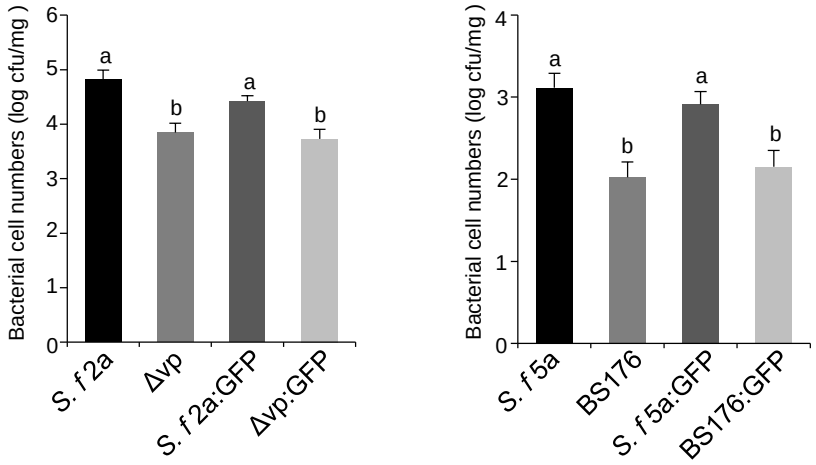
Fig. 8 Co-expression of OspF:GFP suppresses tobacco MEK2^{DD}-triggered cell death. (a) GFP, OspF:GFP, or OspG:GFP was produced in *N. benthamiana* leaves upon infiltration with *Agrobacterium* carrying the appropriate expression constructs (OD_{600} 0.4). The production of the *Shigella* effector proteins in *N. benthamiana* 2 d after the infiltration was evaluated by immunoblotting using anti-GFP antibodies. The RuBisCo protein was stained by Ponceau S as a loading control. (b) Subcellular localization of GFP, OspF:GFP, OspG:GFP, and NLS:RFP (a nuclear localization marker) in *N. benthamiana* leaves. Bar, 50 μm. (c, d) GFP, OspF:GFP or OspG:GFP was co-produced in *N. benthamiana* leaves harboring HA:MEK2^{DD}. (c) Production of OspF:GFP suppressed MEK2^{DD}-dependent cell death by 5 d after the co-infiltration. (d) Production of specific proteins in samples from panel **C** was analyzed by immunoblotting using anti-GFP, anti-HA, anti-phospho-p44/42 (ERK1/2), and anti-ERK1 antibodies. Ponceau S was used to stain the RuBisCo protein (loading control). The asterisk indicates the size of the GFP protein not associated with effector proteins.


(a)
Buffer
DH5α
Pst
Pst hrcC-
S. b
S. s
S. f 2a
S. f 5a
0/9
0/9
7/9
1/9
6/9
8/9
8/9
1/9
3 dpi
0/3
0/3
3/3
0/3
3/3
3/3
3/3
3/3
1 dpi
(b)
Buffer
Pst
Pst hrcC-
S. b
S. s
S. f 2a
S. f 5a
3 dpi
(c)
Bacterial cell numbers (log cfu/mg)
0
1
2
3
4
5
6
7
8
Day 0
Day 3
*
Pst
Pst hrcC-
S. b
S. s
S. f 2a
S. f 5a

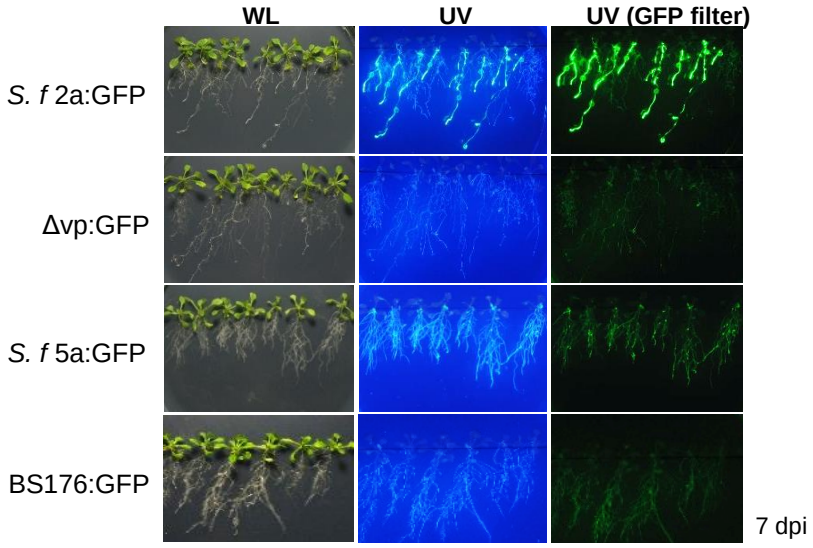

Buffer
Pst
S. b
S. s
S. f 2a
S. f 5a


S. f 5a
S. f 2a
S. s
S. b
Pst
Buffer
5 µm
1 µm

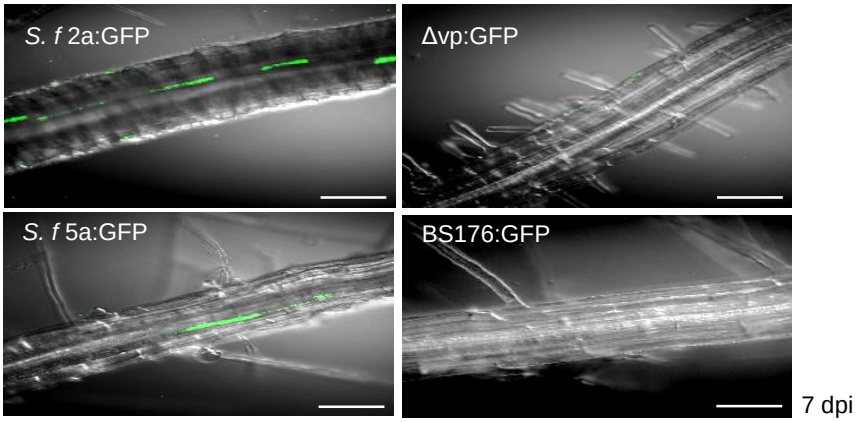
(a)


Bacterial cell numbers (log cfu/mg)
0
1
2
3
4
5
6
a
b
a
b
S. f 2a
Δvp
S. f 2a:GFP
Δvp:GFP
Bacterial cell numbers (log cfu/mg)
0
1
2
3
4
a
b
a
b
S. f 5a
BS176
S. f 5a:GFP
BS176:GFP

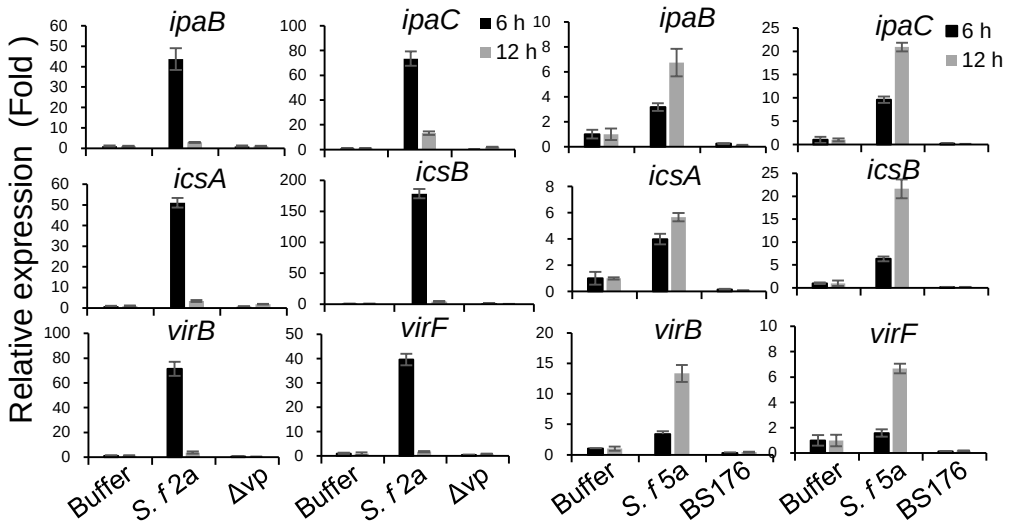
(b)


WL
UV
UV (GFP filter)
S. f 2a:GFP
Δvp:GFP
S. f 5a:GFP
BS176:GFP
7 dpi

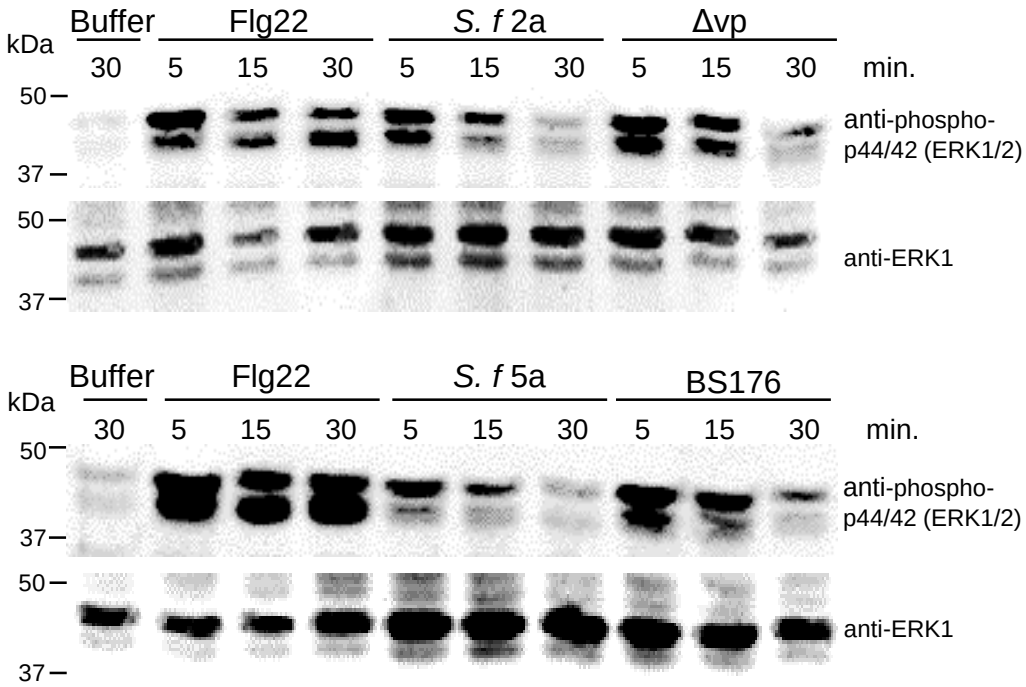
(c)


S. f 2a:GFP
Δvp:GFP
S. f 5a:GFP
BS176:GFP
7 dpi

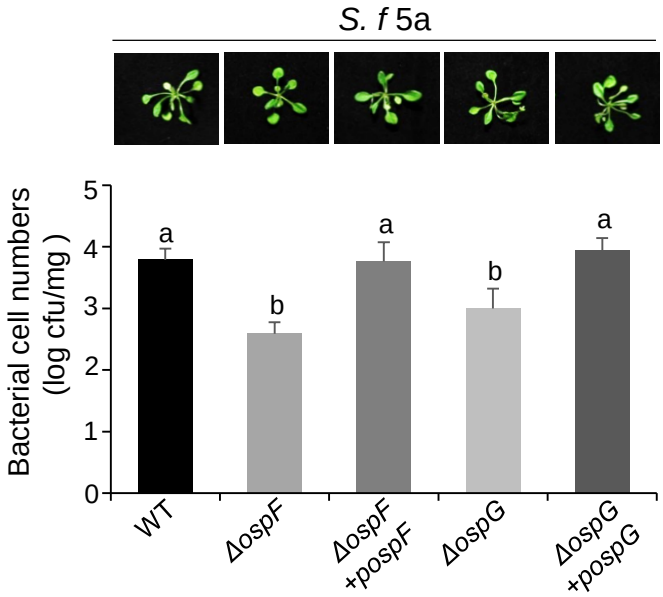
(a)


Relative expression (Fold)
ipaB
ipaC
icsA
icsB
virB
virF
6 h
12 h
Buffer
S. f 2a
Δvp
S. f 5a
BS176

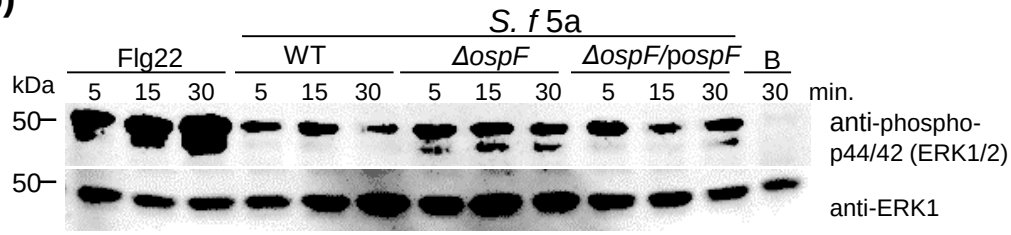
(b)

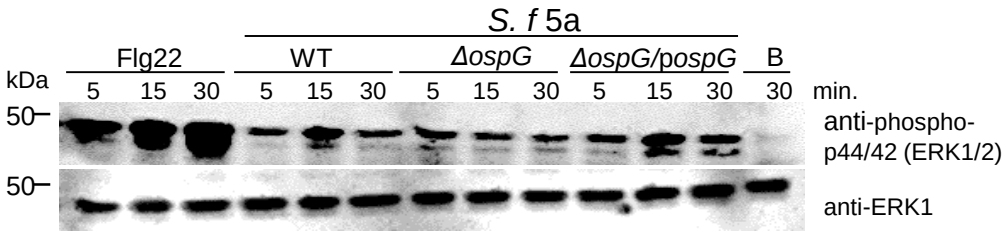

Buffer
Flg22
S. f 2a
Δvp
S. f 5a
BS176
kDa
30 5 15 30 5 15 30 5 15 30 min.
50
37
anti-phospho-p44/42 (ERK1/2)
anti-ERK1

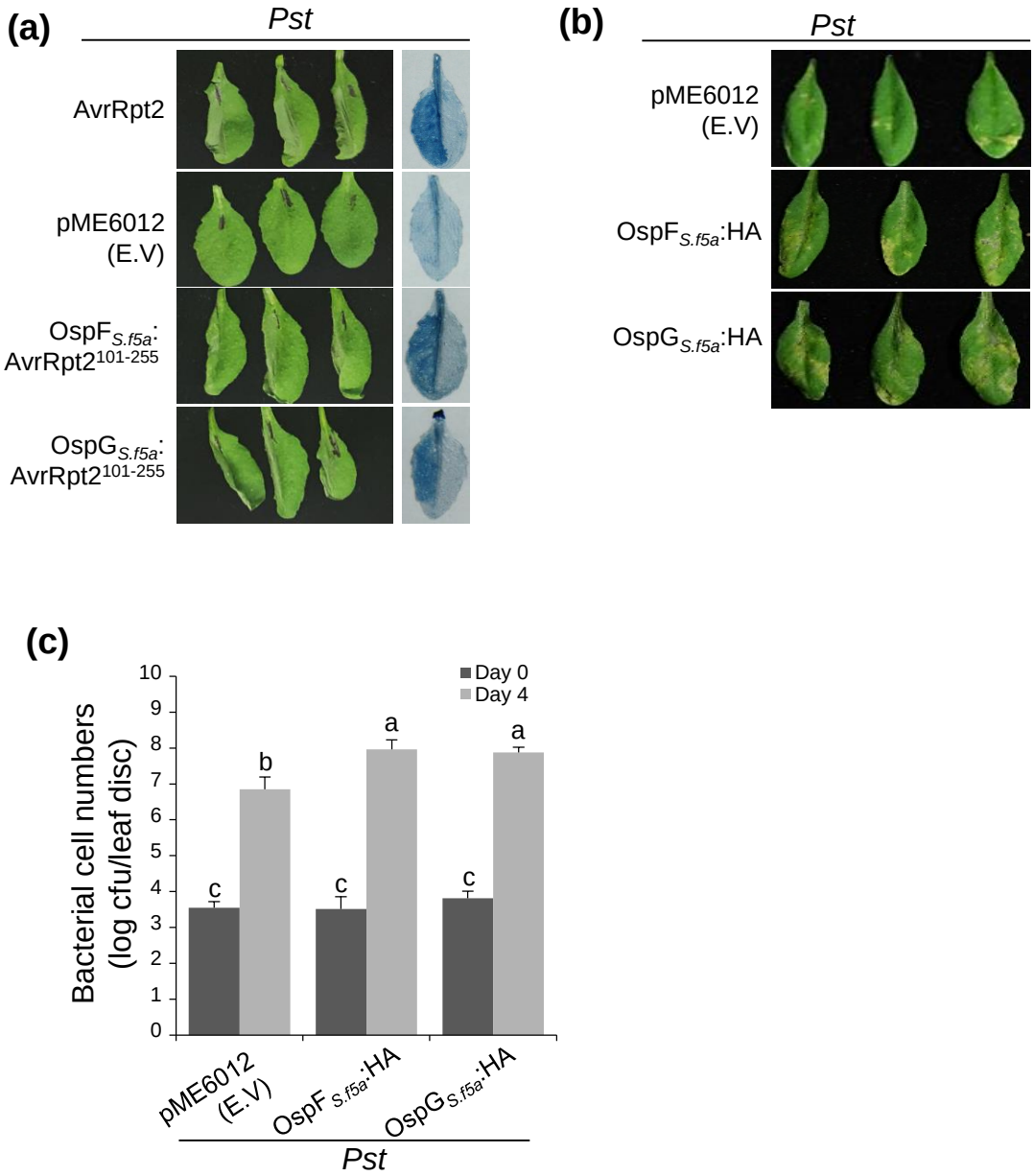
(a)

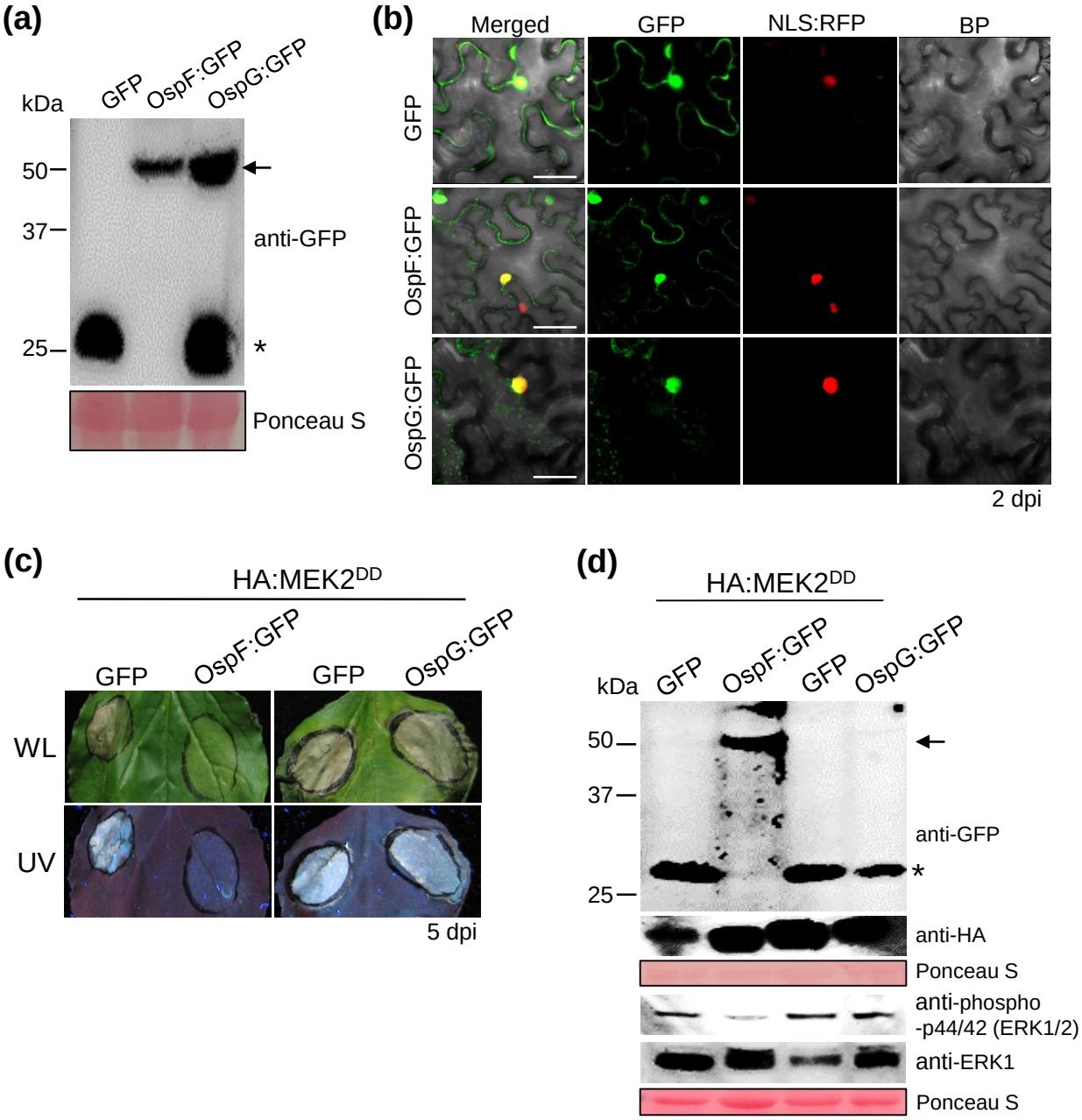

S. f 5a
Bacterial cell numbers
(log cfu/mg)
0
1
2
3
4
5
a
b
a
b
a
WT
ΔospF
ΔospF
+pospF
ΔospG
ΔospG
+pospG

(b)


S. f 5a
Flg22
WT
ΔospF
ΔospF/pospF
B
kDa
5 15 30 5 15 30 5 15 30 5 15 30 30 min.
50
50
anti-phospho-
p44/42 (ERK1/2)
anti-ERK1


S. f 5a
Flg22
WT
ΔospG
ΔospG/pospG
B
kDa
5 15 30 5 15 30 5 15 30 5 15 30 30 min.
50
50
anti-phospho-
p44/42 (ERK1/2)
anti-ERK1


(a)
Pst
AvrRpt2
pME6012 (E.V)
OspF S.f5a: AvrRpt2 101-255
OspG S.f5a: AvrRpt2 101-255
(b)
Pst
pME6012 (E.V)
OspF S.f5a:HA
OspG S.f5a:HA
(c)
Bacterial cell numbers (log cfu/leaf disc)
Day 0
Day 4
0
1
2
3
4
5
6
7
8
9
10
c
b
c
a
c
a
pME6012 (E.V)
OspF S.f5a:HA
OspG S.f5a:HA
Pst


(a)
kDa
GFP
OspF:GFP
OspG:GFP
50
37
25
anti-GFP
*
Ponceau S
(b)
Merged
GFP
NLS:RFP
BP
GFP
OspF:GFP
OspG:GFP
2 dpi
(c)
HA:MEK2DD
GFP
OspF:GFP
GFP
OspG:GFP
WL
UV
5 dpi
(d)
HA:MEK2DD
kDa
GFP
OspF:GFP
GFP
OspG:GFP
50
37
25
anti-GFP
*
anti-HA
Ponceau S
anti-phospho
-p44/42 (ERK1/2)
anti-ERK1
Ponceau S