The human pathogenic bacterium *Shigella* proliferates in *Arabidopsis* plants through type III effector-mediated immune suppression

Sung Hee Jo[1,2], Jiyoung Lee[1,3], Dong Wook Kim[4,5], Dae Hee Lee[2,6], Choong Min Ryu[2,7], and Jeong Mee Park[1,2,*]

[1]*Plant Systems Engineering Research Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), Daejeon, South Korea;* [2]*Department of Biosystems and Bioengineering, KRIBB School of Biotechnology, Korea University of Science and Technology (UST), Daejeon, South Korea;* [3]*Biological Resource Center, KRIBB, Jeongeup, South Korea;* [4]*Department of Pharmacy, College of Pharmacy, Hanyang University, Ansan, South Korea;* [5]*Institute of Pharmacological Research, Hanyang University, Ansan, South Korea;* [6]*Synthetic Biology and Bioengineering Research Center, KRIBB, Daejeon, South Korea;* [7]*Infectious Disease Research Center, KRIBB, Daejeon, South Korea.*

*Address correspondence to:

Plant Systems Engineering Research Center, Korea Research Institute of Bioscience & Biotechnology (KRIBB), 125 Gwahak-Ro, Yuseong-Gu, Daejeon 34141, South Korea

Tel: +82-42-860-4346

Fax: +82-42-860-4468

E-mail: jmpark@kribb.re.kr

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Originality-significance statement

Increased incidence of food-borne disease outbreaks caused by fresh produce contaminated with *Escherichia coli* O157:H7 and *Salmonella* spp. has prompted researchers to examine the interaction between these bacteria and various plant species under different environmental conditions. Although studies show that human enteropathogenic bacteria survive both on the surface of and inside plants, little is known about the molecular mechanism underlying plant invasion and colonization. Here, we examined the interaction between the human pathogenic bacterium *Shigella* and the model plant *Arabidopsis*. We found that four *Shigella* spp. strains proliferated successfully in *Arabidopsis*, causing symptom-like lesions in tissues. Using mutants lacking T3S effectors (i.e., noninvasive human strains), we demonstrated that effectors regulating pathogenesis of shigellosis in humans also play a central role in bacteria-plant interactions. To the best of our knowledge, this is the first study to examine *Shigella*-mediated virulence and host immune suppression in a plant host at a molecular level.

Summary

Although there is debate about whether human intestinal pathogenic bacteria are also plant pathogens, it is clear that these bacteria use plants as an alternative host. *Shigella*, which infects primates, is reportedly transmitted by fresh vegetables; however, its molecular interactions with plants have not been extensively studied. Here, we show that four *Shigella* strains, *S. boydii* (*S. b*), *S. sonnei* (*S. s*), *S. flexneri* 2a (*S. f* 2a), and *S. flexneri* 5a (*S. f* 5a), proliferated at different levels in *Arabidopsis thaliana*. Microscopic studies revealed that these bacteria were present inside leaves and damaged plant cells. GFP-labeled *S. b*, *S. f* 2a, and *S. f* 5a entered plants via guard cells, and *S. f* 2a infiltrated root tissues and colonized roots. Using mutants lacking type III secretion systems (T3SS), we found that T3SS of *Shigella* that regulate the pathogenesis of shigellosis in humans also play a central role in attachment and multiplication in *Arabidopsis.* Furthermore, the immunosuppressive activity of two T3S effectors, OspF and OspG, were needed for the proliferation of *Shigella* in *Arabidopsis.* These findings demonstrate that *Shigella*-mediated virulence determinants are expressed, and pathogenic

55 symptoms are observed, in model plants.

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58 **Keywords: Plant-*Shigella* interaction, *Shigella* spp., Alternative host, Type III secretion system**

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61 **Introduction**

62 *Shigella* is a Gram-negative bacterium from the *Enterobacteriaceae* family. It is a non-motile,
63 rod-shaped, facultative intracellular and invasive pathogen, very closely related to *Escherichia coli*.
64 Based on the carbohydrate composition of the O-antigen, i.e., the polysaccharide component of the
65 lipopolysaccharide molecule that is the major bacterial surface antigen, *Shigella* is classified into four
66 serogroups. These have been given species designations, namely, *S. dysenteriae* 1 (serogroup A), *S.*
67 *flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D) (Lindberg et al., 1991;
68 Schroeder and Hilbi, 2008). *Shigella* spp. are important epidemic pathogens and a serious public
69 health concern in developed and developing countries. Although about 164,300 deaths in all age
70 groups, and 54,900 deaths in children younger than 5 years, were reported globally in 2015, the actual
71 number of infections might be higher because mild symptoms are not reported (Mortality and Causes
72 of Death, 2016). This means that the microorganism may employ a variety of survival strategies not
73 only for infection but also for survival in a restricted host. In recent years, the morbidity or mortality
74 of the infection caused by the emergence of *Shigella* that produces β-lactamase, an enzyme that
75 underpins the resistance to the third-generation antibiotics cephalosporins, has been increasing (Zhang
76 et al., 2011).

77 *Shigella* is a human-adapted pathogen that infects the host via multiple transmission routes.
78 Human hosts are primarily infected by the ingestion of water or food contaminated with bacteria from
79 feces (the fecal-oral route), including ingestion of undercooked contaminated meats and improperly
80 washed contaminated vegetables (Mead et al., 1999; Weir, 2002; Gupta et al., 2004). Fresh fruits and

vegetables, such as lettuce, tomatoes and green peppers, are responsible for the widespread transmission of food-borne infections by *Salmonella* or *Shigella* (Guchi and Ashenafi, 2010; Semenov et al., 2010; Gu et al., 2013). These observations suggest that human pathogenic bacteria use plants as alternative hosts as a stable environmental niche. Several human pathogenic bacteria, including *Salmonella enterica* serovar Typhimurium, *Escherichia coli* O157:H7, and *Pseudomonas aeruginosa*, are known to use plants as alternative hosts (Plotnikova et al., 2000; Semenov et al., 2010). These bacteria can attach to the plant surface, and actively invade and proliferate in plant tissues (Cevallos-Cevallos et al., 2012; Martínez-Vaz et al., 2014). In particular, several enteropathogenic bacteria, including *E. coli* O157:H7 and *Salmonella*, spread within plants through vascular tissues after infection via contaminated water (Solomon et al., 2002). 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 and Dangl, 2006). To suppress PTI, bacteria inject effector proteins into plant cells using type III secretion systems (T3SS). To counteract this PTI evasion response, the plant nucleotide binding-leucine rich repeat proteins recognize the pathogen effectors; effector-triggered immunity is then activated to accompany the hypersensitive response (Jones and Dangl, 2006). For human or animal intestinal bacteria to infect plants, the PTI must first be disabled. *S. enterica* serovar Typhimurium, similar to its activity in the mammalian host, uses T3SS to suppress plant immune responses (Schikora et al., 2011; Schikora et al., 2012). In particular, 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. s* (Holt et al., 2012), *S. b* and *S. f* 2a

(Wei et al., 2003), and *S. f* 5a (Onodera et al., 2012)) to proliferate in *Arabidopsis* plants. We found that the four strains invaded and proliferated differently in plant tissues. Proliferation of mutants lacking the T3S effectors, i.e., noninvasive human strains, was reduced *in planta*. Reverse genetics and molecular biology experiments demonstrated that the immunosuppressive function of *Shigella* T3S effectors OspF and OspG was important for *Shigella* proliferation in plants. These observations indicate that *Arabidopsis* may be useful as a model host for studying the pathogenesis of *Shigella*.

Results

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

To observe the behavior of the human pathogen *Shigella* in plants, we investigated the interaction of four *Shigella* spp. strains representing three serogroups (*S. b*, *S. s*, *S. f* 2a, and *S. f* 5a) with the model plant *A. thaliana*. *Shigella* is a water-borne pathogen, with infection spreading via contaminated water (Pandey et al., 2014). Hence, we chose to use a flood-inoculation approach (Ishiga et al., 2011), which is thought to mimic natural inoculation closely. Similarly to the phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*), when 2-week-old *Arabidopsis* seedlings were inoculated with *S. s* or *S. f* 2a, clear symptoms were observed, such as yellowing and necrosis of the leaves (Fig. 1A). On the other hand, no obvious symptoms were apparent after inoculation with *S. b* and *S. f* 5a (Fig. 1A) (Liu et al., 2015). *Pst* Δ*hrcC*, a mutant lacking the T3SS of *Pst*, and the non-pathogenic bacterium *E. coli* DH5α were used as negative controls for infection. In addition to observing the symptoms, the bacterial growth *in planta* was also evaluated, to detect initial plant adherence and proliferation. Early attachment of all *Shigella* strains and DH5α was more than 10 times lower than that of *Pst* and *Pst* Δ*hrcC* (Fig. 1B). However, at 3 days post-inoculation (dpi), the extent of cell amplification differed depending on the *Shigella* strain; nevertheless, the cell number of all strains increased more than 1 000 times. Notably, *S. s* cell numbers increased over 10^5 times. This level of proliferation was comparable to that of plant pathogens. By contrast, the interaction between *S. f* 5a and *Arabidopsis* (in terms of symptoms and/or proliferation) was similar to that between the

plant and non-pathogenic DH5α.

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

Penetration of the plant leaf surface and subsequent internalization of *Shigella* spp.

Since we observed that *Shigella* proliferate and induce disease-like symptoms in *Arabidopsis*, we used scanning electron microscopy (SEM) and transmission electron microscopy (TEM) to examine whether the bacterium multiplies on the leaf surface or in the intercellular space (apoplast). The cuticle of a plant leaf is the first physical barrier encountered by the pathogen; known plant pathogens enter the host plant cell through a wound, an open stomata, or a vector (Yeats and Rose, 2013). As anticipated, *Pst*, which infects plants via open stomata (Panchal et al., 2016), colonized guard cells at 24 h post-infection (Fig. 2). All tested *Shigella* strains clustered around guard cells and the surface of epidermal cells (Fig. S2). *S. s* and *S. b* formed relatively wide clusters in the surrounding areas, including guard cells (Fig. 2 and S2). In particular, *S. b* and *S. f* 2a intensely colonized guard cells (Fig. 2), leading us to speculate that they enter plants via open stomata, similar to *Pst*.

Most plant pathogenic bacteria *in planta* infect and colonize the apoplast (Abramovitch et al., 2006; Gao et al., 2016). We used TEM to investigate whether *Shigella* is capable of intercellular colonization and causing damage to plant cells by analyzing *Arabidopsis* leaves flood-inoculated with *Shigella* (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 inoculated 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.

Taken together, these results indicated that, although the proliferative capacity of the *Shigella* strains differs, the cells can invade and colonize the apoplast of plants, thereby causing structural damage to the host.

Penetration of Arabidopsis root tissue by *Shigella* strains

Previously published studies demonstrate that several human pathogenic bacteria, including *E. coli* O157:H7 and *Enterococcus faecalis*, invade the leaves and roots of *A. thaliana* (Jha et al., 2005; Deering et al., 2012). To determine whether *Shigella* strains invade through *A. thaliana* roots, we attempted to label the four *Shigella* strains and DH5a with green fluorescent protein (GFP) and then observe them in live plant tissues. The *S. b*, *S. f* 2a, and *S. f* 5a strains were labeled successfully with GFP (the *S. s* strain was not), confirming that GFP labeling did not affect their growth in plants (Fig. S1).

Next, bacterial suspensions were dropped onto the root tips of *Arabidopsis* plants that were grown vertically. Five days later, whole plants and root tissues were photographed under ultraviolet (UV) light to observe the distribution of fluorescently labeled bacteria (Fig. 4). In accordance with the disease phenotypes observed, *Pst* (which is a foliar pathogen) exhibited strong fluorescence throughout the plant, despite the fact that it was applied to root tips (Fig. 4A). To observe *Pst* in root tissues, inoculated roots were washed with sterile water and observed under a fluorescence microscope. GFP fluorescence was observed only in epidermal cells after application of *Pst* to root tips (Fig. 4B). This finding indicates that *Pst* spreads throughout the entire plant along its surface, not by invading root tissues or colonizing the roots. Fluorescence was also observed throughout plants when GFP-labeled *S. b*, *S. f* 2a, and *S. f* 5a were applied to root tips, although the fluorescence intensity differed (Fig. 4A). In root tissues, GFP fluorescence was observed in root endodermal cells only in *S. f* 2a-treated plants (Fig. 4B). Taken together, these results indicate that *Shigella* invades plant leaves and roots in a strain-dependent manner, and then moves along the surface and through the internal vascular tissues of the plant.

In addition, we dropped bacterial solutions onto root tips and observed symptoms in *Arabidopsis* plants for 14 d. Severe inhibition of root growth and leaf yellowing were observed in plants inoculated by the *S. b*, *S. s*, and *S. f 2a* strains (Figure S3). Inoculation of *S. f 5a* caused slight inhibition of root growth, but caused much less damage to the plant than other strains.

Shigella T3S effectors are necessary for attachment and multiplication in _Arabidopsis_

T3SS is the key determinant of the virulence of many Gram-negative bacteria, including animal and plant pathogens. Thus, we investigated if pathogenic proteins, which are involved in animal host infections, are required for invasion and multiplication of *Shigella* in plants. To study the biological role of T3SS in the interaction between *Shigella* and plants, we used noninvasive variants of *S. f* 2a and *S. f* 5a (strains Δvp and BS176, respectively) (Sansonetti et al., 1982; Wenneras et al., 2000; Shim et al., 2007). To facilitate observation of bacterial invasion in living plants, strains *S. f* 2a, *S. f* 5a, and their variants were labeled with GFP, and bacterial growth and expression of effector proteins were verified (Fig. S4). Bacterial proliferation in plants after inoculation with Δvp or BS176 strains was 10 times lower than that after inoculation by parental *Shigella* strains; similar results were observed for GFP-labeled *Shigella* strains and mutants (Fig. 5A).

To examine involvement of T3SS in bacterial invasion of the plant surface, leaves were flood-inoculated with GFP-labeled bacteria and leaf surfaces were observed 24 h later (Fig. 5B). Examination under UV light revealed that the levels of GFP-labeled *S. f* 2a and *S. f* 5a on leaf surfaces were higher than those of Δvp and BS176, especially in open stomata (Fig. 5B). We also confirmed that none of the *Shigella* strains grew in plant culture medium (Fig. S5), and that effector protein expression was maintained in bacteria recovered from plants (Fig. S6).

To determine if other *Shigella* serotypes interact with plants in a T3SS-dependent manner, we attempted to obtain *S. s* and *S. b* mutants that had lost pathogenic plasmids via continuous subculture. A non-pathogenic mutant was successfully obtained for *S. s* (Fig. S7). When plants were inoculated with this T3SS-deficient *S. s* strain, disease phenotypes and bacterial growth were greatly reduced (Fig. S7), as observed with the *S. f* serotype. Altogether, these results suggested that the T3SS

of *Shigella* that operate during an infection in human are also required for interactions with plants.

Expression of *Shigella* T3S effector genes and modulation of *Arabidopsis* MAPK signaling after *Shigella* inoculation

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

To investigate the plant innate immune responses to *Shigella* inoculation, MAPK phosphorylation was evaluated. The activation of MAPK by phosphorylation is a conserved response of the earliest microbe-triggered immune signaling in both plants and animals (Zipfel, 2009). The flg22 peptide is a representative microbe-associated molecular pattern in plants (Bethke et al., 2009). In plants treated with flg22, pronounced MAPK phosphorylation was apparent within 5 min of treatment and this response lasted up to 30 min (Fig. 6B). 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. 6B). 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

243 those elicited by the flg22 treatment (Fig. 6B). These observations indicated that *Shigella* suppresses
244 the innate immunity of *Arabidopsis* via its T3S virulence effectors.
245
246 **Suppression of immune signaling in *Arabidopsis* plants by *Shigella* T3S effectors OspF and**
247 **OspG**
248 The most important role of T3S effector proteins injected into host cells is modulation of the
249 host immune response. Therefore, we chose two *Shigella* effectors, OspF and OspG, to study
250 suppression of plant immune responses by *Shigella* effector proteins. *Shigella* OspF was chosen
251 because of its ability to inhibit MAPK signaling, which is conserved in plants and animals (Arbibe et
252 al., 2007; Li et al., 2007). OspG is an important immunosuppressive effector protein secreted at the
253 later stages of infection; this protein interferes with activation of the NF-κB pathway (Kim et al.,
254 2005), which is absent from plants.
255 To find out whether OspF and OspG have virulence activity in plants, we introduced them
256 into a phytopathogen, *Pst*, and monitored its pathogenicity. First, we used an AvrRpt2-derived T3SS
257 reporter system to check that the introduced OspF or OspG protein was delivered to plant cells via *Pst*
258 T3SS (Mudgett, 2005). When *Arabidopsis* leaves were syringe-infiltrated with various *Pst*-producing
259 AvrRpt$^{101–255}$ constructs, we found that *Pst* producing OspF:AvrRpt2$^{101–255}$ or OspG:AvrRpt2$^{101–255}$
260 induced a cell death response at 1 dpi, while control *Pst* cells containing the empty vector did not.
261 This indicated that the *Shigella* effectors OspF and OspG were delivered successfully into
262 *Arabidopsis* cells via *Pst* T3SS (Fig. S8A, B). Full-length AvrRpt2 was used as a positive control for
263 cell death response. Finally, we examined whether the virulence of *Pst* was increased by expression of
264 *Shigella* OspF or OspG. Plants infected with *Pst* cells producing OspF: HA or OspG: HA showed
265 more severe symptoms than plants infected with the empty vector control (Fig. 7A; Fig. S8C). In
266 addition, the number of bacterial cells was 10 times higher than that in plants infected with the empty
267 vector control (Fig. 7B). Production of OspF:HA and OspG:HA by *Pst* was confirmed by
268 immunoblotting with an anti-HA antibody (Fig. S8D). Taken together, these observations suggest that
269 the *Shigella* effectors OspF and OspG function as virulence factors in plant cells.

We attempted to produce the OspF and OspG proteins in plant cells to verify whether their known function in the human host is conserved in the plant cell. To do this, subcellular localization of the two proteins fused with GFP was evaluated in the plant cells. The DNA fragments encoding the OspF:GFP and OspG:GFP fusions were inserted into a binary plant expression vector and transiently expressed in *Nicotiana benthamiana* leaves using the *Agrobacterium* system (Fig. S9A, B). 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 (Fig. S9C). In the host human cell, OspF localization is nuclear (Zurawski et al., 2006; Arbibe et al., 2007) and that of OspG is nuclear and cytoplasmic (Kim et al., 2005; Zhou et al., 2013; de Jong and Alto, 2014), i.e., their subcellular localization is similar to that of plant cells.

Previously, *in vitro* studies demonstrated that OspF exerts a phosphothreonine lyase activity and irreversibly removes phosphate groups from MAPK (Arbibe et al., 2007; Li et al., 2007). We used a phenotypic screening system involving a MEK2 (a tobacco MAP kinase kinase2) mutant, $MEK2^{DD}$, to investigate whether the virulence associated with *Shigella* OspF in plants was linked to the same mechanism of action as in animals. $MEK2^{DD}$, a constitutively active mutant of MEK2, induces cell death when overproduced in plant leaves (Yang et al., 2001; Kim and Zhang, 2004). 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 a pronounced cell death (Fig. 7C). Co-production of OspF:GFP, but not OspG:GFP, and $MEK2^{DD}$ completely inhibited the $MEK2^{DD}$-induced cell death (Fig. 7C). 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. 7D), even though both proteins were stably produced in the absence of $MEK2^{DD}$ (Fig. S9B). 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. 7D). The production of $MEK2^{DD}$ protein was confirmed in all samples using anti-HA antibodies (Fig. 7D).

These observations strongly suggest that the *Shigella* effector OspF inhibits plant immune responses by inhibiting activation of MAPK (as in humans), and that OspG induces immunosuppression in plants by targeting distinct MAPK pathways.

OspF or OspG affects *Shigella* proliferation in plants

To see whether the OspF or OspG proteins play a role in the interaction between *Shigella* and plants (as in human cells), we inoculated plants with *S. f* 5a mutants lacking the OspF or OspG proteins and examined their growth. Growth of mutants lacking *ospF* or *ospG* was as deficient as that of the virulence plasmid-deficient mutant BS176 (Fig. 8A). Reduced growth of *S. f* 5a *ΔospF* or *S. f* 5a *ΔospG* mutants was restored completely by complementation of the mutation, indicating that these two effector proteins are indeed important for bacterial growth in plants (Fig. 8A). Next, we monitored activation of MAPKs to determine whether plant immune suppression was affected by deletion of *ospF* or *ospG*. As shown in Figure 8B, *S. f.* 5a Δ*ospF* induced stronger phosphorylation of MAPKs than wild-type *S. f* 5a; this was offset by complementation with OspF. By contrast, phosphorylation of MAPK in *S. f* 5a *ΔospG*-inoculated plants was no different from that in plants inoculated with wild-type *S. f* 5a, although phosphorylation of MAPKs increased slightly after complementation with OspG (Fig. 8B). Taken together, these results indicate that *Shigella* effectors OspF or OspG play an important role in increasing bacterial proliferation in both plant and animal hosts.

Discussion

In the current study, we investigated the interaction of the human pathogenic bacterium, *Shigella*, with an alternative host, the *Arabidopsis* plant. We demonstrated that four *Shigella* strains, *S. b*, *S. s*, *S. f* 2a, and *S. f* 5a, invade and colonize *Arabidopsis* to different extents. 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 and Sansonetti, 2007). We demonstrated in the current study that, in addition to the human host, *Shigella* invades *Arabidopsis*, through vascular tissues 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 (Fig. 2; Fig. S2). Interestingly, we found that the four studied strains of *Shigella* associate with the plant cells and induce different plant responses. The bacterial loads of *S. s in planta* were relatively higher than those of the other strains. By contrast, inoculation of *S. f* 5a was associated with lower bacterial proliferation and less severe symptoms than observed for other strains (Figs. 1–4; Fig. S2 and Fig. S3). *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 interactions. 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 and 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 interactions with *Arabidopsis*. We showed that secretion of T3S effectors is required to trigger both symptoms and expression of the invasive phenotypes in plants (Fig. 5; Fig. S7). Furthermore, the effector proteins impacted MAPK-dependent innate immune responses in *Arabidopsis* (Fig. 6–8). 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 bacterial growth inside plants (Shaw et al., 2008). The relevance of T3SS for 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 and Yaron, 2011). By contrast, the T3SS and specific effectors play an important role in proliferation and survival of *Pseudomonas syringae* pv. *syringae* B728a on the plant leaf surface (Lee

et al., 2012). 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 (Park et al., 2017), is required.

Expression of the T3SS of *Shigella* is regulated at the transcriptional level and is activated at a permissive temperature (≥ 32°C) (Tobe et al., 1991; Campbell-Valois and Pontier, 2016). We were able to observe expression of the T3SS genes of *Shigella* under temperatures at which plants grow (22 ± 3°C) (Fig. 6A). A recent study showed that elevation of the temperature increases T3SS-mediated virulence of the phytopathogen *Pst* in plants, which is in contrast with the negative effect of high temperature on expression of the T3SS of *Pst in vitro* (Huot et al., 2017). Regardless of the temperature of host cells, it will be interesting to determine whether *Shigella* regulates T3SS gene expression *in vivo* and to identify factors that influence T3SS gene expression other than plant temperature.

Salmonella strains capable of proliferating on plant leaves and actively entering plant tissues, root hairs, or trichomes were recently shown to exhibit virulence in animals (Barak et al., 2011; Golberg et al., 2011; Schikora et al., 2011). We demonstrated that *Shigella* strains actively colonize the surface of and inside *Arabidopsis* leaves and root tissues (Figs. 1–4) and that bacteria recovered from plants maintain expression of pathogenic proteins (Fig. S6). Collectively, these findings suggest that, similar to *Salmonella*, *Shigella*-inoculated plants are a serious risk to food safety and that contamination of plants is another route underlying infection of *Shigella*, an important human pathogenic bacterium. In this study, we only observed plants artificially inoculated with *Shigella* in a laboratory environment. Therefore, to confirm the food safety concern surrounding *Shigella*-inoculated plants, the ability of *Shigella* to infect a variety of crops grown in the field should be tested. The pathogenicity of plant-isolated *Shigella* in animals should also be investigated.

The current study provides new insights into host invasion mechanisms utilized by *Shigella* to interact with an alternative host, the plant *Arabidopsis*. Studying trans-kingdom pathogenesis involving human-adapted pathogens, such as *Shigella*, may uncover novel pathogenic mechanisms

uniquely activated in response to specific hosts. When we isolated the two *Shigella* effectors OspF and OspG, and produced them in plant cells, their localization coincided with that in the animal cells (Fig. S9C), and it was apparent that the production of both proteins increased the virulence of plant pathogens (Fig. 7). In addition, we confirmed that OspF inhibits the innate immune response of plants via the same enzymatic activity as in animals (Fig. 7 and Fig. 8). In animals, OspG inhibits the host immune response by inhibiting the activity of NF-κB by blocking degradation of IκB (Ashida et al., 2015). Plants possess an IκB-like protein called NIM1 (Ryals et al., 1997); however, no other published studies have investigated whether a NF-κB–induced immune response exists in plants. In the current study, we demonstrated the ability of OspG to increase the phytopathogenicity of non-*Shigella* bacteria, and also observed degradation of OspG:GFP upon constitutive activation of MAPK signaling (Fig. 7). 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.

Experimental procedures

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 and 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

405 immune response in terms of MAPK activity, 1 μM flg22 peptide (#FLG22-P-1; Alpha Diagnostics,
406 Inc., San Antonio, TX, USA) was used as a positive control (Bethke et al., 2009).
407
408 **Bacterial strains, growth conditions, and plasmids**
409 The bacterial strains and plasmids used in the study are described in S1 Table. *Shigella* and
410 *Pseudomonas* strains harboring plasmid pDSK-GFPuv were generated by electroporation, as
411 described previously (Wang et al., 2007; Hong et al., 2016).
412 *Shigella* spp. were grown at 37°C in Luria-Bertani (LB) medium or tryptic soy agar
413 containing 0.003% (w/v) Congo red dye (Sigma-Aldrich, St. Louis, MO, USA) (Runyen-Janecky and
414 Payne, 2002). *Pseudomonas syringae* strains were grown at 28°C (with shaking at 200 rpm) in King's
415 B liquid medium (Sigma-Aldrich) containing appropriate antibiotics (King et al., 1954). *Escherichia*
416 *coli* DH5α was grown in LB medium at 37°C with shaking (Kennedy, 1971) and used as a non-
417 pathogenic control. *Agrobacterium tumefaciens* GV2260 was grown at 28°C in LB broth with shaking
418 at 200 rpm (Shamloul et al., 2014).
419 The coding region of *ospF* or *ospG* was PCR-amplified using *attB*-containing PCR primers
420 (S2 Table). The PCR fragments were cloned into the pDONR™207 vector by BP recombination using
421 the Gateway® BP Clonase™ II kit (Invitrogen, Carlsbad, CA, USA). The products were then
422 transferred to pBAV178 (for AvrRpt2 fusion) or pBAV179 (for HA fusion) vectors by LR
423 recombination (Gateway® LR Clonase™ II, Invitrogen). pBAV178, pBAV179, and pME6012 (empty
424 vector control) were kindly provided by Dr. Jean T. Greenberg (University of Chicago) (Vinatzer et
425 al., 2005). The products were also transferred to pK7FWG2 (obtained from Ghent University,
426 Belgium) by LR recombination to produce the GFP-fused *Shigella* effectors OspF:GFP and
427 OspG:GFP (Karimi et al., 2002).
428
429 **Bacterial inoculation assay *in planta***
430 *Arabidopsis* seedlings (2 weeks old) grown in 1/2 MS medium were used for flood-
431 inoculation (Ishiga et al., 2011). Briefly, 10 *Arabidopsis* seedlings in one container were incubated for

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

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

Microscopy

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

For fluorescence confocal microscopy, inoculated *Arabidopsis* leaves or roots were washed with sterile water. 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* ; S3 Table) (Bando et al., 2010). The qRT-PCR results were normalized to expression of 16s rRNA.

Immunoblotting

Total protein was extracted from *Shigella*- or *Agrobacterium*-infected leaves (from three plants) using denaturing extraction buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% Triton-X, 1× protease inhibitor cocktail (Roche, Basel, Switzerland), 0.4 M DTT, 1 M NaF, and 1 M Na_3VO_3]. The extracted proteins were separated on 12% SDS-PAGE gels and transferred to a PVDF membrane (Pierce, Rockford, IL, USA). Antibodies specific for phospho-p44/p42 MAPK ERK1/2 (#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 Student's t-test (Microsoft Office Excel) and ANOVA (SPSS v.18; IBM, Armonk, NY, USA) (Moon et al., 2016). Asterisks and letters indicate significant differences between samples ($P < 0.05$).

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The authors declare no conflict of interest.

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686

687 **Figure legends**

688 **Fig. 1.** *Shigella* proliferates and induces disease symptoms in *Arabidopsis* plants. **(A, B)** *Arabidopsis*
689 seedlings in 1/2 MS medium were flood-inoculated with sterile water containing 0.025% Silwet L-77
690 (Mock) or bacterial suspensions (5×10^5 cfu/ml). **(A)** The symptoms of *Arabidopsis* inoculated with
691 *Shigella* spp. by flood-inoculation. Photographs of representative symptoms were taken at 3 dpi. **(B)**
692 Bacterial cell numbers were evaluated on Days 0 and 3 after flood-inoculation. The bars represent the
693 mean ± SD of three replicates and the different letters indicate significant differences between
694 samples ($P < 0.05$, one-way ANOVA). All experiments were repeated three times independently and
695 representative results are shown.
696
697 **Fig. 2.** *Shigella* adheres to the *Arabidopsis* epidermis and clusters around guard cells. *Arabidopsis*
698 seedlings were flood-inoculated with bacterial suspensions (5×10^6 cfu/ml). After 24 h, the leaves
699 were fixed and analyzed under a SEM. The *Pst* cells were observed over the stomata. *Shigella* strains
700 were associated with the stomata. Bar, 10 µm. Representative image showing bacteria around guard
701 cells (indicated by white arrows). The results are representative of two independent experiments.
702
703 **Fig. 3.** *Shigella* localizes in the apoplast and affects plant cell structures. *Arabidopsis* seedlings were
704 flood-inoculated with bacterial suspensions (5×10^6 cfu/ml). After 24 h, the leaves were evaluated
705 under a TEM. The *Shigella* strains colonized the intercellular spaces. Bar, 5 µm (left panels). Bar, 1
706 µm (right panels). Each column of micrographs represents a different magnification. TEM images
707 revealed the *Shigella* (white arrows) attached to the cell wall in the intercellular spaces and altered
708 mesophyll cells. Black arrows indicate separation of the plasma membrane from the cell wall, the
709 abnormal organelles and disruption of chloroplasts. The results are representative of two independent
710 experiments.
711
712 **Fig. 4.** *Shigella S. f 2a* invades and colonizes plant roots. **(A, B)** Root tips of *Arabidopsis* seedlings
713 were drop-inoculated with GFP-labeled bacterial suspensions (5×10^7 cfu/ml). **(A)** Bacterial

714 colonization of inoculated plants was photographed under white and UV light at 5 dpi. **(B)** GFP-
715 labeled *Shigella* are localized in the epidermal or endodermal cells of *Arabidopsi*s roots. GFP images
716 were taken using a confocal microscope. Higher magnification micrographs and 3D Raman confocal
717 volume images show internalization of *S. f* 2a in *Arabidopsis* roots. All experiments were repeated at
718 least three times, and representative results are shown.
719
720 **Fig. 5.** The virulence plasmid-deficient mutant of *Shigella* is impaired in the *Arabidopsis*
721 multiplication. **(A, B)** *Arabidopsis* seedlings in 1/2 MS medium were flood-inoculated with DH5α, *S.*
722 *f* 2a, Δvp, *S. f* 5a, or BS176, and their GFP-labeled variants (5×10^5 cfu/ml). **(A)** The bacterial cell
723 numbers *in planta* were determined at 0 and 3 dpi. Bars represent the mean ± SD of three replicates
724 and the different letters indicate significant differences between samples ($P < 0.05$, one-way ANOVA).
725 **(B)** Images of GFP-labeled wild-type *Shigella* in leaf epidermal cells of *Arabidopsis* acquired at 24
726 hpi under a fluorescence confocal microscope. Bar, 10 μm. The blue represents auto-fluorescence of
727 chlorophyll. All experiments were repeated at least three times, and representative results are shown.
728
729 **Fig. 6.** Expression of *Shigella* effectors and MAPK activation in *Arabidopsis* plants in response to
730 virulence plasmid-deficient *Shigella* mutants. **(A, B)** *Arabidopsis* leaves were syringe-infiltrated with
731 buffer, 1 μM flg22, or bacterial suspension (5×10^6 cfu/ml), and samples were collected at the
732 indicated times. **(A)** Transcription of *Shigella* effectors (*ipaB*, *ipaC*, *icsA*, *icsB*, *virB*, and *virF*) in
733 infected *Arabidopsis* leaves was analyzed by qRT-PCR. qRT-PCR results were normalized to
734 expression of 16s rRNA. Expression of effectors by plasmid-deficient mutant strains was compared
735 with that in the WT. Data are expressed as the mean ± SD of three replicates. **(B)** Immunoblotting was
736 performed using either anti-phospho-p44/42 (ERK1/2, upper panels) or anti-ERK1 (lower panels)
737 antibodies. All experiments were repeated at least three times, each with similar results.
738
739 **Fig. 7.** The virulence activityies of *Shigella* effectors OspF or OspG. **(A, B)** *Arabidopsis* plants were
740 sprayed with *Pst* cells carrying an empty vector (pME6012), or with cells producing OspF:HA or

741 OspG:HA (5×10^7 cfu/ml). **(A)** Disease symptoms were monitored for 4 d after spraying. **(B)**
742 Proliferation of *Pst* cells producing *Shigella* effector proteins in *Arabidopsis* at 0 and 4 d after spray-
743 inoculation. Bars represent the mean ± SD of six replicates, and different letters indicate significant
744 differences between samples ($P < 0.05$, two-way ANOVA). All experiments were repeated at least
745 three times. **(C)** Co-expression of OspF:GFP suppresses tobacco $MEK2^{DD}$-triggered cell death. GFP,
746 OspF:GFP, or OspG:GFP was produced with HA:$MEK2^{DD}$ in *N. benthamiana* leaves upon infiltration
747 by *Agrobacterium* carrying the appropriate expression constructs (OD_{600} 0.4). **(D)** Production of
748 specific proteins in samples from panel A was analyzed by immunoblotting using anti-GFP, anti-HA,
749 anti-phospho-p44/42 (ERK1/2), and anti-ERK1 antibodies. Ponceau S was used to stain the RuBisCo
750 protein (loading control). The asterisk indicates the size of the GFP protein not associated with
751 effector proteins.
752
753 **Fig. 8.** The *Shigella* effector proteins OspF and OspG underpin the bacterial virulence in plants. **(A)**
754 The virulence of the OspF- or OspG-deficient *Shigella* mutants is impaired in the *Arabidopsis* model.
755 *Arabidopsis* seedlings in 1/2 MS medium were flood-inoculated with *S. f* 5a, Δ*ospF*, Δ*ospF*+p*ospF*,
756 Δ*ospG*, or Δ*ospG*+p*ospG* (5×10^5 cfu/ml). Photographs of representative disease symptoms were
757 taken, and bacterial cell numbers *in planta* were determined, at 3 dpi. The bars represent the mean ±
758 SD of three replicates. The different letters indicate significant differences between samples ($P < 0.05$,
759 one-way ANOVA). **(B)** MAPK activation in *Arabidopsis* plants in response to infection by ospF or
760 ospG deletion mutants. *Arabidopsis* leaves were infiltrated with each bacterial strain as described
761 previously and then collected at the indicated times. Anti-phospho-p44/42 (ERK1/2, upper panels) or
762 anti-ERK1 (lower panels) antibodies were used for immunoblotting. All experiments were repeated at
763 least three times, with similar results.

A


Mock
DH5α
Pst
Pst hrcC-
S. b
S. s
S. f 2a
S. f 5a
3 dpi

B

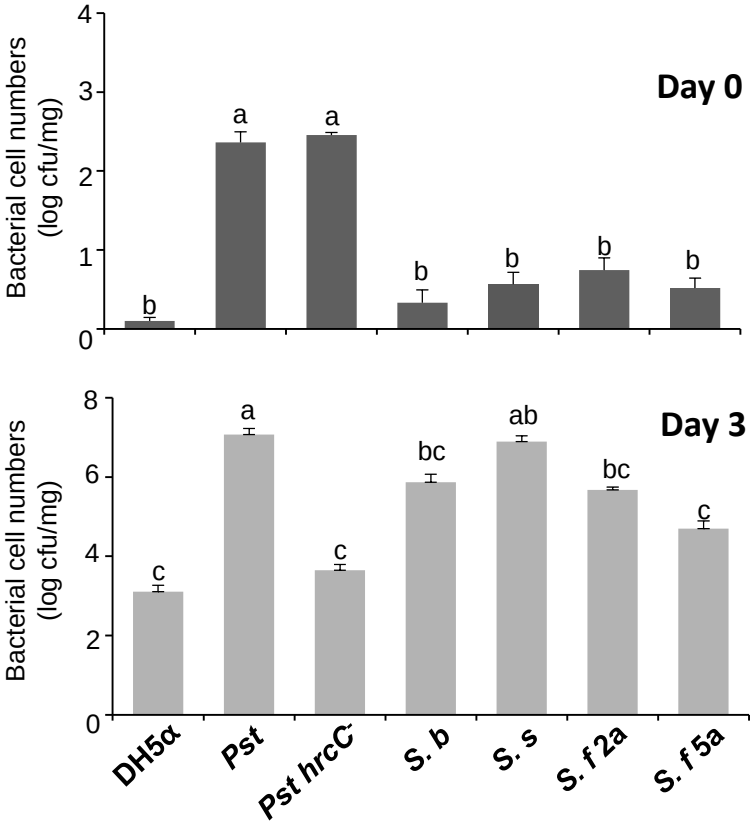

Bacterial cell numbers (log cfu/mg)
Day 0
a
a
b
b
b
b
Bacterial cell numbers (log cfu/mg)
Day 3
c
a
c
bc
ab
bc
c
DH5α
Pst
Pst hrcC-
S. b
S. s
S. f2a
S. f5a

Fig.1

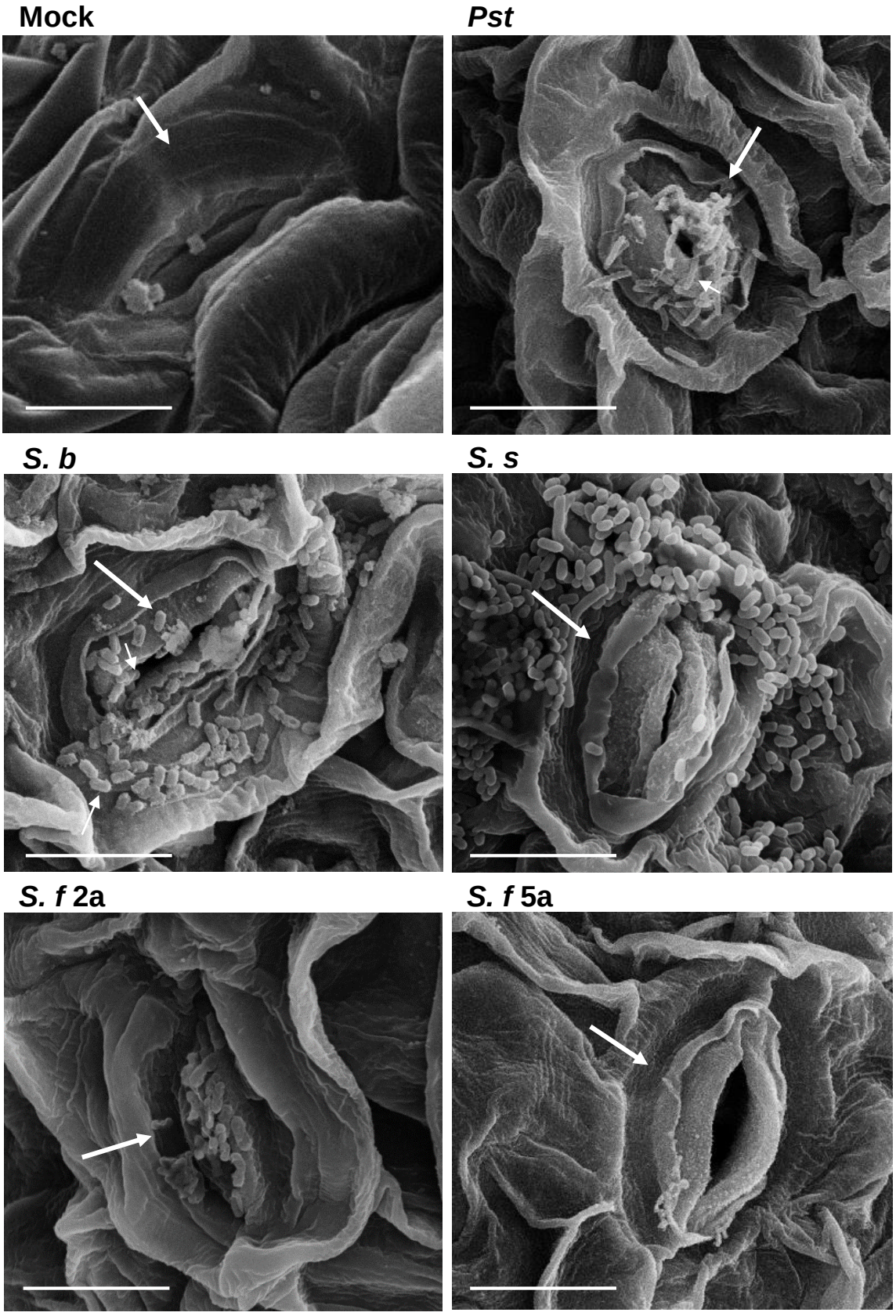


Fig.2

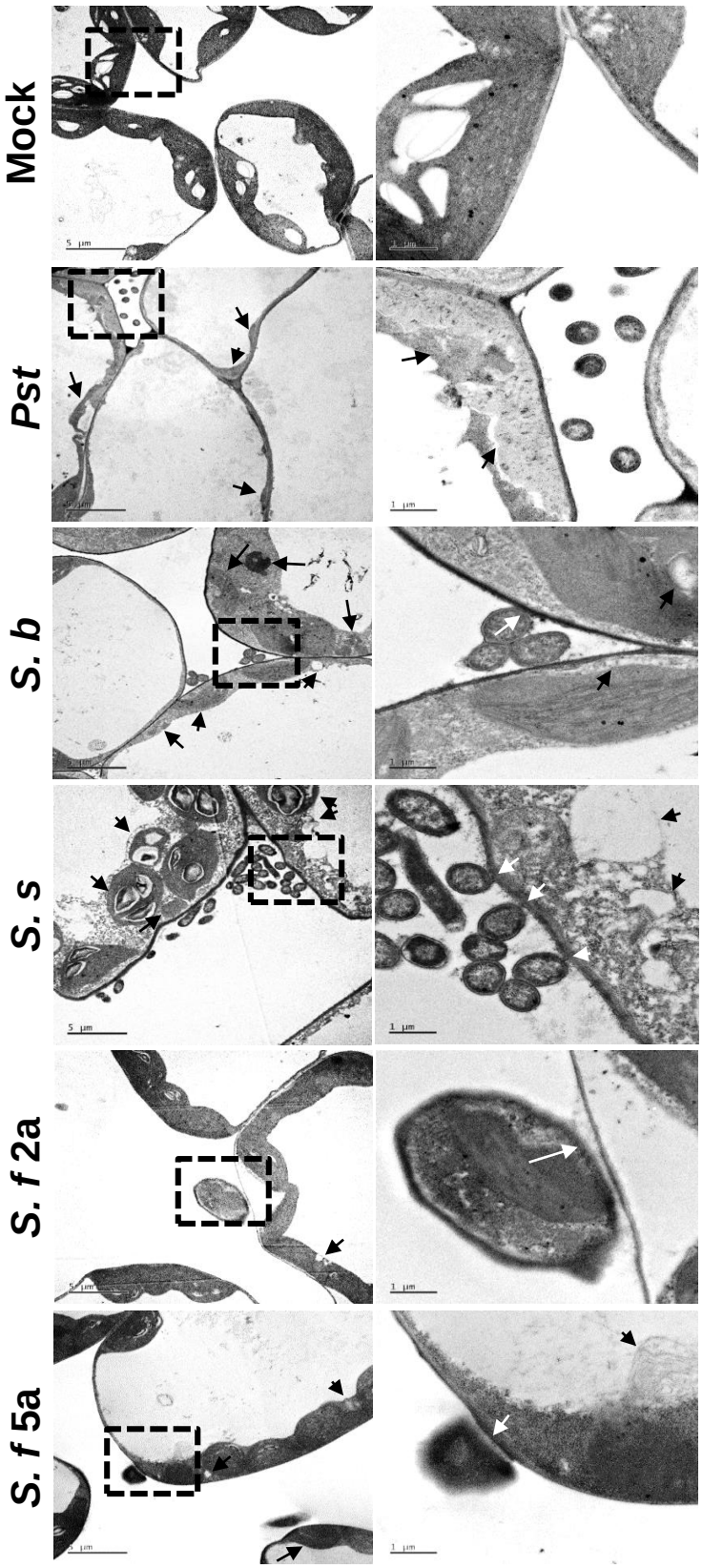


Fig.3

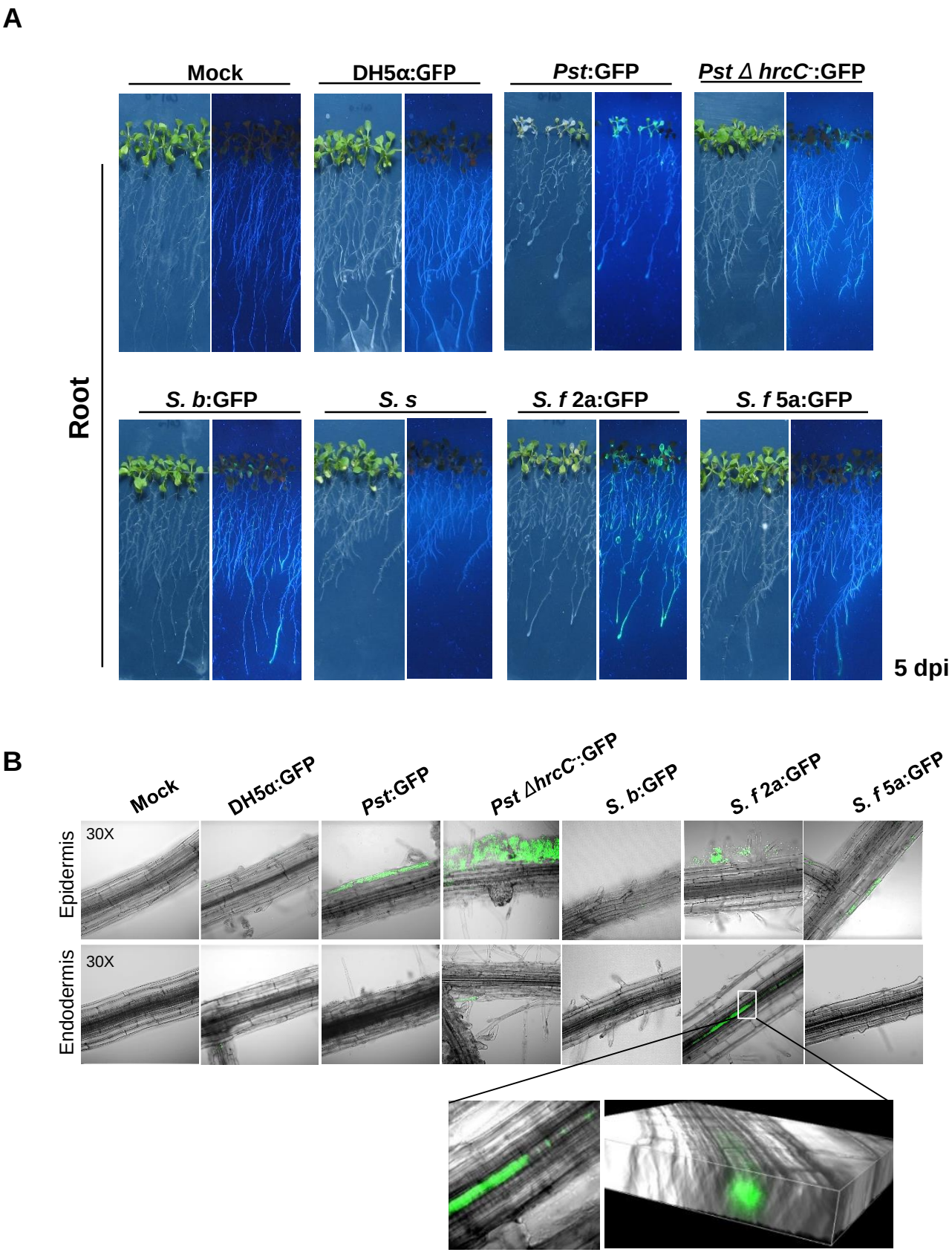

A
Mock
DH5α:GFP
Pst:GFP
Pst Δ hrcC⁻:GFP
Root
S. b:GFP
S. s
S. f 2a:GFP
S. f 5a:GFP
5 dpi
B
Mock
DH5α:GFP
Pst:GFP
Pst ΔhrcC⁻:GFP
S. b:GFP
S. f 2a:GFP
S. f 5a:GFP
Epidermis
30X
Endodermis
30X

Fig.4

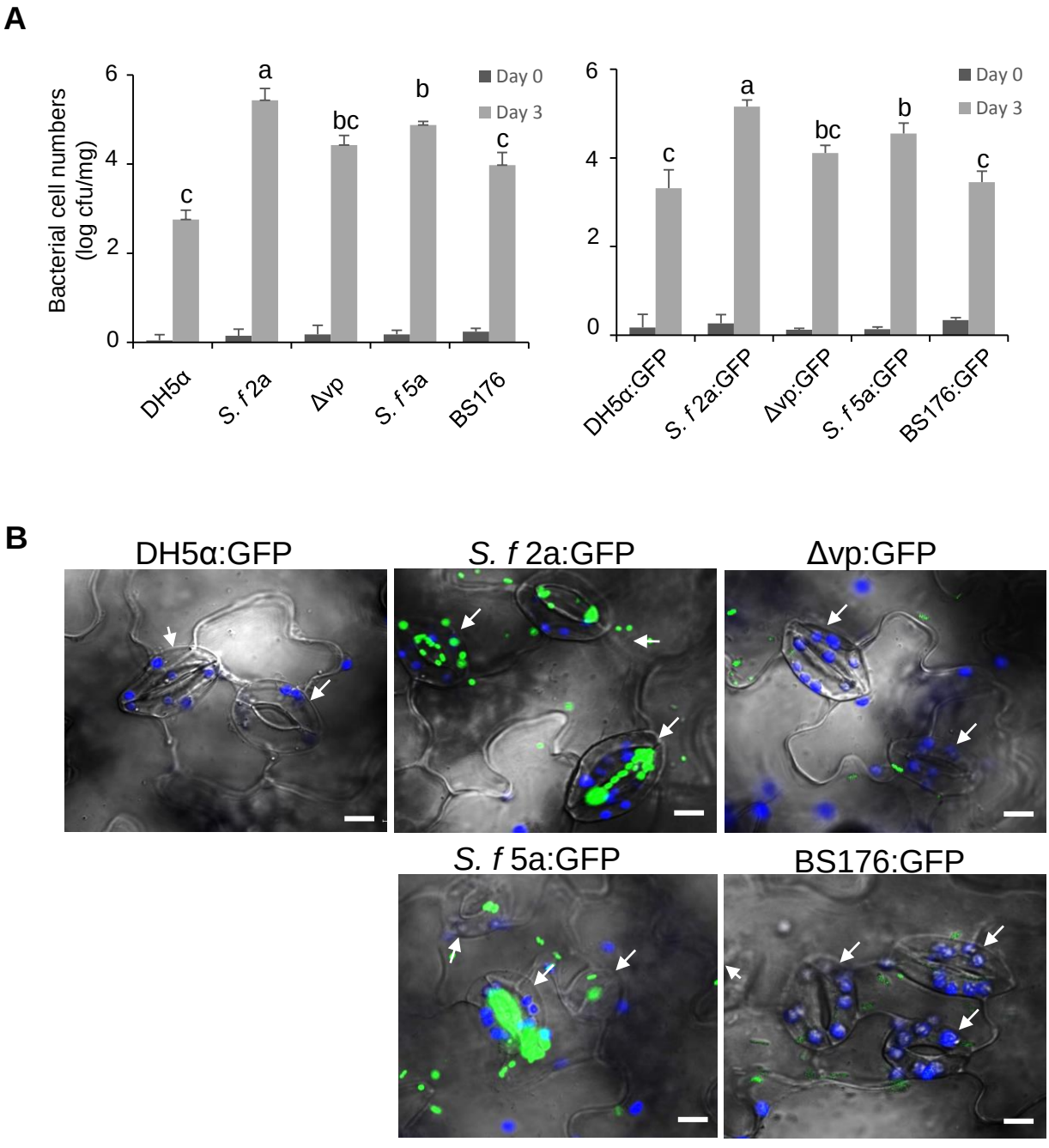
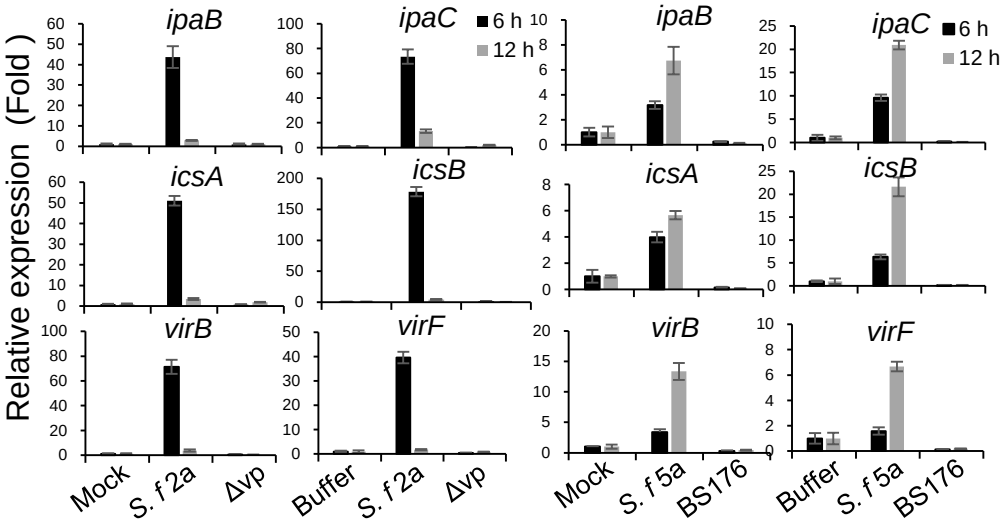

A
Bacterial cell numbers (log cfu/mg)
Day 0
Day 3
DH5α
S. f 2a
Δvp
S. f 5a
BS176
DH5α:GFP
S. f 2a:GFP
Δvp:GFP
S. f 5a:GFP
BS176:GFP
B
DH5α:GFP
S. f 2a:GFP
Δvp:GFP
S. f 5a:GFP
BS176:GFP

Fig.5

A


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

B

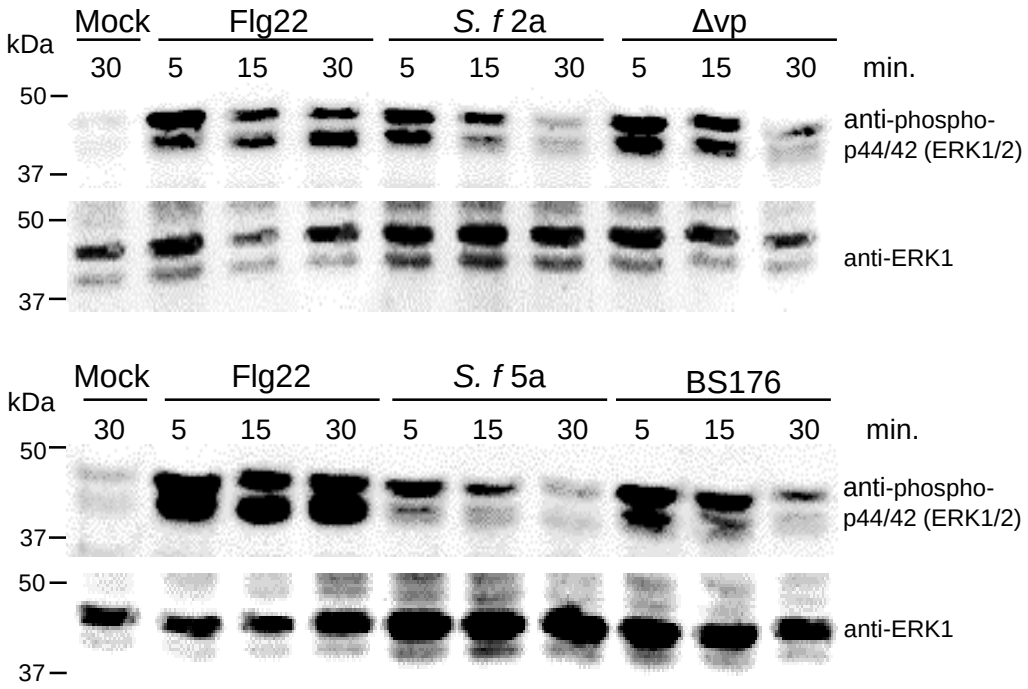

kDa
Mock
Flg22
S. f 2a
Δvp
30
5
15
30
min.
50
37
anti-phospho-p44/42 (ERK1/2)
anti-ERK1
S. f 5a
BS176

Fig.6

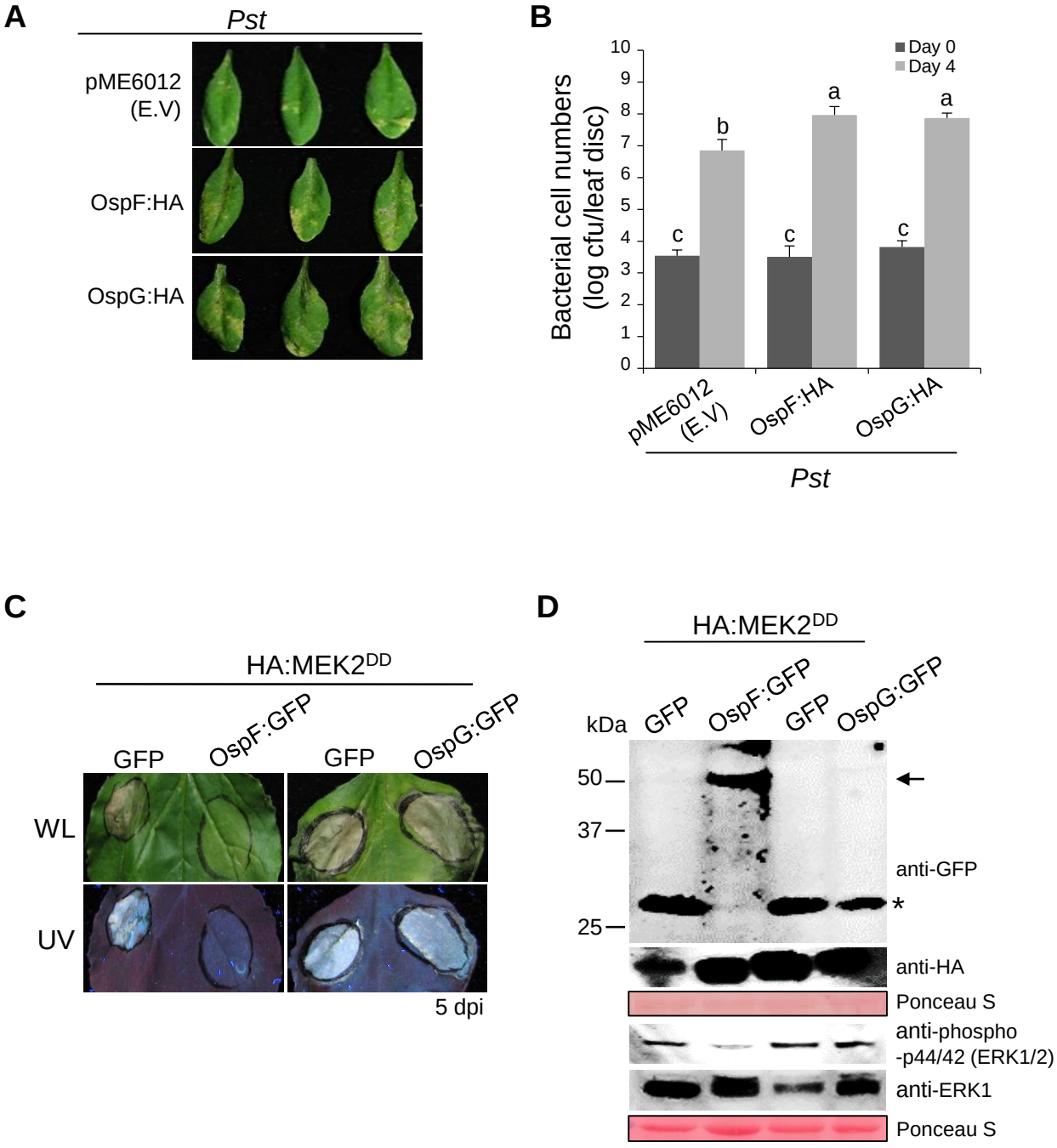

A
Pst
pME6012
(E.V)
OspF:HA
OspG:HA
B
Bacterial cell numbers
(log cfu/leaf disc)
Day 0
Day 4
10
9
8
7
6
5
4
3
2
1
0
a
b
c
c
a
c
pME6012
(E.V)
OspF:HA
OspG:HA
Pst
C
HA:MEK2DD
GFP
OspF:GFP
GFP
OspG:GFP
WL
UV
5 dpi
D
HA:MEK2DD
kDa
GFP
OspF:GFP
GFP
OspG:GFP
50
37
25
anti-GFP
*
anti-HA
Ponceau S
anti-phospho
-p44/42 (ERK1/2)
anti-ERK1
Ponceau S

Fig.7

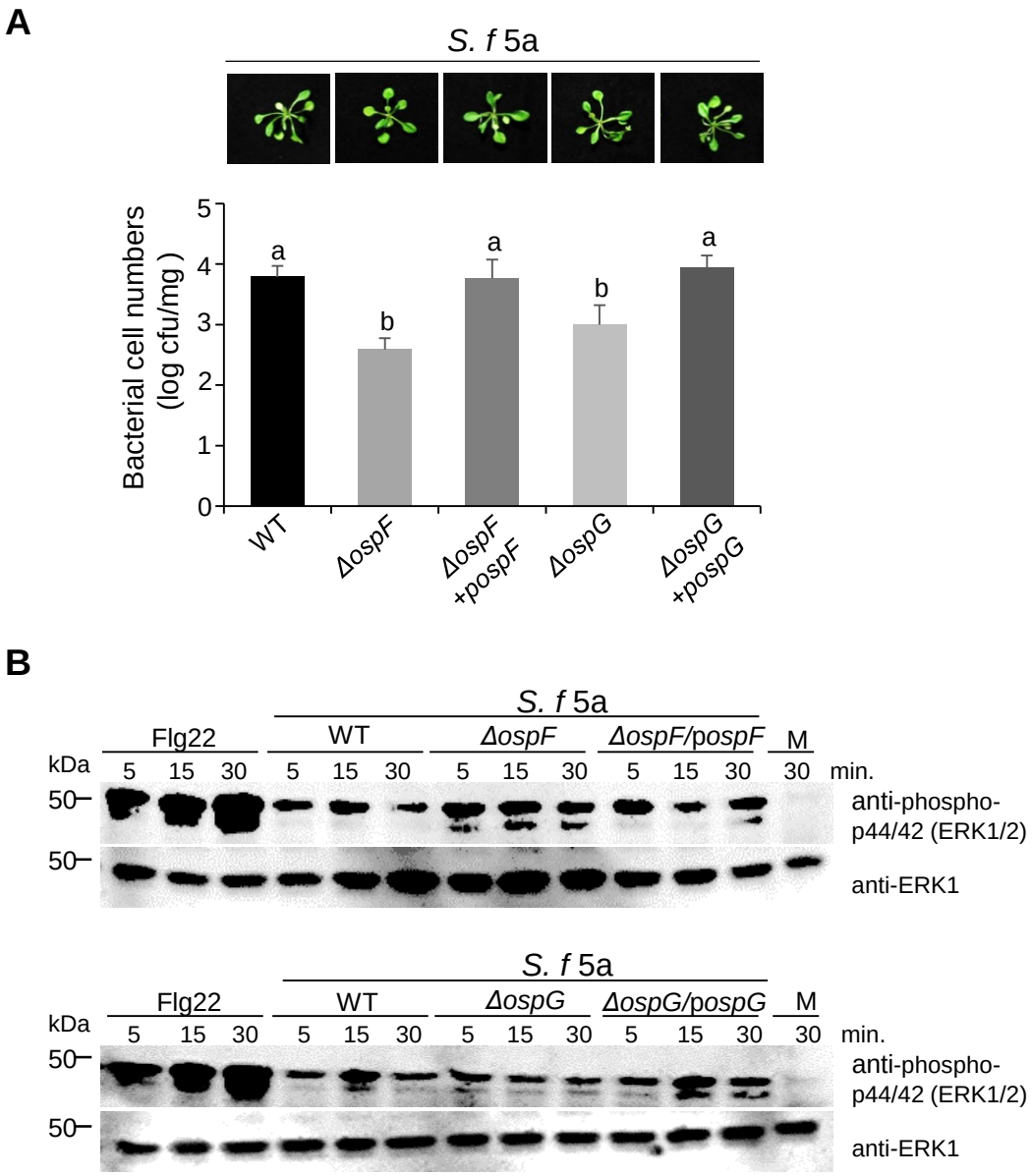

A
S. f 5a
Bacterial cell numbers (log cfu/mg)
5
4
3
2
1
0
a
b
a
b
a
WT
ΔospF
ΔospF +pospF
ΔospG
ΔospG +pospG
B
S. f 5a
Flg22
WT
ΔospF
ΔospF/pospF
M
kDa
5 15 30 5 15 30 5 15 30 5 15 30 30 min.
50
anti-phospho-p44/42 (ERK1/2)
anti-ERK1
S. f 5a
Flg22
WT
ΔospG
ΔospG/pospG
M
kDa
5 15 30 5 15 30 5 15 30 5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

Fig.8