PLANTS AS ALTERNATIVE HOSTS FOR HUMAN AND ANIMAL PATHOGENS

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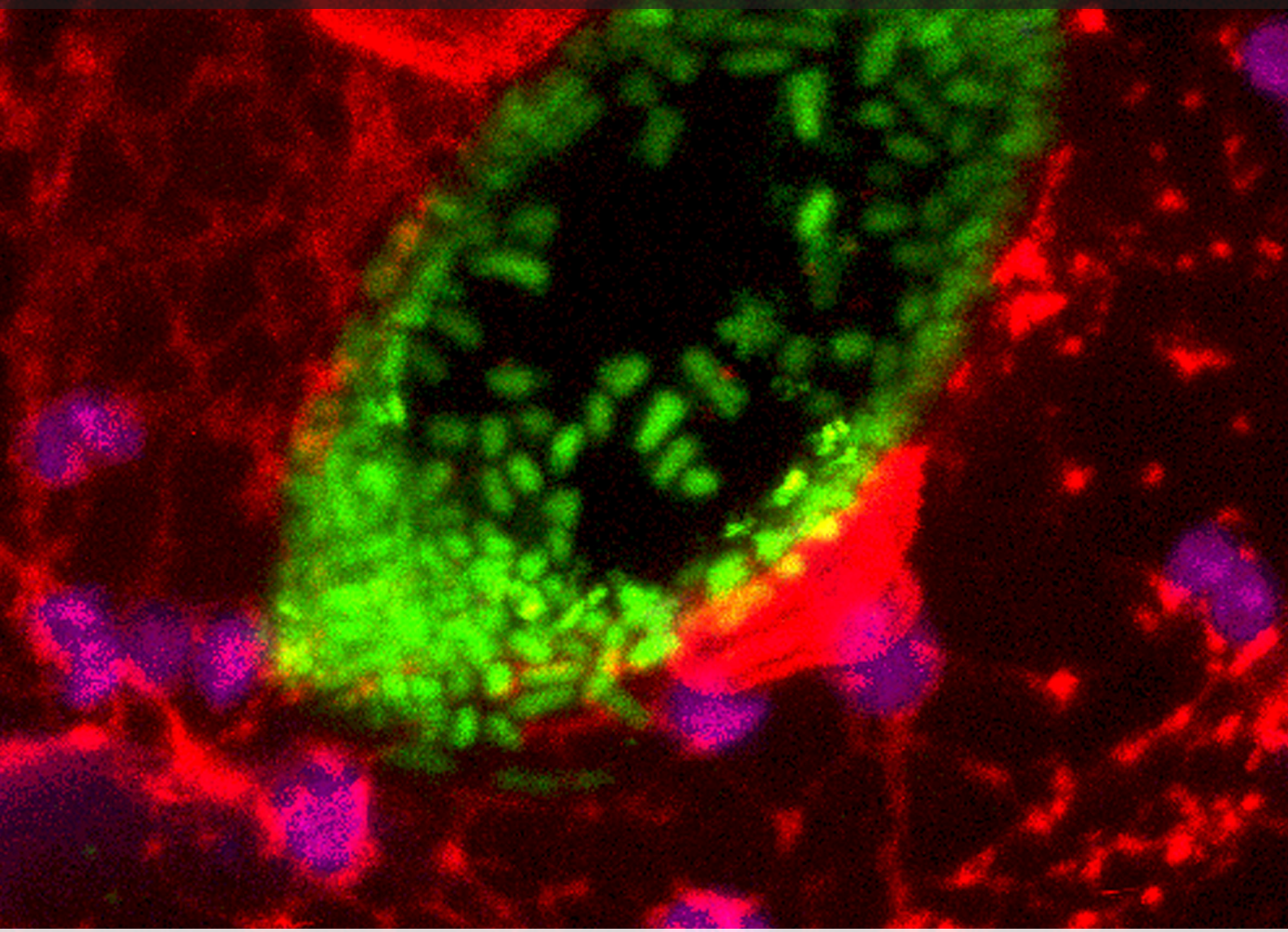

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PLANTS AS ALTERNATIVE HOSTS FOR HUMAN AND ANIMAL PATHOGENS

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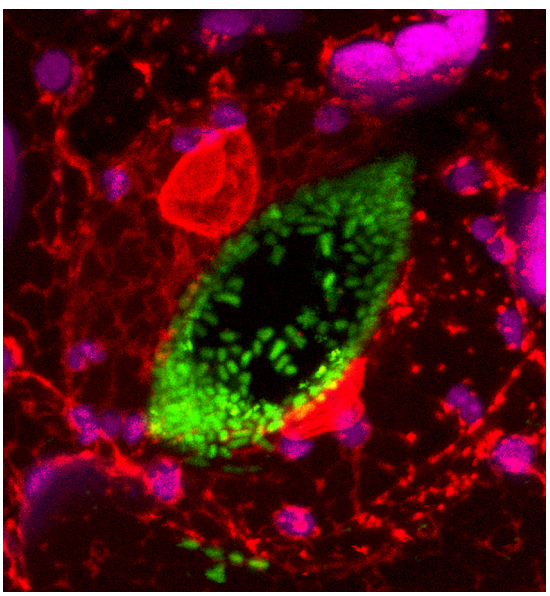

E. coli O157:H7 (Sakai) within a stomatal pore of *A. thaliana*. The bacteria are labeled with GFP (green) and *A. thaliana*, Wave line 138, are labeled with RFP fused to the intrinsic plasma membrane protein PIP1;4. Chloroplasts are false coloured in magenta. (Rossez et al., 2013, doi:10.1111/1462-2920.12315).

Many of the most prevalent and devastating human and animal pathogens have part of their lifecycle out-with the animal host. These pathogens have a remarkably wide capacity to adapt to a range of quite different environments: physical, chemical and biological, which is part of the key to their success. Many of the well-known pathogens that are able to jump between hosts in different biological kingdoms are transmitted through the faecal-oral and direct transmission pathways, and as such have become important food-borne pathogens. Some high-profile examples include fresh produce-associated outbreaks of *Escherichia coli* O157:H7 and *Salmonella enterica*. Other pathogens may be transmitted via direct contact or aerosols are include important zoonotic pathogens. It is possible to make a broad division between those pathogens that are passively transmitted via vectors and need the animal host for replication (e.g. virus and parasites), and those that are able to actively interact with alternative hosts, where they can proliferate (e.g. the enteric bacteria). This research topic will focus on plants as alternative hosts for human pathogens, and the role of plants in their transmission back to humans. The area is particularly exciting because it opens up new aspects to the biology of some microbes already considered to be very well characterised. One aspect of cross-kingdom host colonisation is in the comparison between the hosts and how the microbes are able to use both common and specific adaptations for each situation.

The area is still in relative infancy and there are far more questions than answers at present. We aim to address important questions underlying the interactions for both the microbe and plant host in this research topic.

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EDITORIAL
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Editorial on plants as alternative hosts for human and animal pathogens

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Keywords: ***Salmonella enterica***, ***Escherichia coli***, ***Arabidopsis thaliana***, **fresh produce, PAMP-triggered immunity, effectors, mRNA extraction, microbiome**

Fresh produce, in the form of raw or minimally processed fruits and vegetables, is recognized to be an important source of food-borne disease. Sporadic cases or outbreaks can arise from bacterial, viral, or parasitic contamination. Importantly, while the latter both require animal hosts for proliferation and cannot grow on or within crop plants, bacteria can do so and are able to use plants as secondary hosts. This type of interaction means that human pathogenic bacteria need to undergo adaptation to the plant host, which presents an environment quite distinct to animals or humans. Important differences lie in both physiochemical and biological properties, e.g., physiology, immunity, native microflora, physical barriers, mobility, and temperature. There is now good published evidence to describe different aspects of the interactions between plants and human pathogenic bacteria, and the subsequent effects on the outcome of colonization. However, in comparison to the interactions between the pathogens and their animal hosts, for which we know a great deal about both host and microbial factors, this area is relatively new with many important knowledge gaps. Research in this area is of most relevance to food safety, since the results can be and indeed, are already applied by public health agencies and food producers. It also reveals fascinating aspects of the bacterial life-cycle that were hitherto under appreciated.

One of the important features of plant host colonization is the adaptation of pathogens to the host defense response. Innate immunity is the first line of defense with both physical barriers and biological recognition of human pathogens, and the different layers of the response are reviewed in Melotto et al. (2014). An intriguing aspect is the finding that some human pathogens are able to subvert the plant defense, shown using *Salmonella enterica* and *Arabidopsis* as an example (Garcia and Hirt, 2014). Perception of human pathogens is based on recognition of PAMPs (e.g., flagellin) similar to those in phytopathogens and other plant-associated bacteria; perhaps unsurprising given that the mechanisms of recognition tend to be based on evolutionary conserved proteins. What was unexpected was the fact that some *Salmonella* serovars encode flagellin variants that are no longer recognized by the plant, presumably as a result of evolutionary selection (Garcia and Hirt, 2014).

There are good parallels in microbial suppression of the innate response of animal and plant hosts, for example via effectors secreted through the type 3 secretion (T3S) apparatus. Despite structural differences in the apparatus between human- and phyto-pathogens, the general function of host subversion is shared. Commonalities (and differences) in which pathways in animal and plant hosts are targeted by human pathogens are highlighted in Brunner and Fraiture (2014), showing where there is evolutionary conservation in the cellular hubs. Detailed examination of the function for one of the T3S effectors from *S. enterica*, SpvC, shows that it targets a signaling pathway that is shared in plant and animal hosts (Neumann et al., 2014). This effector is a phosphothreonine lyase able to de-phosphorylate activated MAP kinases, therefore attenuating the immune response in *Arabidopsis*.

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Examination of mechanisms of colonization shows that although some aspects are broadly the same, there are important differences in the interaction between *S. enterica* and either plant or animal hosts (Wiedemann et al., 2015). To better understand the molecular basis of the interaction, it is necessary to determine the protein-protein interactions (PPIs) between the pathogen and the plant host. An important comparison of PPIs between *Salmonella* and *Arabidopsis* proteins to known interactions between bacterial and animal proteins was presented in Schleker et al. (2015). In order to characterize the interactions on a global scale, an approach was used that combines several new as well as previously described algorithms predicting new PPIs (Kshirsagar et al., 2015). However, while this approach yields candidates worthy of further investigation, it also serves to highlight the paucity of reported functional data. To further explore the molecular interactions, methods must be developed that are appropriate to the bacteria-plant system, for example for the analysis of bacterial gene expression. An improved method for the extraction of RNA from mixed samples was presented in Holmes et al. (2014), which allows the analysis of expression patterns in bacteria and the plant host at the same time.

Colonization of plant hosts by human pathogens rarely, if ever, occurs in isolation and successful colonization is dependent on the ability of the bacteria to compete with the native microflora. Microbiome approaches show that crops not obviously associated with human pathogens may harbor potential pathogens. As shown with an example of grapevines, crop plants can support the endophytic growth of human pathogen, in this case *Propionibacterium acnes,* which probably arose from human contamination in the first place (Yousaf et al., 2014). Application of bulky organic fertilizers, such as manure, to crops may increase the chances of transmission of bacteria into the food chain. However, the potential for transmission clearly shows some specificity, not least from differences between species and sub-species of bacteria and the host plants (Hofmann et al., 2014). A combination of axenic system with susceptible plants (e.g., spinach) revealed that 400 bacteria in one ml are sufficient to successfully colonize the crop plant.

This collection of articles helps to highlight the underpinning mechanisms, but also shows the complexity of interactions between human pathogenic bacteria and plant hosts. It is becoming clear that it is not possible to apply a broad set of rules to these interactions: there is a great deal of specificity that depends on multiple factors. This is particularly important in the consideration of how to address issues related to food safety, for practices applied at the pre-harvest stage or for prevention of transmission and contamination during post-harvest processing.

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REVIEW ARTICLE
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Plant innate immunity against human bacterial pathogens

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Certain human bacterial pathogens such as the enterohemorrhagic *Escherichia coli* and *Salmonella enterica* are not proven to be plant pathogens yet. Nonetheless, under certain conditions they can survive on, penetrate into, and colonize internal plant tissues causing serious food borne disease outbreaks. In this review, we highlight current understanding on the molecular mechanisms of plant responses against human bacterial pathogens and discuss salient common and contrasting themes of plant interactions with phytopathogens or human pathogens.

Keywords: leafy vegetables, fresh produce, *Salmonella enterica*, *Escherichia coli* O157:H7, plant defense

INTRODUCTION

Bagged greens in the market are often labeled "pre-washed," "triple-washed," or "ready-to-eat," and look shiny and clean. But are they really "clean" of harmful microbes? We cannot be so sure. Food safety has been threatened by contamination with human pathogens including bacteria, viruses, and parasites. Between 2000 and 2008, norovirus and *Salmonella* spp. contributed to 58 and 11% of forborne illnesses, respectively in the United States (Scallan et al., 2011). In those same years, non-typhoidal *Salmonella* alone was ranked as the topmost bacterial pathogen contributing to hospitalizations (35%) and deaths (28%) (Scallan et al., 2011). In 2007, 235 outbreaks were associated with a single food commodity; out of which 17% was associated with poultry, 16% with beef, and 14% with leafy vegetables that also accounted for the most episodes of illnesses (CDC, 2010).

Apart from the direct effects on human health, enormous economic losses are incurred by contaminated food products recalls. The 8-day recall of spinach in 2006 cost $350 million to the US economy (Hussain and Dawson, 2013). It should be realized that this is not the loss of one individual, but several growers, workers, and distributors. This is a common scenario for any multistate foodborne outbreak. Additionally, the skepticism of the general public toward consumption of a particular food product can lead to deficiencies of an important food source from the diet. Less demand would in turn lead to losses for the food industry. Economic analysis shows that money spent on prevention of foodborne outbreak by producers is much less than the cost incurred after the outbreak (Ribera et al., 2012).

Contamination of plants can occur at any step of food chain while the food travels from farm to table. Both pre-harvest and post-harvest steps are prone to contamination. Contaminated irrigation water, farm workers with limited means of proper sanitation, and fecal contamination in the farm by animals can expose plants to human pathogens before harvest of the edible parts (Lynch et al., 2009; Barak and Schroeder, 2012). After harvest, contamination can occur during unclean modes of transportation, processing, and bagging (Lynch et al., 2009). Mechanical damage during transport can dramatically increase the population of human pathogens surviving on the surface of edible plants (Aruscavage et al., 2008). Control measures to decrease pathogen load on plant surfaces have been defined by the Food Safety Modernization Act (US Food and Drug Administration) and Hazard Analysis and Critical Control Point system (HACCP). Using chlorine for post-harvest crop handling has been approved by US Department of Agriculture (USDA) under the National Organic Program. However, some studies indicated that internalized human pathogens escape sanitization (Seo and Frank, 1999; Saldaña et al., 2011). Thus, understanding the biology of human pathogen-plant interactions is now crucial to prevent pathogen colonization of and survival in/on plants, and to incorporate additional, complementing measures to control food borne outbreaks.

We reasoned that as plants are recognized vectors for human pathogens, enhancing the plant immune system against them creates a unique opportunity to disrupt the pathogen cycle. In this cross-kingdom interaction, the physiology of both partners contribute to the outcome of the interactions (i.e., colonization of plants or not). Bacterial factors important for interaction with plants have been discussed in recent, comprehensive reviews (Tyler and Triplett, 2008; Teplitski et al., 2009; Berger et al., 2010; Barak and Schroeder, 2012; Brandl et al., 2013). Plant factors contributing to bacterial contamination (or lack of) is much less studied and discussed. In this review, we highlight current knowledge on plants as vectors for human pathogens, the molecular mechanisms of plant responses to human bacterial pathogens, and discuss common themes of plant defenses induced by phytopathogens and human pathogens. We have focused on human bacterial pathogens that are not recognized plant pathogens such as *Salmonella enterica* and *Escherichia coli* (Barak and Schroeder, 2012; Meng et al., 2013), but yet are major threats to food safety and human health.

PLANT SURFACE: THE FIRST BARRIER FOR BACTERIAL INVADERS

The leaf environment has long been considered to be a hostile environment for bacteria. The leaf surface is exposed to rapidly fluctuating temperature and relative humidity, UV radiation, fluctuating availability of moisture in the form of rain or dew, lack of nutrients, and hydrophobicity (Lindow and Brandl, 2003). Such extreme fluctuations, for example within a single day, are certainly not experienced by pathogens in animal and human gut. Thus, it is tempting to speculate that animal pathogens may not even be able to survive and grow in an environment as dynamic as the leaf surface. However, the high incidence of human pathogens such as *S. enterica* and *E. coli* O157:H7 on fresh produce, sprouts, vegetables, leading to foodborne illness outbreaks indicate a certain level of human pathogen fitness in/on the leaf.

The plant surface presents a barrier to bacterial invaders by the presence of wax, cuticle, cell wall, trichomes, and stomata. All except stomata, present a passive defense system to prevent internalization of bacteria. Nonetheless, several bacteria are able to survive on and penetrate within the plant interior. The surface of just one leaf is a very large habitat for any bacteria. The architecture of the leaf by itself is not uniform and provides areas of different environmental conditions. There are bulges and troughs formed by veins, leaf hair or trichomes, stomata, and hydathodes that form microsites for bacterial survival with increased water and nutrient availability, as well as temperature and UV radiation protection (Leveau and Lindow, 2001; Miller et al., 2001; Brandl and Amundson, 2008; Kroupitski et al., 2009; Barak et al., 2011). Indeed, distinct microcolonies or aggregates of *S. enterica* were found on cilantro leaf surfaces in the vein region (Brandl and Mandrell, 2002) In addition, preference to the abaxial side of lettuce leaf by *S. enterica* may be is an important strategy for UV avoidance (Kroupitski et al., 2011). Conversion of cells to viable but non-culturable (VNBC) state in *E. coli* O157:H7 on lettuce leaves may also be a strategy to escape harsh environmental conditions (Dinu and Bach, 2011). Hence, localization to favorable microsites, avoidance of harsh environments, and survival by aggregation or conversion to non-culturable state may allow these human pathogens to survive and at times multiply to great extent on the leaf surface.

As stomata are abundant natural pores in the plant epidermis which serve as entrance points for bacteria to colonize the leaf interior (intercellular space, xylem, and phloem), several studies addressed the question as to whether human bacterial pathogens could internalize leaves through stomata. Populations of *E. coli* O157:H7 and *S. enterica* SL1344 in the Arabidopsis leaf apoplast can be as large as four logs per cm^2 of leaf after surface-inoculation under 60% relative humidity (Roy et al., 2013) suggesting that these bacteria can and access the apoplast of intact leaves. Several microscopy studies indicated association of pathogens on or near guard cells. For instance, *S. enterica* serovar Typhimurium SL1344 was shown to internalize arugula and iceberg lettuce through stomata and bacterial cells were located in the sub-stomatal space (Golberg et al., 2011). However, no internalization of SL1344 was observed into parsley where most cells were found on the leaf surface even though stomata were partially open (Golberg et al., 2011). Cells of *S. enterica* serovar Typhimurium MAE110 (Gu et al., 2011), enteroaggregative *E. coli* (Berger et al., 2009b), and *E. coli* O157:H7 (Saldaña et al., 2011) were found to be associated with stomata in tomato, arugula leaves, and baby spinach leaves, respectively. In the stem *E. coli* O157:H7 and *Salmonella* serovar Typhimurium were found to be associated with the hypocotyl and the stem tissues including epidermis, cortex, vascular bundles, and pith when seedlings were germinated from contaminated seeds (Deering et al., 2011a,b).

The plant rhizosphere is also a complex habitat for microorganisms with different life styles including plant beneficial symbionts and human pathogens. Nutritionally rich root exudate has been documented to attract *S. enterica* to lettuce roots (Klerks et al., 2007a). Although bacteria cannot directly penetrate through root cells, sites at the lateral root emergence and root cracks provide ports of entry for *S. enterica* and *E. coli* O157:H7 into root tissues (Cooley et al., 2003; Dong et al., 2003; Klerks et al., 2007b; Tyler and Triplett, 2008), and in some instances between the epidermal cells (Klerks et al., 2007b). High colonization of *S. enterica* has been observed in the root-shoot transition area (Klerks et al., 2007b). Once internalized both bacterial pathogens have been found in the intercellular space of the root outer cortex of *Medicago truncatula* (Jayaraman et al., 2014). *Salmonella enterica* was found in the parenchyma, endodermis, pericycle, and vascular system of lettuce roots (Klerks et al., 2007b) and in the inner root cortex of barley (Kutter et al., 2006). A detailed study on the localization of *E. coli* O157:H7 in live root tissue demonstrated that this bacterium can colonize the plant cell wall, apoplast, and cytoplasm (Wright et al., 2013). Intracellular localization of *E. coli* O157:H7 seems to be a rare event as most of the microscopy-based studies show bacterial cells in the intercellular space only. Bacterial translocation from roots to the phyllosphere may be by migration on the plant surface in a flagellum-dependent manner (Cooley et al., 2003) or presumably through the vasculature (Itoh et al., 1998; Solomon et al., 2002). The mechanism for internal movement of enteric bacterial cells from the root cortex to the root vasculature through the endodermis and casparian strips and movement from the roots to the phyllosphere through the vascular system is yet to be demonstrated.

Several outbreaks of *S. enterica* have also been associated with fruits, especially tomatoes. *Salmonella enterica* is unlikely to survive on surface of intact fruits (Wei et al., 1995) raising the question: what are the routes for human pathogenic bacteria penetration into fruits? It has been suggested that *S. enterica* can move from inoculated leaves (Barak et al., 2011), stems, and flowers (Guo et al., 2001) to tomato fruits. However, the rate of internal contamination of fruits was low (1.8%) when leaves were surface-infected with *S. enterica* (Gu et al., 2011). The phloem has been suggested as the route of movement of bacteria to non-inoculated parts of the plant as bacterial cells were detected in this tissue by microscopy (Gu et al., 2011). **Figure 1** depicts the observed phyllosphere and rhizosphere niches colonized by bacteria in/on intact plants and probable sources of contamination.

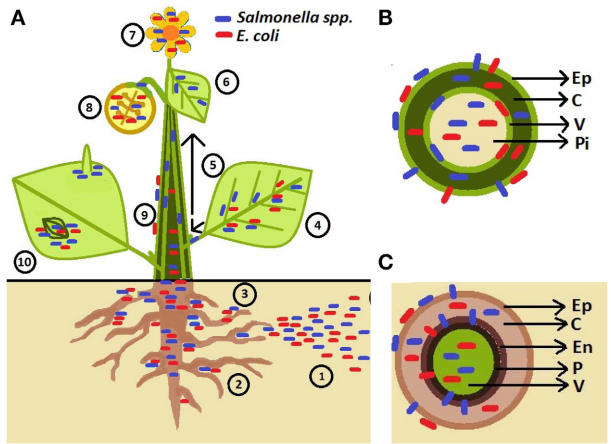

FIGURE 1 | Schematic representation of human pathogen (HP) association with plants. (A) Pathogens are introduced to soil through contaminated irrigation water, fertilizers, manure, and pesticides (**1**). HPs are attracted to rhizosphere (**2**; Klerks et al., 2007a) and penetrate root tissues at the sites of lateral root emergence, root cracks as well as root-shoot transition area (**3**; Cooley et al., 2003; Dong et al., 2003; Klerks et al., 2007b; Tyler and Triplett, 2008). HPs were found to live on the leaf surface near veins (Brandl and Mandrell, 2002), in the leaf apoplast (intercellular space) (Brandl and Mandrell, 2002; Solomon et al., 2002; Niemira, 2007; Kroupitski et al., 2009; Barak et al., 2011; Dinu and Bach, 2011; Gu et al., 2011; Roy et al., 2013), and sometimes with affinity for abaxial side of leaf (e.g., *S. enterica*; (Kroupitski et al., 2011) (**4**). *Salmonella enterica* Typhimurium can enter tomato plants *via* leaves and move through vascular bundles (petioles and stems) (**5**) into non-inoculated leaves (**6**) and fruits (**8**) (Gu et al., 2011). HPs are also found to be associated with flower (**7**; Guo et al., 2001; Cooley et al., 2003). *Salmonella* could travel from infected leaves (**4**), stems (**5**), and flowers (**7**) to colonize the fruit interior (the diagram represents a cross-section of a fruit) and fruit calyx (**8**) Guo et al., 2001; Janes et al., 2005; Barak et al., 2011. *Escherichia coli* O157:H7 has also been observed in the internal parts of the apple and the seeds following contamination of the flower (**8**) (Burnett et al., 2000). Movement on the plant surface has also been observed (**9**; Cooley et al., 2003). Epiphytic *Salmonella* and *E. coli* O157:H7 can aggregate near stomata and sub-stomatal space (**10**; Shaw et al., 2008; Berger et al., 2009a,b; Golberg et al., 2011; Gu et al., 2011; Saldaña et al., 2011), reach the sub-stomatal cavity and survive/colonize in the spongy mesophyll (Solomon et al., 2002; Wachtel et al., 2002; Warriner et al., 2003; Jablasone et al., 2005; Franz et al., 2007). *Salmonella* cells were observed near trichomes (**10**; Barak et al., 2011; Gu et al., 2011). **(B)** Stem cross-section showing bacteria located in different tissues (Ep, epidermis; C, cortex; V, vascular tissue; Pi, pith) (Deering et al., 2011a,b). **(C)** Root cross-section showing bacteria on the root surface, internalizing between the epidermal cells, and colonizing root outer and inner cortex, endodermis (En), pericycle (P) and vascular system (Kutter et al., 2006; Klerks et al., 2007a,b; Jayaraman et al., 2014).

PERCEPTION OF HUMAN PATHOGENS BY THE PLANT IMMUNE SYSTEM

Plants possess a complex innate immune system to ward off microbial invaders (Jones and Dangl, 2006). Plants are able to mount a generalized step-one response that is triggered by modified/degraded plant products or conserved pathogen molecules. These molecules are known as damage or pathogen associated molecular patterns (DAMP/PAMP). In many cases, conserved PAMPs are components of cell walls and surface structures such as flagellin, lipopolysaccharides, and chitin (Zeng et al., 2010). Examples of intracellular PAMPs exist such as the elongation factor EF-Tu (Kunze et al., 2004). PAMPs are recognized by a diverse set of plant extracellular receptors called pattern-recognition receptors (PRRs) that pass intracellular signals launching an army of defense molecules to stop the invasion of the pathogens. This branch of the immune system known as pathogen-triggered immunity (PTI) is the first line of active defense against infection.

Human pathogen on plants (HPOP) is an emerging field that only recently has caught the attention of plant biologists and phytopathologists. A few studies have been reported in the last 5–10 years, which focused on the most well studied PAMPs, flagellin and lipopolysaccharide (LPS), in the interaction of human pathogens with plants. **Table 1** lists the plants, bacterial strains, and method details for such studies.

FLAGELLIN PERCEPTION

Flagellin, the structural component of flagellum in bacteria, is involved in bacterial attachment and motility on the plant (Cooley et al., 2003), is recognized by plant through the FLS2 receptor (Garcia et al., 2014), and induces plant defenses (Meng et al., 2013; Garcia et al., 2014). Similar to the well-studied PTI elicitor flg22 (Felix et al., 1999), the flg22 epitope of *S. enterica* serovar Typhimurium 14028 is also an effective PAMP and elicitor of downstream immune responses in Arabidopsis (Garcia et al., 2014), tobacco, and tomato plants (Meng et al., 2013). Flagellum-deficient mutants of *S. enterica* serovar Typhimurium 14028 are better colonizers of wheat, alfalfa, and Arabidopsis roots as compared to the wild type bacterium (Iniguez et al., 2005) further suggesting that the *Salmonella* flagellum induces plant defenses that may restrict bacterial colonization of several plant organs. However, the *Salmonella* flg22 peptide is not the only PAMP for elicitation of plant immune response as *fls2* mutant of Arabidopsis still shows a low level of PTI activation in response to this PAMP (Garcia et al., 2014).

Purified flagellin or derived epitopes of *E. coli* O157:H7 has not been used to induce plant defenses. However, flagellum-deficient mutant of this strain does not activate the SA-dependent *BGL2* gene promoter as much as the wild type strain and shows larger population in Arabidopsis than the wild type strain (Seo and Matthews, 2012) further suggesting that surface structures in the bacterial cell are perceived by plants.

The differences in responses observed could be attributed to the presence of other microbial signatures eliciting plant defense. Variations in plant response to *S. enterica* flagellin could be owed to host-strain specificity as well. Although flagellin sequences from *S. enterica* strains and other bacteria are highly conserved, even a minor change of five amino acids in the flg22 epitope leads to reduced activation of PTI in Arabidopsis, tobacco, and tomato plants (Garcia et al., 2014). Adding to the specificity, it has also been shown that Brassicaceae and Solanocecae plants recognize specific flagellin (Robatzek et al., 2007; Clarke et al., 2013). Hence, evolving variations in flagellin sequences could be a strategy employed by the pathogens to avoid plant recognition, which in turn leads to the development of pathogen-specific immune responses in the plant.

Flagella also play an important role in bacterial behavior on the plant. Several studies have pointed out to the usefulness of

Table 1 | Experimental conditions used in the studies reporting plant response to pathogenic *Salmonella* and *E. coli*.

References	Plant	Plant genotype	Wild type bacterium	Mutant bacterium	Plant tissue used for detection	Inoculation method	Inoculum concentration	Surface sterilization	Methods	Intact plant tissue for infection
FLAGELLIN AND LPS PERCEPTION										
Cooley et al., 2003	*A. thaliana*	Col-0	*S. enterica* serovar Newport (RM1655); *E. coli* O157:H7 Odwalla (RM1484); *Enterobacter asburiae* (RM3638)	Δ*flhDC, flaN*	Roots and shoots of seedling and adult plants, leaves, flowers, seeds, chaff	Soil, seed, root inoculation	1×10^4 or 1×10^6 cfu/ml for root inoculation; 1×10^8 cfu/ml for seed inoculation and 1×10^8 cfu/g for soil inoculation	No	Microscopy, plating	Yes
*Iniguez et al., 2005	*A. thaliana, M. truncatula,* wheat, *M. sativa*	Wheat Trenton; *M. truncatula* Jester, Jermalong, Gaerten A17, Mutant Sickle; *M. sativa* CUS101; *A. thaliana* Col-0, *npr1-4, nahG*	*S. enterica* serovar Typhimurium 14028s	*spaS, sipB, fliC, fljB*	Root and hypocotyl	Seedling inoculation	dose response	Yes	Plating	Yes
*Shirron and Yaron, 2011	Tobacco		*S. enterica* serovar Typhimurium SL1344, 14028s	Δ*invA,* Δ*rfaH,* Δ*phoP*	Leaf	Syringe infiltration; drip-irrigation	7.5 log cfu/ml	No	Microscopy	Yes
*Seo and Matthews, 2012	*A. thaliana*	Col-0, *npr1-1*	*E. coli* O157:H7 43895, 86-24	Δ*fliC,* Δ*csgD,* Δ*waaI*	Whole plant	Dipping	1×10^8 cfu/ml	No	Plating	Yes
*Garcia et al., 2014	*A. thaliana*	Col-0, *fls2*	*S. enterica* serovar Typhimurium 14028s, SL1344; *S. enterica* serovar Senftenberg 20070885	*prgH, fliC, fljB, hrcC*	Seedling	Seedling inoculation	2×10^8 cfu/ml	No	Plating	Yes
Meng et al., 2013	Tobacco, tomato		*S. enterica* serovar Typhimurium 14028s	Δ*fliC,* Δ*fljB,* Δ*hilA,* ΔhilD, Δ*sirA,* Δ*ssrAB,* Δ*sseA,* Δ*sseB,* Δ*invA,* Δ*prgH*	Leaf	Syringe infiltration	2×10^4 cfu/ml	No	Plating	Yes
STOMATAL IMMUNITY										
Melotto et al., 2006	*A. thaliana*	Col-0, *fls2*	*E. coli* O157:H7		Leaf	Epidermal peels	1×10^8 cfu/ml		Microscopy	No

(Continued)

Table 1 | Continued

References	Plant	Plant genotype	Wild type bacterium	Mutant bacterium	Plant tissue used for detection	Inoculation method	Inoculum concentration	Surface sterilization	Methods	Intact plant tissue for infection
Kroupitski et al., 2009	Lettuce	Iceberg	*S. enterica* serovar Typhimurium SL1344	*fliGHI, cheY*	Leaf	Leaf pieces submersion	1×10^8 cfu/ml	No	Microscopy	No
*Roy et al., 2013	*A. thaliana,* lettuce	*A. thaliana* Col-0 *ost1-2,* Butter Lettuce	*S. enterica* serovar Typhimurium SL1344; *E. coli* O157:H7		Leaf	Dipping	1×10^8 cfu/ml	Yes	Microscopy, plating	Yes
PLANT INTRACELLULAR RESPONSE										
Thilmony et al., 2006	*A. thaliana*	Col-0	*E. coli* O157:H7	*fliC*	Leaf	Vacuum infiltration	1×10^8 cfu/ml			
Klerks et al., 2007b	Lettuce	Tamburo	*S. enterica* serovars Dublin; *E. coli* JM109		Seedling phyllosphere	Manure contami-nationSeedling inoculation	1×10^7 cfu/g manure or 1×10^7 cfu/ml inoculum	Yes	Plating	Yes
Schikora et al., 2008	*A. thaliana*	Col-0	*S. enterica* serovar Typhimurium 14028s		Seedling, leaf	Seedling inoculation, leaf vacuum infiltration	3×10^8 cfu/ml	Yes	Plating	Yes
Saldaña et al., 2011	Baby spinach		*E. coli* O157:H7	*escN, tir, eae, espFu, espP, fliC, qseB, hcpA, ecpA, elfA, csgA, csgD, bscA*	Leaf	Leaf pieces submersion	1×10^7 cells	Yes for some experiments	Microscopy, plating	No
Schikora et al., 2011	*A. thaliana*	Col-0	*S. enterica* serovar Typhimurium 14028s	*prgH, invA, ssaV, ssaF*	Leaf	Syringe infiltration	1.7×10^8 cfu/ml	Yes	Plating	Yes
Üstün et al., 2012	Tomato, tobacco, pepper	Tomato Money maker; tobacco Domin., pepper ECW-10R	Transient expression of *S. enterica* SseF in tobacco		Leaf	Transient expression by syringe infiltration of vector organism	2×10^8 cfu/ml		Plating	Yes
Jayaraman et al., 2014	*M. truncatula*		*S. enterica*. serovars Schwarzengrund, Enteritidis, Mbandaka, Havana, Cubana; *E. coli* O157:H7 serovars Odwalla, EDL933, H2439, C7927, 96A 13466		Root	seedling root	Dose response	Yes for some experiments	Microscopy, plating	Yes

(Continued)

Table 1 | Continued

References	Plant	Plant genotype	Wild type bacterium	Mutant bacterium	Plant tissue used for detection	Inoculation method	Inoculum concentration	Surface sterilization	Methods	Intact plant tissue for infection
GENOTYPIC VARIABILITY										
Barak et al., 2008	Radish, tomato, broccoli, turnip, carrot, lettuce, cilantro, parsley, spinach, radicchio, endive	Lettuce Balady Aswan, Salinas 88, Little Gem, PI251246, Pavane, Valmaine, Iceburg, La Brillante, Paris Island, Parade, Calmar; Tomato Brandywine, Amish Paste, Money Maker, Rose, Soldacki, Stupics, Green Grape, San Marzano, Nyarous, Yellow Pear	*S. enterica* serovars Baildon 05x-02123, Cubana 98A9878, Enteritidis 99A-23, Havana 98A4399, Mbandaka 99A1670, Newport 96E01152C-TX, Poona 00A3563, Schwarzengrund 96E01152C		Seedling phyllosphere	Soil inoculation	1×10^4 cfu/ml	No	Plating	Yes
Brandl and Amundson, 2008	Lettuce	Parris Island	*E. coli* O157:H7 H1827; *S. enterica* serovar Thompson RM1987		Leaf	Dip inoculation	1×10^5 cfu/ml	No	Microscopy, plating	Yes
Mitra et al., 2009	Spinach	Bordeaux, Tyee, Space	*E. coli* O157:H7		Leaf, stem, root	Leaf drop, leaf infiltration, soil drench, stem puncture	1×10^6 cfu/ml	Yes	Microscopy, plating, BAX PCR assay	Yes
Barak et al., 2011	Tomato	H7996, Yellow Pear, Nyagous, LA2838A, LA3172, LA3556, LA0337, LA1049, Micro-Tom, Money Maker	*S. enterica* serovars Baildon 99A 23, Cubana 98A 9878, Enteritidis 05x-02123, Havana 98A4399, Mbandaka 99A 1670, Newport 96 E01153c-TX, Poona 00A 3563, Schwarzenfrund 96 E01152c-TX		Seedling phyllosphere and leaf	Soil inoculation; water inoculation; leaf dipping	1×10^8 cfu/ml	No	Microscopy, plating, BAX PCR assay	Yes
Golberg et al., 2011	Lettuce, arugula, parsley, tomato, basil	Iceberg, Romaine, Red Ruby	*S. enterica* serovar Typhimurium SL1344		Leaf	Leaf pieces/leaf submersion	1×10^8 cfu/ml	No	Microscopy	Leaf pieces and intact leaves

**Indicates articles that have also reported plant intracellular responses to bacteria.*

flagella for attachment to leaf surfaces and movement on plant surfaces (Berger et al., 2009a,b; Xicohtencatl-Cortes et al., 2009; Saldaña et al., 2011; Shaw et al., 2011).

LPS PERCEPTION

Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacterial pathogens of animals and plants. In the animal host, LPS is a well-characterized PAMP that is recognized by host Toll-like receptor 4 (de Jong et al., 2012). In plants however, receptors for LPS have not been discovered yet. Nonetheless, current evidence suggests that human pathogen-derived LPS can be perceived by plants resulting in PTI activation. For instance, on the leaf surface, purified LPS from *Pseudomonas aeruginosa*, *S. Minnesota* R595, and *E. coli* O55:B5 induces strong stomatal closure in Arabidopsis (Melotto et al., 2006). Purified LPS from *Salmonella* triggers of ROS production and extracellular alkalinization in tobacco cell suspension (Shirron and Yaron, 2011) but not on tomato leaves (Meng et al., 2013) suggesting that LPS recognition may be either dependent on experimental conditions or variable among plant species.

Genetic evidence suggests that the high activity of SA-dependent *BGL2* gene promoter in Arabidopsis is dependent on the presence of LPS in *E. coli* O157:H7 as higher activity of this promoter was observed in the wild type bacterial as compared to its LPS mutant (Seo and Matthews, 2012). However, LPS-dependent responses seem not to be sufficient to restrict bacterial survival on plants as the population titer of *E. coli* O157:H7 LPS mutant or wild type in plant is essentially the same (Seo and Matthews, 2012). Additionally, live *S.* Typhimurium cells do not induce ROS in epidermal tissue of tobacco (Shirron and Yaron, 2011) suggesting that, at least *Salmonella*, can suppress LPS-induced ROS and extracellular alkalinization.

Similar to flagellin, the O-antigen moiety of LPS is not only important for plant perception of bacterial cells, but also for bacterial attachment, fitness, and survival on plants (Barak et al., 2007; Berger et al., 2011; Marvasi et al., 2013).

FUNCTIONAL OUTPUT OF BACTERIUM PERCEPTION

One of the earliest PTI responses in plants is stomatal closure that greatly decreases the rate of pathogen entry into plant's internal tissues. This response requires molecular components of PTI including such as flagellin and LPS perception and hormone perception and signaling (Melotto et al., 2006, 2008; Zeng and He, 2010; Sawinski et al., 2013). Stomatal immunity is also triggered by the presence of human pathogens *S. enterica* serovar Typhimurium SL1344 and *E. coli* O157:H7 (Melotto et al., 2006; Kroupitski et al., 2009; Roy et al., 2013), albeit at various levels. For instance, *E. coli* O157:H7 induces a strong stomatal immunity and *Salmonella* SL1344 elicits only a transient stomatal closure in both Arabidopsis (Melotto et al., 2006; Roy et al., 2013) and lettuce (Kroupitski et al., 2009; Roy et al., 2013) suggesting that the bacterial strain SL1344 can either induce weaker or subvert stomata-based defense. Active suppression of stomatal closure by SL1344 may be unlikely because it cannot re-open dark-closed stomata (Roy et al., 2013). However, it is possible that signaling pathways underlying bacterium-triggered and dark-induced stomatal closure are not entirely overlapping and SL1344 acts on immunity-specific signaling to subvert stomatal closure.

PLANT INTRACELLULAR RESPONSE TO HUMAN PATHOGENS

Recognition of PAMPs by PRRs leads to several hallmark cellular defense responses that are categorized based on the timing of response. Zipfel and Robatzek (2010) have discussed that early responses occur within seconds to minutes of recognition including ion fluxes, extracellular alkalinization, and oxidative burst. Intermediate responses occur within minutes to hours including stomatal closure, ethylene production, mitogen-activated protein kinase (MAPK) signaling, and transcriptional reprogramming. Late responses occur from hours to days and involve callose deposition, salicylic acid accumulation, and defense gene transcription.

These hallmark plant cellular defenses have also been tested for both *E. coli* and *S. enterica* (**Figure 2**). In particular, *S. enterica* infection results in the induction of *MPK3*/*MPK6* kinase activity and plant defense-associated genes *PDF1.2*, *PR1*, and *PR2* in Arabidopsis leaves (Schikora et al., 2008) as well as *PR1*, *PR4*, and *PR5* in lettuce (Klerks et al., 2007b). MPK6 activation in Arabidopsis is independent of FLS2 (Schikora et al., 2008), indicating that flagellin is not the only active PAMP of *Salmonella* and plant response to other PAMPs may converge at MAPK signaling. Direct comparison of the *PR1* gene expression in Arabidopsis indicated that both *E. coli* O157:H7 and *Salmonella* SL1344 are able to induce this defense marker gene, however at difference levels (Roy et al., 2013). The *PR1* gene induction is low in SL1344-infected plants indicating that immune responses are either weaker or are suppressed by *Salmonella*.

A few studies (**Table 1**) have addressed the role of plant hormones in response to endophytic colonization of human bacterial pathogens:

ETHYLENE SIGNALING

The ethylene-insensitive mutant of Arabidopsis, *ein2*, supports higher *Salmonella* 14028 inside whole seedlings as compared to the wild type Col-0 plants (Schikora et al., 2008). Furthermore, addition of a specific inhibitor of ethylene mediated signaling, 1-methylcyclopropene (1-MCP), to the growth medium resulted in increased *S. enterica* 14028 endophytic colonization of *Medicago truncatula*, but not *M. sativum*, roots and hypocotyls (Iniguez et al., 2005) suggesting that the role of endogenous ethylene signaling maybe be specific to each plant-bacterium interaction. However, ethylene signaling may play a contrasting role during fruit contamination. Tomato mutants (*rin* and *nor*) with defects in ethylene synthesis, perception, and signal transduction show significantly reduced *Salmonella* proliferation within their fruits as compared to the wild type control (Marvasi et al., 2014).

JASMONIC ACID

Similar to the *ein2* mutant, the coronatine-insensitive mutant of Arabidopsis, *coi1-16*, also supports high *Salmonella* 14028 inside whole seedlings (Schikora et al., 2008). Along with the induction of the jasmonate-responsive gene *PDF1.2* addressed in the same study and mentioned above, it seems that jasmonate signaling is

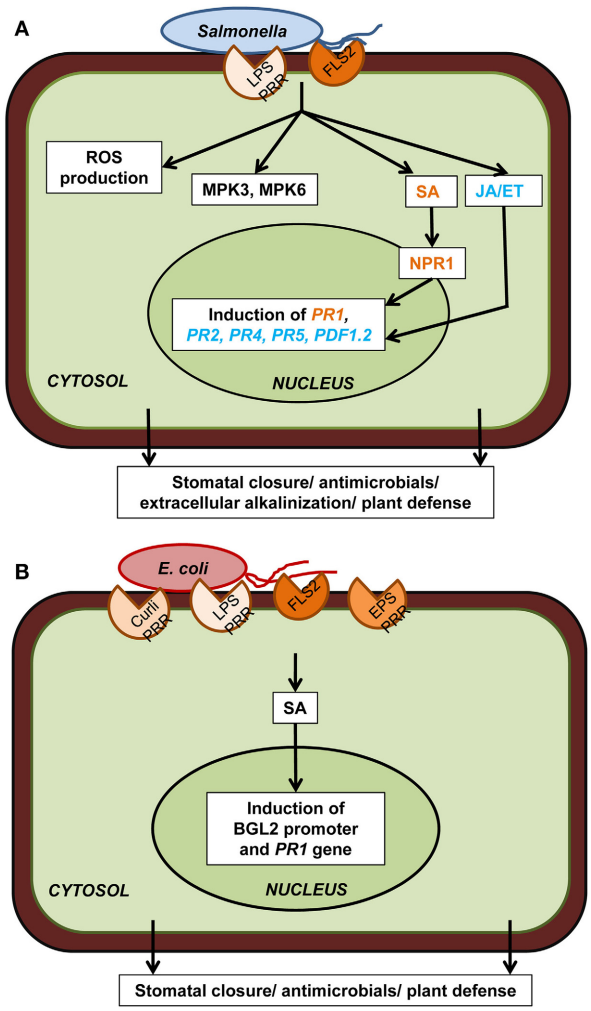

FIGURE 2 | Plant cellular defense responses against human pathogens. **(A)** Upon reception of PAMP (flagellin, LPS) through PRR (FLS2 and putatively others), *Salmonella* spp. trigger downstream plant defense responses which include ROS production, MPK3/6, salicylic acid (SA) signaling through NPR1, jasmonic acid (JA) and ethylene (ET) signaling, defense-associated gene induction, and extracellular alkalinization. All these cellular events ultimately lead to stomatal closure, antimicrobial activity, and plant defense. **(B)** *Escherichia coli* PAMPs (curli, LPS, flagellin, EPS) are also perceived by PRRs (FLS2 and putatively others) present on plant cell surface which triggers the induction of the SA-dependent BGL2 promoter activity and *PR1* gene expression. Only components that have been directly demonstrated experimentally are included in the diagram. Plant defense responses in case of both these human pathogens are strain specific as well as plant cultivar specific.

also an important component to restrict *Salmonella* infection in, at least, Arabidopsis. These results are surprising as *coi1* mutants are well known to have increased resistant to various bacterial pathogen of plants, such as *P. syringae*, but not to fungal or viral pathogens (Feys et al., 1994; Kloek et al., 2001).

SALICYLIC ACID

Two genetic lines of Arabidopsis has been extensively used to determine the role of salicylic acid (SA) in plant defenses against phytopathogens, the transgenic *nahG* plant that cannot accumulate SA (Friedrich et al., 1995) and the null mutant *npr1* that is disrupted in both SA-dependent and -independent defense responses (Ton et al., 2002). Both of these plant lines support higher populations of *Salmonella* 14028 inside their roots (Iniguez et al., 2005) and seedlings (Schikora et al., 2008) as compared to the wild type plant. NPR1-dependent signaling is important reduce the population of the curli-negative strain of *E. coli* O157:H7 43895 but not for the curli-positive strain 86-24 in Arabidopsis leaves (Seo and Matthews, 2012). Although only a few strains of *Salmonella* and *E. coli* have been used, there is an emerging patterns suggesting that SA itself and activation of SA-signaling can potentially restrict HPOP.

In attempts to understand the overall cellular transcriptional response to human bacterial pathogens, global transcriptomic analyses have been used. Thilmony et al. (2006) showed that *E. coli* O157:H7 regulates PTI-associated genes in Arabidopsis leaves, albeit in a flagellin-independent manner. A similar transcriptomic analysis with medium-grown Arabidopsis seedlings 2h after inoculation with *S. enterica* serovar Typhimurium 14028, *E. coli* K-12, and *P. syringae* pv. *tomato* DC3000 showed a strong overlap among genes responsive to each bacterial infection suggesting a common mechanism of plant basal response toward bacteria (Schikora et al., 2011). Gene expression analysis of *Medicago truncatula* seedlings root-inoculated with only two bacterial cells per plant indicated that 83 gene probes (30–40% of each data set) were commonly regulated in response to *S. enterica* and *E. coli* O157:H7 (Jayaraman et al., 2014). All together, these studies indicate that each human pathogenic bacterium can modulate specific plant genes beyond a basal defense response; however the mechanisms for plant-bacterium specificity are largely unknown.

CAN HUMAN PATHOGENIC BACTERIA INDUCE ETI IN PLANT CELLS?

Successful virulent pathogens of plants are able to defeat this army plant defense by employing its own set of artillery (such as the type three secretion system effectors and phytotoxins) and cause disease in the host plant (Melotto and Kunkel, 2013; Xin and He, 2013). In incompatible interactions (i.e., low bacterial colonization and no disease on leaves), the host plant already has pre-evolved molecules (R proteins) that recognize these effectors and cause a specific defense response to this pathogen. This specific response is called effector-triggered immunity (ETI). Because the type 3-secretion system (T3SS) is important for the virulence of both animal and plant pathogenic bacteria on their natural hosts as evidenced by the use of bacterial mutants, it is reasonable to expect that T3SS would be important for HPOP as well. However, animal and plant cell surfaces are structurally different; the plant cells wall seems to be impenetrable by the secretion needle of the extracellular animal pathogens (*Salmonella* and *E. coli*) as discussed by He et al. (2004) raising the question of how these effectors can reach the plant cytoplasm and interfere with plant defenses. To date, there is no evidence for the ability of human pathogens to inject T3SS effectors inside plant cells. It is possible that the T3SS is still active on the plant cell surface and the effectors are secreted into the

plant apoplast. If that is the case, however, plant membrane receptors would be necessary to recognize the effectors and trigger plant cellular responses. Nevertheless, it has been observed that the T3SS mutant of *E. coli* O157:H7, *escN*, has reduced ability to attach to and colonize baby spinach leaves similar to the *fliC* mutant (Saldaña et al., 2011). Furthermore, apoplastic population of T3SS structural mutants of *S. enterica* serovar Typhimurium 14028 (*invA*, *prgH*, *ssaV*, and *ssaJ*) is smaller than that of the wild type bacterium in Arabidopsis leaves (Schikora et al., 2011) and plant defense-associated genes are up-regulated for longer time by the *prgH* mutant than wild type *Salmonella* in Arabidopsis seedlings (Garcia et al., 2014). Contrary to these findings, Iniguez et al. (2005) reported that two *Salmonella* 14028 T3SS-SPI1, the structural mutant *spaS* and the effector mutant *sipB,* hypercolonize roots and hypocotyls of *M. sativum* and fail to induce SA-dependent *PR1* promoter in Arabidopsis leaves. More studies need to be conducted to conclude whether T3SS of *Salmonella* acts as "recognizable" surface structure similar to flagellum and/or as a conduit to deliver effectors in plant tissues and trigger ETI. It is worth mentioning that T3SS and effectors of the phytopathogen *P. syringae* pv. *syringae* have functions on ETI as well as bacterial fitness on plant surface (Lee et al., 2012) and the filamentous T3SS protein EspA is required for *E. coli* O157:H7 attachment to arugula leaves (Shaw et al., 2008).

The *invA* structural mutant, that is defective in all T3SS-1 system-associated phenotypes, induces high ROS and extracellular alkalinizing in tobacco BY-2 cell suspension and hypersensitive reaction (HR) in tobacco leaves as compared to the wild type strain (Shirron and Yaron, 2011) suggesting that T3SS is important for this suppression of immunity. However, Shirron and Yaron (2011) also reported that plant response to the regulatory mutant *phoP* that modulates the expression of many effector proteins and membrane components (Dalebroux and Miller, 2014), is no different to that of the wild type bacterium. These findings raised the question whether the phenotypes observed are due to the T3SS structure itself or due to the translocated effectors. A recent report shows that transient expression of the type three effector of *Salmonella* 14028 SseF in tobacco plants elicits HR, and this response is dependent on the SGT1 protein (Üstün et al., 2012). This study suggests that SseF can induce resistant-like response in plants and requires resistance (R) protein signaling components. Üstün et al. (2012) and Shirron and Yaron (2011) also showed that *Salmonella* 14028, which is able to deliver the SseF effector, cannot induce HR or any disease-like symptoms in tobacco leaves. Thus, it remains to be determined what would be the biological relevance of ETI in the *Salmonella* and other human pathogenic bacteria in their interaction with plants in nature.

GENOTYPIC VARIABILITY IN PLANT-*SALMONELLA* AND PLANT-*E. COLI* INTERACTIONS

Although *S. enterica* and *E. coli* O157:H7 have not been traditionally known to be closely associated with plants and modulate plant's physiology, the evidence tells us otherwise. An arms-race evolution in both the human pathogen and the plant is therefore, expected. A few studies (methodology details described in **Table 1**) have addressed whether genetic variability among plant species or within the same plant species (i.e., cultivars, varieties, and ecotypes) can be correlated with differential bacterial behavior and/or colonization of plants. Barak et al. (2011) described that different tomato cultivars can harbor different levels of *S. enterica* population after inoculation *via* water (sprinkler imitation) indicating plant factors may control the ability of bacterial to colonize the phyllosphere. However, they also found that the cultivar with the smallest *S. enterica* population also had the lowest number of speck lesions when infected with the tomato pathogen *Pst* DC3000 (Barak et al., 2011), suggesting that strong basal defense in this cultivar may account for low bacterial colonization. On a comparative study of *S. enterica* contamination of several crop species, Barak et al. (2008) reported that seedlings from Brassicaceae family have higher contamination than carrot, tomato, and lettuce when grown on contaminated soil. Seedling contamination correlated with the *Salmonella* population in the phyllosphere of all crop species, except tomato.

Golberg et al. (2011) reported variations in internalization of *Salmonella* SL1344 in different leafy vegetables and fresh herbs using confocal microscopy. Internalization incidence (% of microscopic fields containing bacterial cells) was high in iceberg lettuce and arugula, moderate in romaine lettuce, red lettuce, basil, and low in parsley and tomato. Attraction to stomata was seen in iceberg lettuce and basil, not in arugula, parsley, and tomato. Brandl and Amundson (2008) reported that the age of romaine lettuce leaves is correlated with population size of *E. coli* O157:H7 and *S. enterica* Thompson on leaves. Young leaves (inner) harbor greater number of cells than middle aged leaves. These authors also observed that exudates on the surface of younger leaves have higher nitrogen content than that of older leaves, which may contribute to determining the bacterial population size on the leaf. Thus, it is tempting to speculate that the genetic variability existent among plant genotypes regarding the chemical composition of their organ exudates may be a determinant for human pathogen behavior (such as chemotaxis and tropism toward stomata and roots) and ability to colonize plants.

Finally, Mitra et al. (2009) studied the effect of different methods of inoculation on internalization and survival of *E. coli* O157:H7 in three cultivars of spinach. Among the organs studied, the spinach phylloplane and the stem provided the most and least suitable niche for this bacterium colonization, respectively. Although the leaf surface was the best "territory" for *E. coli*, the leaf morphologies of each cultivar affected the ability of this bacterium to survive.

Collectively, all these studies point out that the plant genotype, age, leaf morphology, chemical composition of exudates, and the primarily infected organ affect the outcome of bacterial colonization of plants and the process may not be a generalized phenomenon, consequently shaping specific human pathogen and plant interactions.

CONCLUDING REMARKS

The fundamental understanding of plant association with human bacterial pathogens that do not cause visual or macroscopic symptom in the plant, but yet are major food contaminants, are in its infancy. Both plant and bacterial factors are critical for these cross-kingdom interactions and emerging evidence suggests an overlap between plant molecular responses to human pathogens

and phytopathogens. The future challenge will be to determine how these interactions differ. As this field of research is relatively new, we see differences in conclusions from different laboratories regarding multiplication vs. decline in bacterial populations overtime and disease-like symptoms vs. HR on inoculated plants. These differences are mainly associated with differences in methods of inoculation, bacterial strains, inoculum concentration, plant age, and plant cultivation methods (e.g., growth on medium, soil, or hydroponic solutions). Standard procedures for model systems, consensus, and collaborations must be developed among food scientists, microbiologists, plant pathologists, and molecular biologists to elucidate the specificity of each plant-bacterium interaction and avoid discrepancies in making general conclusions. A major point to be resolved is whether the observed plant defenses against *Salmonella* and its PAMPs are due to low recognition and/or active suppression. If *Salmonella* suppression of the plant immunity is a cause of weak defense responses, the major question becomes what is the responsible factor? This line of research might lead to a whole new paradigm that otherwise could not be revealed by only studying plant associations with its own natural pathogens.

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Salmonella enterica induces and subverts the plant immune system

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Infections with *Salmonella enterica* belong to the most prominent causes of food poisoning and infected fruits and vegetables represent important vectors for salmonellosis. Although it was shown that plants raise defense responses against *Salmonella*, these bacteria persist and proliferate in various plant tissues. Recent reports shed light into the molecular interaction between plants and *Salmonella*, highlighting the defense pathways induced and the means used by the bacteria to escape the plant immune system and accomplish colonization. It was recently shown that plants detect *Salmonella* pathogen-associated molecular patterns (PAMPs), such as the flagellin peptide flg22, and activate hallmarks of the defense program known as PAMP-triggered immunity (PTI). Interestingly, certain *Salmonella* strains carry mutations in the flg22 domain triggering PTI, suggesting that a strategy of *Salmonella* is to escape plant detection by mutating PAMP motifs. Another strategy may rely on the type III secretion system (T3SS) as T3SS mutants were found to induce stronger plant defense responses than wild type bacteria. Although *Salmonella* effector delivery into plant cells has not been shown, expression of *Salmonella* effectors in plant tissues shows that these bacteria also possess powerful means to manipulate the plant immune system. Altogether, these data suggest that *Salmonella* triggers PTI in plants and evolved strategies to avoid or subvert plant immunity.

Keywords: *S. enterica*, flagellin, PAMP-triggered immunity, effector, plants

INTRODUCTION

Several reports have demonstrated that certain human pathogens can colonize plants both at pre- and post-harvest stages, which is the cause of various outbreaks of foodborne human illnesses (Fletcher et al., 2013). These findings have expanded the research interest on so-called human pathogens on plants (HPOPs) as a means to explore and develop new avenues to increase food safety. One important HPOP is the Gram-negative bacterium *Salmonella enterica*, the causative agent of diseases such as gastroenteritis and typhoid fever that every year is responsible of outbreaks related to the consumption of raw fruits and vegetables (Centers for Disease Control and Prevention; http://www.cdc.gov/foodsafety/outbreaks/multistate-outbreaks/outbreaks-list.html). Indeed, non-typhoidal *S. enterica* serovars can be internalized and persist in several plant species. Using GFP-labeled bacteria, it was shown that *S. enterica* can enter plant leaves through natural openings, such as hydathodes in tomato (Gu et al., 2013) and stomata in lettuce (Kroupitski et al., 2009), or by forcing themselves into plant tissues via "weak points" such as lateral root junctions (Cooley et al., 2003). Once in the intercellular space named apoplast, *S. enterica* is safe from regular sanitizer treatments, but in order to persist inside plant tissues it needs to cope with the plant immune system.

Plants lack an adaptive immune system as exists in higher animals but they have multilayered defense mechanisms that resist infection by a large variety of potential pathogenic microorganisms. The first layer of induced defenses is mediated by plasma membrane localized pattern-recognition receptors (PRRs) that detect conserved microbial features termed pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs). Most characterized PRRs possess an extracellular sensing domain, a transmembrane region and an intracellular protein kinase domain that activates a chain of signaling events upon recognition of external molecules (Monaghan and Zipfel, 2012).These signaling events start with the rapid formation of a receptor complex at the plasma membrane, the activation of kinase cascades involving mitogen-activated protein kinases (MAPKs) and the production of reactive oxygen species (ROS) within minutes but also include slower events such as a transcriptional reprogramming and production of the defense hormones salicylic acid (SA) and ethylene (ET; Monaghan and Zipfel, 2012). Altogether these signaling events lead to the so-called pattern-triggered immunity (PTI) which is usually sufficient to stop microbial invasion. Host-adapted pathogens are able to deliver effectors to the apoplast or inside the host cell using delivery systems, such as the bacterial type III secretion system (T3SS), to inactivate PTI components and thereby enable host colonization. A second layer of plant immunity is mediated by intracellular nucleotide-binding leucine-rich repeat receptors (NLR) that recognize the presence or the activity of specific microbial effectors and initiate effector-triggered immunity (ETI). ETI amplifies PTI responses and is normally associated with the appearance of localized cell death lesions known as hypersensitive response (HR; Heidrich et al., 2012). Furthermore, plants need to tailor their defense responses according to the lifestyle of the

pathogenic microorganism. Whereas SA-based defenses are efficient to fight biotrophic pathogens that depend on living cells, necrotrophic pathogens that feed on dead tissue induce defense responses mediated by the hormones jasmonic acid (JA) and ET and many other plant hormones further influence the outcome of plant−pathogen interactions (Robert-Seilaniantz et al., 2011).

Several reports have shown that plants are able to detect and mount defense responses to *S. enterica* and recent studies started to shed light on the bacterial features recognized and the plant receptors involved. Here we will review new data concerning the molecular interaction between *S. enterica* and plants, and highlight key aspects of the interaction that are still unclear.

Salmonella enterica INDUCES PTI IN PLANTS

The recognition of *S. enterica* in animals occurs through its O antigen, reflecting variation in the lipopolysaccharide (LPS), and its H antigen, reflecting variation in flagellin, and is essential for activating animal innate immunity (Broz et al., 2012). Most *S. enterica* serovars carry two flagellin-encoding genes, *fliC* and *fljB*, and have the capability of "phase variation" through which *Salmonella* alternate between the expression of the two flagellar genes (Silverman and Simon, 1980). Bacterial flagellin constitutes the best studied PAMP recognition system in plants, whereas LPS perception and induced signaling cascades are less characterized (Zipfel et al., 2004; Sun et al., 2012). In plants, flagellin is recognized through direct binding of a conserved N-terminal domain called flg22 by the LRR receptor kinase FLS2 (flagellin-sensing 2) (Gomez-Gomez and Boller, 2000; Zipfel et al., 2004). Recently, a second domain at the N-terminal region of *Pseudomonas syringae* flagellin, termed flgII-28, was shown to be recognized in certain solanaceous species (Cai et al., 2011; Clarke et al., 2013).The N-terminal region spanning the plant recognized domains are identical in the two flagellin proteins encoded by *S. enterica* serovar *Typhimurium* (*S. Typhimurium*) and present some amino acid differences with respect to the sequences shown to be recognized in plants (Garcia et al., 2013; Meng et al., 2013).

Various studies indicated that *S. enterica* possesses PAMPs that are recognized in plants (**Figure 1**). In a similar approach to that used for the identification of flagellin as a PAMP in plants (Felix et al., 1999), the treatment of tobacco cell cultures with heat-killed *S. Typhimurium* elicited rapid ROS accumulation (Shirron and Yaron, 2011). Furthermore, inoculation of *S. enterica* serovars to *Arabidopsis thaliana* seedlings triggered MAPK activation and defense gene expression to a similar extent as that provoked by *P. syringae* inoculation (Schikora et al., 2008, 2011; Garcia et al., 2013). Using a cell death suppression assay Meng et al. (2013) further confirmed that *S. enterica* induces PTI when infiltrated into *Nicotiana benthamiana* leaves. The induction of PTI hallmarks was reduced in *Arabidopsis fls2* mutant seedlings and in *N. benthamiana* leaves silenced for *FLS2* (Garcia et al., 2013; Meng et al., 2013). Furthermore, *S. enterica* flagellin mutants triggered reduced defense responses in *Arabidopsis* and tomato, and colonized *Medicago* spp. to higher numbers compared to wild type bacteria (Iniguez et al., 2005; Garcia et al., 2013; Meng et al., 2013). Together, these results demonstrated that *S. enterica* flagellin is recognized in plants via FLS2. PTI induction was mostly dependent on the *fliC* gene, the most widespread flagellin-encoding gene in *S. enterica* populations (Meng et al., 2013).The *S. enterica* flg22 sequence shows five amino acid changes with respect to the

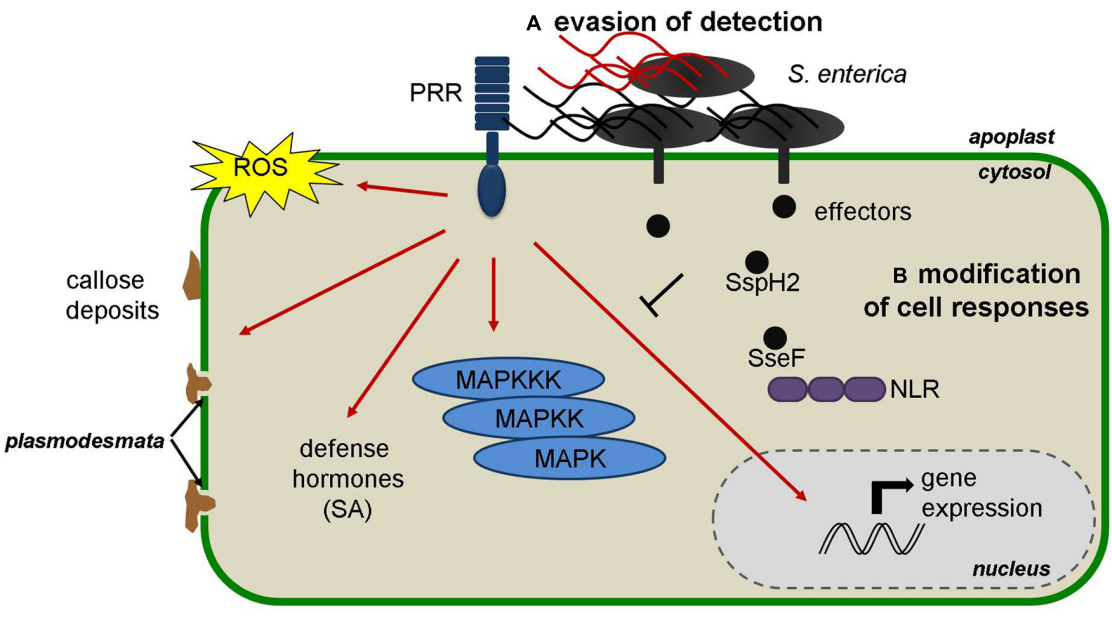

FIGURE 1 | Representation of the defense responses induced upon *S. enterica* perception in plants and the hypothetical mechanisms that could be used by *S. enterica* to accomplish plant colonization: (A) evasion of detection by PRRs through modification of PAMPs such as flagellin and (B) delivery of effectors to modify cell responses. NLR, nucleotide-binding leucine-rich repeat receptor; PRR, pattern-recognition receptor; ROS, reactive oxygen species; SA, Salicylic Acid.

canonical flg22 from *P. aeruginosa* but nevertheless the purified peptide corresponding to the *S. enterica* flg22 sequence (flg22-ST) activated several PTI hallmarks, such as ROS accumulation, callose deposition, growth reduction, and resistance, to a similar extent as *Pseudomonas* flg22 (Garcia et al., 2013; Meng et al., 2013). Interestingly, a recent report demonstrated that *S. Typhimurium* triggers stomatal closure, another PTI-related defense response that limits pathogen entry into the apoplast (Roy et al., 2013). Bacteria-induced stomatal closure is largely mediated by FLS2-mediated recognition of flagellin (Melotto et al., 2006; Zeng and He, 2010). It is therefore intriguing that *S. Typhimurium* treatment of *Arabidopsis* and lettuce leaves triggered reduced stomatal closure as compared with *Escherichia coli* (Roy et al., 2013), even if both bacteria carry the same flg22 sequence (Garcia et al., 2013). Furthermore, *Salmonella* treated leaves showed stronger stomatal reopening 4 h after bacterial inoculation (Roy et al., 2013). These differences could be due to the action of effector molecules or phytotoxins of *Salmonella* that interfere with plant stomatal immunity and that may be absent or less efficient in *E. coli*. Otherwise, it is also possible that the stronger responses triggered by *E. coli* can be attributed to the recognition of other PAMPs such as LPS, which also trigger stomatal closure (Melotto et al., 2006).

Interestingly, previous studies suggested the existence of a different flg22 sequence in strains of *S. enterica* serovar Senftenberg that is more divergent from the canonical flg22 (Tankouo-Sandjong et al., 2008; Berger et al., 2011). The corresponding flg22 peptide (flg22-SS) displayed reduced PTI activity in *Arabidopsis* and therefore suggests that certain *S. enterica* strains may have evolved divergent flagellin sequences to avoid plant recognition (Garcia et al., 2013; **Figure 1**). Other reports have already shown the existence of intra-species variation in the flg22-encoding regions of the *fliC* genes of *P. syringae* and *Xanthomonas campestris* (Sun et al., 2006; Cai et al., 2011; Clarke et al., 2013), providing evidence that PAMPs are less conserved than usually assumed and can evolve to avoid activation of plant defense responses.

The results gathered so far also indicate that other *S. enterica* PAMPs besides flagellin are recognized in plants, as certain PTI activation was still observed in the *Arabidopsis fls2* mutant or after inoculation with *S. enterica* flagellin mutants (Garcia et al., 2013; Meng et al., 2013). In this line, it was reported that *S. enterica* strains carrying the O antigen 1,3,19 induce leaf chlorosis and wilting when infiltrated into *Arabidopsis* leaves (Berger et al., 2011). Furthermore, purified LPS from *S. Typhimurium* (carrying another O antigen) induced ROS accumulation in tobacco (Shirron and Yaron, 2011). On the contrary, LPS purified from other serovars did not activate PTI when infiltrated into *N. benthamiana* leaves or induce ROS in tomato (Meng et al., 2013). This suggests that either a specific O antigen is recognized only by a reduced group of plant species or that other molecule(s) in the strains carrying the O antigen 1,3,19 is recognized in *Arabidopsis*. Synthetic peptides representing conserved regions of cold shock proteins (CSPs) were also inactive in *N. benthamiana* but presented a mild activity in ROS assays in tomato (Meng et al., 2013). Altogether these results indicated that the flagellin flg22 domain is the most prominent PAMP from *S. enterica* recognized in plants. Other PAMPs such as CSPs and flgII-28 seem to contribute to PTI activation to a lesser extent (Meng et al., 2013) and the recognition of *S. enterica* LPS in plants is still unclear.

Data suggests that defense hormones are also involved in the interaction between *S. enterica* and plants. Indeed, Arabidopsis mutants or transgenic lines affected in SA as well as in JA and ET signaling pathways showed increased *S. enterica* colonization (Iniguez et al., 2005; Schikora et al., 2008). Furthermore, pre-treatment with the ET precursor ACC (1-Aminocyclopropane-1-carboxylic acid) reduced the colonization of *Arabidopsis* and alfalfa (*Medicago sativa*) roots by *S. Typhimurium*, as well as by other bacterial endophytes (Iniguez et al., 2005). Recently, it was shown that treatment of *Arabidopsis* seedlings with *S. Typhimurium* wild type and *prgH*- mutant leads to a mild but significant increase in SA accumulation and the reprogramming of several marker genes of the SA pathway (Garcia et al., 2013; **Figure 1**). Phytohormones can mold plant–microbe interactions in a positive or negative way depending on the pathogen, which in turn can be exploited by microbes to induce susceptibility (Robert-Seilaniantz et al., 2011). Therefore, whereas it is clear that defense hormones play a role in the interaction between *S. enterica* and plants, further information is needed to depict which hormones contribute to resistance and if certain hormones are induced by *S. enterica* to increase host susceptibility.

A ROLE FOR *Salmonella enterica* EFFECTORS IN PLANT TISSUES

Salmonella has two pathogenicity islands, SPI-1 (*Salmonella* pathogenicity island 1) and SPI-2, which encode a T3SS (T3SS-1 and T3SS-2) and a suite of effectors. These two secretion systems contribute to different stages of the animal infection process: while the T3SS-1 is expressed at the extracellular stage the T3SS-2 is induced after internalization into animal cells. More than 30 Salmonella effectors have been studied and shown to play important functions for virulence in animal cells by manipulating diverse host cell functions (McGhie et al., 2009). Recently, *Salmonella* T3SSs and effectors were proposed to contribute to the plant colonization process. *S. Typhimurium* mutants in T3SS-1 and T3SS-2 induced stronger cell death and chlorosis symptoms and proliferated to lower levels in *Arabidopsis* leaves (Schikora et al., 2011). Furthermore, the *S. Typhimurium prgH*-mutant, carrying a mutation in a structural component of the SPI-1-encoded T3SS needle complex, triggered enhanced expression of defense genes in *Arabidopsis* seedlings (Schikora et al., 2011; Garcia et al., 2013). Besides, the *S. Typhimurium invA*- mutant, another T3SS-1 defective mutant, induced stronger ROS accumulation than wild type bacteria in tobacco BY-2 cells (Shirron and Yaron, 2011). These results suggested that *S. enterica* effector delivery may be important for plant colonization by dampening the plant immune system (**Figure 1**). On the contrary, the initial colonization of alfalfa roots was higher when using *S. enterica* mutants in the SPI-1-encoded *spas* and *sipB* genes, with defects in effector delivery, which indicates the possibility that certain SPI-1 encoded molecules are recognized and trigger defense responses in this host (Iniguez et al., 2005). Finally, it was recently reported that

the *S. Typhimurium prgH*- mutant can multiply to similar levels as the wild type strain in tomato leaves, which led the authors to conclude that SPI-1 is not important for tomato colonization and that tomato plants do not recognize SPI-1 products (Meng et al., 2013). The different results obtained in different plant species are intriguing and may point to different mechanisms of interaction.

So far no study has demonstrated the *Salmonella*-mediated delivery of effectors into plant tissues but two recent studies suggest that *S. enterica* effectors are functional in plant cells. Ustun et al. (2012) used *Agrobacterium tumefaciens* to monitor the effect of several *S. Typhimurium* effectors when expressed in *N. benthamiana* and identified the SPI-2-encoded effector SseF as an inducer of HR-like cell death lesions. The appearance of cell death lesions was specific and accompanied by the upregulation of several cell death and defense-related genes. Furthermore, SseF also triggered HR-cell death and ETI when delivered into *N. benthamiana* leaves in a T3SS-dependent manner by inoculation with *X. campestris* pv. *vesicatoria*. Interestingly, the SseF-triggered cell death was compromised by silencing the *N. benthamiana* genes coding for the co-chaperone SGT1 (suppressor of G2 allele of skp1) and the plasma-membrane localized protein NDR1 (non-race-specific disease resistance 1), two proteins normally required for resistance mediated by NLRs carrying an N-terminal coiled-coil domain (Austin et al., 2002; Knepper et al., 2011; Ustun et al., 2012). Altogether, these data suggested that the *S. Typhimurium* SseF effector is recognized by an NLR in *N. benthamiana*. Recently, another SPI-2-encoded effector, SspH2, was proposed to perform functions conserved in plant and animal cells (Bhavsar et al., 2013). It was demonstrated that SspH2, an E3 ubiquitin ligase, interacts with and increases the activity of animal and plant SGT1 proteins, which in turn increases NLR-mediated cell death (Bhavsar et al., 2013). The functional significance of this interaction is unclear, but it demonstrates that *S. enterica* effectors are able to manipulate plant and animal immune components (**Figure 1**).

FURTHER HOST ADAPTATION MECHANISMS

The colonization of plants can also lead to molecular and phenotypical changes in *S. enterica* cells, which can help us to identify the determining factors governing the interaction between *S. enterica* and plants. The genome sequences of several *S. enterica* serovars are known and transcriptome analyzes have successfully been used to assess gene expression changes in environmental conditions associated to the animal infection processes (Hébrard et al., 2011; Kroger et al., 2013). Recently, the first transcriptome analysis of *S. enterica* cells in contact with plants was reported (Goudeau et al., 2013). Given that *S. enterica* proliferation levels are higher in soft-rotted tissues, the authors analyzed the transcriptional changes in *S. Typhimurium* upon inoculation of *Dickeya dadantii*-macerated cilantro (*Coriandrum sativum*) and lettuce (*Lactuca sativa*) leaves. This analysis revealed significant changes in gene expression that includes the upregulation of various nutritional and metabolic pathways, suggesting that *S. enterica* reacts to and benefits from the enhanced nutrient availability in the soft-rotted leaves (Goudeau et al., 2013). It is still intriguing to know how the plant environment impacts on the expression of other virulence related genes, such as the *Salmonella* pathogenicity islands and the encoded T3SS and effectors. For instance, SPI-1 genes are known to be induced by high osmolarity, low oxygen levels and short chain fatty acids (Hautefort et al., 2003) and using a promoter-reporter fusion, it was recently shown that the SPI-1 gene *prgH* is expressed when in contact with *Arabidopsis* root cells (Garcia et al., 2013).

Furthermore, genetic screens monitoring the efficiency of *S. enterica* mutants in different steps of the plant colonization process have proved useful to identify bacterial genes that are important for the interaction with plants (Barak et al., 2005, 2009). These analyzes pointed to the synthesis of cellulose and aggregative fimbriae (curli), involved in the so-called "rdar" (red dry and rough) phenotype, as being important for plant attachment. Interestingly, several non-rdar mutants have been recovered from produce-related disease outbreaks (Brandl et al., 2013). Furthermore, *S. Typhimurium* non-rdar mutants originating by sequential passages through tomatoes showed enhanced fitness in plants as compared to the parental strain, despite being less competitive on common laboratory media (Zaragoza et al., 2012). Altogether these results indicate that *S. enterica* has the ability to rapidly evolve and adapt to specific host environments such as the hostile plant apoplast. Further gene expression analyzes and mutant screens will allow us to gain insight into the virulence mechanisms used by *S. enterica* to colonize and survive in plant tissues.

PERSPECTIVES

In the past few years, the research on HPOPs has expanded and demonstrated that *S. enterica* serovars are able to colonize and persist in different plant tissues and species. These studies have also suggested that the ability to colonize different hosts is variable and governed by various genetic and environmental factors. A few studies reported varied proliferation levels (one to five logs) of *S. Typhimurium* inside plant tissues (Cooley et al., 2003; Schikora et al., 2008, 2011; Barak et al., 2011; Garcia et al., 2013; Meng et al., 2013) and evidence suggests that *S. enterica* titers inside the plant apoplast are highly dependent on the environmental conditions (Cooley et al., 2003; Roy et al., 2013). It is clear that the increased interest in HPOPs will reveal the mechanisms used by these microorganisms to exploit plants as secondary hosts and should help to develop strategies to reduce *S. enterica* populations in plants and thereby disease outbreaks.

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Killing two birds with one stone: trans-kingdom suppression of PAMP/MAMP-induced immunity by T3E from enteropathogenic bacteria

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Within the past decade, remarkable similarities between the molecular organization of animal and plant systems for non-self discrimination were revealed. Obvious parallels exist between the molecular structures of the receptors mediating the recognition of pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) with plant pattern recognition receptors strikingly resembling mammalian Toll-like receptors. Mitogen-activated protein kinase cascades, leading to the transcriptional activation of immunity-associated genes, illustrate the conservation of whole molecular building blocks of PAMP/MAMP-induced signaling. Enteropathogenic *Salmonella* and *Escherichia coli* use a type three secretion system (T3SS) to inject effector proteins into the mammalian host cell to subvert defense mechanisms and promote gut infection. Lately, disease occurrence was increasingly associated with bacteria-contaminated fruits and vegetables and common themes have emerged with regard to whether and how effectors target innate immune responses in a trans-kingdom manner. We propose that numerous *Salmonella* or *E. coli* effectors may be active *in planta* and tend to target central components (hubs) of immune signaling pathways.

Keywords: PAMP/MAMP, innate immunity, mammals, plants, enteropathogenic bacteria, *Salmonella*, *Escherichia coli*, type three effectors

INTRODUCTION

Animals and plants are able to discriminate between self and non-self, which is the key feature to fight against microbial pathogens. The first line of the innate immune response in both animals and plants is induced by the perception of common pathogen or microbe-associated molecular patterns (PAMPs/MAMPs) that are absent from host cells (Nurnberger et al., 2004; Ausubel, 2005; Akira et al., 2006; Boller and Felix, 2009; Macho and Zipfel, 2014). PAMP/MAMP sensing is mediated by specific pattern recognition receptors (PRRs), localized in different sub-cellular compartments. In mammals, the transmembrane Toll-like receptor family (TLRs) and the cytoplasmic nucleotide-binding oligomerization domain (NOD)-like receptor family (NLRs) are the most representative PRRs (Kawai and Akira, 2011; Kumar et al., 2011). In plants, typical PRRs belong to the class of transmembrane receptor-like kinases or membrane bound- receptor like proteins with extracellular leucine-rich repeat- (LRR-RLKs/LRR-RLPs) or lysine motif- (LysM-RLKs/LysM-RLPs) containing domains (Boller and Felix, 2009; Macho and Zipfel, 2014). PAMP/MAMP binding induces intracellular signaling cascades leading to cellular re-programming. In mammals, the immune response is associated with the transcriptional activation of immunity-associated genes and production of cytokines including interleukins (ILs) or tumor necrosis factors (TNFs) and antimicrobial peptides. In plants, anti-microbial metabolites (phytoalexins) and proteins (Pathogenesis-Related proteins) are produced in response to infection. The ultimate outcome of the process, called inflammatory response in animals and PAMP/MAMP-triggered immunity (PTI/MTI) in plants, is pathogen clearance.

Successful pathogens have learned to subvert PAMP/MAMP triggered immune responses by producing effectors that contribute to virulence. Many effectors originated from human pathogenic enterobacteria, e.g. *Salmonella, shigella, Yersinia,* and *Escherichia coli* or plant pathogenic bacteria e.g. *Pseudomonads* and *Xanthomonads* were shown to manipulate host PTI/MTI signaling (Gohre and Robatzek, 2008; Brodsky and Medzhitov, 2009; Dean, 2011; Dou and Zhou, 2012; Johannessen et al., 2013; Raymond et al., 2013). Although enterobacteria do not represent a threat to agriculture, cumulative evidence support the view that, against the general dogma, *Salmonella* and some *E. coli* strains (O157:H7 serogroup) can actively invade, proliferate and spread in plants (Holden et al., 2009; Schikora et al., 2012). A prerequisite for *Salmonella* or *E. coli* growth on host plants would be the ability to cope with the immune system, notably through the evolution of a functional type three secretion system (T3SS) capable to breach through the cell wall and inject type three effectors (T3E) that manipulate immunity-associated components.

Here, we will review the parallels (and differences) between PAMP/MAMP-induced immune signaling in plants and animals with emphasis on the molecular components that are functionally conserved between both kingdoms. Further, we will discuss the potential of candidate effectors from animal- and plant-adapted

enteropathogenic bacteria to suppress the evolutionary conserved PAMP/MAMP-triggered immune response.

PAMP/MAMP-TRIGGERED IMMUNITY IS EVOLUTIONARY CONSERVED ACROSS KINGDOMS

The identification and functional characterization of PAMPs/MAMPs and their corresponding PRRs in mammals and plants underwent a burst of interest in the past two decades, contributing to drastically increase our fundamental knowledge about the molecular mechanisms of host adaptation to microbial infection. Best-studied PAMPs/MAMPs in mammals are lipopolysaccharides (LPS) from Gram- bacteria, peptidoglycan (PGN) from Gram+ bacteria, flagellin (the major constituent of bacterial flagella), double stranded RNA of viruses or bacterial DNA. In mammals, transmembrane TLRs and cytoplasmic NOD proteins with leucine rich repeats (LRR) are responsible for the recognition of PAMPs/MAMPs (Akira et al., 2006; Kawai and Akira, 2011; Kumar et al., 2011). The heterodimerization between different TLRs increases the potential of recognized PAMPs/MAMPs (Ozinsky et al., 2000). TLR5 is a good representative of the TLR family in mammals (**Figure 1**). It senses flagellin from a variety of different Gram+ and Gram- bacteria (Nempont et al., 2008) by recognizing the highly conserved N-terminal 99 amino acids and C-terminal 416-444 amino acids of the protein (Smith et al., 2003). Flagellin-induced signaling recruits adaptor proteins such as MyD88 (Myeloid Differentiation primary response gene 88). The adaptor then recruits IRAK1 and IRAK4 (IL-1R-associated kinases) causing their autophosphorylation and the association with TRAF6 (TNF-receptor-associated factor 6). TRAF6 activates the TAK complex (TAB1,2–TAK1 binding protein 1,2- and TAK1–TGFβ activated Kinase 1) which then induces the activation of MEK1/2, MKK3/6, 4, 7 (MAP kinase kinase 1/2, 3/6, 4, 7) and of the IKKγ(NEMO)/IKKα/IKKβ complex (IKK complex). The activated MKKs in turn activate ERK (Extracellular signal Regulated Kinase), JNK (c-Jun N-terminal Kinase), and p38 MAP kinase which ultimately induce the expression of immunity-associated genes like IL-1 (interleukin-1), IL-2, IL-6, and IL-12 through stimulation of the transcription factor AP-1 (Activator Protein 1). The activation of the IKK complex results in the degradation of I-κB (Inhibitor of kappaB) and the translocation of NF-κB (Nuclear Factor-kappaB) to the nucleus where it regulates the expression of genes encoding TNFs (Akira et al., 2006; O'Neill and Bowie, 2007; Kumar et al., 2011).

Most of the PAMPs/MAMPs, which are recognized in mammals, also trigger defense responses in plants. Like in animals, the perception is executed by cell surface receptors that are structurally very similar to TLRs. One of the best-studied PRR in plants is FLS2 (Flagellin Sensitive 2) from *Arabidopsis thaliana*, a protein with an extracellular leucine rich repeat domain (LRR) responsible for flagellin binding and an intracellular kinase domain involved in signal transduction (Gomez-Gomez and Boller, 2000; **Figure 1**). FLS2 homologs are present in nearly all the plant species that were tested for responsiveness to flagellin (Boller and Felix, 2009). FLS2 binds a conserved 22 amino acid peptide (flg22) located in the N-terminal part of flagellin. Flg22 binding leads to the recruitment of BAK1 (BRI1-Associated Kinase 1), another transmembrane receptor-like kinase, to form a functional receptor complex (Chinchilla et al., 2007; Heese et al., 2007; Sun et al., 2013). The FLS2-BAK1 complex undergoes several events of cross-phosphorylation at multiple serine/threonine and tyrosine residues that are located in the kinase domain (Schulze et al., 2010; Schwessinger et al., 2011; Yan et al., 2012). These phosphorylation events are thought to contribute to signaling initiation and specificity but their functions remain largely hypothetical (Macho and Zipfel, 2014). The receptor-like cytoplasmic kinase BIK1, constitutively associated with FLS2 in absence of flg22 also becomes phosphorylated and dissociates from the receptor complex (Lu et al., 2010; Zhang et al., 2010). The NADPH oxidase RbohD has been identified recently as a substrate of BIK1. Thus, a direct mechanistic link could be established between the formation of the receptor complex and the production of reactive-oxygen species (Kadota et al., 2014; Li et al., 2014). In contrast, the activation mechanism of MAP kinase cascades comprising MEKK1, MKK1/MKK2, MPK4 and MKK4/MKK5, MPK3/MPK6 downstream of the FLS2 receptor remains elusive (Asai et al., 2002). Activated MAP kinases are translocated to the nucleus where they regulate the expression of immunity-associated genes, for example by activating the transcription of genes encoding pathogenesis-related (PR) proteins.

FLS2 and TLR5 recognize different epitopes in flagellin. Therefore it is considered that animals and plants have developed their perception systems for flagellin independently. It is generally admitted that the similarity between the components present in the plant and animal innate immune signaling pathways is likely based on convergent evolution. Plants do not have downstream signaling components homologous to the MyD88 adaptor protein and intracellular TIRAP (Toll-interleukin receptor containing adaptor protein). The Rel family of transcription factors, which includes NF-κB in mammals, is also missing in plants. On the contrary, animals do not have transcription factors homologous to the WRKY family found in *Arabidopsis*.

BACTERIAL TYPE THREE EFFECTORS MANIPULATING PAMP/MAMP-TRIGGERED IMMUNE SIGNALING COMPONENTS

Successful pathogens are able to manipulate PAMP/MAMP-triggered immunity in both animals and plants by producing effectors. Most of the Gram- bacteria use a sec-independent protein delivery system, the so-called type three secretion apparatus (T3SS), to translocate type three effectors (T3E) into the cytosol of host cells.

The molecular mechanisms underlying *Salmonella* and *E. coli* infection in humans are well-studied and the functions of T3Es become apparent. More than thirty T3Es have been identified in *Salmonella* and some of them have been shown to manipulate key cellular functions implicated in immunity (McGhie et al., 2009; Dean, 2011; Figueira and Holden, 2012). Many effectors are GTPase-activating proteins (GAPs) that interact and alter the function of the Rho family GTPases, proteins regulating numerous cellular processes including immune responses and

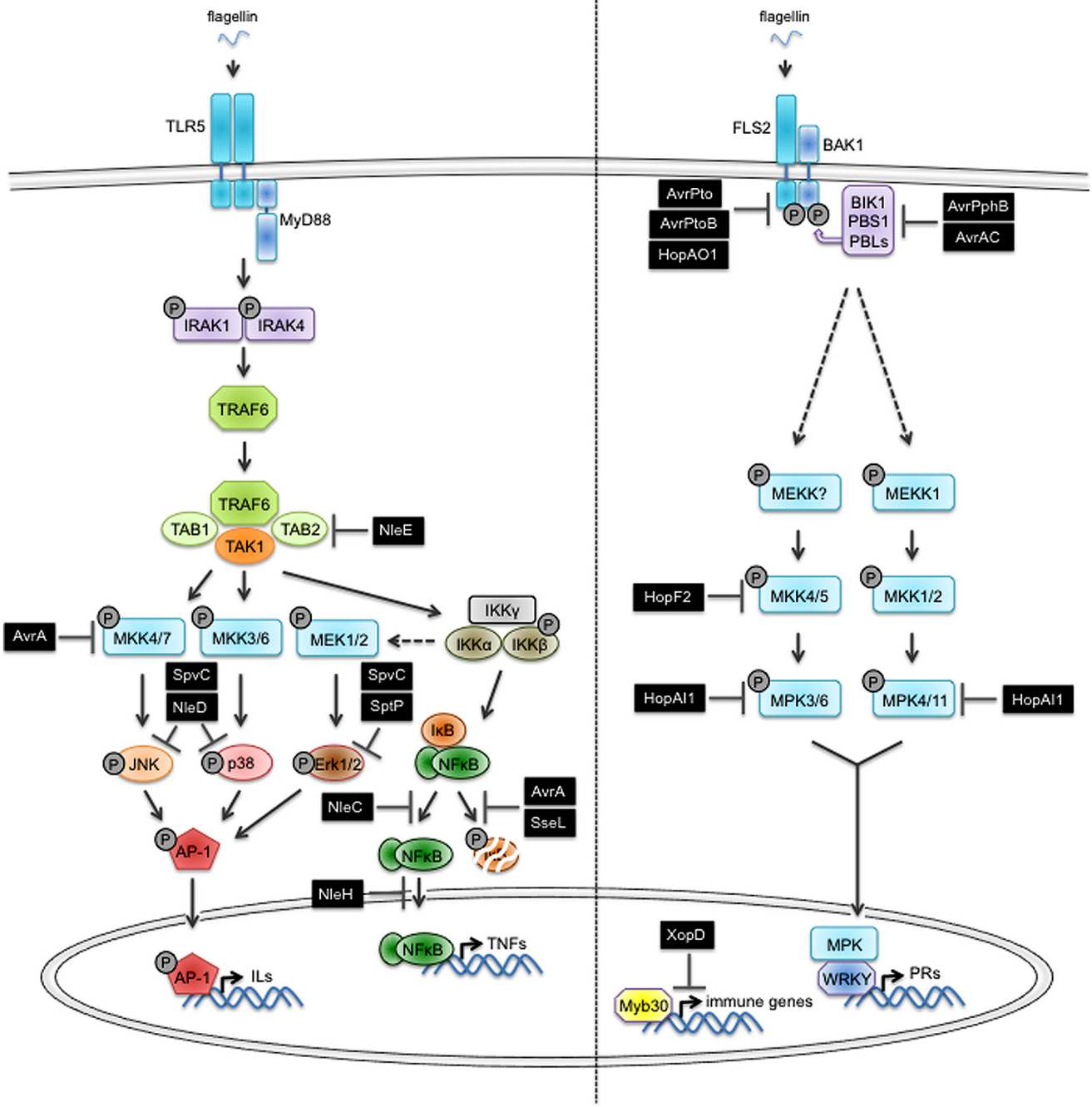

FIGURE 1 | Overview of the PTI/MTI-suppressing function of type three effectors from enterobacteria and phytopathogenic bacteria. The perception of pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) by pattern recognition receptors (PRRs) triggers signaling pathways conferring immunity (PTI/MTI) that are conserved in mammals and plants. Host-adapted bacterial pathogens use the type three secretion system (T3SS) to inject effectors (T3E) that compromise PTI/MTI. Components of basic plant defense and interfering pathogen effectors (black box) are depicted. See main text for additional details. Abbreviations used in the figure: TLR5, Toll-like receptor 5; FLS2, Flagellin Sensitive 2; MyD88, Myeloid Differentiation primary response gene 88; BAK1, BRI1-Associated receptor Kinase 1; BIK1, Botrytis-Induced Kinase 1; PBS1, AvrPphB susceptible 1; PBL, AvrPphB susceptible 1-like; IRAK1,4, Il-1 receptor associated kinase 1,4; TRAF6, TNF receptor associated factor 6; TAB1,2, TAK1 binding protein 1,2; TAK1, TGFβ activated Kinase 1; MEKKs, mitogen-activated protein kinase kinase kinases; MEKs/MKKs, mitogen-activated protein kinase kinases; MPKs, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinase; p38, p38 mitogen-activated protein kinase; Erk, extracellular signal regulated kinase; IKKs, IkappaB kinases; IκB, inhibitor of kappa B; NF-κB, nuclear factor-kappa B; AP-1, activator protein 1; WRKY, WRKY transcription factor; MYB30, MYB domain protein 30.

cytoskeleton re-arrangements (Bokoch, 2005; Aktories, 2011). The *Salmonella* effector SptP contains a GAP domain and was shown to inhibit the downstream activity of Rac1 and Cdc42, two Rho family GTPases (Fu and Galan, 1999; Bruno et al., 2009). In addition, the C-terminal domain of SptP displays tyrosine phosphatase activity that blocks the MAP kinase pathway by inhibiting Raf kinase activation (Lin et al., 2003).

SpvC, a homolog of OspF from *Shigella flexneri*, is an effector with phosphothreonine lyase activity that exerts its inhibitory action on active MAP kinases and downregulates the expression of cytokines in infected cells (Mazurkiewicz et al., 2008). The MAP kinases are irreversibly inactivated through β-elimination of the phosphate group from a conserved phosphothreonine residue in the activation loop (Li et al., 2007; Zhu et al., 2007).

Another well-studied *Salmonella* effector, AvrA, is a close homolog of YopJ from *Yersinia spp.* It was shown that AvrA possesses acetyltransferase activity. Through its interaction with MKK4 and MKK7, it inhibits the activation of the JNK pathway without interfering with the activation of the NF-κB or p38 pathways (Jones et al., 2008). Interestingly, AvrA itself is activated through phosphorylation that is dependent on the stimulation of the extracellular signal-regulated kinases 1/2 (Erk 1/2) pathway upon *Salmonella* infection (Jones et al., 2008; Du and Galan, 2009). In addition, it was reported that AvrA has deubiquitinase activity and removes ubiquitin from IκB and β-catenin, two inhibitors of NF-κB, thereby preventing their degradation by the proteasome. The resulting increase of association with NF-κB attenuates the translocation of the transcription factor to the nucleus and attenuates the inflammatory response and cellular apoptosis (Ye et al., 2007). NF-κB functions as an important cellular hub of immune responses that is preferentially targeted by many effectors. Hence, *Salmonella* SseL is acting via a similar mechanism than AvrA (Le Negrate et al., 2008).

E. coli is producing a range of effectors with different functions and acting on different targets of the NF-κB signaling pathway. NleE displays methyltransferase activity and methylates TAB2/3, which in turn inhibits TRAF6-induced activation of the NF-κB pathway (Nadler et al., 2010; Newton et al., 2010; Vossenkamper et al., 2010). NleC is a zinc metallo-protease that degrades the NF-κB p65 subunit, thus blocking the induction of IL-8 (Yen et al., 2010). Another effector, NleH, blocks translocation of NF-κB into the nucleus without affecting IkBα degradation (Gao et al., 2009; Pham et al., 2012). NleD is a zinc-dependent metallo-protease specifically targeting the MAP kinases JNK and p38 but not Erk1/2, thereby blocking the nuclear translocation of the transcription factor AP-1 (Baruch et al., 2011).

Like *Salmonella* and *E. coli*, typical plant pathogenic bacteria such as *Pseudomonas spp.* or *Xanthomonas spp.* produce effectors that are translocated into the host cell via the T3SS. Genetic and functional analyzes have shown that a large number of T3E suppress defense responses that are normally induced by T3SS-deficient bacteria or by flg22 (Gohre and Robatzek, 2008; Dou and Zhou, 2012). These T3E affect PAMP/MAMP-induced signaling at different steps, from the early recognition events at the plasma membrane involving the PAMP/MAMP receptor complex to the late defense responses associated with the *de novo* synthesis of anti-microbial metabolites and proteins or callose deposition for cell wall reinforcement. AvrPto from *Pseudomonas syringae* interacts with the kinase domain of FLS2 (and other PAMP/MAMP receptors) *in planta* (Xiang et al., 2008) and/or with its co-receptor BAK1 (Shan et al., 2008). This interaction causes the inhibition of the kinase activity that is essential for the initiation of the signaling cascade (Xing et al., 2007). Very recently, it was shown that the tyrosine phosphatase activity of HopAO1 affects early immune signaling triggered by the receptor-like kinase EF-Tu Receptor (EFR) – and potentially FLS2 -, which perceives the elf18 peptide derived from the bacterial elongation factor Tu (EF-Tu) (Macho et al., 2014). Another effector from *P. syringae*, AvrPtoB, is a E3 ubiquitin ligase that causes degradation of FLS2 (Gohre et al., 2008). BIK1 is targeted by effectors originated from different phytopathogenic bacteria. *P. syringae* AvrPphB is a cysteine protease that causes degradation of BIK1 and related receptor-like cytoplasmic kinases such as PBS1 and PBLs (Zhang et al., 2010) whereas *Xanthomonas campestris* AvrAC displays uridylyl-transferase activity to interfere with the activation of BIK1 upon FLS2-BAK1 association (Feng and Zhou, 2012). Many T3E were shown to suppress PAMP/MAMP-dependent signal transduction downstream of the PRR complex. HopAI1, an effector with phosphothreonine lyase activity inactivates MPK3, MPK6, and MPK4 by dephosphorylating them (Zhang et al., 2007). HopF2 inhibits PAMP/MAMP-induced signaling by targeting MKK5 via its ADP-ribosyltransferase activity (Wang et al., 2010). There are increasing evidence that the nuclear transcriptional and post-transcriptional machinery represent a key target of bacterial effectors for the suppression of immunity-associated genes. For example, *X. campestris* XopD blocks the activity of the transcription factor MYB30 resulting in the suppression of basal immune responses (Kim et al., 2008; Canonne et al., 2011).

EVOLUTIONARY CONSERVED CELLULAR HUBS OF IMMUNE RESPONSES AS PUTATIVE TARGETS OF ENTEROBACTERIA TYPE THREE EFFECTORS

A large yeast two-hybrid interaction matrix analysis with effectors from *P. syringae* and the filamentous oomycete *Hyaloperonospora arabidopsisdis*, both pathogens of *Arabidopsis*, suggests that taxonomically distant microorganisms have independently evolved effectors targeting an overlapping subset of proteins that are central components of the plant immune network (Mukhtar et al., 2011). By extension, cellular hubs that are evolutionary conserved among kingdoms are potentially prime choice targets of effectors. So far, a few studies show experimental evidence in favor of enterobacteria T3E being functional in plant cells. Notably, Ustün et al. (2012) showed that SseF from *S. enterica* induces a hypersensitive response (HR)-like cell death when transiently expressed in *Nicotiana benthamiana* (Ustün et al., 2012). Importantly, the HR-like phenotype is dependent on the co-chaperone SGT1 (Suppressor of G2 allele of skp1) and the protein NDR1 (Non-race specific Disease Resistance 1), which are essential for effector-triggered immunity , a form of race-cultivar specific resistance that is mediated by the recognition of effectors (mostly T3E)

from adapted phytopathogenic bacteria by cytoplasmic plant resistance (R) proteins of the CC-NB-LRR (coiled-coil – nucleotide binding – leucine rich repeat domain) type. The function of R proteins is to monitor changes occurring to cellular components that are modulated by effectors. In the case of SseF, no plant interactors have been reported yet, thus, the virulence function of SseF, especially its ability to interfere with PTI/MTI remains elusive. SGT1 is conserved in both mammals and plants and in another study, it was shown that SspH2, an E3 ubiquitin ligase, interacts and modifies the activity of SGT1 to subvert immunity (Bhavsar et al., 2013). Effector-mediated protein ubiquitination to trigger degradation seems to be a common strategy adopted by mammals and plant pathogens to re-program cellular functions. SspH2 and other *Salmonella* ubiquitin ligases, e.g., SspH1, SlrP, or SopA might very well work *in planta* and target components of the PTI/MTI signaling pathway, like AvrPtoB does at the level of the PRR complex.

The MAP kinase cascades represent an obvious target of T3E action. SpvC interacts with *Arabidopsis* MPK6 by blocking the flg22-dependent post-translational activation of MPK3/6 and consequently the induction of the expression of typical PAMP/MAMP-induced marker genes (A. Schikora, personal communication). Because of the functional similarity with HopAI1, it is assumable that SpvC inactivates the plant MAP kinases through its phosphothreonine lyase activity.

Enterobacteria produce many effectors that modulate the function of the Rho family GTPases. Also the ROP (Rho of plant) family of GTPases is playing a determinant role in plant defense to pathogen attack (Moeder et al., 2005; Chen et al., 2010; Hoefle et al., 2011). It will be a very exciting challenge to elucidate to what extent the interaction between SptP and the *Arabidopsis* Rac1 homolog AtROP2 (A. Schikora, personal communication) affects the biochemical and biological function of this protein.

Overall these research findings give rise to the concept that *Salmonella* has deployed an arsenal of effectors able to manipulate both animal and plant basal immune defenses, which would be requested for successful growth and host colonization.

CONCLUDING REMARKS

Until very recently it was assumed that disease caused by fruits and vegetables contaminated by enteropathogenic bacteria was due to post-harvesting handling. However, reports indicating that *Salmonella* and *E. coli* are able to actively grow and replicate in soil-grown vegetables suggest that plants may provide a niche for these bacteria (Holden et al., 2009; Schikora et al., 2012). In the case of *Salmonella*, it is still controversial whether the bacterium is able to enter the plant cell and replicate in specialized structures (the *Salmonella*-containing vacuoles) as it does in human epithelial cells. To date, none of the common plant pathogenic Gram-bacteria (*Pseudmonads, Xanthomonads*) was reported to penetrate into the cell but rather grow in the apoplast in an epiphytic modus. The reason for that is the presence of the plant cell wall, which may prevent bacterial uptake by macropinocytosis. Also, it is not proven whether the T3SS from *Salmonella* is adapted for translocation of effectors into plant cells. A major function of the T3E is to manipulate PAMP/MAMP-triggered immunity. As the molecular blocks of the signaling cascades are conserved between mammals and plants, it is tempting to speculate that *Salmonella* and *E. coli* have evolved effectors targeting components of the immune signaling pathway that are conserved among plants and animals. The biological relevance of pathogenic enterobacteria T3E manipulating signaling components of the plant immune system needs, however, to be addressed. Besides this aspect, these effectors can represent useful molecular tools to study the function of interacting plant proteins in their natural genetic background. This also suggests to search for the potential of effectors from other human and animal pathogens to re-program cellular functions in plants.

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The *Salmonella* effector protein SpvC, a phosphothreonine lyase is functional in plant cells

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Salmonella is one of the most prominent causes of food poisoning and growing evidence indicates that contaminated fruits and vegetables are an increasing concern for human health. Successful infection demands the suppression of the host immune system, which is often achieved via injection of bacterial effector proteins into host cells. In this report we present the function of *Salmonella* effector protein in plant cell, supporting the new concept of trans-kingdom competence of this bacterium. We screened a range of *Salmonella* Typhimurium effector proteins for interference with plant immunity. Among these, the phosphothreonine lyase SpvC attenuated the induction of immunity-related genes when present in plant cells. Using *in vitro* and *in vivo* systems we show that this effector protein interacts with and dephosphorylates activated *Arabidopsis* Mitogen-activated Protein Kinase 6 (MPK6), thereby inhibiting defense signaling. Moreover, the requirement of *Salmonella* SpvC was shown by the decreased proliferation of the Δ*spvC* mutant in *Arabidopsis* plants. These results suggest that some *Salmonella* effector proteins could have a conserved function during proliferation in different hosts. The fact that *Salmonella* and other *Enterobacteriaceae* use plants as hosts strongly suggests that plants represent a much larger reservoir for animal pathogens than so far estimated.

Keywords: T3SS, trans-kingdom pathogenicity, *Salmonella*, plant infection

INTRODUCTION

Various pathogenic bacteria such as *Salmonella enterica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* O157:H7, and *Listeria monocytogenes* are able to proliferate on both animal and plant organisms (Prithiviraj et al., 2005; Milillo et al., 2008; Schikora et al., 2008, 2011; Haapalainen et al., 2009; Holden et al., 2009). *Salmonella* is a genus of Gram-negative enteropathogenic bacteria that colonizes a wide range of hosts, including humans. These bacteria are the causal agents of gastroenteritis and typhoid fever (Pang et al., 1995). The most common mode of infection in humans is the ingestion of contaminated food or water. Whereas 0.3% of fresh products were contaminated with *Salmonella* bacteria in 2007 in the European Union (Westrell et al., 2009), the proportion of raw-food related outbreaks reached 25% in the USA in recent years (Rangel et al., 2005).

The study of the *Salmonella* infection mechanism was until recently mainly driven by its medical aspect; therefore the mouse and human epithelial cell models are the best studied to date. Today, it is still poorly understood how these bacteria successfully proliferate in such diversified hosts as animals or plants. However, important insights were obtained during last years. Stomata openings were identified as possible entry points of bacteria into the inner layers of the mesophyll (Kroupitski et al., 2009). Interestingly, while some plant species (e.g., arugula) allow the *Salmonella enterica* subsp. *enterica* ser. Typhimurium (*S.* Typhimurium) strain SL1344 to internalize, some others (e.g., parsley) seem to be capable of preventing internalization (Golberg et al., 2011). In a previous report, we showed that in *Arabidopsis thaliana*, roots and especially root hair cells can be colonized by *Salmonella* (Schikora et al., 2008).

Studies of the infection mechanisms in animals revealed that, besides remodeling the host cell architecture, *Salmonella* actively suppresses the host immune system by injecting a cocktail of effector proteins. These effectors are delivered by Type III Secretion Systems (T3SSs). *S.* Typhimurium possesses two

distinct T3SSs, T3SS-1, and T3SS-2, encoded by two *Salmonella* Pathogenicity Islands, SPI-1 and SPI-2, respectively. To date, about 44 *Salmonella* effectors have been described and the function of many of them is known [reviewed in Heffron et al. (2011)]. In addition to SPIs, some *Salmonella* serovars carry plasmids with a common locus called salmonella plasmid virulence (*spv*) (Boyd and Hartl, 1998). The *spv* operon encodes further effector proteins responsible for full virulence in humans and in the mouse model (Montenegro et al., 1991; Fierer et al., 1992; Gulig and Doyle, 1993; Chu and Chiu, 2006).

Even though some *Salmonella* effectors have homologs in plant pathogenic bacteria, the role of *Salmonella* T3SS-dependent effectors in the modulation of the plant immune system and their contribution to plant host susceptibility are less understood. Plants induce defense mechanisms after recognition of pathogens. This recognition may occur at two levels: (i) at the cell surface, where Pattern Recognition Receptors (PRRs) recognize conserved microbial structures called Pathogen-Associated Molecular Patterns (PAMPs), and (ii) in the cytoplasm where Resistance (R) proteins recognize bacterial effectors injected into plant cells. Both recognition events initiate immune responses referred to as Pattern-Triggered Immunity (PTI) [renamed from PAMP-triggered immunity (Boller, 2012)] or Effector-Triggered Immunity (ETI), respectively. An activation of MAPKs and enhanced expression of *Pathogenesis Related* (*PR*) genes are hallmarks of both: the PTI and the ETI responses. Both responses were already observed after inoculation with *Salmonella* (Schikora et al., 2008; Meng et al., 2013; Garcia et al., 2014). Recently, the suppression of plant defense by *Salmonella* was reported in two different systems. In contrast to living *S.* Typhimurium, treatment with dead or chloramphenicol-treated bacterial cells elicited an oxidative burst and changes in apoplastic pH in tobacco (Shirron and Yaron, 2011). Similar responses were provoked by inoculation with the *invA* mutant, which has no functional T3SS-1, showing that T3SS-deficient or dead bacteria induce defense reactions while living wild-type bacteria actively suppress their induction. We observed a very similar phenomenon in *Arabidopsis* plants (Schikora et al., 2011). Inoculation with wild-type *S.* Typhimurium strain 14028s provoked changes in expression of 249 and 1318 genes at 2 and 24 h after infection, respectively (Schikora et al., 2011). However, inoculation with the *prgH* mutant, which has no functional T3SS-1, changed the expression of over 1600 genes at 24 h. Gene ontology (GO) term enrichment analysis of the 649 *prgH*-specific genes revealed an overrepresentation of genes related to pathogen responses and ubiquitin-mediated protein degradation (Schikora et al., 2011; Garcia et al., 2014). Interestingly, this set includes *BAK1*, *BIK1*, *WRKY18*, *WRKY33*, *EIN3*, *PR4*, *FRK1*, *4CL*, *Sec61,* and *PUB23*, all of which are up-regulated upon inoculation with pathogen or PAMP treatment. The higher expression levels of these genes after inoculation with the *prgH* mutant compared to the wild-type imply that the mutant is lacking an effective suppression mechanism to hinder plant defense. A powerful response to pathogen attack is the hypersensitive response (HR). This induced cell death is often the reaction to bacterial proteins present in the host cytoplasm (Jones and Dangl, 2006). In respect to *Salmonella* effector proteins, SseF was the first effector reported to induce HR-like symptoms in tobacco plants (Ustun et al., 2012). The fact that SseF-induced HR-like symptoms can be suppressed by RNAi-mediated silencing of *SGT1* (*Suppressor of G2 allele of Skip1*) indicates an R-protein-mediated response, identical to ETI.

In this report, we present two functional screens of *Salmonella* effector proteins and virulence factors in plants. Our screens resulted in the identification of the phosphothreonine lyase SpvC, which was able to suppress PTI. Using *in vitro* and *in vivo* systems we showed that this effector protein actively interacts with and dephosphorylates activated *Arabidopsis* Mitogen-activated Protein Kinase 6 (MPK6). MAPKs are important regulators of the immune response in animals and plants and the dephosphorylation of MPK6 hinders the induction of defense-related genes in *Arabidopsis*. Moreover, we showed that bacterial fitness on *Arabidopsis* plants is compromised in mutants lacking the *SpvC* gene. These results strengthen the notion that some *Salmonella* effectors may be equally applied in plant and animal systems to suppress the respective host immune systems.

MATERIALS AND METHODS

PLANT GROWTH CONDITIONS

Arabidopsis thaliana Colombia-0 (N60000) plants were cultivated on soil under stable climate conditions: 8 h light/16 h dark at 20°C, 40–60% humidity, ∼120 μE m-2 s-1 light intensity. Leaves from 4-week old plants were used for protoplast preparation and analysis of transient gene expression. Alternatively *Arabidopsis* seedlings were germinated on sterile half-strength MS agar medium and cultivated for 2 weeks in short-day conditions (at 21°C, 60% humidity) in growing chambers. *Nicotiana benthamiana* plants were germinated and cultivated on soil, in a greenhouse under long-day conditions (16 h light at 22°C, 40–60% humidity) for 4 weeks.

CLONING OF *SALMONELLA* VIRULENCE FACTORS AND SPI-DEPENDENT EFFECTOR PROTEINS

Fifty-four *Salmonella* virulence genes, which when mutated caused the attenuation of virulence in the mouse model, and genes coding 18 SPI-1- or SPI-2-encoded effectors from *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain 14028s (*S.* Typhimurium) were cloned into the versatile Gateway (Invitrogen) vector system. All open reading frames (ORFs) were constructed in two versions: one including the native stop codon: the STOP version and a second without the stop codon: the END version. Cloning was based on the ATOME cloning strategy (http://urgv.evry.inra.fr/ATOMEdb). The consequential entry clones were sequenced and those with correct ORFs were used for further studies. For the screen in *Arabidopsis* protoplasts, the ORFs were further recombined into p2GW7 (VIB, University of Ghent). *SpvC* was additionally cloned into p2FGW7 (VIB, University of Ghent) for expression of the N-terminal GFP fusion protein GFP-SpvC.

BACTERIAL MUTAGENESIS

The *SpvC* mutant Δ*spvC* of the *S.* Typhimurium 14028 strain was obtained using the λ-Red mutagenesis system as described by Datsenko and Wanner (2000). The sequences of the primers used were: 5′ATGCCCATAAATAGGCC

TAATCTAAATCTAAACATCCCTCCTTTGAATATGTGTAGGCT GGAGCTGCTTC3′ and 5′TTACTCTGTCATCAAACGATAAAAC GGTTCCTCACGTAAAGCCTGTCTCTCATATGAATATCCTCCT TAG3′.

AGROBACTERIUM-MEDIATED TRANSFORMATION

The Gateway compatible pGreen derivative vectors pJC005 and pJC001 for expression of 10xMyc- or 3xHA-tagged recombinant proteins, respectively, carrying *Salmonella* ORFs, were transformed into the *Agrobacterium tumefaciens* strain GV3101, pMP90. Transformed bacteria were cultivated until stationary phase, washed in infiltration medium (10 mM $MgCl_2$, 10 mM MES-KOH, pH 5, 4, 200 μM acetosyringone) and incubated for 2 h in the dark. OD_{600} of the infiltration solution was then adjusted to 0.3. Leaves of *N. benthamiana* were infiltrated one-sided.

PROTOPLAST TRANSFORMATION

The preparation of *Arabidopsis* mesophyll protoplasts was performed according to the protocol from Yoo et al. (2007) with minor changes (Fraiture et al., 2014). Briefly, thin leaf stripes were dipped into 1.5% cellulose "Onozuka" R10—0.4% macerozyme R10 solution (Yakult Pharmaceutical Industry), vacuum-infiltrated for 30 min and digested for 3 h at 20°C in the dark. After two subsequent washing steps with W5 buffer *Arabidopsis* protoplasts were suspended to a concentration of 2×10^5 cells/ml in MMG buffer and subjected to polyethylene glycol-mediated transfection. 100 μg plasmid DNA/ml protoplast suspension was used during transfection. Protoplasts samples were then incubated in W1 buffer at 20°C in the dark for 12–16 h allowing plasmid gene expression.

LUCIFERASE REPORTER GENE ASSAYS

Luciferase gene assays were conducted to screen for immunity-suppressing effects of effector proteins from *Salmonella* (Fraiture et al., 2014). For this, *Arabidopsis* protoplasts were co-transfected with *pFRK1-Luciferase* (*pFRK1-Luc*) and a candidate effector gene in p2GW7 (or empty p2FGW7 serving as GFP control). For the assay, luciferin was added to 600 μl transfected protoplast solution to a final concentration of 200 μM. Protoplasts were transferred to an opaque 96-well plate (100 μl per well). For each sample, flg22 was added to 3 wells to a final concentration of 500 nM. The remaining 3 replicates were left untreated. The luminescence reflecting the luciferase activity was measured at different time-points using a Berthold Mithras LB 940 luminometer.

RNA ISOLATION AND QUANTITATIVE REAL-TIME PCR

Total RNA from 400 μl protoplast solution was extracted with TRI reagent (Ambion) and treated with DNase I (Macherey-Nagel) following the suppliers' protocols. Poly A-tailed RNA (1 μg) was converted to cDNA using the RevertAid reverse transcriptase (Fermentas) and oligo-dT primers. qRT-PCR reactions were performed in triplicates with the Maxtra SYBR Green Master Mix (Fermentas) and run on a Biorad iCycler according to the manufacturer's instructions. The primers used for the qRT-PCR are presented in Supplementary Table S1. Relative gene expression was determined with a serial cDNA dilution standard curve. The actin transcript was used as an internal control in all experiments. Data was processed with the iQ software (Biorad) (Zheng et al., 2014).

IMMUNOBLOT ANALYSIS

To monitor the activation of MAPKs, *Salmonella* effector-gene transformed protoplasts were challenged with 500 nM flg22 (Zheng et al., 2014). Pellets from 100 μl protoplast solution were collected 0, 15, and 30 min after treatment and dissolved in denaturating protein loading buffer. Proteins were separated by SDS-PAGE, blotted onto nitrocellulose membranes (Hybond–ECL, Amersham) and stained with 0.1% Ponceau S to visualize equal sample loading. The membranes were incubated with anti-phospho-p44/42 MAPK antibody (Cell Signaling Technology) diluted 1/1000 in 5% BSA TBS-T. The expression of GFP-tagged *Salmonella* virulence proteins and effectors was assessed in *Arabidopsis* protoplasts collected 24 h after transformation using an anti-GFP antibody. The immunoblot was revealed in NBT/BCIP detection solution.

PROTEIN PURIFICATION

Recombinant GST-SpvC and 6xHis-SpvC proteins were produced in *E. coli* BL21 bacteria using the pDEST15 and pDEST17 vectors (Invitrogen). Protein expression was induced with 1 mM IPTG overnight at 30°C. Cells were lysed and protein purified accordingly to the manufacturers' protocols (Macherey-Nagel for GTH-beads and Qiagen for Ni-beads purifications).

PULL-DOWN ASSAY

For the pull-down assay 50 μg of purified recombinant proteins were incubated with 50 μg of total *Arabidopsis* protein extract in the presence (or absence) of 80 μg BSA for 30 min in a final volume of 200 μl at 21°C together with the corresponding beads. Beads were washed 3 times and Ni- or GTH- binding complexes separated by SDS-PAGE. Anti-MPK6, anti-MPK3, or anti-MPK4 antibodies (Sigma-Aldrich) were used to visualize the binding between SpvC and MAPKs.

BiFC

Bimolecular fluorescence complementation (BiFC) assay was performed using the full-length versions of *MAPKs* and *SpvC* cloned down-stream of N-terminal or C-terminal part of gene coding for the *Yellow Fluorescent Protein* (*YFP*) in both combinations, using pBIFC1-4 vectors. *Arabidopsis* epidermal cells were co-transformed with vectors carrying those constructs and vector carrying *p35S-mCherry*. Fluorescence was observed 24–48 h after transformation. Expression of *mCherry* was used as readout for successful transformation. Reconstitution of functional YFP was observed with the 510–540 nm band pass filter on a Leica SP2 confocal laser-scanning microscope.

IN VITRO DEPHOSPHORYLATION ASSAY

The phosphatase activity of SpvC on activated MAPKs was assessed using 25 μg purified recombinant GST-SpvC or 6xHis-SpvC proteins and 50 μg of total protein extract from *Arabidopsis* seedlings treated or not (control) with 1 μM flg22 for 15 min. Recombinant effector proteins and *Arabidopsis* proteins were co-incubated for 30 min at 21°C. Samples were precipitated using

a chloroform/methanol procedure and separated by SDS-PAGE. The presence of the phosphorylated pTEpY epitope was probed with anti-pERK1/2 antibody (see above).

PATHOGENICITY ASSAY

To assess the *Salmonella* proliferation rate in plants, soil-grown, 4-week old *Arabidopsis* Col-0 plants were infiltrated with wild-type *Salmonella enterica* subsp. *enterica* ser. Typhimurium strain 14028s or its isogenic mutant Δ*spvC*, using syringe infiltration. Bacteria were grown in LB medium until early log phase, washed and re-suspended in 10 mM $MgCl_2$. Infiltration solution was adjusted to $OD_{600} = 0.01$ (1.7×10^6 bacteria/ml). Bacterial population was monitored during 4 days post-infiltration as described in Schikora et al. (2008).

INCOMPATIBLE INTERACTION

To test the breach of non-host resistance, leaves from soil-grown *Arabidopsis* plants were transformed with *p35S-GFP-SpvC* or *mCherry* via particle bombardment and inoculated with *Blumeria graminis f. sp. hordei* (*Bgh*) conidia. After 48 h, leaves were stained with calcofluor to visualize fungal growth. The outcome of interaction was counted on cells transformed either with *mCherry* (control) or plasmid carrying *GFP-SpvC*.

RESULTS

A DUAL SCREEN FOR *SALMONELLA* VIRULENCE FACTORS AND EFFECTOR PROTEINS ACTIVE IN PLANT CELLS

To identify the important factors for *Salmonella* pathogenicity on plants we decided to follow a two-screening-strategy through a set of *Salmonella* virulence factors (SVFs) and *Salmonella* Pathogenicity Islands (SPIs)-encoded effector proteins. We chose 54 SVF genes, which when mutated caused an attenuation of virulence in the mouse model (PHI-base, www.phi-base.org), and 18 SPI-1- or SPI-2-encoded effectors (Heffron et al., 2011) from *Salmonella enterica* subsp. *enterica* ser. Typhimurium strain 14028s (*S.* Typhimurium) for cloning into the versatile Gateway (Invitrogen) vector system. Cloned genes resulted in a set of *Salmonella* ORFs used for further studies. In a first step, 37 ORFs were successfully cloned into binary vectors for *Agrobacterium*-mediated expression in tobacco (*Nicotiana benthamiana*) leaves. *Salmonella* SVFs and effectors were expressed as N-terminal 10xMyc or 3xHA fusion proteins and symptoms caused by the expression were observed during 5 days after infiltration. We identified eight proteins (SseF, OrgA, Orf4, SsaI, SsaQ, HilC, SicA, and SseG), which caused chlorosis, wilting or hypertrophy on tobacco leaves (**Figure 1**), while the expression of the others, provoked no visible symptoms (**Table 1**).

Next, we tested the potential of the proteins inducing visible changes in tobacco leaves for suppressing early defense responses. Additionally, we tested 5 selected *Salmonella* effectors (AvrA, SptP, SlrP, SseL, SpvC) for which the biochemical function and/or suppression of immunity in mammals has well been characterized (Heffron et al., 2011). We used a protoplast-based system in *Arabidopsis* in which transiently expressed effectors were evaluated for their capability to suppress PAMP-triggered activation of luciferase (Luc) activity. In our screen *Luc* expression was driven by the *FRK1* promoter, which is strongly induced upon treatment with the PAMP flg22, a 22 amino acid long peptide derived from the N-terminal part of flagellin and conserved in many pathogenic bacteria including *Pseudomonas aeruginosa*, *Escherichia coli* and *S.* Typhimurium (Felix et al., 1999). Out of the 13 tested *Salmonella* proteins, a strong suppression of *pFRK1-Luc* activity 6 h after flg22 treatment was observed when co-expressing the SpvC effector protein ($p < 0.01$, one-way ANOVA and Dunnett's test) compared to the GFP control (**Figure 2A** and Supplementary Figure S1). The suppression effect was comparable ($p > 0.05$) with AvrPto from *Pseudomonas syringe*, an effector that is known to interfere with early PAMP signaling (He et al., 2006). In addition, significant suppression ($p < 0.01$) was observed when expressing *SseL*, *SseG,* and *SseF* (**Figure 2A**). In order to confirm our observations we performed a time-course experiment, in which we analyzed *pFRK1-Luc* activity in *SpvC*-transformed protoplasts during 8 h after induction with flg22 (**Figure 2B**). SpvC and AvrPto have similar effects on the activity of *pFRK1* suggesting that SpvC may, similarly to AvrPto, affect PAMP signaling at an early stage (4 h or earlier). Comparable observations were made when the fusion protein Green Fluorescent Protein-SpvC (GFP-SpvC) protein was expressed (**Figure 2A**). The localization analysis performed with GFP-SpvC fusion protein indicated that the effector protein localizes to the cytoplasm and nucleus when present in plant cells (**Figure 2C**). In the following experiments we decided to focus on SpvC, because of its well-known inhibitory effect on immunity during animal infection (Mazurkiewicz et al., 2008).

SALMONELLA SpvC EFFECTOR SUPPRESSES THE EXPRESSION OF PAMP-INDUCED GENES

In a next step, we analyzed the effect of SpvC on the activity of the endogenous *FRK1* promoter. To this end, we measured the expression of *FRK1* in *Arabidopsis* protoplasts transformed with *SpvC*, *AvrPto,* or *GFP* after 1 and 3 h challenge with flg22. Equally to previous experiments and in accordance with the literature (Asai et al., 2002), expression of *FRK1* was induced upon treatment with flg22. Expression of *AvrPto* efficiently suppressed this induction in *Arabidopsis* protoplasts (He et al., 2006). Likewise, SpvC also abolished the induction of endogenous *FRK1* expression after flg22 treatment (**Figure 3**). We extended our analysis to other PAMP-induced genes. Similar to *FRK1*, the transcription factor *WRKY17* and the gene encoding for the protein transport protein Sec61 were induced upon flg22 treatment and hindered in their inductions by AvrPto and SpvC (**Figure 3**). Remarkably, SpvC did not suppress all tested PAMP-induced genes. The *4CL* gene encoding a 4-coumarate-CoA ligase was induced after flg22 treatment and repressed in the presence of AvrPto. However, in contrast to AvrPto, SpvC was not able to restrain its flg22-driven induction (**Figure 3**). These results suggest that the *Salmonella* effector SpvC interferes only with a subset of flg22-induced defense related genes (*FRK1*, *WRKY17,* and *Sec61*, but not *4CL*).

INTERACTION BETWEEN *ARABIDOPSIS* MPK6 AND *SALMONELLA* SpvC

Inhibition of flg22-induced gene expression by bacterial effectors can occur at many levels from the flg22 receptor complex

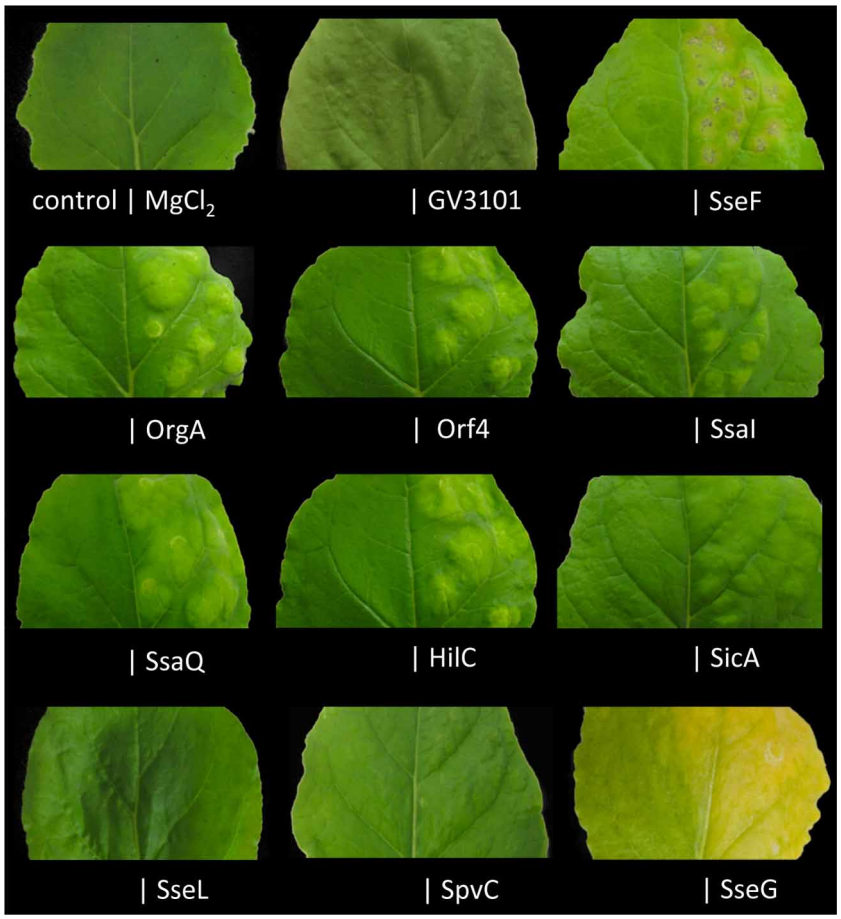

FIGURE 1 | Screen for *Salmonella* proteins that induce HR-like symptoms in plants. SVF and effector genes were cloned into plant expression vectors and expressed as HA- and Myc-tagged versions in *N. benthamiana* leaves *via Agrobacterium*-mediated transformation. Symptoms were observed 5 days after infiltration. Expression of eight proteins resulted in macroscopic changes in leaf morphology when compared to the non-transformed parts of the leaf (control), transformation with *Agrobacterium* GV3101 or infiltration with 10 mM $MgCl_2$. The experiment was repeated 4 times with both versions of bacterial fusion protein. Only the right side of the leaf was infiltrated.

(FLS2-BAK1), through the MAPK cascade, down to transcriptional regulation of defense genes. MAPK cascades play a key role in flg22 signal transduction and in pathogen defense. Among the 20 *Arabidopsis* MAPKs, MPK3, MPK4, and MPK6 are strongly activated by flg22 (Asai et al., 2002; Pitzschke et al., 2009). Based on the functional characteristics of SpvC during animal infection as well as the function of other members of the OspF family [e.g., HopAI1 (Zhang et al., 2007)], we hypothesized that SpvC targets plant MAPKs. To test our hypothesis, we analyzed possible protein-protein interactions between SpvC and *Arabidopsis* MAPKs. Recombinant 6xHis-SpvC and GST-SpvC proteins were expressed and purified from *E. coli* BL21 cells. The recombinant proteins were subsequently co-incubated with total protein extract from *Arabidopsis* seedlings and either Ni- or GTH-coated beads were used to precipitate the respective Ni- or GTH-binding complexes. Pull-down samples were probed for the presence of MAPKs in immunoblot assays. In the presence of His-tagged, but not GST-tagged SpvC, we detected the MPK6 in the pulled-down protein complex (**Figure 4A**), suggesting the interaction between SpvC and MPK6. This interaction was observed even in the presence of an excess of BSA. However, we did not detect MPK3 or MPK4, indicating a specific interaction between SpvC and MPK6.

The *in vitro* SpvC-MPK6 interaction was tested also in bimolecular fluorescent complementation (BiFC) assays. Full-length cDNAs of *SpvC* and the three *MAPKs* were cloned downstream of sequences encoding either the N- or C-terminal part of the Yellow Fluorescent Protein (YFP) and subsequently transiently expressed in *Arabidopsis* epidermal cells via particle bombardment. Both tested combinations: (i) *YFPn-MPK6* with *YFPc-SpvC* and (ii) *YFPn-SpvC* with *YFPc-MPK6*, when expressed together, resulted in reconstitution of a functional YFP protein (**Figure 4B**). We co-expressed the constructs with *p35S-mCherry* plasmid, allowing normalization of the interaction events (**Figure 4C**). Eighteen percent of all transformed cells showed visible interaction between SpvC and MPK6 when *YFPn-MPK6* was co-expressed with *YFPc-SpvC*, and 34% of all cells when *YFPn-SpvC*, and *YFPc-MPK6* were used as interaction

Table 1 | Salmonella ORFs cloned for screens in plants.

Gene	Tobacco assay		Protoplast assay
	10xMyc	3xHA	
***Salmonella* VIRULENCE FACTORS**			
ssrB	–	–	
ssaB	–	–	
sseE	–	–	
sseF	HR	HR	PTI suppression
sifA	–	–	
sirA	–	–	
orgA	Hypertrophy	Hypertrophy	–
orf2	–	–	
ttrB	–	–	
ssaM	–	–	
orf2	–	–	
orf4	Hypertrophy	Hypertrophy	–
ssaE	–	–	
sseD	–	–	
sscB	–	–	
ssaI	Hypertrophy	Hypertrophy	–
ssaJ	–	–	
ssaK	–	–	
ssaM	–	–	
ssaQ	Hypertrophy	Hypertrophy	–
yscR	–	–	
ssaS	–	–	
ssaT	–	–	
hilC	Hypertrophy	Hypertrophy	–
prgK	–	–	
prgJ	–	–	
iagB	–	–	
sicA	Hypertrophy	Hypertrophy	Weak PTI suppression
invI	–	–	
invE	–	–	
spvR	–	–	
***Salmonella* T3SS-1 AND T3SS-2 DEPENDENT EFFECTORS**			
avrA	–	–	–
sptP	–	–	–
slrP	–	–	–
sseL	–	–	PTI suppression
spvC	–	–	PTI suppression
sseG	Yellowing	Yellowing	PTI suppression

Symptoms observed on tobacco leaves 5 days after infiltration with Agrobacterium tumefaciens (tobacco assay) or suppression of the pFRK1-Luc activity in protoplasts challenged with flg22 (protoplast assay). HR, hypersensitive response; –, represents no difference to controls.

partners. *Arabidopsis* MPK6 localizes to the cytoplasm and nucleus, but accumulates in the nuclear compartment after activation (Bethke et al., 2009). The observed cytoplasmic and nuclear localization of SpvC-MPK6 complex had similar localization. Moreover, this localization overlapped with the localization of the GFP-tagged versions of SpvC in epidermal cells (**Figure 2C**). Similarly to the *in vitro* assay, we did not observe an interaction between SpvC and the other MAPKs (MPK3 and MPK4) (**Figure 4B**). Hence, these results indicate that SpvC interacts with *Arabidopsis* MPK6.

ACTIVATED MAPKs ARE DEPHOSPHORYLATED BY SpvC

By monitoring *in vitro* the phosphorylation status of MAPKs after activation with flg22 in the presence of SpvC, we tested the assumption that SpvC might dephosphorylate the double phosphorylated active forms of MAPKs. The recombinant proteins 6xHis-SpvC and GST-SpvC were expressed in *E. coli* BL21 cells and purified with the respective affinity chromatography (Ni-sepharose or GTH-agarose columns, respectively). *Arabidopsis* MAPKs were activated by challenge of intact seedlings with flg22. Total proteins from those seedlings were incubated with recombinant SpvC protein (**Figure 5A**). The activation of the MAP kinases can be efficiently detected by means of an anti-pERK1/2 antibody that recognizes the phosphorylated T and Y residues in the activation loop (pTEpY) of MAPKs (Hamel et al., 2005). In **Figure 5**, the upper panel presents the phosphorylation status of MPK6 (upper band) and MPK3 (lower band) as detected by means of the anti-pERK1/2 antibody (αpERK1/2). Twenty minutes after treatment of *Arabidopsis* seedlings with flg22, the detected signals indicated active, phosphorylated MAPKs. It should be noted that the 30 min incubation time, which is necessary to carry out this assay, did not affect the phosphorylation on the TEY epitope. When 6xHis-SpvC or GST-SpvC proteins were added to the *Arabidopsis* protein extract, the phosphorylated pTEpY epitope of the MAPK was no longer detectable (**Figure 5A**, αpERK1/2 blot). This result suggests that SpvC is able to dephosphorylate activated plant MAPKs *in vitro*.

In the next step we sought to verify the result in an *in vivo* system. To achieve this aim, we expressed *SpvC* as native or GFP-tagged protein in *Arabidopsis* protoplasts, and subsequently assessed the phosphorylation status of MAPKs after flg22 treatment. As controls we transformed the protoplasts with *GFP* or the effector *AvrPto*, which is known to inhibit MPK6 phosphorylation (He et al., 2006). In *GFP*-expressing protoplasts, flg22-triggered transient activation of MAPKs was peaking after 15 min (**Figure 5B**). In contrast, protoplasts expressing *AvrPto*, *SpvC* as well as *GFP-SpvC* showed complete inhibition of MAPK activity (**Figures 5B,C**). These results are in line with the dephosphorylation activity of SpvC observed *in vitro*, as well as the suppressing effect on defense gene activation. Moreover, they support the idea that SpvC interferes with plant defense signaling upstream or at the level of the MAPKs.

EXPRESSION OF SpvC BREACHES THE NON-HOST RESISTANCE IN *ARABIDOPSIS*

MAPKs are key components of immune signaling in plants. Accordingly, we assumed that manipulation and inactivation of MAPKs by SpvC might affect plant resistance. To verify this, we analyzed the resistance of epidermal cells transformed with *SpvC* toward the fungal pathogen *Blumeria graminis f. sp. hordei* (*Bgh*). *Arabidopsis* is a non-host for *Bgh* and copes easily with this fungus either by papillae formation or by hypersensitive response (HR) at the infection site. *SpvC* was transiently expressed in *Arabidopsis* epidermal cells under the control of the constitutive 35S promoter

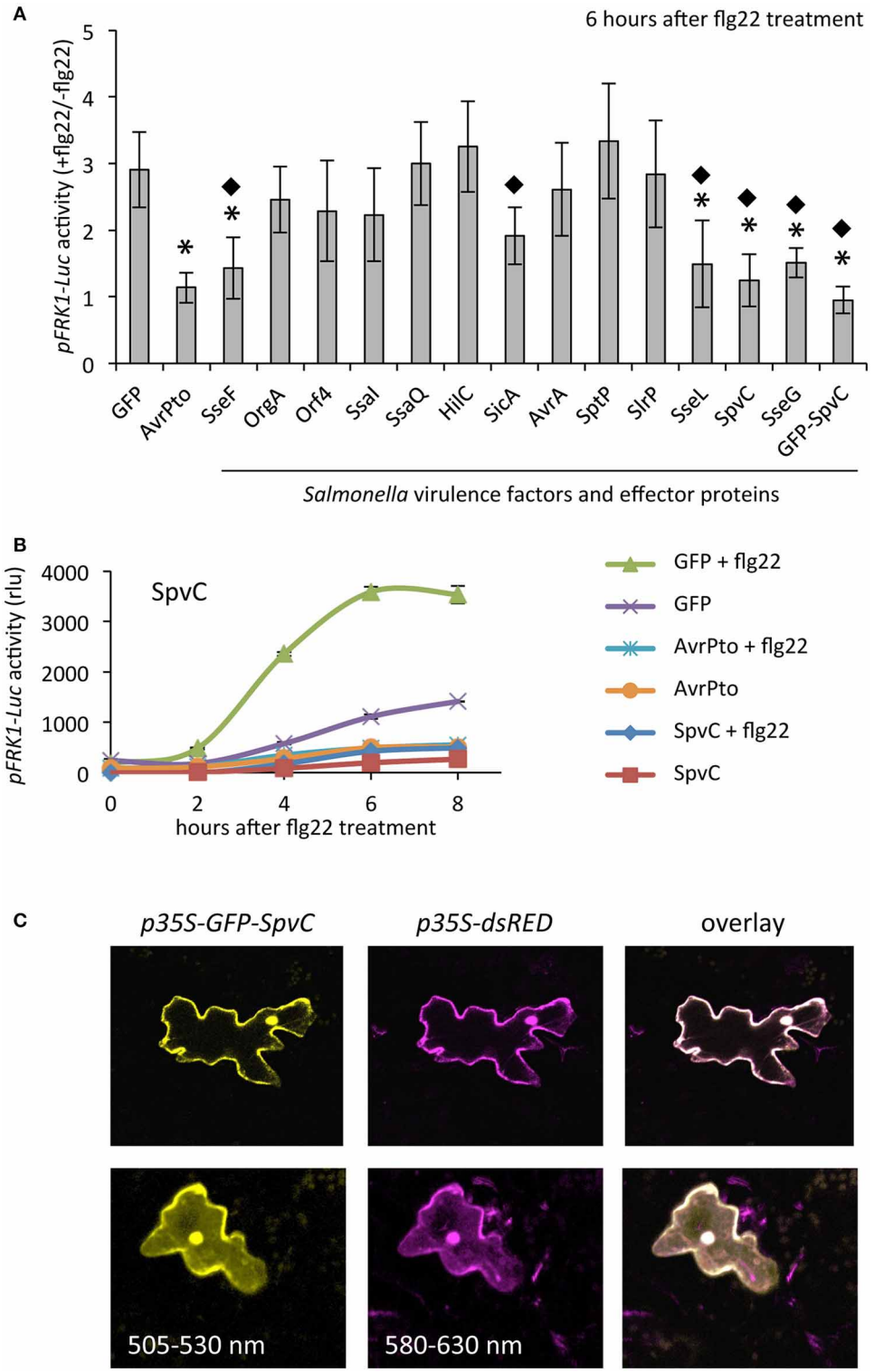

FIGURE 2 | Suppression of flg22-induced *pFRK1-Luc* expression by *Salmonella* proteins. (A) Mesophyll protoplasts from *Arabidopsis thaliana* Col-0 were co-transformed with pFRK1-Luc and p35S-Salmonella-ORF plasmids. Co-transformations of pFRK1-Luc with p2FGW7 (GFP) and with p2GW7-AvrPto (AvrPto) plasmids served as controls. Protoplasts were subsequently treated with flg22 or left untreated. The ability to suppress the flg22-driven activation of *pFRK1-Luc* of chosen virulence factors and effectors was assessed 6 h later by measuring luciferase (Luc) activity. Results are presented as ratio between flg22-treated and non-treated samples (+flg22/−flg22). For each effector, at least four independent experiments with three technical replicates were carried out. All data were pooled. Mean values ± SD are plotted. One-way ANOVA followed by Dunnett's multiple comparison test was performed to assess significant differences between

(Continued)

FIGURE 2 | Continued
the GFP control and the virulence factor- and effector- protein-producing samples. An asterisk marks data sets with $p < 0.01$. The same test was performed to assess the difference between *Salmonella* effectors and AvrPto. A diamond represents those proteins, which have similar effects to AvrPto at $p > 0.05$. **(B)** Representative time-course experiment of flg22-mediated *pFRK1-Luc* activity in *Arabidopsis* protoplasts expressing *GFP*, *AvrPto* or *SpvC*. Luciferase activity was measured every 2 h for 8 h after flg22 challenge. The data represents mean values ± SD from three technical replicates. rlu; relative light units. **(C)** Localization study of a GFP-SpvC fusion protein produced under the 35S promoter in *Arabidopsis* leaves transformed via particle bombardment. Cytoplasmic and nuclear localized dsRED protein was used as a control. Images present two exemplary cells (lower and upper panels, respectively) expressing the GFP-SpvC fusion protein.

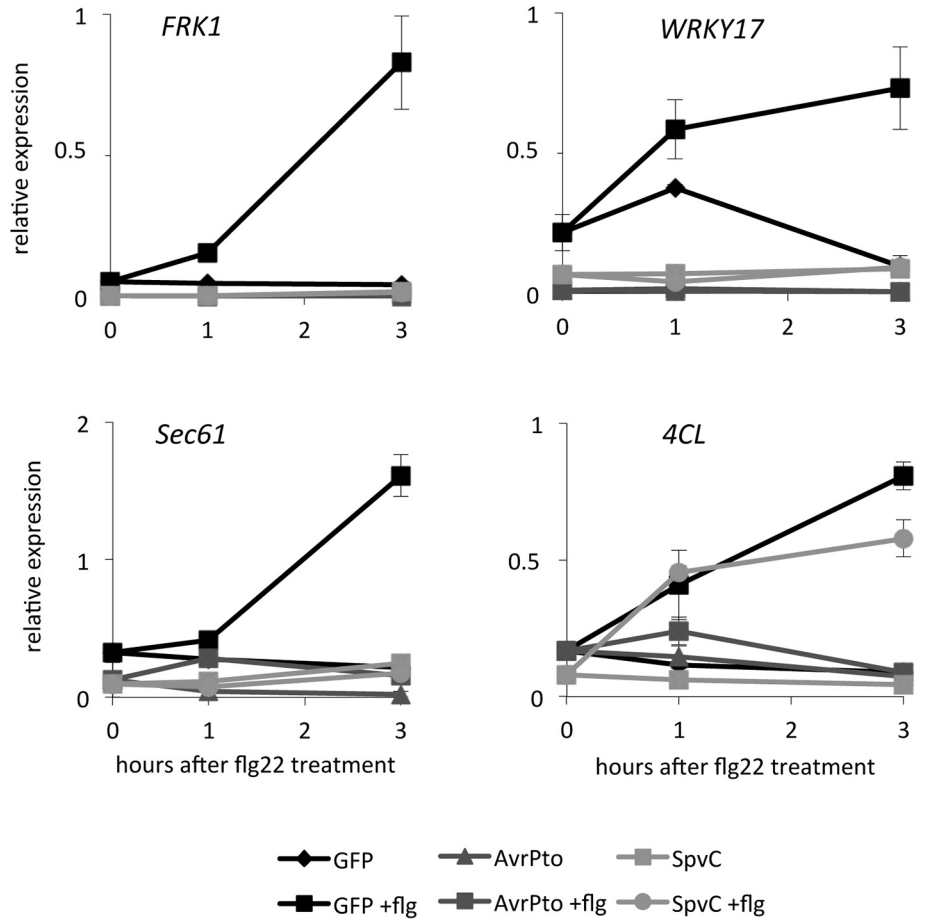

FIGURE 3 | SpvC attenuates flg22-induced defense responses in protoplasts. *Arabidopsis* mesophyll protoplasts were transformed with p2FGW7 (GFP), p2GW7-AvrPto (AvrPto) or p2GW7-SpvC (SpvC) plasmids and subsequently challenged with flg22. Samples were collected 1 and 3 h after treatment. Relative expression levels of *FRK1*, *WRKY17*, *Sec61*, and *4CL* were assessed using quantitative RT-PCR and normalized to the expression of the house-keeping gene *actin*. The graphs show one representative experiment out of three. Data is presented as mean values ± s.e.m. of three technical replicates. Expression of *FRK1*, *WRKY17*, and *Sec61* was attenuated in protoplasts in the presence of SpvC. However, SpvC had no impact on the expression of *4CL*.

as a GFP-tagged version (*GFP-SpvC*) and transformed leaves were inoculated with *Bgh* conidia. On control-transformed (*mCherry*) cells about 48% of *Bgh* conidia germinated 24 h after inoculation, though all of the germinated conidia died or did not develop any further in the following 24 h (**Figure 6A**). In contrast, in cells expressing *GFP-SpvC* the percentage of germinated conidia increased to 66% and the later developed into secondary hyphae was observed in 11% of the transformed cells (**Figure 6A**). These results suggest that *Bgh* successfully penetrated into part of the epidermal cells that expressed *GFP-SpvC*. We conclude that the efficient defense mechanism against *Bgh* is at least partially compromised when SpvC is present in the cell, most likely due to its effect on MAPKs and the subsequent inhibition of PTI.

SpvC IS REQUIRED FOR FULL VIRULENCE OF *SALMONELLA* TOWARD PLANTS

The Δ*spvC* mutant is characterized by attenuated virulence in the mouse model (Mazurkiewicz et al., 2008) and SpvC is thought to play a crucial role in systemic bacteremia in humans [reviewed in Guiney and Fierer, 2011]. To assess the question whether

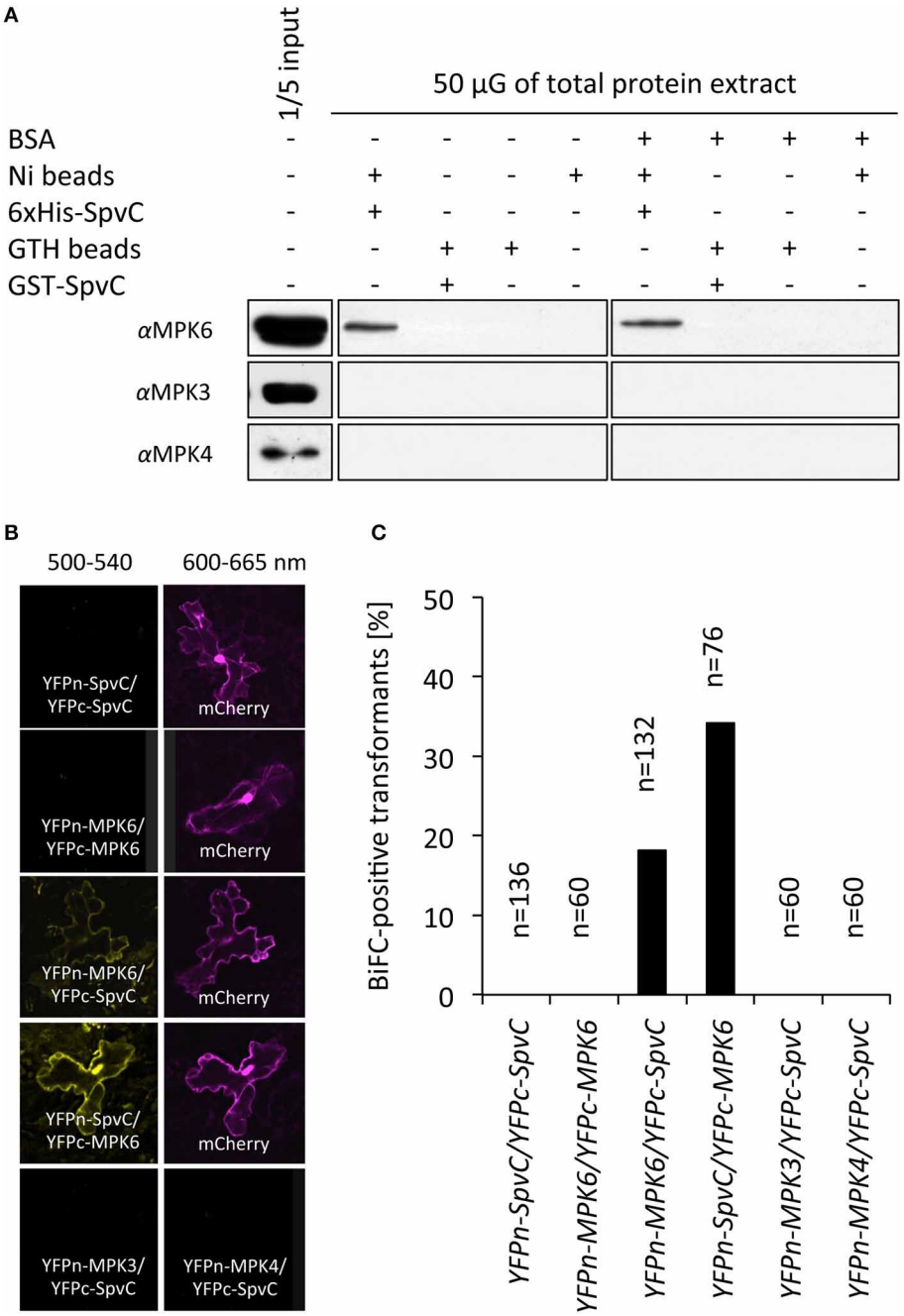

FIGURE 4 | Arabidopsis MPK6 interacts with the SpvC effector. (A) In a pull-down assay, recombinant 6xHis-SpvC or GST-SpvC proteins were co-incubated with total protein extract from *Arabidopsis* seedlings and Ni- or GTH-coated beads, respectively, as indicated by "+". Anti-MPK6, anti-MPK3, and anti-MPK4-specific antibodies were used to visualize the presence of the respective kinases in Ni- or GTH-binding complexes in an immunoblot analysis. Only MPK6 was detected, indicating a specific binding between MPK6 and 6xHis-SpvC, however not GST-SpvC, proteins. **(B)** Bimolecular fluorescence complementation (BiFC) assay was performed with full-length versions of *MAPKs* and *SpvC* cloned down-stream of a portion of the *Yellow Fluorescent Protein* (*YFP*) gene encoding the N-terminal or C-terminal part of YFP in all four possible combinations. *Arabidopsis* epidermal cells were co-transformed with vectors carrying those constructs and vector carrying *35S-mCherry* via particle bombardment. Fluorescence was observed 48 h after transformation. **(C)** Quantification of the interaction between SpvC and MAPKs as percentage of the transformed cells. mCherry-positive cells from four independent experiments were counted. The diagram represents the percentage of YFP-positive cells among transformed cells.

SpvC plays a significant role during proliferation in plants, we tested the performance of the Δ*spvC* mutant on *Arabidopsis* plants. The Δ*spvC* mutant was constructed by replacing the *SpvC* gene with a chloramphenicol resistance cassette in the wild-type *S.* Typhimurium strain 14028s (Datsenko and Wanner, 2000). Six-week old, soil-grown *Arabidopsis* plants were syringe-infiltrated and the bacterial populations were monitored during 4 days. The wild-type *S.* Typhimurium 14028s strain reached about

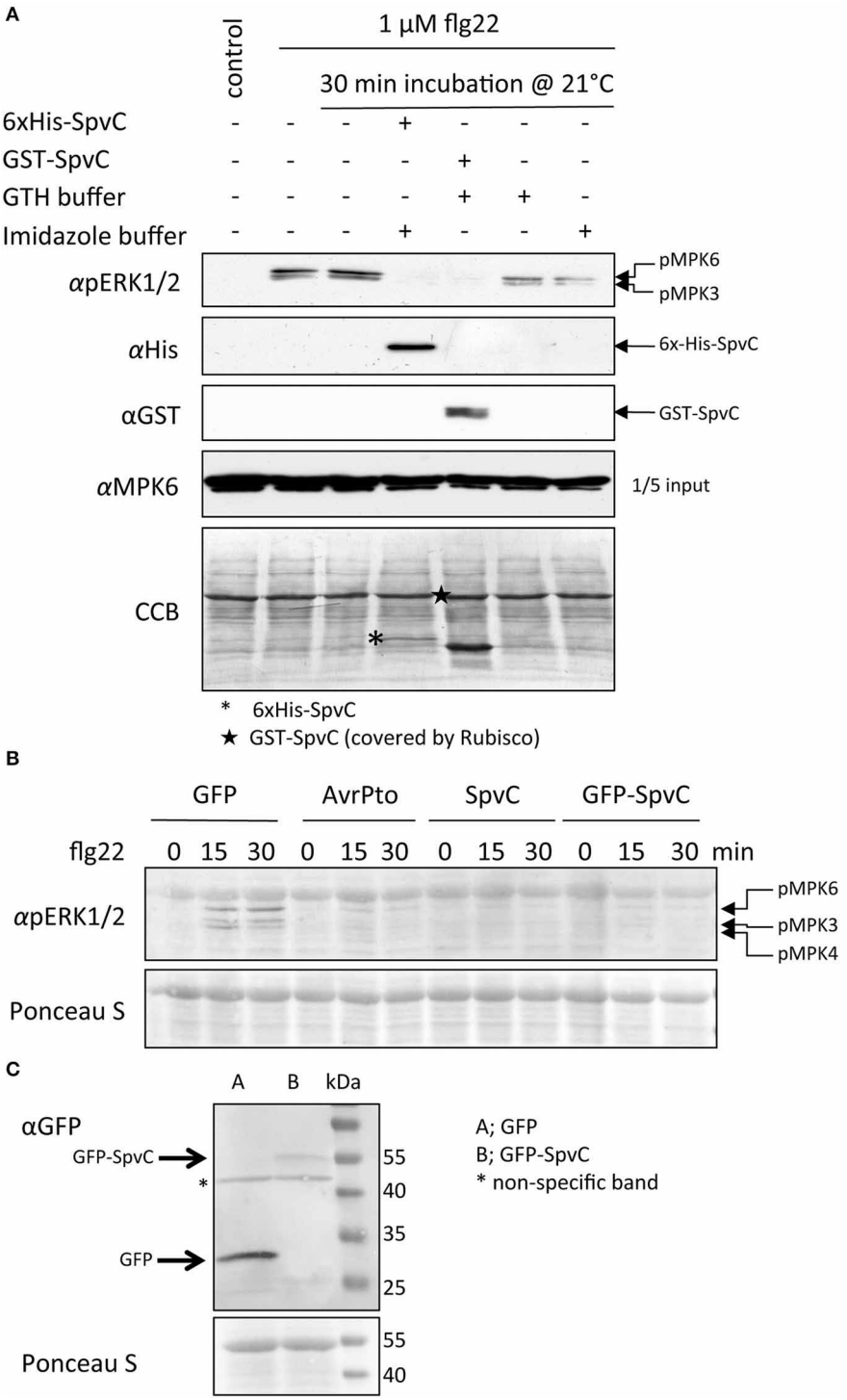

FIGURE 5 | Active MAPKs are dephosphorylated by SpvC. (A) MAPKs were activated by treatment of 2-week old seedlings with flg22 for 20 min prior to the extraction of total soluble proteins. Extracted proteins were then incubated for 30 min with purified recombinant 6xHis-SpvC or GST-SpvC proteins. The phosphorylation status of *Arabidopsis* MAPKs was analyzed by immunoblotting using an antibody raised against the phosphorylated form of EKR1/2 (αpERK1/2). The recombinant proteins were probed with anti-His and anti-GST antibodies (αHis and αGST, respectively). Specific anti-MPK6 (αMPK6) antibody was used to assess the presence of MPK6. CCB stain was used to monitor the equal sample loading. **(B)** *Arabidopsis* protoplasts were transformed with p2FGW7 (GFP), p2GW7-AvrPto (AvrPto) or p2GW7-SpvC (SpvC) and subsequently treated with flg22 for 0, 15 or 30 min. The phosphorylation status of MAPKs was assessed with the αpERK1/2 antibody. Treatment with flg22 caused phosphorylation of MPK6 and MPK3 as visible by the appearing bands at 15 and 30 min after treatment. However, signals are missing in protoplasts expressing *AvrPto* or *SpvC*. **(C)** Expression and stability of GFP-SpvC fusion protein in *Arabidopsis* protoplasts. Ponceau S staining was used to show equal sample loading.

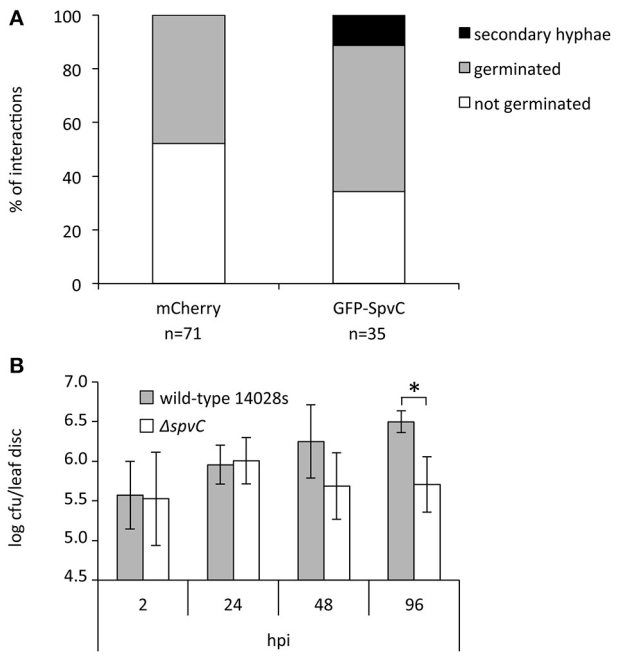

FIGURE 6 | Expression of SpvC breaches the non-host resistance in *Arabidopsis* to powdery mildew fungus and its lack renders *Salmonella* bacteria less virulent toward plants. (A) Leaves from soil-grown *Arabidopsis* plants were co-transformed with p35S-GFP-SpvC and p35S-mCherry plasmids or transformed with p35S-mCherry plasmid alone, and inoculated with *Blumeria graminis f. sp. hordei* (*Bgh*) conidia. 48 h after inoculation, leaves were stained with calcofluor to visualize fungal growth. The outcome was counted on cells transformed with mCherry or GFP-SpvC. Three types of interaction were observed: non-germination, germination without further development, and formation of secondary hyphae. The experiment was repeated three times with similar results. **(B)** Proliferation of the Δ*spvC* mutant *in planta* was tested on 4-week old *Arabidopsis* plants, syringe-infiltrated with bacterial solutions. The data represents mean values ± SD from five biological replicates; * represents $p < 0.05$ in Student's *t*-test, hpi; hours post-infection.

10^7 colony-forming units (cfu) in a leaf disc. In contrast to the wild-type, the Δ*spvC* mutant showed a decreased ability to proliferate in *Arabidopsis* (**Figure 6B**), suggesting that SpvC plays a important role for *Salmonella* when present in a plant host.

DISCUSSION

In this report we performed a functional screen of *Salmonella* effector proteins and virulence factors in plants. We demonstrated that the function of the *Salmonella* effector protein SpvC is conserved in hosts originating from different kingdoms. In analogy to the infection in the animal system, SpvC interacts with plant MAPKs and dephosphorylates their active form, thus attenuating defense mechanisms. The presence of *SpvC* in *Arabidopsis* cells repressed the induction of several defense-related genes and breached the non-host resistance toward *B. graminis*. Moreover, the mutant lacking *SpvC* was less virulent on *Arabidopsis* plants when compared to the wild-type strain *S.* Typhimurium 14028s.

Among known *Salmonella* effectors, some are encoded on plasmids within a shared common locus called salmonella plasmid virulence (*spv*) (Boyd and Hartl, 1998). The *spv* operon is absolutely required for the development of a lethal systemic infection in the mouse model (Montenegro et al., 1991; Fierer et al., 1992; Gulig and Doyle, 1993). The expression of the *spv* operon (encoding five proteins: SpvR, A, B, C, and D) is strongly induced in intracellular bacteria and is regulated by the positive transcriptional regulator SpvR and the sigma factor RpoS (Fang et al., 1991; Krause et al., 1992). SpvC is a phosphothreonine lyase that dephosphorylates the double phosphorylated pTXpY activation loop in the kinases ERK1/2, as well as in p38 and probably JNK (Li et al., 2007; Mazurkiewicz et al., 2008; Haneda et al., 2012). In consequence, SpvC blocks the pro-inflammatory function of the MAPK pathway, facilitating the cell-to-cell spread of bacteria. In contrast to the dual-specificity phosphatases, which cleave the C-P bond, SpvC cleaves the C-O bond, promoting the formation of β-methyldehydroalanine, which cannot be re-phosphorylated. The enzymatic activity of SpvC is common to the OspF family, named after the first characterized effector protein OspF from *Shigella flexneri* (Arbibe et al., 2007; Li et al., 2007; Smith et al., 2009). Interestingly, also plant pathogens possess members of the OspF family. HopAI1 from *Pseudomonas syringe* is a close homolog to OspF/SpvC, and has similarly to the *Salmonella* protein, a phosphothreonine lyase activity. Previously, HopAI1 has been shown to dephosphorylate activated MPK3 and MPK6 in *Arabidopsis* plants (Zhang et al., 2007). Recently, MPK4 was also shown to be targeted and dephosphorylated by HopAI1 (Zhang et al., 2012).

Here, we demonstrate that SpvC dephosphorylates three activated MAPKs (MPK3, MPK4, and MPK6) in *Arabidopsis*. In both performed tests, the presence of SpvC caused loss of the phosphorylated pTEpY epitope on the MAPKs. On the one hand, the assumption that the biochemical action (cleavage of C-O bond) of SpvC on active plant MAPKs is similar to its action on pERK1/2 is very tempting, remains however to be verified. On the other hand, the dephosphorylation of MAPK3/4/6 by SpvC is clearly coupled to the attenuation of the plant defense responses. When present in *Arabidopsis* protoplasts, SpvC hinders the expression of several defense-associated genes. It also lowers the resistance of *Arabidopsis* cells against the biotrophic, non-host pathogen *Bgh*, a phenomenon observed in defense-compromised mutants [reviewed in Lipka et al., 2008)]. Similarly to the situation in animal cells, where SpvC blocks the pro-inflammatory pathway and therefore the actual defense response, inhibition of MAPKs in plants seems to block the otherwise efficient defense strategy. How specific the particular MAPKs are targeted by SpvC could not be answered. The dephosphorylation assays clearly showed the possibility to dephosphorylate MPK3, MPK4, and MPK6, a situation similar to HopAI1 (Zhang et al., 2012). Nevertheless, in contrast to MPK6, interaction of SpvC with MPK3 or MPK4 could be verified neither in BiFC nor in pull-down assays, which indicates a high affinity of SpvC toward MPK6 or that interaction with MPK3 or MPK4 requires yet other components.

During animal infection, SpvC induces late macrophage apoptosis. However, no cell death-inducing activity could be detected in plants. In contrast to macrophage apoptosis used by *Salmonella*

to facilitate the cell-to-cell spread in animal organism, cellular death in plants (hypersensitive response; HR) is very often a defense mechanism induced by recognition of pathogen effector proteins by the plant intracellular R proteins. Despite the fact that SpvC is a T3SS-translocated effector in mammalian cells, the described above screen in tobacco leaves suggests that SpvC does not induce the hallmark of effector-triggered immunity (ETI) in plants, the HR, implying that SpvC is not recognized by R protein(s). We also exclude the possibility that SpvC is recognized by surface located receptors by testing its PAMP activity. Growth inhibition and production of reactive oxygen species, both hallmarks of pattern-triggered immunity (PTI), were studied in plants after contact with SpvC (Supplementary Figure S2). Our results suggest that SpvC is not toxic for plant cells when externally present and that plants do not recognize SpvC by potential surface receptor(s).

As described above, the intracellular presence of SpvC attenuated the activation of MPK3/4/6 and expression of several defense-related genes. Whether, besides inhibition of those two aspects of plant defense, SpvC actively suppresses the HR response remains to be verified in future experiments. Furthermore, the translocation of *Salmonella* effector proteins into plant cytoplasm was not yet demonstrated. The function of SpvC requires its presence in the host cytoplasm, therefore a direct evidence of translocation of this effector (or/and others) needs to be provided in future work, as this would certainly help to understand how these bacteria suppress plant immune responses. Interesting was the observation that expression of other *Salmonella* effectors *in planta* induced visible changes. SseF and SseG, both SPI-2 encoded effector proteins involved in the trafficking of *Salmonella* Containing Vacuole (SCV) in animal cells, induced HR-like (SseF) or yellowing (SseG) symptoms in tobacco leaves, when expressed via *Agrobacterium*-mediated transformation. It confirms the observation made by Ustun et al. (2012), who showed that SseF from *S. enterica* triggers HR-like symptoms in tobacco plants when expressed transiently via *Agrobacterium* infiltration or delivered via the T3SS from *Xanthomonas campestris pv. vesicatoria*. Moreover, the ability of SseF to trigger HR-like symptoms was lost upon silencing of *SGT1* (suppressor of G2 allele of skp1), which is required for HR induction in tobacco. These results indicate that *Salmonella* SseF is recognized in *N. benthamiana via* an R protein-mediated mechanism and triggers ETI in consequence. Surprisingly, expression of *SptP* or *SlrP*, both postulated to be key effectors of *Salmonella* with the highest number of predicted protein-protein interactions (Schleker et al., 2012), induced no visible symptoms in tobacco leaves nor had an effect on the induction of *pFRK1-Luc* in *Arabidopsis* protoplasts.

In summary, an increasing number of evidence indicates that plants evolved diverse mechanisms to recognize *Salmonella* bacteria using surface receptors as well as intracellular R proteins. Our study supports the view that *Salmonella* also evolved means to interfere with plant immunity by efficiently employing its repertoire of effector proteins to succumb plant immune responses. Consequently, *Salmonella*, and possibly other human pathogenic bacteria, seems to possess effective tools for suppression of the plant immune system.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00548/abstract

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Interactions of *Salmonella* with animals and plants

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Salmonella enterica species are Gram-negative bacteria, which are responsible for a wide range of food- and water-borne diseases in both humans and animals, thereby posing a major threat to public health. Recently, there has been an increasing number of reports, linking *Salmonella* contaminated raw vegetables and fruits with food poisoning. Many studies have shown that an essential feature of the pathogenicity of *Salmonella* is its capacity to cross a number of barriers requiring invasion of a large variety of cells and that the extent of internalization may be influenced by numerous factors. However, it is poorly understood how *Salmonella* successfully infects hosts as diversified as animals or plants. The aim of this review is to describe the different stages required for *Salmonella* interaction with its hosts: (i) attachment to host surfaces; (ii) entry processes; (iii) multiplication; (iv) suppression of host defense mechanisms; and to point out similarities and differences between animal and plant infections.

Keywords: *Salmonella* infections, adhesion, invasion mechanisms, multiplication, host defense strategies

INTRODUCTION

The genus *Salmonella* consists of only two species, *S. bongori* and *S. enterica*, and the latter is divided into six subspecies: *enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *indica*. *S. enterica* subsp. *enterica* includes more than 1,500 serotypes, which despite their high genetic similarity vary greatly in their host range and disease outcome ranging from enteritis to typhoid fever (Ohl and Miller, 2001). *Salmonella enterica* subsp. *enterica* is an important economic and public health problem throughout the world.

The degree of adaptation to hosts varies between *Salmonella* serotypes and determines the pathogenicity. Serotypes adapted to humans, such as *S.* Typhi and *S.* Paratyphi A, B, C, cause systemic typhoid fever. These serotypes are not pathogenic for animals. Similarly, *S.* Gallinarum and *S.* Abortusovis, which are specifically adapted to poultry and ovine, respectively, are responsible for severe systemic infections in these animals. However, *S.* Choleraesuis, for which pigs are the primary hosts, also causes severe systemic illness in humans. Ubiquitous serotypes, such as *S.* Enteritidis or *S.* Typhimurium, generally cause gastrointestinal infections in humans but can induce other diseases in animals (Hoelzer et al., 2011). For example, they can produce typhoid-like infections in mice, systemic infection in humans or asymptomatic intestinal colonization in chickens and pigs (Velge et al., 2012). Some of them are responsible for chlorosis on plant leaves sometimes causing death (Klerks et al., 2007b; Schikora et al., 2008, 2011; Gu et al., 2013b).

Disease in mammals occurs after ingestion of contaminated food or water. *Salmonella* infection of animals and humans depends on the ability of bacteria to survive the harsh conditions of the gastric tract before entering the intestinal epithelium and subsequently colonizing the mesenteric lymph nodes and internal organs in the case of systemic infections. In order to enter non-phagocytic cells and survive within the host environment, *Salmonella* has evolved mechanisms to interact with host cells and to induce its own internalization (Vazquez-Torres et al., 1999; Rosselin et al., 2012).

Salmonella usually enters agricultural environments via animal feces. Animals can directly contaminate plants or surface water used for irrigation and pesticide or fertilizer diluent through contaminated feces. Recently, there has been an increasing number of reports, linking *Salmonella* contaminated raw vegetables and fruits with food poisoning (Heaton and Jones, 2008). *Salmonella* is able to adapt to different external conditions including low pH or high temperature, allowing it to survive outside the host organism (Samelis et al., 2003; Semenov et al., 2007). Indeed, *Salmonella* is able to attach and adhere to plant surfaces before actively infecting the interior of different plants, leading to colonization of plant organs (Klerks et al., 2007a; Gu et al., 2011), and suppression of the plant immune system (Schikora et al., 2012). In addition, *Salmonella* originating from plants retains virulence toward animals (Schikora et al., 2011). Thus, plants are an alternative host for *Salmonella* pathogens, and have a role in its transmission back to animals.

Currently it is poorly understood how *Salmonella* successfully infects hosts as diversified as humans, animals, or plants. Here, our current understanding of the strategies used by *Salmonella* to colonize mammals and plants will be summarized. The gap in our knowledge about the differences in host colonization between animals and plants will be discussed.

COLONIZATION

Salmonella infection requires different stages: attachment and adhesion to host surfaces, and production of bacterial factors, which facilitate invasion, initial multiplication, and ability to overcome or bypass host defense mechanisms.

ADHESION TO HOST SURFACES

One of the first crucial events in successful colonization by *Salmonella* is adhesion to tissues. Two steps can be distinguished in the adhesion process: an initial adhesion that is reversible followed by a tight attachment which depends on bacterial factors and that is irreversible (Cevallos-Cevallos et al., 2012). This first contact is decisive whatever the host infected. However, this step is not exactly the same in animals and plants. In animals, bacterial adhesion occurs when *Salmonella* interacts with eukaryotic cells prior to invasion or when bacteria initiate biofilm formation on host surfaces, such as the intestinal epithelium or gallstones. In contrast, to date bacterial adhesion has been described only at the plant surface level and not at the plant cell level. Nevertheless, as in animals, biofilm formation on plant tissue has been observed to play an important role in plant colonization by *Salmonella* often in association with other plant pathogens as described in Section "The Different Multiplication Areas of *Salmonella*." To strongly adhere to surfaces, *Salmonella* serotypes use several surface components depending on the surface to which they will attach. The different adhesive structures of *Salmonella*, their host receptor when known and the current knowledge about their role in the interaction of *Salmonella* with animals and plants are described below.

Fimbrial structures

Fimbriae are proteinaceous surface appendages of 0.5–10 μm in length and 2–8 nm in width (**Figure 1**), which have, at their distal part, a protein which interacts with its host receptor thus mediating the adhesion of the bacteria to the host or inert surfaces. So far, more than 10 fimbrial operons have been identified in *Salmonella* genomes and the number and types of fimbrial operons depends on the serotype (van Asten and van Dijk, 2005). Horizontal gene transfer and deletion events have created unique combinations of fimbrial operons among *Salmonella* serotypes (Baumler et al., 1997; Townsend et al., 2001). The combination of adhesins used by each serotype affects its ability to adhere to different cell types and therefore contributes to the ability of this serotype to colonize different niches or hosts. Thirteen fimbrial operons have been identified in *S.* Typhimurium: *agf* (also called *csg*), *fim*, *pef*, *lpf*, *bcf*, *saf*, *stb*, *stc*, *std*, *stf*, *sth*, *sti*, and *stj*. Until now, studies of fimbriae have been slowed down by the fact that only one of them, the Type I fimbriae (also called Fim fimbriae or SEF21), is expressed in commonly used laboratory culture conditions. This can in part be related to a post-transcriptional control of other fimbrial gene expression via the 5′untranslated region of the *fimAICDH* transcript or to a negative control of their expression as observed for the *std* operon that is repressed by Dam, SeqA, HdfR, and RosE (Chessa et al., 2008a; Sterzenbach et al., 2013). There is, however, evidence that these adhesive structures can be expressed *in vivo*. Indeed, BcfA, FimA, LpfA, PefA, StbA, StcA, StdA, StfA, and StiA have been shown to be expressed after inoculation of bovine ileal loops with *S.* Typhimurium. Moreover, antibodies against the same fimbrial proteins and also against AgfA and SthA have been observed after inoculation of mice with *S.* Typhimurium (Humphries et al., 2003, 2005).

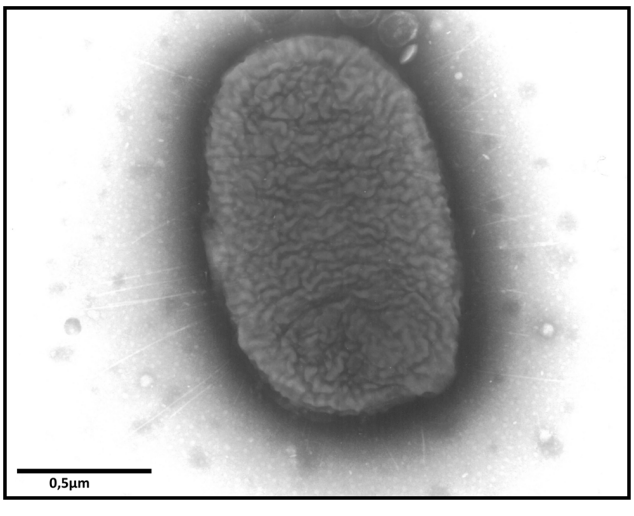

FIGURE 1 | Transmission electron microscopy image showing fimbriae of *S.* Enteritidis after culture on Sven Gard plates. Bar represents 0.5 μm.

Due to the difficulties encountered to study fimbriae, their respective cell receptor and targeted cell types in their animal hosts are known for only a few of them. Type I fimbriae are characterized by hemagglutination, yeast agglutination, and binding to eukaryotic cells expressing the α-D-mannose receptor (Korhonen et al., 1980). Long polar fimbriae mediate the adhesion of *S.* Typhimurium to murine Peyer patches while Pef fimbriae, whose binding carbohydrate is the Lewis X blood group antigen, are involved in adhesion to murine villous small intestine (Baumler et al., 1996; Chessa et al., 2008b). Std fimbriae bind terminal Fucα1-2 moieties present in the mucus layer of the murine caecum mucosa or on the surface of cells such as Caco-2 cell line (Chessa et al., 2009) and thin aggregative fimbriae (also called Tafi or Curli), encoded by *agf* operon, interact with the extracellular matrix glycoproteins. While the interactions of fimbriae with animal cells are not well characterized, several studies have shown that fimbriae are involved in the colonization of different animals. Type I fimbriae contribute to mouse, pig, and chick intestinal colonization (Dibb-Fuller and Woodward, 2000; Althouse et al., 2003). *lpf*, *bcf*, *stb*, *stc*, *std*, and *sth* encoded fimbriae lead to the long-term persistence of *S.* Typhimurium in resistant mice ($Nramp^{+/+}$) (Weening et al., 2005; Lawley et al., 2006). In chicks, *pef*, *std*, *sth*, *sef*, and *agf*-encoded fimbriae are also involved in spleen and intestinal colonization by *S.* Typhimurium and *S.* Gallinarum (Morgan et al., 2004; Shah et al., 2005). Usually, the absence of expression of only one fimbriae type does not greatly reduce *Salmonella* virulence. However, multiple mutations have a greater impact. For example, in *Salmonella* susceptible mice ($Nramp^{-/-}$), a *S.* Typhimurium strain where the three *pef*, *lpf* and *agf* operons are deleted, has a 29-fold higher 50% lethal dose (LD_{50}) and is less able to colonize the intestine than the wild-type strain or than strains with a single mutation after oral inoculation, thus highlighting the synergistic action of fimbriae to colonize the intestine (van der Velden et al., 1998).

Some fimbriae also contribute to biofilm formation in animals and plants, particularly curli fimbriae. These fimbriae are required for biofilm formation on epithelial cells and chicken

intestinal surfaces and favor the attachment and the persistence of the biofilm-associated *Salmonella* on alfalfa sprouts, parsley, and tomato leaflets (Barak et al., 2005, 2007; Ledeboer et al., 2006; Jonas et al., 2007; Lapidot and Yaron, 2009; Cevallos-Cevallos et al., 2012). They also promote survival of *Salmonella* inside plants (Gu et al., 2011). A role of Pef and Lpf fimbriae has also been observed in biofilm formation on animal surfaces. As curli fimbriae, Pef fimbriae have been shown to be required for biofilm formation on inert, epithelial cells and chicken intestinal surfaces, while Lpf fimbriae appear to be more involved in biofilm formation on chicken intestinal tissue than on plastic or tissue culture cells (Ledeboer et al., 2006; Jonas et al., 2007). In addition to the curli fimbriae involved in plant colonization, fimbriae encoded by the *stf* operon have been shown to increase the persistence of *S.* Typhimurium on intact but not on damaged lettuce leaves after cold storage but this seems not to be related to an attachment defect on leaf tissue (Kroupitski et al., 2013).

Non-fimbrial adhesins

Two types of non-fimbrial adhesins have been described in *Salmonella* according to their secretion pathway: BapA and SiiE are each secreted by a Type-1 secretion system, while ShdA, MisL, and SadA are autotransporters also known as Type-V secretion systems.

BapA (386 kDa) and SiiE (595 kDa) are the largest proteins of *Salmonella* and share the characteristics of having numerous bacterial Immunoglobulin-like domains. The genes encoding these two proteins are highly conserved among *Salmonella* serotypes (Biswas et al., 2011; Suez et al., 2013). BapA has been shown to be involved in biofilm formation in *S.* Enteritidis. Its expression is co-regulated with the two other essential components of *Salmonella* biofilms, i.e., thin aggregative fimbriae and cellulose, by the central transcriptional regulator AgfD (Latasa et al., 2005). In mice, BapA is involved in the first steps of the infectious process as a *bapA S.* Enteritidis mutant was less able to colonize mice ileal loops than the wild-type strain and was shown to be less virulent for mice than its parent only when orally inoculated (Latasa et al., 2005). The role of BapA in *S.* Typhimurium is less clear (Latasa et al., 2005; Jonas et al., 2007). In plants, no studies have been performed, but the role of BapA in biofilm formation supports a possible role of this protein in *Salmonella*/plant interactions.

SiiE is an adhesin encoded by *Salmonella* Pathogenicity Island-4. The *siiABCDEF* operon encodes the adhesin and the proteins required for the biosynthesis of its Type-I secretion system. The SiiE protein mediates the initial adhesion of *Salmonella* to the apical side of polarized epithelial cells via multiple interactions with glycostructures with terminal *N*-acetyl-glucosamine and/or α 2,3-linked sialic acid. This SiiE-mediated adhesion is required for subsequent Type III-secretion-system-1 (T3SS-1) invasion of these cells (detailed in Section "T3SS-1 Dependent Mechanism"; Gerlach et al., 2008; Wagner et al., 2014). In line with the cooperation of SiiE and the T3SS-1, the *siiABCDEF* operon is co-regulated with the *Salmonella* Pathogenicity Island-1 (SPI-1) genes involved in T3SS-1 biosynthesis (Gerlach et al., 2007b; Main-Hester et al., 2008). Contrary to BapA, SiiE has not been shown to contribute to biofilm formation even if a role of SPI-4 in the virulence of *Salmonella* has been observed in animals (Latasa et al., 2005). Indeed, mutants in the *siiABCDEF* operon, including a *siiE* mutant, were attenuated for colonization of mice after oral but not after intraperitoneal infection compared to their wild-type parents (Morgan et al., 2004; Kiss et al., 2007). However, no attenuation was observed by Gerlach et al. (2007a) in a similar model. The role of this giant adhesin in plant colonization remains to be determined.

The ShdA adhesin is a monomeric fibronectin and collagen-I binding protein that is encoded by *shdA* carried on the CS54 island (Kingsley et al., 2004). This gene is present in *Salmonella* serotypes isolated from human and warm-blooded animals but not from cold-blooded animals (Kingsley et al., 2000). ShdA was shown to mediate adhesion to the epithelium of the murine caecum (Kingsley et al., 2002) and to contribute to the colonization of this organ and of the Peyer's patches of the terminal ileum of mice. A *shdA* mutant also had a reduced persistence in the cecum and a fecal shedding defect in this animal model but not in a pig model (Kingsley and Baumler, 2000; Kingsley et al., 2003; Boyen et al., 2006). No data on the role of ShdA in plant colonization is available.

The MisL adhesin, encoded by a gene within SPI-3, shares several characteristics with ShdA. MisL is a monomeric adhesin that is not expressed under standard *in vitro* cultures but its expression can be induced by the transcriptional regulator MarT encoded on SPI-3 (Blanc-Potard et al., 1999; Tükel et al., 2007). In addition, as ShdA, MisL binds fibronectin and is involved in the colonization of the cecum and in the persistence of *S.* Typhimurium in mice after oral inoculation (Dorsey et al., 2005). A *misL* mutant has also been shown to be altered in the intestinal colonization of chicks and in the attachment to lettuce leaves (Morgan et al., 2004; Kroupitski et al., 2013). The latter phenotype could be related to a reduced ability of the mutant to form biofilms on inert surfaces (Kroupitski et al., 2013).

Little is known about the trimeric SadA adhesin. Contrary to ShdA and MisL, this protein is expressed under *in vitro* standard growth cultures and is surface-exposed on *S.* Typhimurium. Its expression on bacterial cells deprived of *O*-antigen mediates autoaggregation and biofilm formation on inert surfaces. Moreover, SadA has been shown to increase the adherence and invasion of an *Escherichia coli* strain lacking smooth lipopolysaccharide (LPS) into human intestinal Caco-2 cells. However, no binding with extracellular matrix molecules collagen I, collagen III, collagen IV, elastin, fibronectin, and laminin has been observed and no role of SadA in the virulence of *S.* Typhimurium in mice and in *C. elegans* models has been demonstrated (Raghunathan et al., 2011).

Other structures

Flagella and LPS are bacterial factors whose main function is not to mediate adhesion. Flagella confer motility and chemotaxis and stimulate the host innate immune response (Vijay-Kumar and Gewirtz, 2009). LPS is a major component of the outer membrane of most Gram-negative bacteria, and protects them from toxic compounds, such as antibiotics or bile salts. LPS is composed of three parts: the lipid A, which is embedded in the

bacterial membrane, the core oligosaccharide, and the most external moiety, the *O*-antigen. It is also an endotoxin responsible for septic shock in animal hosts and, as flagella it stimulates the innate immune response (Tan and Kagan, 2014). However, several papers describe a role of these structures in the adhesion of *Salmonella* to animal or plant tissues. For flagella, the reduced adhesion of *Salmonella* described in some papers in animal models is related to a defect in the motility function conferred by flagella (Jones et al., 1981; Khoramian-Falsafi et al., 1990). This could be explained by the fact that a strain with reduced motility is less likely to enter in contact with its target host cells/tissues and consequently has a reduced attachment/entry rate into cells. However, in other papers, flagella, *per se*, were shown to be involved in adhesion, as mutants in flagellar structure proteins were shown to be impaired in adhesion to chick gut explants and in biofilm formation on cholesterol-coated surfaces, unlike paralyzed mutants (Allen-Vercoe and Woodward, 1999; Crawford et al., 2010). In plants, a role of flagella in the adhesion to basil and lettuce leaves has also been reported (Berger et al., 2009; Kroupitski et al., 2009). In addition, it is important to note that two open reading frames involved in swarming motility are also involved in plant colonization (Barak et al., 2009).

A few papers describe a role of the LPS in the adhesion of some *Salmonella* serotypes. Indeed, *S.* Choleraesuis and *S.* Typhi, rough mutants, i.e., with an *O*-antigen defect, were altered in the attachment and invasion of polarized epithelial monolayers of Madin Darby canine kidney (MDCK) cells and HeLa cell monolayers, respectively (Finlay et al., 1988; Mroczenski-Wildey et al., 1989). However, the absence of *O*-antigen expression was shown to have the opposite effect in *S.* Typhimurium and *S.* Enteritidis serotypes (Kihlstrom and Edebo, 1976; Baloda et al., 1988). In the latter case, the strongest ability of rough mutants to adhere to eukaryotic cells was suggested to be related to the highest hydrophobicity properties of these mutants compared to their wild-type parents, thus allowing hydrophobic interactions between the bacterial and the host cell membranes. In plants, the *O*-antigen capsule was shown to be involved in the colonization of alfalfa sprouts, while colonic acid, another extracellular polysaccharide, was not. Indeed, a mutant defective in the assembly and translocation of the *O*-antigen capsule had a reduced ability to adhere to alfalfa sprouts (Barak et al., 2007). However, *O*-antigen capsule production did not confer a selective advantage to *S.* Typhimurium for red ripe tomatoe colonization (Noel et al., 2010).

As mentioned above, biofilm formation is an important property for *Salmonella* adhesion to plants. In line with this, cellulose, which is the main exopolysaccharide of the biofilm matrix, is involved in the adhesion and colonization to/of lettuce and parsley leaves and alfalfa sprouts (Barak et al., 2007; Lapidot and Yaron, 2009; Kroupitski et al., 2013).

Most *Salmonella* adhesive structures are expressed only *in vivo* thus rendering difficult their study. Even if the constitutive expression of these surface components and the study of the regulation of their expression have promoted *in vitro* studies in the last few years, much work is still required to understand the role of each of them and their potential cooperation and/or redundancy in mediating *Salmonella* interaction with their hosts.

INVASION

In animals, *Salmonella* has developed different mechanisms to induce its own internalization in different cell types in order to survive, multiply, and spread through the host (Rosselin et al., 2012). Until recently, it was assumed that *Salmonella* could enter cells using its T3SS-1(Ibarra and Steele-Mortimer, 2009). However, recent research has shown that *Salmonella* infection may occur independently of the T3SS-1 (Rosselin et al., 2011). While the internalization of *Salmonella* is demonstrated in animal cells, the presence of *Salmonella* inside plant cells remains controversial.

Salmonella have been found inside different plant tissues and even in the seeds inside fruits (Klerks et al., 2007a; Schikora et al., 2008; Gu et al., 2011). In addition, it has been shown that *Salmonella* is able to move within plants (Gu et al., 2011, 2013a,b). Several leaf structures have been postulated as the possible entry sites of *S.* Typhimurium (Kroupitski et al., 2009; Barak et al., 2011; Golberg et al., 2011). One report suggests that the trichomes are preferential colonization sites (Iniguez et al., 2005). However, Kroupitski et al. (2009) have shown that the preferential sites for *Salmonella* entry are the stomata, a natural opening on the leaf surface. Moreover, it has been postulated that this process depends on flagella. In addition, light seems to be required for *Salmonella* to move toward stomatal openings, because an artificial opening of the stomata in the dark had no effect on *Salmonella* internalization. Whether *Salmonella* is able to enter plant cells is still controversial. However, two laboratories observed intracellular localization of *Salmonella*: *S.* Typhimurium bacteria were observed inside rhizodermal cells of *Arabidopsis thaliana* and were shown to enter protoplasts of *Nicotiana tabacum* cells *in vitro*, although at a relatively low level (Samelis et al., 2003; Schikora et al., 2008). In addition, *S.* Typhimurium has been recovered from both lettuce leaves and surface-sterilized parsley leaves, supporting the hypothesis that *Salmonella* is able to invade the inner layers of leaf tissue (Franz et al., 2007; Kisluk and Yaron, 2012). However, in the latter case, the bacterial localization in plant cells was not demonstrated and requires more study.

T3SS-1 dependent mechanism

The SPI-1 island encodes structural components of the secretory machinery, chaperones, regulators, and some effectors involved during mammalian host invasion. When *Salmonella* reaches the intestinal environment, the SPI-1 genes are expressed, allowing assembly of the T3SS-1 at the bacterial surface (Kubori et al., 1998). After an interaction between the host cell and the bacteria the T3SS-1 translocates into host cells at least 15 proteins encoded within the SPI-1, SPI-5 pathogenicity islands, and prophages (Garner et al., 2002; Hayward et al., 2005; McGhie et al., 2009). Among these effectors, SopE, SopE2, SopB, SipA, SipC, and SptP have been shown to be required for cell invasion by *Salmonella*. The synergistic activity of SopE, SopE2, SopB, SipA, and SipC induces actin recruitment and polymerization at the entry site, which results in the formation of "ruffles" at the membrane surface (**Figure 2**; McGhie et al., 2009). These ruffles extend from the cell surface and internalize the bacteria in the host cell in a vacuole. After ruffle formation, the endocytic vacuole closes and the cellular cytoskeleton of the host cell returns to its initial state, allowing the cell to return to its original morphology

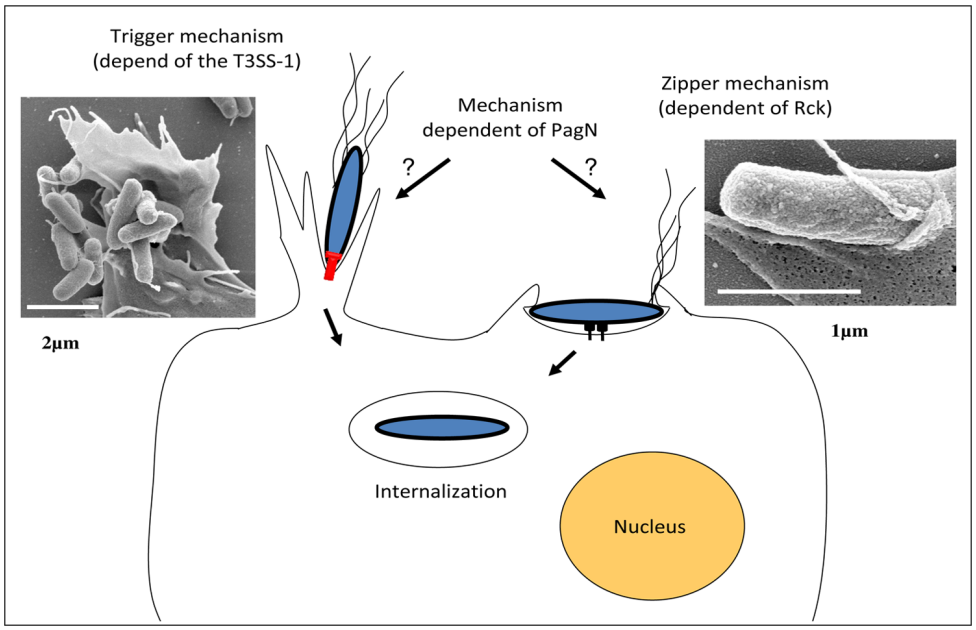

FIGURE 2 | Models of *Salmonella* invasion mechanisms. *Salmonella* uses T3SS-1 to translocate effector proteins directly into host cells (left side; Bar of the transmission electron microscopy image represents 2 µm). Several of these effector proteins modulate host cell actin cytoskeleton, leading to an intense membrane ruffling and internalization of the bacteria into a modified phagosome or *Salmonella*-containing-vacuole (SCV). *Salmonella* can also invade cells via a T3SS-1-independent mechanism, which is induced by the *Salmonella* Rck membrane protein interacting with its receptor on the host cell plasma membrane and characterized by the induction of thin membrane extensions (right side; Bar of the transmission electron microscopy picture represents 1 µm). The membrane rearrangements induced by the *Salmonella* invasin PagN have not been studied yet.

(Fu and Galán, 1999). The effector SptP allows this restoration by reorganizing the actin cytoskeleton (Fu and Galán, 1998, 1999). This T3SS-1 invasion process is referred to as a "Trigger mechanism" and has only been studied in mammalian cells (Velge et al., 2012).

Nevertheless, T3SS-1 contribution to *Salmonella* pathogenesis depends on the model used. In bovine, rabbit, and murine models, the T3SS-1 of *S.* Dublin and *S.* Typhimurium serotypes is essential for intestinal colonization (Wallis and Galyov, 2000). However, some *Salmonella* lacking the T3SS-1 remain pathogenic in different *in vivo* infection models such as a SPI-1 mutant of *S.* Gallinarum in adult chicken (Jones et al., 2001) or *S.* Typhimurium and *S.* Enteritidis mutants in one week-old chicks or Balb/C mice (Coombes et al., 2005; Jones et al., 2007; Karasova et al., 2010). Furthermore, *S.* Seftenberg strains lacking SPI-1 have been isolated from human clinical cases, suggesting that for this serotype, the T3SS-1 is not required to establish infection in humans (Hu et al., 2008).

Interestingly, reducing the virulence of *Salmonella* by removing T3SS-1 increased colonization of alfalfa roots and wheat seedlings (Iniguez et al., 2005). However, these results contrast with the reduced proliferation observed for *prgH* mutants, lacking a functional T3SS-1, in *A. thaliana* (Schikora et al., 2011). In addition, more apparent symptoms in *Arabidopsis* plants are observed with these mutants, suggesting that, in this case, the hypersensitive response (HR) seems to be prevented by the effectors secreted by the T3SS-1 (detailed in Section "Host Defenses"). Overall, this suggests that the T3SS-1-dependent successful colonization seems to be plant-species-specific and that *Salmonella* strains may have different pathogenicity toward plants.

The role of the T3SS-1 in *Salmonella*–plant interactions raises many questions concerning the signals which induce expression of the T3SS-1 in plants and the mechanisms set up by *Salmonella* to deliver effectors.

T3SS-1 independent mechanisms

To date, two *Salmonella* invasins called Rck and PagN have been identified. Moreover, studies have revealed that invasion systems of *Salmonella* are not restricted to the PagN, Rck, and T3SS-1. A *Salmonella* mutant unable to express the T3SS-1, Rck, or PagN was indeed still able to enter different animal cell lines (Rosselin et al., 2012).

Rck invasin. Rck invasin is encoded by the *rck* gene located on the *S.* Enteritidis and *S.* Typhimurium large virulence plasmid (Rotger and Casadesús, 1999). Rck belongs to a family of outer membrane proteins (OMP) associated with virulence functions, including PagC which is involved in *Salmonella* intracellular survival and Ail, a *Yersinia* invasin (Heffernan et al., 1992). The role of Rck in the invasion of *Salmonella* in animal cells has been well described *in vitro* and demonstrated through different methods. Rosselin et al. (2010) have shown that *rck* deletion in *S.* Enteritidis leads to more than a twofold decrease in animal epithelial cell invasion without altering the bacteria attachment to the cells. In addition, it has been shown that Rck alone is able to trigger cell invasion

in a receptor-dependent manner by using Rck-coated latex beads and initially non-invasive *E. coli* strain overexpressing Rck. The minimal region of Rck required to induce invasion corresponds to the G113-V159 peptide. At the cellular level, the interaction of Rck with its receptor expressed on an animal cell membrane leads to a signaling cascade, involving cellular proteins which promote local accumulation of actin and weak and closely adherent membrane extensions. This process is referred to as a "Zipper" mechanism and has only been studied in animal cells (**Figure 2**; Rosselin et al., 2010).

However, in animal *Salmonella* pathogenesis, the role of Rck is still poorly understood. The regulation of Rck regulated by quorum sensing via SdiA, suggests that Rck may play an intestinal role (Ahmer et al., 1998). Dyszel et al. (2010) have reported that Rck confers a selective advantage for intestinal colonization in mice when it is expressed. Moreover, as *rck* is regulated by an unidentified system, which is independent of SdiA at 37 and 42°C (Smith et al., 2008), it is conceivable that Rck has a role which is not only restricted to the gastrointestinal tract and which could be induced in only some animal species.

The role of quorum sensing in *Salmonella* pathogenesis in animals and its impact on Rck expression is still poorly characterized. However, in plants, the quorum sensing which allows plant pathogen colonization of rhizosphere and phyllosphere has been well documented (Daniels et al., 2004; Dulla and Lindow, 2009). A study of the possible role of Rck in plant colonization is ongoing.

PagN invasin. In addition to Rck and the T3SS-1, an OMP called PagN is involved in *Salmonella* animal host invasion (Lambert and Smith, 2008). *PagN* is similar to both the Hek and Tia invasion proteins of *E. coli.* This OMP is encoded by the *pagN* gene, which is located on the centisome 7 genomic island. PagN protein is widely expressed among the different *Salmonella enterica* serotypes (Folkesson et al., 1999). Lambert and Smith (2008) have shown that the deletion of *pagN* in *S.* Typhimurium leads to a significant decrease in animal cell line invasion without altering the bacteria-cell adhesion. In addition, expression of PagN in a non-invasive *E. coli* strain resulted in adhesion to and invasion of animal cell lines. At the cellular level, it was shown that PagN-dependent invasion requires an interaction of PagN with the cell surface heparin sulfate proteoglycans, which could lead to actin polymerization at the entry site (Lambert and Smith, 2008, 2009). However, the membrane proteoglycans are diverse and only a few membrane proteoglycans can transduce a signaling cascade. Another hypothesis is that they could play a role as a co-receptor for invasion and not as a receptor itself.

In a mouse model, it has been shown that PagN is required for *Salmonella* survival (Heithoff et al., 1999) and that spleen colonization of a *pagN* mutant is lower than that of its parental strain (Conner et al., 1998). However, the precise role of PagN in *Salmonella* animal and plant pathogenesis remains unknown. The PhoP/PhoQ two-component regulatory system activates *pagN*, leading to a maximal expression under the conditions found in the intracellular *Salmonella*-containing vacuole (SCV), which is known to downregulate T3SS-1 expression (Conner et al., 1998; Heithoff et al., 1999; Eriksson et al., 2003). Thus, *Salmonella* could express a high level of PagN when the bacteria exit the SCV and the cell, which may facilitate interactions with other cells that the pathogen encounters (Lambert and Smith, 2008). However, the role of PagN in plants remains to be studied.

Non-identified invasion factors. In animals, recent research has shown that invasion factors in *S.* Enteritidis and *S.* Typhimurium are not limited to PagN, Rck, and the T3SS-1. Rosselin et al. (2011) have demonstrated that a strain which does not express the T3SS-1, PagN, or Rck, is still able to significantly invade some animal cells. This idea is reinforced by the study performed by Aiastui et al. (2010) and van Sorge et al. (2011) who showed that a *Salmonella* strain lacking the T3SS-1 which does not express PagN and Rck, was still able to enter different cell types (epithelial, endothelial, and fibroblasts cells). In addition, *S.* Typhimurium invasion studies of a 3-D intestinal epithelium have also supported the idea that *Salmonella* expresses invasion factors, which have not yet been characterized (Radtke et al., 2011).

MULTIPLICATION

Once internalized into the tissue, *S.* Typhimurium is able to multiply. The ability to colonize plants may be an effective survival and multiplication strategy for *Salmonella* as it provides a link between its excretion in the environment via animal feces and the recontamination of herbivorous and omnivorous hosts. Many studies have been conducted on the behavior and multiplication of *Salmonella* in animal hosts, some on plants, especially on the foliage of plants, but very few have been conducted within plant cells (Barak and Liang, 2008). *Salmonella* can also multiply in the rhizosphere (Semenov et al., 2009).

The different multiplication areas of Salmonella

In order to effectively colonize plants, bacteria need to grow and spread. Growth requires bacteria to either synthesize indispensable metabolites or acquire essential nutrients from their environment. *Salmonella* is unable to liberate nutrients from plant cells as plant pathogens do because they lack enzymes to degrade plant cell walls (Teplitski et al., 2009). However, they often grow using nutrients liberated by plant cell lysates and root exudates after action of plant pathogens (Barak and Schroeder, 2012). In this context, *Salmonella* has to adapt to both the plant phyllosphere and rhizosphere, which are heterogeneous environments varying in physical conditions and nutrient availability (Barak and Schroeder, 2012). The leaf surface is, for example, a harsh environment for bacteria due to UV radiation, the heterogeneity of nutrient availability and rapid fluctuations in temperature, and free water availability. However, plant surfaces are not homogenous and contain various microsites that represent oases of available nutrients and which may support multiplication of human pathogens after contamination events (Brandl et al., 2013). Indeed, *Salmonella* has been shown to preferentially move on leaves toward open stomata and colonize the vein areas, the bases of trichomes and damaged leaf areas, which may provide shelter and increase nutrient and water availability (Monier and Lindow, 2005). In addition, inoculation of leaves with *S.* Typhimurium can result in contamination of tomato fruit through internal movement of the bacteria from leaves into the fruit (Gu et al., 2011).

Salmonella appear to be successful secondary colonists, benefiting from the action of phytopathogens, e.g., suppression of plant defenses and plant tissue damage (lesions, water soaking, and soft rots). Numerous studies have shown that soft-rot bacteria promote proliferation of *Salmonella* in plants. Biotrophic plant pathogens, like *P. syringae* and *Xanthomonas campestris*, can promote growth or survival of *Salmonella* and enterohaemorrhagic *E. coli* on plants (Barak and Liang, 2008; Aruscavage et al., 2010; Potnis et al., 2014). Formation of lesions on leaves by both these phytopathogens has been associated with an increase availability of total sugars, specifically, innositol and sucrose (Aruscavage et al., 2010). Moreover, *Salmonella* can benefit from the immune-suppressing action of plant pathogenic bacteria like *Pseudomonas syringae* pv. tomato (Meng et al., 2013) and *Xanthomonas perforans*, which suppress the pathogen-associated molecular pattern (PAMP)-triggered immunity (Potnis et al., 2014). *Salmonella* found in preexisting plant bacteria biofilms was more likely to survive dry conditions on lettuce and cilantro leaves than solitary bacteria (Rastogi et al., 2012). These observations suggest that *Salmonella* may find refuge not only in particular physical microsites on plants but also in microbial conglomerates where protection from adverse conditions outweighs potential competition and antibiosis from other plant colonists. For example, Goudeau et al. (2013) observed that population sizes of *S.* Typhimurium increased 56-fold when inoculated alone onto cilantro leaves, compared to more than 2,800-fold when co-inoculated with *Dickeya dadantii*, a prevalent pathogen that macerates plant tissue. The global gene expression profile of *Salmonella* in soft-rotted tissue showed that there was a lack of competition for nutrients between these two bacterial species due to resource partitioning. Moreover, 29% of the genes that were upregulated in cilantro macerates had also previously been observed to have increased expression levels in the chicken intestine (Goudeau et al., 2013). Commonalities between soft rot lesions and the intestine such as anaerobic conditions and nutritional resources indicate an important overlap in the ecological niche and may explain the adaptation of *Salmonella* to both kingdoms (Goudeau et al., 2013).

The gastrointestinal tract represents a vast mucosal surface vulnerable to attack by enteropathogens. It is fortified with a variety of physical and immunological defense barriers. The colonizing microbiota represents a major protective shield. This dense population is thought to provide both a physical barrier for the attachment of bacterial pathogens to surfaces, and to compete for essential nutrients (Caricilli et al., 2014). The microbiota is also able to produce a nutritional environment unfavorable to growth of bacterial pathogens. This protective mechanism has been termed "colonization resistance" and helps to prevent infection (Van Immerseel et al., 2005). In addition to colonization resistance, the microbiota mediates *S.* Typhimurium clearance from the gut lumen (Endt et al., 2010). However, other reports have shown that *Salmonella* uses ingenious mechanisms to hijack the mucosal inflammation for its own benefit, with detrimental effects for the host and the microbiota (Fabrega and Vila, 2013). For example, using the T3SS virulence factors, *S.* Typhimurium is able to elicit a host inflammatory response, which ultimately helps the pathogen. The intestinal microbiota produces hydrogen sulfide, which normally becomes detoxified to thiosulphate by host cells. The inflammatory response, induced by *Salmonella*, leads to the migration of neutrophils into the intestinal lumen and the subsequent release of reactive oxygen species (ROS). When thiosulphate is exposed to ROS it is oxidized to tetrathionate, which can be used by *S.* Typhimurium as an alternative electron acceptor. Thus the utilization of tetrathionate as a terminal electron acceptor in respiration is a far more efficient process for energy generation than fermentation used by anaerobic microbiota (Winter et al., 2010). This respiratory pathway allows *S.* Typhimurium to use ethanolamine, which does not support growth of intestinal microbiota (Thiennimitr et al., 2011). Thus inflammation leads to a marked boost in *S.* Typhimurium growth.

Similar to the protective role of microbiota in intestinal tract, plants have protective microbial communities. In the rhizosphere, plant growth-promoting bacteria fend off invaders by activating the induced systemic resistance (ISR) response in plants, through the production of antibiotics and competition for nutrients and iron (Pieterse et al., 2014; Sang et al., 2014). Within plants, endophytic bacteria also defend the plant against pathogens. Gu et al. (2013a) have suggested that invasion of tomato plants by *S.* Typhimurium is inversely correlated to the diversity of endophytic bacteria.

Besides the mechanisms of metabolic cooperation or competition between plant or intestine microbiota and *Salmonella*, cell-to-cell signaling in multispecies microbial communities plays an important role in both plants and gut habitats. The contribution of signaling via quorum sensing circuits mediated by either *N*-acyl homoserine lactones (AHL) or the autoinducer-2 (AI-2) to the behavior of *Salmonella* in plant-associated bacterial communities and in animal intestines has already been demonstrated (Ahmer and Gunn, 2011; Brandl et al., 2013). However, the importance of AHL and AI-2-based signaling in *Salmonella* during the interactions of *Salmonella* both with plant and animal bacteria requires further investigation (Thomanek et al., 2013).

Proliferation of *Salmonella* in some plant tissues has been reported to cause disease-like symptoms. In *Arabidopsis*, immersion of seedlings in a dense suspension of *Salmonella* or infiltration of leaves with the pathogen can elicit chlorosis, wilting, or tissue necrosis (Schikora et al., 2008; Berger et al., 2011). The symptoms elicited by *Salmonella* were related to the presence of SPI-1 and SPI-2, which also play a key role in host animal infection (Schikora et al., 2011). Generally, it was believed that *Salmonella* survived on plant tissues after contact with contaminated water or animal manures. However, endophytically present *Salmonella* was observed in the vascular system of *S.* Typhimurium-inoculated tomato leaves (Gu et al., 2011). Moreover, *Salmonella* was observed intracellularly in *A. thaliana* protoplasts and in cultured tobacco cells (Schikora et al., 2008; Shirron and Yaron, 2011). However, very little is known on the intracellular multiplication mechanisms in plant cells. It has been shown that several T3SS *Salmonella* mutants have reduced proliferation in plants, compared to the wild-type strain (Schikora et al., 2011). The same study demonstrated that symptoms caused by the T3SS mutants in *Arabidopsis* plants were more pronounced, suggesting that plants can react to *Salmonella* infection with a HR and that T3SS mutants were unable

to hamper the induced HR (Schikora et al., 2011). In animals, numerous studies have analyzed the multiplication mechanisms at the cell level and especially the role of the T3SS-2 in intracellular multiplication of *Salmonella* Typhimurium.

Intracellular multiplication within animal cells

Salmonella can enter host cells through its T3SS-1 or to its Rck and PagN invasins (detailed in Section "T3SS-1 Independent Mechanisms"). However, unlike for the T3SS-mediated entry process, no studies have examined the intracellular behavior of *Salmonella* internalized in animal or plant cells via the invasin-mediated processes. Following entry in host cells, thanks to the T3SS-1, the majority of *Salmonella* resides in a membrane-bound compartment known as the SCV. Biogenesis and maturation of the SCV has been extensively studied in many cell types and mainly for *S.* Typhimurium (Bakowski et al., 2008; Figueira and Holden, 2012; Fabrega and Vila, 2013). The SCV, which allows bacterial growth, is distinct from a classical phagosome (**Figure 3**). *S.* Typhimurium in the SCV delivers into the host cell cytosol more than 30 effectors encoded by different *Salmonella* pathogenicity islands or the large virulence plasmid using a second type three secretion system called T3SS-2 (Fabrega and Vila, 2013). The T3SS-1, with its associated effectors, is expressed early, and is critical for cell invasion, early SCV biogenesis and the intestinal phase of infection and in particular induction of inflammation (Lostroh and Lee, 2001). The T3SS-2 is expressed a few hours following entry into cells and is responsible through effectors for SCV maturation, intracellular bacterial survival and the systemic phase of infection (Hensel, 2000). Improved understanding of these two secretion systems and of the interplay between effectors translocated by

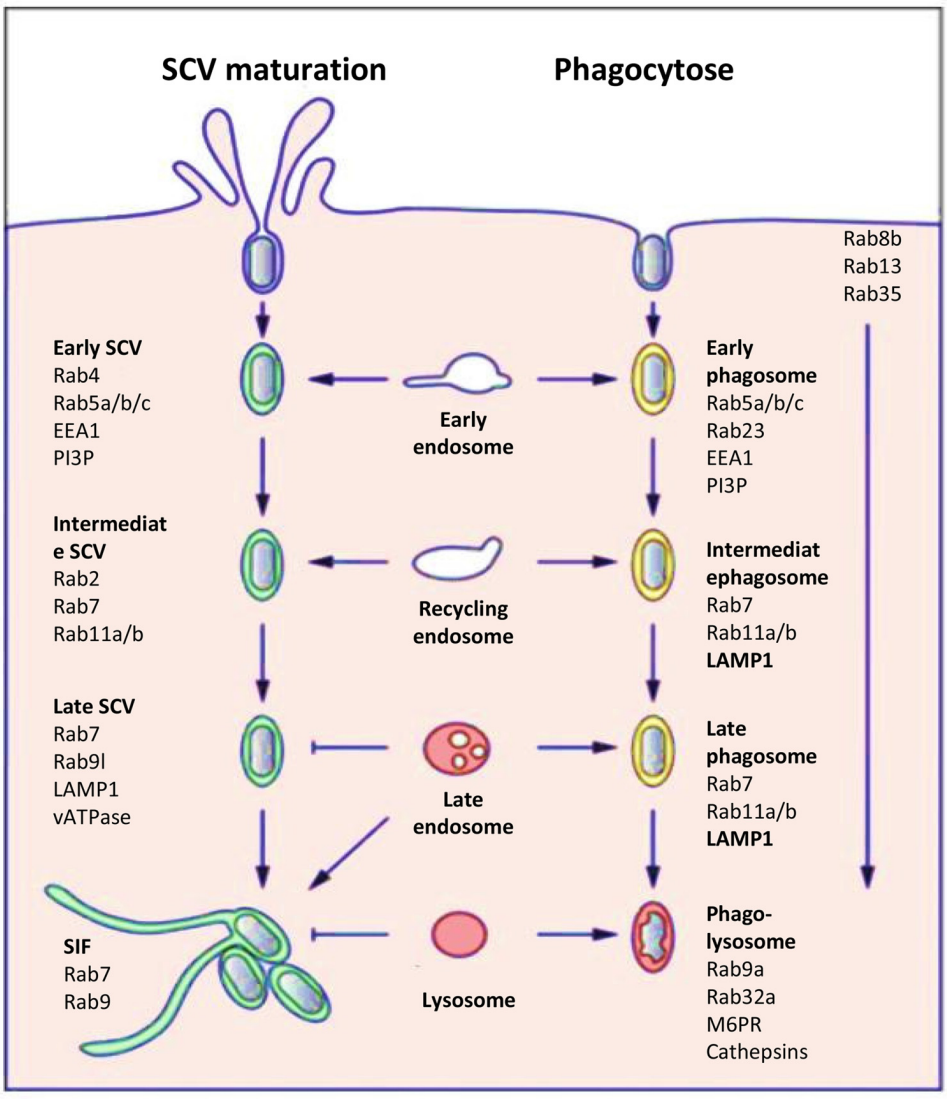

FIGURE 3 | Host cell markers present on the SCV (left) or on a phagosome (right). Comparison of the host cell markers, which characterize the classic endosome process and the biogenesis and maturation of the SCV (Figure modified from Bakowski et al., 2008).

each T3SS has shown that their roles are not so clearly separated. For *S.* Typhimurium, it has recently been shown that the T3SS-2 play a role during the intestinal phase of infection, while the T3SS-1 translocated effectors also act in late stages of intracellular multiplication and SCV maturation (Bakowski et al., 2008). The full repertoire of T3SS-2 effectors is not present in all *Salmonella enterica* serotypes. However, loss of function of the T3SS-2 in different serotypes induces a strong virulence defect characterized by an intracellular growth defect or a loss of systemic infection ability.

Once internalized, the next event triggered by *Salmonella* is the maintenance of the SCV by preventing delivery of antimicrobial host factors such as proteases and free-radical-generating complexes and by remodeling the organization of the host cell cytoskeleton to impair vesicular transport (Rajashekar and Hensel, 2011). The SCV is considered as a unique organelle that diverts from the normal endocytic pathway and allows *Salmonella* to survival and replication intracellularly. Numerous host cell markers associated with this endocytic pathway have been identified and the pattern of recruitment/retention of individual cell markers on the SCV induced by a virulent *Salmonella* strain is in part distinct from that of the classic model of phagosome, which for example, contains a non-virulent bacteria which is degraded in the phagolysosomes (**Figure 3**; Bakowski et al., 2008).

Compartments containing phagocytized material initially appear as early endosomes with markers such as early embryonic antigen 1 (EEA1), ARF6, Rab4, the transferrin receptor, and Rab5a and Rab5b GTPases (Smith et al., 2005). The maturation continues with the loss of early endosome markers and acquisition of late endosome markers like lysosomal glycoproteins (lpgs) such as LAMP1 and Rab7, Rab11a GTPases. The default maturation of phagosomes progresses toward the phagolysosomes with the presence of lpgs, the mannose-6-phosphate receptor (M6PR), Rab9a, and Rab32a. Acquisition of vATPase on phago-lysosomes results in continuous acidification of the phagosomal vacuole. Through interaction with lysosomes, hydrolytic enzymes, in particular cathepsins are delivered into the vacuole and enzymatic activity results in the killing and degradation of internalized non-pathogenic bacteria. This maturation is usually completed within a time frame of two to three hours. To a certain extent, the SCVs show similar maturation and are initially integrated within the early endocytic pathway (Drecktrah et al., 2007). However, the compartments appear arrested in the late endosomal state with some features of late endosomes. They have an acidified lumen and express lysosomal membrane glycoproteins such as LAMP1, but the SCVs are not enriched in lysosomal hydrolases and thus do not express the M6PR, which delivers lysosomal hydrolases to the endosomal system (Steele-Mortimer et al., 1999). *Salmonella* T3SS-2 effectors trigger these modifications in host endocytic trafficking and functions in order to avoid complete fusion with secondary lysosomes. Here, the delivery of T3SS-2 effectors to the host cell cytosol is a precisely controlled process (Figueira and Holden, 2012). Two T3SS-2-related effectors, SigD and SpiC, have been reported to interact with this cell endocytic trafficking to escape from the classic degradation pathway (Uchiya et al., 1999). Moreover, by interacting with host cell proteins, SifA has been reported to compete in binding with Rab9, a small GTPase involved in modulation of cell endocytic trafficking (Jackson et al., 2008). Several hours after bacterial uptake, *Salmonella* induces *de novo* formation of an *F*-actin meshwork around bacterial vacuoles. This process is termed vacuole-associated actin polymerization (VAP) and is important to maintain the integrity of the SCV membrane (Meresse et al., 2001). Different experiments have revealed that not only the T3SS-2-dependent effectors SspH2, SseI, and SpvB but also the T3SS-1 effector SipA are involved in this process (Brawn et al., 2007). As the SCV matures and is surrounded by actin, it migrates toward a perinuclear position, which depends on the balanced activity of two microtubule proteins controlling microtubule formation: kinesin and dynein. This movement occurs, indeed, along microtubules in the direction of the microtubule-organizing centre (MTOC), where Golgi stacks accumulate (Ramsden et al., 2007). This position could allow acquisition of nutrients and membranes. Once SCV is correctly positioned, bacteria start replicating and initiate formation of *Salmonella* induced filaments (SIF) which are driven by the T3SS-2 effectors SifA, SipA, SseF, SseG and SseJ, in balance with the action of other effectors like PipB2 and SpvB (Fabrega and Vila, 2013). This process could control the integrity of the SCV membrane and its expansion, which is necessary for bacterial cell division. It is also possible that by controlling vesicular fusion on the SCV, these bacterial proteins ensure delivery of nutrients to the SCV, thereby facilitating bacterial replication.

The phenotypes and biochemical activity of several effectors reveal that their apparently opposing activities actually work together to control SCV membrane dynamics. It is thus remarkable that selective pressure and convergent evolution have triggered T3SS effectors to interfere both positively and negatively with the two major forms of post-translational modifications within eukaryotic cells: ubiquitination (SspH1, SspH2, SlrP)/deubiquitination (SseL), and phosphorylation (SteC)/dephosphorylation (SpvC) (Figueira and Holden, 2012).

Heterogeneity of Salmonella *behavior within animal cells*

The analysis of bacterial invasion process in animal host cells has revealed that intracellular *S.* Typhimurium populations are heterogeneous. The majority of bacteria reside in SCV which mature into replicative compartments. However, a fraction of the intracellular *Salmonella* encounters different fates, which seem to be controlled by different SCV maturations (**Figure 4**). Although *S.* Typhimurium generally excludes markers of mature lysosomes from the SCV, a few SCVs do acquire them. Indeed the protein hydrolase cathepsin D, and the fluid-phase marker-labeled lysosomes have been found to associate with a small fraction of intracellular bacteria (Garvis et al., 2001). *S.* Typhimurium in lysosome marker positive SCV seems to fail to overcome host cell defenses, leading to SCV–lysosome fusion and bacterial killing (Bakowski et al., 2008). However, data acquired by live-cell imaging in HeLa cells and using dextran as a general marker of the lysosomal compartment, showed that the classic SCV interacts with the endosomal system and associates with lysosomes without inducing death of bacteria (Drecktrah et al., 2007). These differences could also be related to SCV membrane damage, which could induce: (i) SCV–lysosome fusion (Viboud and Bliska, 2001),

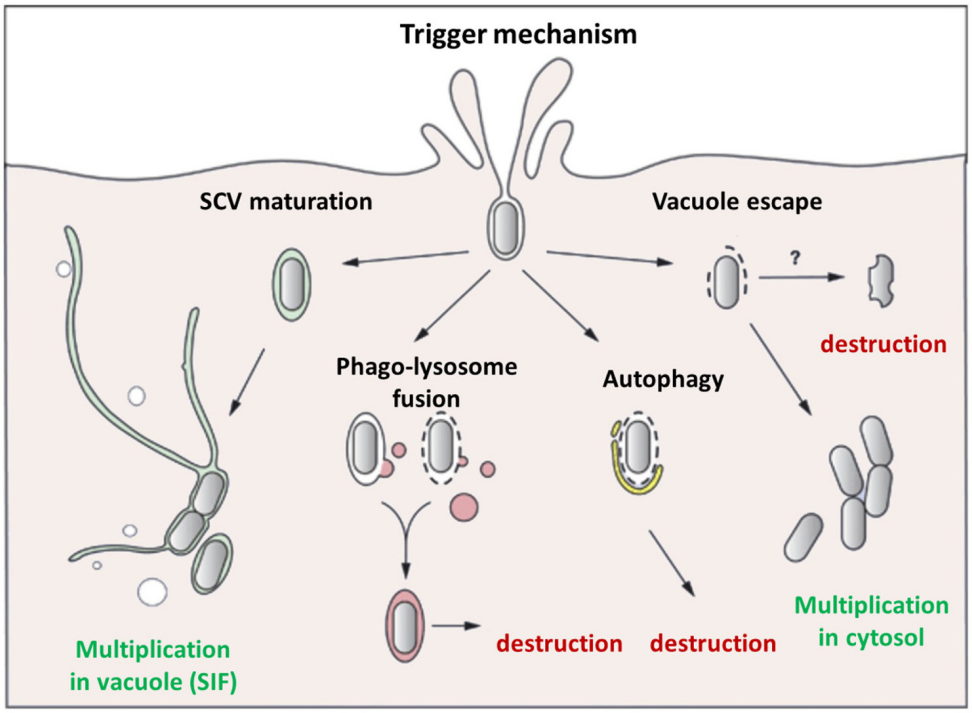

FIGURE 4 | Different behaviors of internalized *Salmonella*. The majority of *Salmonella* strains, internalized within an animal cell by the Trigger mechanism mediated by the T3SS-1, are enclosed in a canonical SCV where they can multiply, and form SIF, which allow delivery of nutrients. However, in some cases the bacteria do not have the time (or the capability) to modify the vacuole leading to the fusion of the SCV with phago-lysosome triggering intra-vacuole destruction or autophagy. In other cases, *Salmonella* damages the SCV membrane triggering vacuole destruction, allowing bacteria to escape into the cytosol, where they can be destroyed, particularly in activated macrophages, or multiply extensively especially in epithelial cells. No data have been obtained for the Zipper mechanism induced by Rck.

(ii) autophagy, a mechanism of capture of either cytosol-adapted or vacuolar bacteria which redirect them to the lysosomal compartment for killing (Knodler and Celli, 2011), or (iii) bacteria escape into the host cell cytosol where they are linked with ubiquitinated proteins (Perrin et al., 2004; Knodler et al., 2010). Once in the cytosol, *S.* Typhimurium behavior depends on the type of cell in which they reside. In epithelial cell lines, escape into the cytosol leads to extensive bacterial proliferation, greater to that observed in SCV (Knodler et al., 2010), whereas in macrophages, the cytosol exhibits a bactericidal activity, leading to bacterial killing. In fibroblasts very limited proliferation of the pathogen has been described (Cano et al., 2001). *Salmonella* contributes to this limited proliferation, since bacterial overgrowth is observed upon inactivation of the PhoP/PhoQ two-component system which also controls expression of the T3SSs.

To date, it is still unknown whether these phenomena, observed mainly *in vitro*, reflect events occurring *in vivo*, and are of additional significance in terms of *S.* Typhimurium pathogenesis in animals. Whether these additional bacterial populations represent a successful host cell clearance mechanism or an *in vitro* artifact remains to be explored. A feature that distinguishes the *in vivo* behavior of intracellular bacteria is their limited capacity to proliferate inside host cells where only three to four individuals per infected cell have been observed (Sheppard et al., 2003). The most widely accepted model indicates that *S.* Typhimurium colonizes mouse organs by increasing the number of infection foci rather than increasing the number of intracellular bacteria per cell. Repetitive cycles of limited proliferation inside host cells followed by cell lysis and infection of neighboring cells may account for the increase in infection foci (Sheppard et al., 2003). It should be noted here that all these phenomena have not been described for plant cells, and the multiplication, localization strategies used by *Salmonella* in plant cells remain poorly understood.

All together, the different results presented in this chapter show that *Salmonella* could have multiple behaviors depending on the cells and the hosts considered. Moreover, we now know that *Salmonella* can enter cells through different mechanisms that could lead to different intracellular behavior. The impacts that these different intracellular behaviors have on host responses and stimulation of immune responses will undoubtedly be a new challenge in the future.

HOST DEFENSES

Animals and plants differ quite extensively in the way they perceive and respond to invading organisms. However, for certain aspects they exhibit similarities. In both animal and plant kingdoms, when pathogens enter an organism, a rapid innate immune response is induced to impede the spreading of the pathogen. This response relies on both germline-encoded membrane-bound and intracellular receptors. In animals, this first line of defense is followed by

an adaptive response in which genes encoding immune receptors and antibodies are subjected to somatic rearrangements, which allow the recognition of very specific epitopes of the pathogen. Specialized immune cells are activated and migrate upon production of soluble factors such as cytokines and chemokines. Finally, during adaptive response an immunological memory is developed which allows the production of an enhanced response in the event of a subsequent encounter with the same pathogen. In plants there are no specialized immune cells and their defense relies on the ability of the infected cell to recognize the pathogen and induce the adequate response. A zigzag model has been proposed to describe the general immune system in plants (Jones and Dangl, 2006). In this model conserved PAMP are recognized by membrane bound receptors triggering the PAMP triggered-immunity (PTI). Thereafter, successful pathogens inject effectors into the cell with the objective of interfering with PTI. These effectors are recognized by intracellular receptors, which launch the effector-triggered immunity (ETI), which eventually culminates in cell death known as HR. In addition, plants can develop two types of systemic resistance in which contact with pathogenic or non-pathogenic beneficial microorganisms induces resistance in distal parts of the plant. Systemic acquired resistance (SAR) is induced upon infection with pathogenic bacteria or fungi and protects the plant against a broad spectrum of pathogens. ISR, the second systemic resistance type, is the result of an interaction between soil rhizobacteria or mycorrhizal fungi and the host plant. The detection of *Salmonella* by animals as well as the induced defense response has been the object of abundant literature (Broz et al., 2012; Ruby et al., 2012; Wigley, 2013). In plants, due to the increasing number of outbreaks of disease associated with consumption of contaminated fruit or vegetables, more and more studies have recently focused on *Salmonella*–plant interactions and particularly on the host response (Fraiture and Brunner, 2014; Garcia and Hirt, 2014; Melotto et al., 2014).

Receptors of the innate immune response

Extracellular receptors. In both animals and plants membrane-embedded receptors are in charge of detecting pathogens in the extracellular environment. They recognize conserved motifs within bacterial, viral, or fungal structures. In animals there are two main classes of these receptors: the C-type lectin receptors and the Toll-like receptors (TLR). TLR are by far the most studied because they play a key role in bacterial clearance. They are composed of three domains: a leucin-rich repeat (LRR) which is responsible for ligand fixation inducing homo- or hetero-dimerization of the receptor, a transmembrane, and an intracellular domain, which initiates the signaling cascade leading to activation of the host response. In plants there are two categories of extracellular receptors. The receptor-like kinases (RLK) encompass an extracellular domain, which may be an LRR, a lectin or a LysM domain, a transmembrane and an intracellular kinase domain. The receptor-like proteins (RLP) have an extracellular LRR and a transmembrane domain but lack the cytoplasmic part. A large difference in the number of extracellular receptors is observed between animals, where 13 TLRs have been described in the mouse, and plants where 200 RLPs and 600 RLKs genes have been identified in *Arabidopsis*.

In animals, TLR4 recognizes the LPS (Hoshino et al., 1999) in a complex multiprotein process involving at least four partners. The lipid A moiety of LPS is recognized by the LPS binding protein, then a ternary complex is formed with CD14 and finally LPS is delivered to the TLR4-MD2 complex (Park and Lee, 2013). TLR4 also recognizes the fibronectin (Okamura et al., 2001) and taxanes originating from plants and used as anti-tumor agents (Kawasaki et al., 2000). Very recently it has been shown that PrgI and SsaG, respectively, two structural proteins of *Salmonella* T3SS-1 and T3SS-2 needles, activate the innate response through TLR4 and also TLR2 (Jessen et al., 2014). In plants, despite the proven role of LPS in host defense (Shirron and Yaron, 2011), no associated receptor has so far been identified. In contrast to animals, both lipid A and the core oligosaccharide moieties of LPS are responsible for its immunostimulatory properties, the core oligosaccharide being involved in an early phase of the response and the lipid A in a later one (Silipo et al., 2005). Moreover Berger et al. (2011) have strongly suggested that the *O*-antigen from *Salmonella* may be considered as a PAMP in plants.

In both animals and plants, flagellin is an important PAMP recognized by extracellular receptors. A mutant strain of *Salmonella* deficient for the expression of flagellin has been shown to be able to colonize more efficiently *Medicago sativa* suggesting that *Salmonella* flagellin is recognized by the plant (Iniguez et al., 2005). *Arabidopsis* flagellin insensitive 2 (FLS2), a receptor of the RLK family, recognizes a 22-amino acid long peptide (flg22) from the N-terminus of the flagellin from different pathogens including *Salmonella* (Felix et al., 1999; Garcia et al., 2013; Meng et al., 2013). In animals, two conserved regions in the N and C terminal domains are recognized by TLR5. However, the flg22 motif is unable to activate innate immunity in animal cells (Donnelly and Steiner, 2002).

Intracellular receptors. In order to enter the host cell and to survive, bacteria produce effector proteins which are translocated into the cytosol of the host cell through the T3SS-1 or the T3SS-2 apparatus. In animal and plant cells, cytosolic receptors have the ability to detect these effectors. In animals, receptors belonging to the TLR, the Nod-like receptor (NLR), the RIG-I like receptors (RLR), and the IFI200/HIN-200 (PYHIN) families are involved in detecting non-self determinants. In plants, the nucleotide-binding site-LRRs (NB-NLR) family encoded by the R-genes encompasses two subclasses of receptors the CC-NB-LRR and the TIR-NB-LRR. NLRs in animals and plants have a similar architecture with the LRR moiety conferring effector recognition specificity, a central domain responsible for receptor dimerization upon ligand fixation and an N-terminal domain which interacts with downstream signaling partners. To be fully functional, intracellular receptors are associated in multi-protein complexes. For example, infection of macrophages with *Salmonella* leads to the formation of a macromolecular complex encompassing ASC, NLRP3, NLRC4 caspase-1, caspase-8, and pro-IL-1β (Man et al., 2014). Interestingly, it has been shown that some members of vertebrate NLR and of plant NB-LRR receptors are both physically associated with HSP90 and SGT1 chaperones which are essential for the activation of innate immunity (Mayor et al., 2007). As for extracellular receptors, the number of NB-LRR in plants exceeds the number in

animals with about 150 genes identified in *Arabidopsis* compared to around 20 in animals.

The question arises of how a limited number of receptors, especially in animals, can cope with the incredible diversity of non-self structures presented by pathogens. So far, direct interaction between NLR and their ligands has not been observed in animals. In plants there are at least two examples of direct recognition of effectors by R-protein. In *Arabidopsis*, a direct interaction has been shown between RRS1-R and the effector PopP2 (Deslandes et al., 2003) and in rice between the effector AvrPita and Pita (Jia et al., 2000). An interesting model, in which receptors detect modified self-proteins, has emerged from studies in plants (Dangl and Jones, 2001). In the guard model, the receptor is the guardian of a cellular protein (the guardee) it detects effector-induced modification of this protein and activates ETI. In this economy of means model, one receptor is able to detect modification of a host protein which may be the target of several pathogens. A given protein may be guarded by different receptors. The response of *Arabidopsis* to effectors from *Pseudomonas syringae* is one of the examples illustrating the guard model. In this model, the guardee protein RIN4 is targeted by different unrelated effectors (AvrRpm1, AvrRpt2, AvrB, or HopF2). AvrRpt2 is a protease, which cleaves RIN4. This cleavage is detected by the RPS2 receptor, which induces ETI. Both AvrB and AvrRpm1 phosphorylate RIN4, the receptor RPM1 recognizes the phosphorylated RIN4 protein and triggers ETI (Mackey et al., 2002, 2003; Wilton et al., 2010). The response of mice to the T3SS-1 SopE effector from *Salmonella* is evocative of the guard model in plants. When injected into the cytosol, SopE activates the small RhoGTPases Rac1 and Cdc42, this activation is detected by the receptor NOD1 and leads to the development of an inflammatory response (Keestra et al., 2013). In animals some *Salmonella* effectors are recognized by intracellular receptors. The T3SS-1 effector SipA activates NOD1/NOD2 (Keestra et al., 2011), while the T3SS-1 protein PrgJ and flagellin are recognized by the inflammasomes NLRC4-NAIP2 and NLRC4-NAIP5, respectively (Zhao et al., 2011; Halff et al., 2012). In *Nicotiana* benthamiana, the T3SS-2 effector SseF is probably recognized by a NB-NLR receptor (Ustun et al., 2012). In both animals and plants, recognition of effectors by their receptors launches signaling cascades which eventually lead to pathogen clearance.

Suppression of innate immune response by Salmonella

In animals, the interaction of innate immune receptors with their ligands may have two outcomes. The first is the activation of the key transcription factor NF-κB or of the MAPKs cascade, which ends with the transcriptional activation of numerous genes involved in inflammation, such as IL-6, iNOS, or TNFα. The second is the assembly of multiproteic scaffoldings, the inflammasomes, in which pro-caspase 1 is recruited and activated in an autocatalytic process leading to the maturation of pro-inflammatory cytokines like IL-1β or IL-18 and to a cell death known as pyroptosis. Plants possess a large family of MAPKs, some of which are involved in signaling cascades pivotal in PTI and ETI (Meng and Zhang, 2013). However important information on the intermediary signaling components which link receptor activation and the MAPK cascades is still missing. Induction of PTI and ETI induces overlapping responses including the production of ROS, antimicrobial compounds, signaling molecules like ethylene, salicylic acid, and jasmonic acid or enhanced expression of *pathogenesis-related* (*PR*) genes. In addition to these responses, HR is the usual outcome of ETI.

Salmonella can induce PTI in plants. For example, the MAPK cascade is activated in *Arabidopsis* inoculated with this bacterium (Schikora et al., 2008). On the other hand, inoculation of *Arabidopsis* with a *spiB* mutant leads to a higher number of bacteria in the roots compared to inoculation with wild-type *Salmonella*, raising the possibility that the T3SS-1 encodes proteins recognized by the plant immune system (Iniguez et al., 2005). Expression of the SseF in *Nicotiana benthamiana* induces the HR, a hallmark of ETI (Ustun et al., 2012). In tobacco, living *Salmonella* does not induce signs of defense response, while LPS from *Salmonella* or killed bacteria do, indicating that the bacterium is able to suppress the response, and this suppression is T3SS-1-dependent (Shirron and Yaron, 2011). *Arabidopsis* inoculated with a T3SS-1 mutant overexpressed genes associated with defense response when compared to inoculation with a wild-type *Salmonella* (Schikora et al., 2011).

However, *Salmonella* has implemented different strategies to overcome the defense response. In animals different *Salmonella* effectors may inhibit immune signaling pathways like NF-κB, the MAPK cascade or the transcription factor Syk through direct interaction with some signaling components (**Table 1**). *Salmonella* may also use some cellular intermediaries to inhibit the response. An unidentified protein from *Salmonella* activates the NLRP12 inflammasome, which in turn down-regulates NF-kB (Zaki et al., 2014). The bacterium may also target directly the receptor involved in its recognition. It has been shown that *Salmonella* down-regulates the expression of the intracellular receptor NLRC4 in B lymphocytes preventing the production of IL-1β and pyroptosis, allowing bacteria to stay hidden in lymphocytes (Perez-Lopez et al., 2013). Very recent data have uncovered different strategies used by *S.* Typhi to circumvent immune response. *S.* Typhimurium induces gastroenteritis and triggers inflammation with recruitment of neutropils to the intestine; in contrast, S. Typhi is associated with a systemic disease with little intestinal inflammation and few neutrophils. Typhi

Table 1 | *Salmonella* effectors which inhibit immune signaling pathways.

Effector	Translocated by	Inhibit	Reference
AvrA	T3SS-1	NF-κB	Collier-Hyams et al. (2002)
		MAPK	Wu et al. (2012)
SseL	T3SS-2	NF-κB	Le Negrate et al. (2008)
SseK	T3SS-2	NF-κB	Li et al. (2013)
SspHI	T3SS-1	NF-κB	Haraga and Miller (2003)
SpvC	T3SS-1	MAPK	Mazurkiewicz et al. (2008)
SptP	T3SS-1	Syk	Choi et al. (2013)

and Typhimurium serovars differ, with the former having a *via* locus. The *S.* Typhi *tviA* regulator gene indirectly downregulates the expression of HilA, a master regulator of the T3SS1, preventing recognition of SopE and activation of NK-κB (Winter et al., 2014). At the same time, chemotactism of the c5a component of the complement toward neutrophils is impaired by the Vi capsular antigen encoded in the *via* locus (Wangdi et al., 2014). In plants, there are few examples of modulation of immune response by *Salmonella*. The serovar Senftenberg, which differs in its canonical flg22 peptide, displays a reduced PTI when inoculated in *Arabidopsis* seedlings (Garcia et al., 2013) suggesting that some *Salmonella* strains may have evolved to escape recognition by FSL2. It has been shown that a mutant of *Salmonella* unable to assemble its T3SS1 apparatus is unable to suppress the expression of genes related to response to pathogens (Schikora et al., 2011) suggesting that some suppressor factors are injected in the cell by the T3SS1. Another study (Shirron and Yaron, 2011) has highlighted the suppressive activity of *Salmonella*: live bacteria do not produce oxidative burst in tobacco while heat killed bacteria or *Salmonella* LPS are able to do so. There is also an interesting example of cross-kingdom modulation of the immune response by the T3SS2 effector SspH2 (Bhavsar et al., 2013). In animal cells, this E3 ubiquitin ligase forms a ternary complex with STG1 which is a co-chaperone of the NLR NOD1; formation of this complex induces ubiquitination of NOD1, increases its activity, and stabilizes the SspH2 effector. STG1 which is highly conserved within eucaryotes, also interacts with SspH2 in plants enhancing their immune response.

CONCLUSION

The ability of *Salmonella* to persist outside its hosts is a critical trait that enables this pathogen to occasionally contaminate fresh produce and therefore cause food-borne disease outbreaks. The ability of the human enteric pathogens to exploit plants as alternative hosts has emerged as an important area of research in the last decade. It has become apparent that *Salmonella* not only passively survives on or within plants but also actively infects them. However, contrary to *Salmonella* with animals or animal cells, these interactions have not been well characterized. Some common features have been identified such as the use of the T3SS or the way animals and plants detect this pathogen. Future studies are required to investigate whether mechanisms employed by *Salmonella* to infect animals and plants are similar. These studies should lead to improved understanding of the evolution of host specificity and will have important impacts on risk assessment and food protection.

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Techniques for transferring host-pathogen protein interactions knowledge to new tasks

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We consider the problem of building a model to predict protein-protein interactions (PPIs) between the bacterial species *Salmonella* Typhimurium and the plant host *Arabidopsis* thaliana which is a host-pathogen pair for which no known PPIs are available. To achieve this, we present approaches, which use homology and statistical learning methods called "transfer learning." In the transfer learning setting, the task of predicting PPIs between *Arabidopsis* and its pathogen *S.* Typhimurium is called the "target task." The presented approaches utilize labeled data i.e., known PPIs of other host-pathogen pairs (we call these PPIs the "source tasks"). The homology based approaches use heuristics based on biological intuition to predict PPIs. The transfer learning methods use the similarity of the PPIs from the source tasks to the target task to build a model. For a quantitative evaluation we consider *Salmonella*-mouse PPI prediction and some other host-pathogen tasks where known PPIs exist. We use metrics such as precision and recall and our results show that our methods perform well on the target task in various transfer settings. We present a brief qualitative analysis of the *Arabidopsis-Salmonella* predicted interactions. We filter the predictions from all approaches using Gene Ontology term enrichment and only those interactions involving *Salmonella* effectors. Thereby we observe that *Arabidopsis* proteins involved e.g., in transcriptional regulation, hormone mediated signaling and defense response may be affected by *Salmonella*.

Keywords: protein interaction prediction, host pathogen protein interactions, plant pathogen protein interactions, machine learning methods, transfer learning, kernel mean matching

1. INTRODUCTION

Understanding the workings of plant responses to pathogens is an important fundamental questions that also has enormous economic importance due to the role of pathogens in food production and processing. While "classical" plant pathogens cause crop losses during production by impacting on plant health, processing of plant-based food can lead to contamination by opportunistic pathogens. It is becoming increasingly supported by experimental evidence that some human bacterial pathogens can colonize plants and cause disease (Kirzinger et al., 2011). *Salmonella* is one of these bacterial species with extremely broad host range that infects not only animals, but also plants (Hernandez-Reyes and Schikora, 2013). Evidence increases that *Salmonella* can utilize plants as alternative host and can be considered as a bona fide plant pathogen. In this respect it has been reported that (a) *Salmonella* actively invades plant cells, proliferates there and can cause disease symptoms (Schikora et al., 2008; Berger et al., 2011) (b) the plant recognizes *Salmonella* and plant defense responses are activated (Iniguez et al., 2005; Schikora et al., 2008) and (c) that functional Type Three Secretion Systems (TTSS) 1 and 2 are important for *Salmonella* pathogenicity in plants with respect to bacterial proliferation and suppression of plant defense responses (Iniguez et al., 2005; Schikora et al., 2011; Shirron and Yaron, 2011). *Salmonella* TTSS-1 and 2 encode proteins, so called effectors, which are known to be translocated into the animal host cell in order to manipulate host cell mechanisms mainly via PPIs (Schleker et al., 2012). Hence, it may be assumed that *Salmonella* utilizes the same proteins during its communication with animals and plant. However, the details of this communication are not known. A critical component of the communication between any host and its pathogen are PPIs. However, the infection of plants by *Salmonella* is only a nascent field, so there are no known PPIs for *Salmonella* with any plant reported yet. Even for the well established pathogen-host pair, *Salmonella*-human, relatively few interactions are known (Schleker et al., 2012). Only 62 interactions between *Salmonella* and mostly human proteins (some *Salmonella* interactions involve other mammalian species, such as mouse and rat) are known to date. Because there exists no plant-*Salmonella* interactions data, we need to rely on computational methods to predict them [reviewed in the accompanying paper (Schleker et al., 2015)].

In this paper, we describe techniques to build computational models to predict interactions between the model plant, *A. thaliana*, and *S.* Typhimurium. Since there is no labeled data of this host-pathogen pair available, we aim to transfer knowledge from known host-pathogen PPI data of other organisms. We use various statistical methods to build models for predicting host-pathogen PPIs. In each case, we cast the PPI prediction

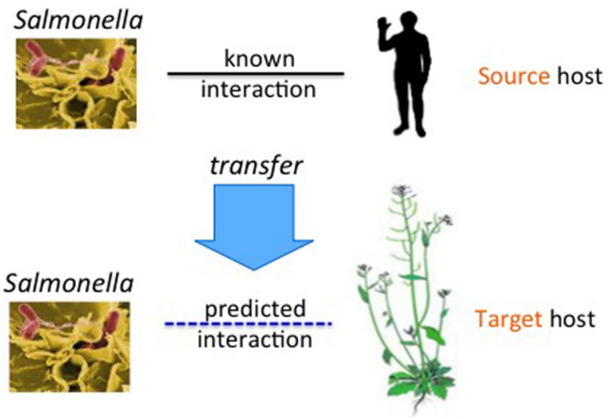


FIGURE 1 | Transfer of PPIs from the source host (for ex: human) to another host, the target host (for example *Arabidopsis*), for the common pathogen, *Salmonella*.

problem as a binary classification task, where given two proteins the goal is to learn a function that predicts whether the pair would interact or not. We derive features on every protein pair using protein sequence data. Each host-pathogen PPI prediction problem is considered as one task. **Figure 1** shows our problem setting. The upper host-pathogen task with *Salmonella* as pathogen and human as the host is the *source* task. The lower task is the target task. The arrow shows the direction of knowledge transfer.

In order to transfer knowledge from one organism to another, we need to utilize some measure of similarity between them. This similarity can be defined between smaller units such as individual proteins or genes from the organisms or higher level units. The higher the similarity, the greater the information transfer between them. Hence the notion of similarity is very critical to the results we obtain from such a transfer based method and should be biologically motivated. Our methods enable the transfer of knowledge using the following mechanisms:

- We use the structural similarity between the individual proteins of the two hosts measured using protein sequence alignment. This follows from the biological intuition that structurally similar proteins in two different organisms are very likely to have similar functions. Hence a pathogen that wants to disrupt a specific function will target structurally similar proteins in different hosts.
- Interactome-level similarity, comparing the human PPI graph with the plant PPI graph. Any biological process in an organism involves the participation of several proteins and more importantly the interactions between these. By comparing the interactomes of different hosts, we are comparing them at the biological process-level. The components of the two graphs that are highly similar will most likely correspond to similar processes in the two organisms.
- Distributional similarity between the protein pairs: here, we identify which of the human-*Salmonella* protein pairs are the most similar (hence most relevant) to the plant-*Salmonella* protein pairs. This similarity is computed using the features of the protein-pairs. Since it is distributional similarity, it involves a comparison over all protein pairs from both organisms. Only the most relevant human-*Salmonella* protein pairs are used to build a model.

The main contributions of this paper are:

(1) We present methods that combine known PPIs from various sources to build a model for a new task
(2) We evaluate our methods quantitatively and our results show the benefits in performance that are possible if we incorporate the similarity information discussed in the previous paragraphs
(3) We present the first machine learning based predictions for plant-*Salmonella* PPIs.

In the rest of the paper, we start by describing the host-pathogen PPI datasets we use in Section 2, followed by a detailed description of our methods in Section 3 and a quantitative and qualitative analysis of the results in Section 5.

2. SOURCE TASKS

As source tasks we used the known PPIs between various other hosts and pathogens. Many of these interactions were obtained from the PHISTO (Tekir et al., 2012) database which reports literature-curated known interactions. For PPIs between human and *Salmonella* we use the manually literature-curated interactions reported in Schleker et al. (2012). Please note that all of these interactions come from biochemical and biophysical experiments. The details of the dataset used in each approach are shown in **Table 1** and they are available for download from http://www.cs.cmu.edu/~mkshirsa/data/frontiers2014/data.zip. Our first approach is a rule-based approach and it uses human-*Salmonella* PPIs from two sources: the 62 experimentally generated PPIs reported in Schleker et al. (2012) and the predicted PPIs from Kshirsagar et al. (2012). Please note that this is the only method that uses any predicted PPIs as "ground truth." All other methods discussed in subsequent sections of this paper do not use any predicted PPIs as source. They use only PPIs validated experimentally by biochemical and biophysical methods.

2.1. *SALMONELLA* SPECIES/STRAINS CONSIDERED

The source data that we use for human-*Salmonella* from Schleker et al. (2012) comes from two different strains: *Salmonella* Typhimurium strain LT2 and *Salmonella* Typhimurium strain SL 1344. One of our three approaches (row-1 of **Table 1**) uses human-*Salmonella* predicted PPIs. These predicted PPIs from Kshirsagar et al. (2012) contain *Salmonella* proteins from two additional strains: *Salmonella* enteritidis PT4 and *Salmonella* Typhi. From henceforth, for the sake of brevity, we will refer to proteins from all strains as *Salmonella* proteins. For *Salmonella* proteins, we used the UniprotKB database (The UniProt Consortium, 2014) to obtain all proteins from the various strains. For *Arabidopsis* thaliana proteins, we used the TAIR database (Lamesch et al., 2012).

Table 1 | Datasets used in the various approaches, their sizes and the appropriate citations.

Approach(es)	Source task(s)	Number of interactions	Citation for interactions data	Feature set
1. Homology based	Human-*Salmonella* known PPI	62	Schleker et al., 2012*	No feature set. Heuristics are used to infer interactions
	Human-*Salmonella* predictions	190,868	Kshirsagar et al., 2012	
2. T-SVM#	Human-*Salmonella* known PPI	62	Schleker et al., 2012*	(a) Protein sequence k-mers (b) Gene expression (from GEO) (c) GO term similarity
3. KMM†-SVM	Human-*Salmonella* known PPI	62	Schleker et al., 2012*	Protein sequence k-mers
	Human-*Francisella tularensis*	1380	PHISTO* (Tekir et al., 2012)	
	Human-*E.coli*	32		
	A. thaliana - *Agrobact. tumefaciens*	22		
	A. thaliana - *E. coli*	15		
	A. thaliana - *Pseudomonas syringae*	13		
	A. thaliana - *Synechocyctis*	23		

†*KMM, Kernel Mean Matching;* # *SVM, Support Vector Machine; GO, Gene Ontology.*

* *This source reports PPIs validated experimentally by biochemical and biophysical methods.*

3. METHODS

In the previous section, we described the dataset used in our various approaches. We now describe the details of the methods we use.

3.1. APPROACH-1 : HOMOLOGY BASED TRANSFER

In this approach, we use the sequence similarity between the plant and human protein sequences to infer new interactions. We use two techniques to predict interactions between plant and *Salmonella* proteins. The first technique uses plant-human orthologs and the second is based on plant-human homology (sequence alignment scores). Both techniques use two sources of interactions: true PPIs from Schleker et al. (2012) and predicted PPIs from Kshirsagar et al. (2012). Please note that this is the only method that uses any predicted PPIs as "ground truth." All other methods discussed in subsequent sections of this paper do not use any predicted PPIs as source.

Homologs and Orthologs: Homologous pairs of genes are related by descent from a common ancestral DNA sequence. These can be either orthologs: genes that evolved from a common ancestral gene by speciation or paralogs: genes separated by the event of genetic duplication. We obtained orthologs from the InParanoid database (Ostlund et al., 2010). To find homologous pairs of proteins, we used BLAST sequence alignment with an *e*-value threshold of 0.01.

(a) **Host ortholog based predictions**: We start with the known human-*Salmonella* PPIs. For each interaction, we search for an ortholog of the human protein in *Arabidopsis*. If one exists, we infer an interaction between the *Salmonella* and the *Arabidopsis* protein. This is similar to finding interologs, with the exception that we restrict ourselves to orthologs of the host protein rather than considering all possible homologs of both the host and pathogen proteins. **Figure 2** illustrates this simple heuristic. There are 62 human-*Salmonella* PPIs in our dataset. Using this ortholog based inference for the host proteins, we obtained a total of 25 plant-*Salmonella* PPIs as some of the human proteins did not have any plant orthologs. The orthologous *Arabidopsis* proteins for the human proteins were obtained from the InParanoid database (Ostlund et al., 2010).

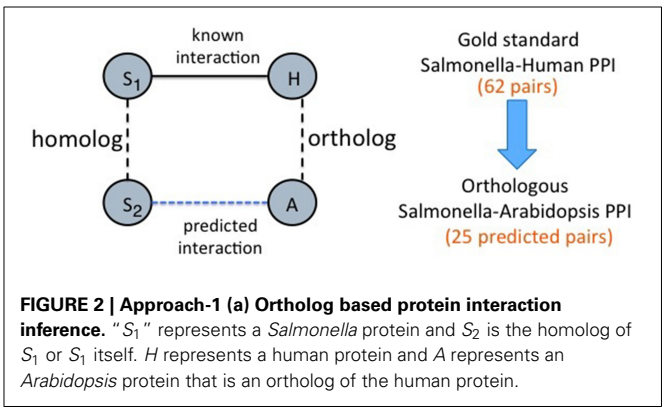


FIGURE 2 | Approach-1 (a) Ortholog based protein interaction inference. "S_1" represents a *Salmonella* protein and S_2 is the homolog of S_1 or S_1 itself. *H* represents a human protein and *A* represents an *Arabidopsis* protein that is an ortholog of the human protein.

(b) **Host graph alignment based predictions**: This method uses homologs between the human and plant proteins. Since the set of known PPIs is very small (62 interactions), here we use them to generate "bootstrap" interactions. The known 62 PPIs are used to build a classifier using the method published in Kshirsagar et al. (2012) to generate a total of 190,868 human-*Salmonella* PPI predictions. These predicted PPIs form the "bootstrap" PPIs and will be used in a graph-based transfer approach. In this graph-based transfer method, we first align the PPI graphs of the two host organisms using NetworkBlast (Sharan et al., 2005). The human PPI network was obtained from the HPRD database (Prasad et al., 2009) and the plant-plant PPIs from TAIR database (Lamesch et al., 2012). The algorithm aligns the human PPI graph with the plant PPI graph using the pairs of homologous proteins

between the two organisms. To find the homologous proteins, we used BLAST sequence alignment with an *e*-value threshold of 0.01. Next, we use NetworkBlast to find the graph components that are the most similar across the two graphs. We call them the "enriched components." By comparing the interactomes of the two hosts, we are comparing them at the biological process-level. The components of the two graphs that are highly similar will most likely correspond to similar processes in the two organisms. NetworkBlast finds a total of 2329 enriched protein complex pairs between the two host organisms. **Figure 3** shows one such enriched protein complex pair: the complex on the left is from *Arabidopsis* and the one on the right is from human. Using these we determine the plant proteins that are the most likely targets for the different *Salmonella* proteins as shown in the **Figure 3**.

For each PPI between a human protein from an enriched protein complex, we infer an equivalent PPI between the corresponding plant protein and the *Salmonella* protein (example, *sipA* in the **Figure 3**). This filtering procedure gives us a final of 23,664 plant-*Salmonella* PPIs. The biological relevance for using the enriched graph components lies in the premise that clusters of similarly interacting proteins across the two organisms will represent biological processes that have been conserved in the two organisms. Hence, the proteins in these components are also likely to be conserved as pathogen targets.

3.2. APPROACH-2: TRANSDUCTIVE LEARNING

This method considers the target task i.e., plant proteins while building a model. It provides a way of incorporating the target task information during model construction. Conventional inductive learning approaches such as the Support Vector Machine classifier use only the training examples to build a model. Transductive learning approaches also use the distribution of the unlabeled test examples. They jointly learn the labels on the test examples while minimizing the error on the labeled training examples. This often results in a good performance, as the classifier has additional information about the unseen test data. In our work here, we use transductive learning for transfer learning in particular the Transductive Support Vector Machine algorithm (T-SVM) (Joachims, 1999). The training examples are the source task examples, i.e., human-*Salmonella* protein interactions. We use the target task examples as the test data.

Training negatives: Since there are 62 known PPIs in the source task, we sample a set of random 6200 human-*Salmonella* protein pairs to maintain the positive:negative class ratio at 1:100.

Figure 4 depicts this setting. This method thus builds a model by using data from both hosts. The optimization function of T-SVM jointly minimizes the training error on the known human-pathogen interactions and the label assignments on the unknown plant-pathogen interactions. The set of target examples can not be used entirely as it is very large and makes the T-SVM algorithm very computationally expensive. Hence we randomly sample 1 percent of the target dataset. For the T-SVM based algorithm to be effective, the kernel function that is used to compute the similarity between examples matters a lot. We use a homology-based kernel function that incorporates the BLAST similarity score between the proteins. Let x_s^i be the feature-vector representing a source task example: the protein pair $< s_s, h_s >$ where s_s is the *Salmonella* protein (i.e., the pathogen protein) and h_s is the host protein. Let the target task example be the protein pair $< s_t, a_t >$ where a_t is the *Arabidopsis* protein; and the corresponding feature vector be x_t^k. The kernel function that computes the similarity between the given two pairs of proteins (i.e., their feature vectors) is defined as shown below.

$$k(x_s^i, x_t^k) = \text{sim}(p_s, p_t) + \text{sim}(h_s, a_t) \quad \text{where}$$

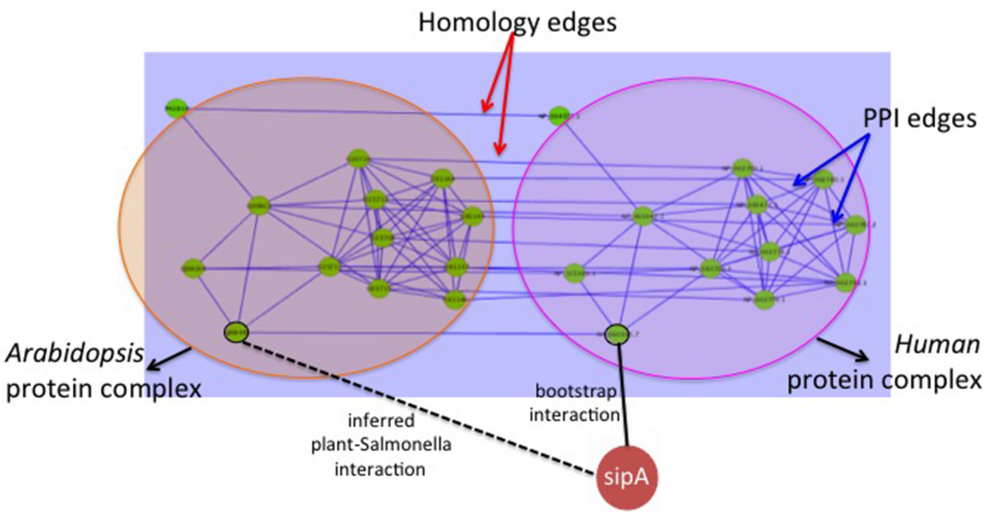


FIGURE 3 | Approach-1(b) Graph based interaction transfer. The big circles show the two protein complexes found to be enriched by Network Blast : the *Arabidopsis* protein complex on the left, and the human protein complex on the right. The edges within a protein complex are the PPIs within the host organism. The edges connecting the two protein complexes (i.e., the two circles) are the homology edges. The solid line connecting *sipA* with a human protein node is a bootstrap interaction. We use this to infer the new plant-*Salmonella* interaction indicated by the dotted line.

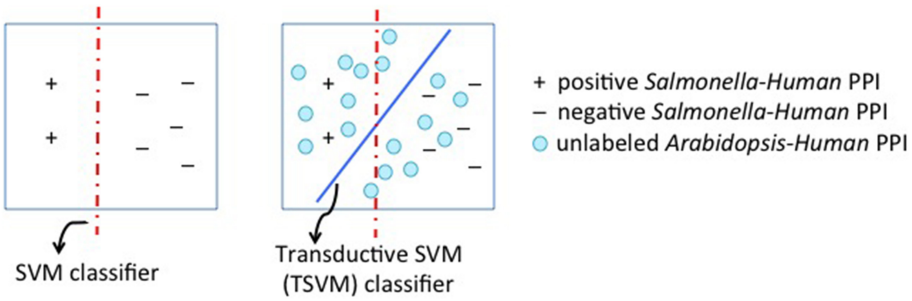


FIGURE 4 | Transductive Support Vector Machine (SVM) for transfer learning. The first panel shows the conventional SVM classifier. The second panel shows T-SVM with circles representing unlabeled examples. We use examples from the target task i.e., *Arabidopsis-Salmonella* protein pairs as the unlabeled examples to influence the classifier boundary.

$$\text{sim}(m, n) = \text{normalized} - \text{BLAST} - \text{score}(m, n)$$

$$k(x_s^i, x_s^j) = dot(x_s^i, x_s^j) \text{ and } k(x_t^i, x_t^j) = dot(x_t^i, x_t^j)$$

The first equation is used in the case where the two protein pairs come from different tasks. We use homology-distance between the pathogen proteins and the host proteins to compute the kernel. The homology distance itself is simply the BLAST protein sequence alignment score. The next two equations show the computation when the examples both come from the same task. Here we simply take the dot product of the two feature vectors. This kernel is symmetric. The similarity between two sequences $sim(m, n)$ is computed using the bit-score from BLAST sequence alignment, normalized using the sequence length of the larger protein. We used the SVM^{light} package (Joachims, 2008) and incorporated our kernel function into it. The parameter tuning for T-SVM (the regularization parameter C) was done using cross validation on the PPIs where we have the true labels. We found $C = 0.1$ was the best setting. This best model is subsequently used to generate predictions on all *Arabidopsis-Salmonella* protein-pairs. The model outputs a score indicating the distance from the classifier hyperplane. A positive score indicates that the protein-pair is on the positive side of the hyperplane and hence closer to the known interacting protein-pairs. All such protein-pairs will be considered as potential interactions predicted by this model.

3.3. APPROACH-3: KERNEL MEAN MATCHING

Our transfer learning scenario here consist of the following setting: multiple "source" tasks with small amounts of labeled data, a single "target" task with no labeled data. The first challenge is to pick the best instances from the source tasks, such that the resultant model when applied on the target task generates high confidence predictions. Toward this, we use the instance reweighting technique Kernel Mean Matching (KMM). The reweighted source task instances are used to build a kernelized support vector machine (SVM) model, which is applied on the target task data to get the predicted PPIs. This brings forth the second challenge—selecting appropriate hyperparameters while building a model for a task with no labeled data. For simplicity we also use the same set of features across all tasks (protein sequence features). However the data distribution will be different across tasks due to the different organisms involved.

This approach is based on instance-transfer where the goal is to pick from each of the source tasks, the most relevant instances w.r.t the target task. We use a two-step process: (1) the first step does the instance weighting on the source tasks. (2) the second step uses the reweighted instance to build several SVM classifier models—one model for each hyper-parameter setting. To deal with the second challenge, we present two heuristic methods to select the best set of hyperparameters.

3.3.1. Step-1: Instance reweighting

The similarity between the source and target data can be expressed using the similarity in their distributions $\mathbf{P}_S(x, y)$ and $\mathbf{P}_t(x, y)$. Here $\mathbf{P}_S$ represents the joint distribution of all source tasks. Since we do not have access to the labels y on the target, we make a simplifying assumption that there is only a covariate shift between the source and target tasks—i.e., the conditional distribution $P(y|x)$ is the same for both tasks. Mathematically, $\frac{\mathbf{P}_S(x,y)}{\mathbf{P}_t(x,y)} = \frac{\mathbf{P}_S(x)}{\mathbf{P}_t(x)} = r(x)$. Many methods have been proposed for estimating the ratio r. Sugiyama et al. (2008) proposed an algorithm Kullback-Leibler Importance Estimation Procedure (KLIEP) to estimate r directly without estimating the densities of the two distributions.

We use the nonparametric Kernel Mean Matching (KMM) (Huang et al., 2007), which was originally developed to handle the problem of covariate shift between the training and test data distributions. KMM reweighs the training data instances such that the means of the training and test data distributions are close in a reproducing kernel Hilbert space (RKHS). This approach does not require distribution estimation. Let $x_i^S \sim P_S$ and n_S be the number of source instances from all source tasks. Let $x_i^t \sim P_t$ and n_t be the number of target instances. Let β_i represent the "importance" of the source instances. KMM uses a function based on the *maximum mean discrepancy* statistic (MMD). In the form written below, it minimizes the difference between the empirical means of the joint source and target distributions.

$$\min_{\beta} \left\| \frac{1}{n_S}\sum_{i=1}^{n_S}\beta_i\Phi(x_i^S) - \frac{1}{n_t}\sum_{j=1}^{n_t}\Phi(x_j^t) \right\|^2$$

$$\Leftrightarrow \min_{\beta} \ \frac{1}{n_S^2}\beta^T K\beta - \frac{2}{n_S^2}\kappa^T\beta \ + C$$

$$\text{subject to } \beta_i \in [0, B] \text{ and } \sum_i \beta_i \leq n_S$$

$$\text{where } K_{i,j} = k(x_i^S, x_j^S) \text{ and } \kappa_i = \frac{n_S}{n_t}\sum_{j=1}^{n_t} k(x_i^S, x_j^t) \qquad (1)$$

K is the kernel matrix over all the source examples. The function (1) is a quadratic program and can be efficiently solved using sequential minimal optimization (SMO), projected gradient based methods. We use the KMM implementation from the Shogun (Sonnenburg et al., 2010) package.

3.3.1.1. Selecting an appropriate set of source and target instances. Using all instances in the optimization problem in equation (1) is infeasible for two reasons. The optimization involves the computation of the gram matrix K of $O(n^2)$ where n is the number of instances. Typically the total number of protein-protein pairs between a host-pathogen are of the order of 100 million. Secondly, the total number of labeled source instances is quite small ($\approx$ 1500). This set is likely to get underweighted (i.e., $\beta_i \approx 0$) if there are too many unlabeled source instances. To represent the source's empirical mean, in addition to the labeled instances we randomly sample four times as many unlabeled instances. For the target, we randomly sampled n_S instances.

3.3.2. Step-2: Model learning

Once we have the optimal set of source instances, we can train a Kernel-SVM model using these. Along with the first step, we thus call this two step process KMM-SVM. We pick a kernel-based learning algorithm since we plan to extend our work to deal with different feature spaces across the tasks. In such a scenario, the only mechanism to operate on the target data is via similarities, i.e., the kernel. The dual formulation for the weighted version of SVM solves the following problem, where the weights β_i were obtained in Step-1.

$$\min_{\alpha} \sum_{i=1}^{n_S}\alpha_i - \frac{1}{2}\sum_{i,j}\alpha_i\alpha_j y_i y_j K(x_i^S, x_j^S) \ \text{ subject to } \sum_i \alpha_i y_i = 0$$

$$\text{and} \quad \beta_i C \geq \alpha_i \geq 0$$

3.3.3. Model selection

Parameter tuning and selecting the best model in the absence of labeled data is a very hard problem. The model built on the source data cannot be tuned using cross validation on the source data because doing so will optimize it for the source distribution. Hence we developed two heuristic approaches to select the best hyperparameters. The first one uses the expected class-skew on the target task while the second uses reweighted cross-validation.

Class-skew based parameter selection: We first built several models by doing a grid-search on the classifier hyper-parameters. There are 3 parameters to tune for the Kernel-SVM: the kernel width γ, the cost parameter C, the weight parameter for the positive class w_+. The total number of parameter combinations in our grid-search were 50. We thus had 50 models trained on the reweighted source data obtained after KMM in Step-1 (Section 3.3.1). We applied each model on the target data and computed the predicted class-skew r_{pred} using the predicted class labels. The expected class skew based on our understanding of the PPI experimental literature is roughly 1:100 ($= r_{true}$). We ranked all 50 models on the statistic $|r_{pred} - r_{true}|$. The top k models were selected based on this criteria and a weighted voting ensemble was built using them. This ensemble was used to get the final class label on the target data. We used $k = 5$.

Aggregating the models and assigning interaction scores: In our experiments, we used $k = 5$ to pick the best models w.r.t the ranking statistic described above. Note that each model gives us a classifier score for every protein-pair in the test data, which can be considered to be the probability of interaction. For $k = 5$, we have five scores for each test protein-pair. These scores were aggregated using two criteria:

(a) The majority vote over the five models where each model votes "yes" if the output probability score is greater than or equal to 0.5.
(b) The averaged of all five probability scores.

3.3.4. Spectrum RBF kernel

We used a variant of the spectrum kernel, based on the features used by Dyer et al. (2007) for HIV-human PPI prediction. The kernel uses the n-mers of a given input sequence and is defined as: $k_{sp}^n(x, x') = \exp\{-\frac{\|\phi_{sp}^n(x) - \phi_{sp}^n(x')\|^2}{\sigma^2}\}$, where x, x' are two sequences over an alphabet Σ. Instead of using the 20 amino acids as the alphabet Σ, we used a classification of the amino-acids. There are seven classes based on the electrostatic and hydrophobic properties of proteins, i.e., $|\Sigma| = 7$. Here ϕ_{sp}^n transforms a sequence s into a $|\Sigma|^n$-dimensional feature-space. One dimension of ϕ_{sp}^n corresponds to the normalized frequency of one of the 7^n possible strings in s. We use $n = 2, 3, 4, 5$.

4. NEGATIVE EXAMPLES AND FEATURE-SET

Classification techniques need a negative class (set of non-interactions) in order to identify the special characteristics of the positives (i.e., interactions). Since there is no published experimental evidence about "non-interacting" host-pathogen proteins for any plant with *Salmonella*, we construct the negative class using random pairs of proteins sampled from the set of all possible host-pathogen protein pairs. The number of random pairs chosen as the negative class is decided by what we expect the interaction ratio to be. It is a parameter that can be changed as our knowledge of the size and nature of the host-pathogen interactome improves.

The interaction ratio/ negative examples are used in different ways as described below. The homology-based transfer method does not directly use any negative examples/ interaction ratios. In the case of T-SVM, while training the transductive model, we

use negative examples from the source task. In the case of KMM-SVM, the data used to build the model comes from the source tasks, where negative examples from each source task are used. Next, during the model selection phase we pick the best models based on the interaction ratio of the model over the predictions on the target task (See Section 3.3.3 for details). No explicit negative examples are used in this part; the interaction ratio is simply used to pick the best model.

We initially chose a positive:negative class ratio of 1:100 meaning that we expect 1 in every 100 random bacteria-human protein pairs to interact with each other. This has been a common practice in host-pathogen PPI prediction in the past (Dyer et al., 2007; Tastan et al., 2009). Recently published work (Mukhtar et al., 2011) involving a yeast-2-hybrid study on plant-bacterial PPIs suggests a higher interaction ratio of around 1:1000. Our choice of 1:100 as the class-skew is an overestimate when considering interactions with all *Salmonella* genes, but if we restrict the binding partners to only the so-called *Salmonella* effector proteins, the ratio we use is reasonable. (There are ≈85 known *Salmonella* effector genes). Also note that, while the exact examples that we choose as negative data may not be true negatives, we expect the false negative rate to be low enough (≈ 1%) to justify our choice of this heuristic.

The class skew is an important parameter in any machine learning method. The choice of this parameter determines the properties of the resultant model. A very balanced class skew of 1:1 will result in a model that is over-predictive i.e., has a very high false positive rate when applied on the target task. On the other hand, a very skewed setting of 1:1000 could give a lower false positive rate but is likely to have a poor recall as compared to models with lower class skews. This parameter thus offers a trade-off between the precision and recall of the resultant model. Our choice of a class ratio of 1:100 will result in a higher recall as compared to models trained on higher class skews. It will however, have some false positives. From a statistical perspective, a model trained with a high class skew such as 1:1000 will capture the distribution of the negatives since they hugely outnumber the positives. Since the negative class examples are not true negatives, the goodness of a model which depends mostly on noisy negatives is debatable. Computationally, the time required for training a model increases as we increase the number of examples. In the case of a high class skew such as 1:1000, there will be thousand times as many examples as the number of positives. This makes training a model very slow, especially for the Kernel-SVM algorithm and Transductive SVM models that are used by our methods. Nonetheless, we also calculated the predictions for a higher skew of 1:500. The results are described in Section 5.

The features used in each approach are shown in **Table 1**. A detailed description of each feature and the biological significance of it follows. We derive protein sequence based features similar to the ones derived by Dyer et al. (2011) for HIV-human PPI prediction.

- **Protein sequence *n*-mer or *n*-gram features**: Since the sequence of a protein determines its function to a great extent, it may be possible to predict PPIs using the amino acid sequence of a protein pair. Shen et al. (2007) introduced the "conjoint triad model" for predicting PPIs using only amino acid sequences. Shen et al. (2007) partitioned the twenty amino acids into seven classes based on their electrostatic and hydrophobic properties. For each protein, they counted the number of times each distinct three-mer (set of three consecutive amino acids) occurred in the sequence. To account for protein size, they normalized these counts by linearly transforming them to lie between 0 and 1 (see Shen et al. (2007) for details). They represented the protein with a 343-element feature vector, where the value of each feature is the normalized count for each of the 343 (7^3) possible amino acid three-mers. We use two-, three-, four-, and five-mers. For each host-pathogen protein pair, we concatenated the feature vectors of the individual proteins. Therefore, each host-pathogen protein pair had a feature vector of length at most 98, 646, 4802, and 33614, in the cases of two-, three-, four-, and five-mers, respectively.
- **Gene expression features**: These features depend only on the human protein (gene) involved in a human-*Salmonella* protein pair. We selected 3 transcriptomic datasets from GEO (Barrett et al., 2011), which give the differential gene expression of human genes infected by *Salmonella*. The 3 datasets (GDS77, GDS78, GDS80) give us a total of 7 features representing differential gene expression of human genes in 7 different control conditions. The intuition behind this feature is that genes that are significantly differentially regulated are more likely to be involved in the infection process, and thereby in interactions with bacterial proteins. Note: these were used in only the human-*Salmonella* task.
- **GO similarity features**: These features model the similarity between the functional properties of two proteins. These were used in only the human-*Salmonella* task. Gene Ontology (Ashburner et al., 2000) provides GO-term annotations for three important protein properties: molecular function (F), cellular component (C) and biological process (P). We derive 6 types of features using these properties. For each of "F," "C," and "P," two types of GO similarity features were defined: (a) pair-level similarity and (b) similarity with human protein's binding partners. The similarity between two individual GO terms was computed using the G-Sesame algorithm (Du et al., 2009). This feature is a matrix of all the GO term combinations found in a given protein pair: $< p_s, p_h >$, the rows of the matrix represent GO terms from protein p_s and the columns represent GO terms from p_h. Analogously, the second feature type-(b) computes the similarity between the GO term sets of the *Salmonella* protein and the human protein's binding partners in the human interactome. We used HPRD to get the human interactome.

Code: The executable files from the packages used to build our methods, and the scripts that we used to run these can be downloaded here: http://www.cs.cmu.edu/~mkshirsa/data/frontiers2014/code.zip.

5. RESULTS AND DISCUSSION

A quantitative evaluation on the target task i.e., plant-*Salmonella* is currently not feasible as there is no known PPI data. Hence for

the purpose of evaluation, we used some of the PPI datasets as "sources" for building a model and one as the "target." We evaluate the machine-learning based methods in two settings of transfer: *pathogen-level transfer*, where the host is fixed to be human and the pathogen is one of various bacterial species. The second setting *host-level transfer*, is more relevant and refers to the case where the pathogen is fixed to be *Salmonella* and we modify the host species. Since there are few known PPIs involving *Salmonella*, we are only able to experiment with mouse as an alternate host. There are 14 known mouse-*Salmonella* PPIs. Interestingly they involve mouse proteins whose human homologs also interact with the same *Salmonella* proteins—i.e., these 14 PPIs have interologs in the human-*Salmonella* dataset.

Our evaluation criteria does not use accuracy (which measures performance on both the positives and negatives). Our PPI datasets are highly imbalanced with a large number of negative samples, and a trivial classifier that calls all protein pairs as "negative" will achieve a very good performance. So we instead use precision (P), recall (R) and F-score(F1) computed on the interacting pairs (positive class).

$$\text{Precision(P)} = \frac{\text{true positives}}{\text{predicted positives}};$$

$$\text{Recall(R)} = \frac{\text{true positives}}{\text{total true positives in data}};$$

$$\text{F1 score (F1)} = \frac{2PR}{P+R}.$$

The source tasks (i.e., training data) and target task (i.e., test datasets) are shown in the **Table 2**. Parameters for all methods are tuned using a class-skew based model selection similar to the one described in Section 3.3.3 for the KMM-SVM method. We compare the following machine-learning based methods:

1. Inductive Kernel-SVM (Baseline): This model assumes that the source and target distributions are identical. All source data is pooled together and used to build a single model. For the kernel we used the RBF-spectrum kernel.
2. Transductive SVM (T-SVM): This is the method described in Section 3.2.
3. KMM-SVM: This method is discussed in Section 3.3.

The host-level transfer performance is shown in the first two rows of **Table 2**. The KMM-SVM based method performs much better while transferring from *Salmonella*-human to *Salmonella*-mouse. The recall is very high at 93.7 since the mouse-pathogen PPIs are interologs of the human-pathogen PPIs. The precision is not as high as some additional positives are predicted and we found that they had a high classifier score. These "false positives" are likely to be true interactions. For the reverse setting, T-SVM does slightly better than the KMM-SVM and 2 points higher than the baseline. Note that here, the source data is very small in size with only 14 PPIs. In the pathogen-level transfer, on the *Salmonella*-human target task, the F1 of the KMM-SVM method is the highest at 19.9 and is 5 points better than the other two methods. On the *E.coli*-human task, the performance is 18.4 which is 5.7 points better than the other methods.

Table 2 | Performance of the machine learning based methods on various transfer settings.

Source task(s) (training data)	Target task (test data)	Method	P†	R†	F1†
HOST-LEVEL TRANSFER					
Salmonella-human	*Salmonella*-mouse	Baseline	42.8	**93.7**	58.8
		T-SVM	45.4	**93.7**	61.2
		KMM-SVM	**51.7**	**93.7**	**66.7**
Salmonella-mouse	*Salmonella*-human	Baseline	95.4	33.8	50
		T-SVM	67.5	43.5	**52.9**
		KMM-SVM	**100**	**35.5**	52
PATHOGEN-LEVEL TRANSFER					
Francisella-human, *E.coli*-human	*Salmonella*-human	Baseline	17.8	12.9	14.9
		T-SVM	15	14.5	14.7
		KMM-SVM	**25.7**	**16.1**	**19.9**
Francisella-human, *Salmonella*-human	*E.coli*-human	Baseline	12.9	12.5	12.7
		T-SVM	10.4	15.6	12.5
		KMM-SVM	**15.9**	**21.9**	**18.4**

We compare them with a simple baseline: inductive kernel-SVM. We report precision (P), recall (R) and f-score (F1). The data that was used to build each of the models is shown in the first column. The second column shows the target task—the data on which we evaluate the model. The numbers in bold font indicate the highest performance in that column (i.e., for that metric).

†Computed using the default classifier threshold: 0.5.

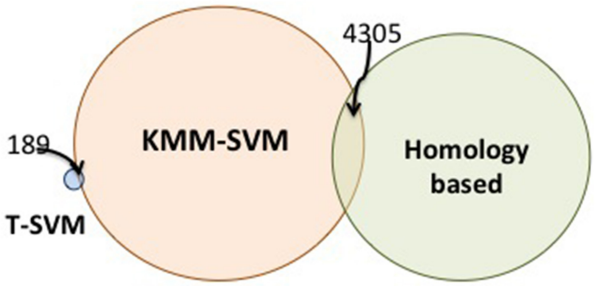
The positive:negative class ratio in all datasets was 1:100.

The performance of a random classifier would be F-score = 1.

A very interesting observation to make from the table is, the performance on the target task: *Salmonella*-human in the two settings. In the host-level transfer, the F1 is 52 whereas in the pathogen-level transfer it is much lower at 19.9. The hosts human and mouse are much more similar than the group of bacterial species namely: *Salmonella*, *E. coli* and *F. tularensis*. The source tasks are indeed very critical in determining the performance on the target.

5.1. ANALYSIS

We apply the models trained using the procedures from previous sections on *Arabidopsis*-*Salmonella* protein-pairs to get predictions for potential interactions. The homology based approach does not assign any confidence scores to the predictions while both T-SVM and KMM-SVM allow us to obtain a score for every predicted interaction. All predictions from T-SVM with a positive score (>0) are considered to be interacting. For the KMM-SVM method, we filter the predictions using a threshold of 0.7 on the averaged probability-score. (See Section 3.3.3 for details on the probability score computation for the KMM-SVM method). We chose this threshold of 0.7 since all positives in our training data are assigned a score $\geq$0.7 by the classifier model. The full lists of predicted interactions from all three approaches are available at the following link: http://www.cs.cmu.edu/~mkshirsa/data/frontiers2014/predictions.zip.



Approach	Homology	T-SVM	KMM-SVM
# predicted interactions	106,807	1088	163,644
# PPIs involving effectors	72461	718	163,397
# unique arabidopsis genes	1107	92	25124
# unique *Salmonella* genes	221	34	31

FIGURE 5 | Overlap amongst the novel PPI predictions from each approach. All predictions from the homology based approach and the T-SVM are shown. For the KMM-SVM method, we filter the predictions using a threshold of 0.7 on the interaction probability reported by the classifier. We picked this threshold based on the interaction probabilities reported on the known interactions.

The total number of PPI predictions based on the score thresholds described above are: 106,807 for homology-based, 1088 for T-SVM and 163,644 from KMM-SVM. Hundreds of thousands of interacting pairs may not be likely and we therefore expect that many of the predictions are likely to be false positives (FPs). We would like to emphasize that, by ranking the predictions on the classifier scores and picking only the top few we are likely to filter out most of the false positives, since the machine learning models are expected to score FPs lower than the true positives. The threshold of 0.7 for KMM-SVM was chosen just to ensure consistency with the threshold that we observed in the training data (i.e., in the known interactions). If one considers say the top 10% of the predictions from the KMM-SVM method, we have 1636 PPIs over ≈1300 unique *Arabidopsis* proteins and 5 *Salmonella* proteins. Choosing by thresholding the prediction score is one way to select potential interactions for further scrutiny. Another approach is to analyze the predictions based on the biological functions one is interested in. To demonstrate the type of biological functions that are represented in the predictions, we performed GO term enrichment analysis of the *Arabidopsis* proteins involved in the predictions. We can then look at *Arabidopsis* genes with the most enriched GO terms and what their predicted *Salmonella* partners are.

A Venn diagram depicting the overlap between the predicted pairs of proteins interacting according to the three approaches is shown in **Figure 5**. The PPIs reported by each approach are quite different from the others. Only 189 are shared between T-SVM and KMM-SVM and 4305 between the homology approach and KMM-SVM. No overlap was found between the homology approach and the T-SVM approaches. These relatively small overlaps are due to the different input sources (tasks) used by each approach. Further, the machine-learning based approaches KMM-SVM and T-SVM use a discriminative model which employs negative examples whereas the heuristics based approach does not use any such negative data and hence has a small overlap with the other two. The two machine-learning based approaches differ due to the use of different kernels. The KMM-SVM approach is the only approach that shows overlap in predictions to both, the heuristics and the T-SVM approaches, and the results are therefore discussed in detail in the accompanying paper (Schleker et al., 2015).

Because the ratio of 1 positive to 100 negative pairs likely overestimates the number of interactions, we next changed this ratio to 1:500 and generated a new model. As expected, a much smaller number of pairs are predicted namely, 6035. This is a more manageable list and the predictions of the new model are provided at http://www.cs.cmu.edu/∼mkshirsa/data/frontiers2014/predictions_class_skew_500.txt.

5.2. QUALITATIVE ANALYSIS OF PREDICTED INTERACTIONS

As with any predictions, experimental validation is ultimately needed to verify them. The choice depends on the interest of the experimentalist. Here we have chosen for discussion a few predictions that are interesting to us, but we encourage the reader to look at the list of predictions for others of potential biological interest.

We calculated Gene Ontology (GO) enrichment in the *Arabidopsis* proteins predicted to be targeted by the *Salmonella* proteins. We are interested in analyzing the characteristics of the plant proteins predicted to be the most popular targets for pathogenesis. We defined the "popular targets" using the following criteria: (a) the *Arabidopsis* protein is predicted to be targeted by at least 3 *Salmonella* effectors with a probability greater than 0.9 and (b) the GO term annotations of the *Arabidopsis* protein are significantly enriched [with a p-value of <0.001 as obtained by GO enrichment analysis using FuncAssociate (Berriz et al., 2003)]. There are a total of 5247 *Arabidopsis* proteins satisfying these criteria. In **Table 3**, we show 20 *Arabidopsis* genes selected randomly from this set of highly targeted *Arabidopsis* proteins. In **Table 4**, we show the list of all enriched GO terms.

For each gene we show the description and the enriched GO annotations. Among the presented *Arabidopsis* proteins, nearly one third are transcription factors. These function e.g., in hormone-mediated signaling pathways. It has been reported that jasmonic acid and ethylene signaling pathways are involved in plant defense response against *Salmonella* (Schikora et al.,

Table 3 | GO terms that were enriched in the most targetted *Arabidopsis* proteins in our predictions.

Arabidopsis (TAIR id)	Protein name/gene	Enriched Gene Ontology annotations	Enriched GO terms (corresp. to column 3)
AT1G01030	B3 domain containing transcription factor	Sequence-specific DNA binding transcription factor activity ; regulation of transcription, DNA-templated	GO:0003700 GO:0006355
AT1G06160	Ethylene-responsive transcription factor ERF094	DNA binding ; sequence-specific DNA binding transcription factor activity ; regulation of transcription from RNA-polymerase II promoter ; response to jasmonic acid stimulus	GO:0003677 GO:0003700 GO:0006355 GO:0009753
AT1G01060	Myb-related putative transcription factor	Response to cadmium ion ; response to salt stress ; response to auxin stimulus ; response to cold	GO:0046686 GO:0009651 GO:0009733 GO:0009409
AT1G13180	Actin-related protein 3	Actin binding	GO:0003779
AT2G40220	Ethylene-responsive transcription factor ABI4. Protein glucose insensitive 6	DNA binding ; response to water deprivation ; positive regulation of transcription, DNA-dependent ; sequence-specific DNA binding	GO:0003677 GO:0009414 GO:0045893 GO:0043565
AT2G46400	Putative WRKY transcription factor 46	Response to chitin	GO:0010200
AT1G01080	Ribonucleoprotein, putative	nucleic acid binding ; RNA binding	GO:0003676 GO:0003723
AT3G12110	Actin-11	Chloroplast stroma	GO:0009570
AT3G56400	Probable WRKY transcription factor 70	Response to salicylic acid stimulus ; sequence-specific DNA binding transcription factor activity ; protein amino acid binding	GO:0009751 GO:0003700 GO:0005515
AT1G01090	Pyruvate dehydrogenase E1 component subunit alpha-3, chloroplastic	Chloroplast stroma	GO:0009570
AT4G09570	Ca-dependent protein kinase 4	protein amino acid binding	GO:0005515
AT1G01150	Homeodomain-like protein with RING-type zinc finger domain	Zinc ion binding ; regulation of transcription, DNA-templated	GO:0008270 GO:0006355
AT4G18170	Probable WRKY transcription factor 28	Regulation of transcription, DNA-templated ; sequence-specific DNA binding transcription factor activity	GO:0006355 GO:0003700
AT1G01160	GRF1-interacting factor 2	Protein amino acid binding	GO:0005515
AT1G01200	Ras-related protein RABA3	GTP binding; small GTPase mediated signal transduction ; protein transport	GO:0005525 GO:0007264 GO:0015031
AT5G47220	Ethylene-responsive transcription factor 2	Positive regulation of transcription, DNA-dependent ; ethylene mediated signaling pathway	GO:0045893 GO:0009873
AT1G01250	Ethylene-responsive TF ERF023	Sequence-specific DNA binding transcription factor activity ; nuclear envelope	GO:0003700 GO:0005634
AT1G01350	Zinc finger CCCH domain-containing protein 1	Nucleic acid binding ; zinc ion binding	GO:0003676 GO:0008270
AT1G01370	Histone H3-like centromeric protein HTR12	DNA binding ; protein amino acid binding	GO:0003677 GO:0005515

To get this list, we performed a GO term enrichment analysis using the FuncAssociate tool (Berriz et al., 2003). We then procure the set of Arabidopsis genes which correspond to the enriched GO terms; i.e., GO terms with a p-value of <0.001. We further filter this set to include only those Arabidopsis genes predicted to interact with at least 3 Salmonella effector proteins. In this table, we show around 20 such Arabidopsis genes for the lack of space. The remaining are available via the download link.

Table 4 | List of all enriched GO terms obtained by applying enrichment analysis tool FuncAssociate (Berriz et al., 2003) on the set of highly targeted *Arabidopsis* proteins (i.e., *Arabidopsis* proteins predicted to interact with at least 3 *Salmonella* effectors).

GO term	Description
GO:0003676	Nucleic acid binding
GO:0003677	DNA binding
GO:0003700	Sequence-specific DNA binding TF activity
GO:0003723	RNA binding
GO:0003735	Structural constituent of ribosome
GO:0003755	peptidyl-prolyl cis-trans isomerase activity
GO:0003779	Actin binding
GO:0003899	DNA-directed RNA polymerase activity
GO:0004298	Threonine-type endopeptidase activity
GO:0004693	Cyclin-dependent protein serine/threonine kinase activity
GO:0004842	Ubiquitin-protein transferase activity
GO:0004871	Signal transducer activity
GO:0005484	SNAP receptor activity
GO:0005507	Copper ion binding
GO:0005509	Calcium ion binding
GO:0005515	Protein binding
GO:0005525	GTP binding
GO:0005576	Extracellular region
GO:0005622	Intracellular region
GO:0005634	Nuclear envelope
GO:0005839	Proteasome core complex
GO:0005840	Ribosome
GO:0006351	Transcription, DNA-templated
GO:0006355	Regulation of transcription, DNA-templated
GO:0006412	Translation
GO:0006413	Translational initiation
GO:0006457	Protein folding
GO:0006511	Ubiquitin-dependent protein catabolic process
GO:0007264	Small GTPase mediated signal transduction
GO:0007267	Cell-cell signaling
GO:0008233	Peptidase activity
GO:0008270	Zinc ion binding
GO:0008794	Arsenate reductase (glutaredoxin) activity
GO:0009408	Response to heat
GO:0009409	Response to cold
GO:0009414	Response to water deprivation
GO:0009570	Chloroplast stroma
GO:0009579	Thylakoid
GO:0009651	Response to salt stress
GO:0009733	Response to auxin
GO:0009737	Response to abscisic acid
GO:0009739	Response to gibberellin
GO:0009751	Response to salicylic acid
GO:0009753	Response to jasmonic acid
GO:0009828	Plant-type cell wall loosening
GO:0009873	Ethylene mediated signaling pathway
GO:0010200	Response to chitin
GO:0015031	Protein transport
GO:0015035	Protein disulfide oxidoreductase activity
GO:0016491	Oxidoreductase activity
GO:0016607	Nuclear speck
GO:0016762	Xyloglucan:xyloglucosyl transferase activity
GO:0022626	Cytosolic ribosome
GO:0022627	Cytosolic small ribosomal subunit
GO:0042254	Ribosome biogenesis
GO:0042742	Defense response to bacterium
GO:0043565	Sequence-specific DNA binding
GO:0045454	Cell redox homeostasis
GO:0045892	Negative regulation of transcription, DNA-templated
GO:0045893	Positive regulation of transcription, DNA-templated
GO:0046686	Response to cadmium ion
GO:0046872	Metal ion binding
GO:0051726	Regulation of cell cycle

(Continued)

The shown terms had a p-value less than 0.001.

2008). Other examples that highlight the role of transcription factors in plant-pathogen interaction are e.g., that a *Xanthomonas* effector protein targets an ethylene responsive transcription factor (ERF) in tomato to inhibit ethylene induced transcription (Kim et al., 2013) and systemic immunity in barley induced by *Xanthomonas* and *Pseudomonas* bacteria may involve WRKY and ERF-like transcription factors (Dey et al., 2014). Further, actin-11 and actin-related proteins involved in actin polymerization and depolymerization are obtained. It is well known that *Salmonella* translocates effectors into the mammalian host cell in order to interact with actin and e.g., modify the cell cytoskeleton to allow bacterial entry (for review see Schleker et al., 2012). Our analysis revealed growth regulating factor 1 (GRF1)-interacting factor 2, a transcriptional co-activator which is part of a regulatory complex with GRF1 and microRNA (miRNA) 396. MiRNAs are involved in plant disease resistance to bacteria and miRNA396 has been shown to be upregulated in plants upon flg22 treatment (Li et al., 2010). Liu et al. (2014) reported that putative GRF1 targets in *Arabidopsis* are heavily involved in biosynthetic and metabolic pathways, e.g., phenylpropanoid, amino acids and lignin biosynthesis as well as plant hormone signal transduction indicating the role of GRF1 in plant defense mechanisms. Other examples of predicted interactions and more details of their possible relevance in *Salmonella*-plant interplay are discussed in the accompanying paper (Schleker et al., 2015).

5.3. LIMITATIONS AND FUTURE WORK

In this paper, we addressed the challenge of predicting the *Salmonella-Arabidopsis* interactome in the absence of any experimentally known interactions. Previous work in this area was based purely on homology between human and *Arabidopsis* proteins and was therefore limited to proteins that do display sequence similarity. Due to the large divergence between the two organisms, this approach neglects a large fraction of potential *Arabidopsis* targets. We therefore presented here three different sophisticated computational and machine learning methods to

predict hereto unknown *Salmonella*-plant interactions from a relatively small list of known *Salmonella*-human interactions. This is a very challenging task because it is not possible to quantitatively validate the predictions. Nonetheless, the predictions provide a gold-mine for discovery because they provide experimentally testable hypotheses on the communication mechanisms between plant and *Salmonella* without restriction to known effectors in the pathogen or sequences of similarity to those observed in better studied eukaryotic organisms. With these advantages comes a set of limitations to be aware of.

Since machine learning methods need some known interactions to evaluate the models on, and to pick the best set of predictions, their application in the current paper has limitations. For example, we can obtain different predictions from our methods by varying the parameters, especially the class skew (we studied the ratios 1:100 and 1:500 in this paper). Because there are currently no known *Salmonella*-plant interactions, we are not able to quantify which of these sets of predictions is more reliable. Augmenting the predictions with some other biological information from the target task can help in picking the most plausible PPIs. This is a direction for future research. Further,

1. The interactome predicted by each method is not the true interactome, but is a set of predictions. There will be false positive and false negative interactions. Thus, each individual prediction has to be considered a hypothesis not a fact.
2. In line with point 1 above, the size of the predicted interactomes does not necessarily relate to the true interactome. We dont know how many interactions to expect. Our different predictions vary greatly in size, with one method predicting only one thousand interactions, while others predict more than 100,000 interactions. While it is more likely that smaller numbers of interactions are more likely, it does not mean that this method is inherently better than the other methods.
3. The size of the predicted interactions list also depends on a critical parameter, the positive to negative class ratio. This parameter is important but it is tuneable, so the methods validity is not dependent on its choice. However, it is important to appreciate that the predictions will differ greatly when this parameter is changed. Thus, biological insight in choosing predictions to validate still needs to be applied, regardless of the prior choice of ratio in generating the model.

These general limitations in the context of the specific results of the models presented here translate to the following issues, pointed out by a reviewer of this paper: The data presented for the KMM-SVM model indicate that 163,644 PPIs are predicted (**Figure 5**). This is of the same order of magnitude as the number of false positives that would be predicted, given the reported false positive rate of the method that indicate ≈180,000 false positive PPIs would be expected. This raises the possibility that the bulk of the predictions may be false positives. The data presented for the KMM-SVM model also indicates that 25,124 distinct *Arabidopsis* genes participate in PPIs with 31 distinct *Salmonella* genes (**Figure 5**). This implies that 91% of the *Arabidopsis* protein-coding gene complement (TAIR10: 27,416 genes— http://www.arabidopsis.org/portals/genAnnotation/gene_structural_annotation/annotation_data.jsp) enters into productive interaction with only 31 *Salmonella* proteins. It also implies that, on average, each interacting *Salmonella* protein is capable of productive interaction with over 5000 *Arabidopsis* proteins. It is unlikely that this is the case, again suggesting that a large number of false positives have to be expected.

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Comparing human–*Salmonella* with plant–*Salmonella* protein–protein interaction predictions

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Salmonellosis is the most frequent foodborne disease worldwide and can be transmitted to humans by a variety of routes, especially via animal and plant products. *Salmonella* bacteria are believed to use not only animal and human but also plant hosts despite their evolutionary distance. This raises the question if *Salmonella* employs similar mechanisms in infection of these diverse hosts. Given that most of our understanding comes from its interaction with human hosts, we investigate here to what degree knowledge of *Salmonella*–human interactions can be transferred to the *Salmonella*–plant system. Reviewed are recent publications on analysis and prediction of *Salmonella*–host interactomes. Putative protein–protein interactions (PPIs) between *Salmonella* and its human and *Arabidopsis* hosts were retrieved utilizing purely interolog-based approaches in which predictions were inferred based on available sequence and domain information of known PPIs, and machine learning approaches that integrate a larger set of useful information from different sources. Transfer learning is an especially suitable machine learning technique to predict plant host targets from the knowledge of human host targets. A comparison of the prediction results with transcriptomic data shows a clear overlap between the host proteins predicted to be targeted by PPIs and their gene ontology enrichment in both host species and regulation of gene expression. In particular, the cellular processes *Salmonella* interferes with in plants and humans are catabolic processes. The details of how these processes are targeted, however, are quite different between the two organisms, as expected based on their evolutionary and habitat differences. Possible implications of this observation on evolution of host–pathogen communication are discussed.

Keywords: host–pathogen interactions, systems biology, prediction, pathways, interactome

INTRODUCTION

Salmonella are Gram-negative bacteria comprising more than 2500 known serovars (Abraham et al., 2012). The pathogen has an unusually broad host range infecting diverse species, including humans, sheep, cows, reptiles, and plants. *Salmonella* causes severe worldwide health problems in developing as well as developed countries and constitutes one of the main causes of foodborne diseases in humans (World Health Organization, 2014). The bacteria can be transmitted to humans, e.g., through infected animals, contaminated meat, fish, and egg products, water, vegetables, and fruits. Diseases caused by *Salmonella* are divided into typhoid fever which is caused by *S.* Typhi and *S.* Paratyphi and Salmonellosis (diarrheal diseases) caused by a variety of non-typhoidal *Salmonella* (NTS) serovars, mainly *S.* Enteritidis and *S.* Typhimurium. In the USA alone, more than one million cases are reported annually (Centers for Disease Control and Prevention, 2014b). Besides reports on *Salmonella* outbreaks due to the consumption of contaminated animal products, there are many cases where an outbreak was ascribed to contaminated vegetables and fruits or processed plant products. For instance, in 2011, the consumption of mung bean sprouts lead to a Salmonellosis outbreak in Germany (Abu Sina et al., 2012). In the USA, 25% of all reported multistate outbreaks of *Salmonella* in 2012 and 2013 have been linked to plant products, e.g., peanut butter, mangoes, and cucumbers (Centers for Disease Control and Prevention, 2014a).

While *Salmonella* may not require active invasion of plant tissue as part of its transmission mechanism, it is now well established that they are able to invade plant cells and proliferate inside plants (Schikora et al., 2008). This makes plants a bona fide host for *Salmonella*, and poses the question to what extent the communication between *Salmonella* and its plant host are related to the better established interaction with the human host. One means of communication between any pathogen and its hosts are via protein–protein interactions (PPIs). *Salmonella*–human interactions are much better studied than *Salmonella*–plant interactions, and the question is to what extent the knowledge can be transferred from the human to the plant system. For its mammalian

hosts, it is known that *Salmonella* exerts its pathogenicity by injection of proteins, called effectors, into the host cell. These effectors interact with host proteins and thereby influence host mechanisms for the pathogen's benefit. The best characterized set of *Salmonella* effectors are those encoded on *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and -2). These gene clusters contain the genetic information of the proteins building the type three secretion systems 1 and 2 (TTSS-1 and -2) which *Salmonella* utilizes to translocate the SPI-1 and -2 encoded effectors into the host cell. These effectors then interact with host proteins, the host–pathogen interactome, which is of major importance for the functional interplay between the two organisms.

Here, we will first review the evidence for experimentally demonstrated functional interactions between *Salmonella* proteins and plant cellular targets (see Evidence for Plants as Bona Fide Hosts for *Salmonella*). We will then briefly summarize the current state of knowledge on the *Salmonella*–human interactions (see Known *Salmonella*–Human Protein–Protein Interactions), which is the best studied host organism and has been extensively reviewed elsewhere (Haraga et al., 2008; McGhie et al., 2009; Heffron et al., 2011; Schleker et al., 2012b). Because the number of known interactions, even for human, is still small, we will then review recent results on the predicted *Salmonella*–human entire interactome (see The *Salmonella*–Human Predicted Interactome), followed by the extrapolation to the plant host (see The *Salmonella*–*Arabidopsis* Predicted Interactome). We will then compare the *Salmonella*–plant interactome with available plant experimental data (see Comparison of Plant–*Salmonella* PPI Prediction Results with Experimental Data) and finally compare the two host (plant and human) responses with each other (see Comparison of Plant and Human Host Responses to *Salmonella* Challenge).

EVIDENCE FOR PLANTS AS BONA FIDE HOSTS FOR *Salmonella*

Although *Salmonella* is better known as a human and animal pathogen, and the majority of human infections are incurred via animal product routes such as meat and eggs, there is growing evidence that *Salmonella* actively interacts with plants and utilizes these organisms as alternative hosts making *Salmonella* a true plant pathogen. Infection studies coupled to fluorescence microscopy demonstrated that *Salmonella* actively invades the interior of alfalfa sprouts (Gandhi et al., 2001; Dong et al., 2003), lettuce (Klerks et al., 2007b) and enters *Arabidopsis* cells and propagates there (Schikora et al., 2008). While some infected plants may not always show symptoms of infection (Gandhi et al., 2001; Charkowski et al., 2002; Islam et al., 2004; Barak et al., 2005; Klerks et al., 2007a; Lapidot and Yaron, 2009; Noel et al., 2010; Shirron and Yaron, 2011), a reduction in biomass production, wilting, chlorosis, and death of infected organs has clearly shown that plants can exert disease symptoms due to *Salmonella* infection (Klerks et al., 2007b; Schikora et al., 2008). Furthermore, contact of *Salmonella* with plants activates plant defense responses and the expression of pathogenesis-related (PR) genes. Flagella of *S.* Typhimurium are recognized by plants and the bacteria activate salicylic acid (SA)-dependent and -independent defense responses (Iniguez et al., 2005). Moreover, kinase activity assays and qRT-PCR analysis revealed that *S.* Typhimurium activates mitogen-activated protein kinase (MAPK) cascades in *Arabidopsis*. Furthermore, SA, jasmonic acid (JA), and ethylene (ET) signaling pathways contribute to the defense response (Schikora et al., 2008). Several PR genes are upregulated in infected plants including PR1, 2, and 4 and the plant defensin PDF1.2 (Iniguez et al., 2005; Schikora et al., 2008) with the enhanced expression of PR1 being dependent on a functional TTSS-1 (Iniguez et al., 2005). TTSS-1 and -2 effectors are clearly important for *Salmonella* pathogenicity in plants as evidenced by the observation that mutants compromised in these secretion systems proliferate slower in *Arabidopsis* compared to the wild type (WT). Further, these mutants seem to be unable to inhibit the plant hypersensitive response (HR; Schikora et al., 2011; Shirron and Yaron, 2011). Thus, functional TTSS-1 and -2 are necessary to suppress plant defense responses implying that *Salmonella* utilizes the same proteins to communicate with its mammalian and plant hosts.

KNOWN *Salmonella*–HUMAN PROTEIN–PROTEIN INTERACTIONS

Protein–protein interactions constitute an important part of the communication between a host and its pathogen and thus, are fundamental to understanding the biological processes occurring during infection. Interactions between primarily human and *Salmonella* and their biological significance have been reviewed previously (Haraga et al., 2008; McGhie et al., 2009; Heffron et al., 2011; Schleker et al., 2012b). A recent extensive literature and data base survey, screening more than 2200 journal articles and over 100 databases, revealed that there is relatively little published information available: only 58 direct and 3 indirect PPIs between 22 *Salmonella* TTSS-1 and -2 effectors and 49 mammalian proteins (including 40 human proteins) could be retrieved by our literature survey (Schleker et al., 2012b). In the meantime two more interactions have been published adding two *Salmonella* effectors and two human proteins to the list of *Salmonella*–human interactions (Spano et al., 2011; Odendall et al., 2012). With these interactions *Salmonella* interferes with a variety of host cellular processes for its benefit. For instance, these PPIs trigger the modification of the actin cytoskeleton, recruitment of vesicles, the formation of the *Salmonella* containing vacuole (SCV) and tubulation and interfere with cytokine secretion, inflammatory response, antigen presentation by major histocompatibility complexes (MHC) I and II and apoptosis.

THE *Salmonella*–HUMAN PREDICTED INTERACTOME

Because our knowledge of known *Salmonella*–mammalian interactions is limited, several publications describe the prediction of PPIs between *Salmonella* and human proteins. These include three interolog approaches where putative *Salmonella*–human PPIs are obtained by sequence and/or domain comparison to proteins of known interactions (Krishnadev and Srinivasan, 2011; Arnold et al., 2012; Schleker et al., 2012a). Thus, interolog approaches make use of available information from sequence, domain, and PPI databases. Whereas Krishnadev and Srinivasan (2011) used iPfam and DIP databases as information input to their approach, Arnold et al. (2012) used DIMA 3.0 and

SIMAP databases and Schleker et al. (2012a) obtained domain information from iPfam and 3DID databases, protein sequence information from uniprot and known PPIs from BIANA that integrates available data from 10 PPI databases. Due to the fact that the retrieved list of putative *Salmonella*–human PPIs is unranked, techniques to filter the predictions are needed in order to obtain a subset of interesting and relevant PPIs. In the three interolog studies, the predicted interactions were filtered by different methods according to properties of the *Salmonella* protein (Krishnadev and Srinivasan, 2011; Schleker et al., 2012a). For example, these properties may include whether the protein contains a predicted transmembrane helix or an extracellular signal peptide (Krishnadev and Srinivasan, 2011), or the predicted list of human interaction candidates was ranked according to the degree of these candidates in the human intraspecies network thereby underlying the assumption that *Salmonella* effectors tend to interact with host hub proteins (Arnold et al., 2012). Another filtering approach was to apply the GUILD method (Guney and Oliva, 2012; Schleker et al., 2012a). GUILD is a genome-wide network-based prioritization framework based on known human disease genes and disease-gene prioritization algorithms. Thereby, GUILD indicates the putative human target proteins that are involved in human pathology and assigns a score to each target.

Conceptually different from the interolog approaches, Kshirsagar et al. (2012) described a supervised machine learning approach that integrates information from diverse sources, including but not limited to interolog information. Here, the set of known interactions (Schleker et al., 2012b) is used as gold standard and diverse data such as tissue expression, transcriptomic, gene ontology (GO), sequence, and domain information are used as features. In a so-called classification approach, a model is learnt from this gold standard and feature input to differentiate the two classes, interact and not interact. Because not all features are equally well studied, such integration is challenging and many features display what is known as the missing value problem, where values are available only for a subset of the interactions. Kshirsagar et al. (2012) therefore made use of techniques for missing value imputation in order to overcome the problem that often certain attributes are unavailable in the used data sources. Kshirsagar et al. (2012) used data from other related bacterial species, in addition to information from *Salmonella*. To transfer the information from the other closely related species to *Salmonella*, protein sequence alignment was carried out to define a measure of similarity between the proteins from the two species. Nearest-neighbor-based methods were then employed to combine such cross-species data. This approach proved superior to prediction techniques using generic imputation methods.

The best scored PPIs of the machine learning approach (score = 1) were with the *Salmonella* effectors SlrP and SspH2. Functional enrichment analysis for GO biological processes revealed that the putative human targets are involved in cellular processes related to, e.g., catabolic processes, proteolysis, cell death, regulation of transcription, regulation of kinase cascades, cytoskeleton organization, and cell cycle. Within the small subset of filtered PPIs obtained by Arnold et al. (2012) in the interolog approach, putative human interactors of SlrP and SspH2 are involved in the same processes as predicted by the machine learning approach. Whereas these studies concentrated on *Salmonella* TTSS-1 and -2 effectors, Krishnadev and Srinivasan, 2011 and Schleker et al. (2012a) additionally looked for putative interactions with any *Salmonella* protein and *Salmonella* virulence factor, respectively. The top 100 GUILD scored putative human targets were found to function in cell death, immune response, cytokine production and secretion, protein secretion, transport and localization, peptidase activity, and kinase cascades (Schleker et al., 2012a).

THE *Salmonella–Arabidopsis* PREDICTED INTERACTOME

To our knowledge, to date there has been no report on the experimental identification of any interaction between a *Salmonella* effector protein and a plant protein. We therefore have to rely on predictions for the time being. The above described interolog approach was also utilized to predict interactions between *Salmonella* and *Arabidopsis* (Schleker et al., 2012a). The resulting putative interactome highlighted the *Salmonella* effectors SptP, SspH1, SspH2, and SlrP as the proteins with the highest number of interactions (hub proteins). Further, a comparison of the putatively targeted human and *Arabidopsis* proteins indicated that similar processes appear affected by the infection in these two hosts. Considering that the function of more than half of the *Arabidopsis* protein-coding genes is unknown, this comparison could help to elucidate the biological functions of so far uncharacterized *Arabidopsis* proteins, for example, to identify pathogen recognition receptors (Schleker et al., 2012a).

The *Salmonella–Arabidopsis* interactome has also been predicted by several machine learning approaches (Kshirsagar et al., 2015). As no data on known *Salmonella*–plant PPIs is available, this technique builds a prediction model based on the known *Salmonella*–human PPIs (Schleker et al., 2012b) and knowledge of plant interactions with other pathogenic bacteria such as *E. coli* (Kshirsagar et al., 2015). In one approach a two-step procedure involving kernel mean matching (KMM) and a support-vector-machine (SVM) based classifier model was applied to infer high confidence *Salmonella–Arabidopsis* predictions. The predictions of the five best models were aggregated by taking into account (a) the majority voting for a given protein pair to interact or not and (b) the average probability score indicating the confidence that a predicted PPI occurs. Some of the *Arabidopsis* proteins of high-scoring PPIs are predicted to interact with many different effectors. The number of predicted interactions by the KMM model is very large (∼160,000 when using a cut-off of 0.7; see Kshirsagar et al., 2015 and the full list of predictions is available in the supplementary of this paper). Even when using a very stringent cut-off of 0.9 there are ∼66,000 and 0.98 there are still ∼6200. Additionally, Kshirsagar et al. (2015) increased the stringency of the prediction model by exchanging the setting of the parameter "ratio between protein interact to non-interact" from 1:100 to 1:500 thereby obtaining a smaller subset of the predicted PPIs assumed to potentially occur with higher confidence (high class skew model). Because these predictions are not experimentally verified, it is very challenging to choose individual predictions. The choice is inherently biased by interest and expertise of the investigator. We have chosen specific pairs of our interest for which there was also supporting evidence in the literature from

the predictions with the aim of demonstrating the utility of the predictions in generating biological hypothesis. Kshirsagar et al. (2015) had performed GO functional term enrichment analysis that has identified general trends, and we here further perform MapMan analysis for the set of ∼6200 to assist in analysis. From the general trends identified, we then chose specific pairs that are discussed in greater detail. For example, abscisic acid (ABA) insensitive 4 (ABI4) (also predicted by the high class skew model), an ET-responsive transcription factor involved in ABA signaling leading to callose deposition and stomata closure. ABI4 has been reported to thereby mediate resistance against the necrotrophic pathogens *Alternaria brassicicola*, *Plectosphaerella cucumerina*, and *Sclerotinia sclerotiorum* (Guimaraes and Stotz, 2004; Ton and Mauch-Mani, 2004). In the next section, we further interpret the prediction results from the KMM–SVM model from a biological point of view by comparing with published experimental data.

COMPARISON OF PLANT–*Salmonella* PPI PREDICTION RESULTS WITH EXPERIMENTAL DATA

Here, we provide a discussion on the biological significance of the available KMM–SVM model predictions (Kshirsagar et al., 2015) based on comparison with available experimental data. In particular, Schikora et al. (2011) had analyzed the transcriptomic response of *Arabidopsis* plants infected with four different bacteria: *S.* Typhimurium WT, a *prgH*$^-$ mutant with deficiency in expression of TTSS-1 genes, *Pseudomonas syringae*, and *E. coli* DH5alpha. About 30 *Arabidopsis* genes were exclusively differentially regulated upon infection with *Salmonella*. This included, for example, cytoskeleton-associated proteins. When comparing *Salmonella* WT and the *prgH*$^-$ mutant, a large portion of *Arabidopsis* genes specifically upregulated in the *prgH*$^-$ mutant were involved in the ubiquitin-dependent protein degradation process as well as cell wall, defense response and WRKY transcription factor clusters. The KMM–SVM classification approach predicts that proteins involved in these processes are putative targets of *Salmonella* effectors (Kshirsagar et al., 2015). Examples of how *Salmonella* interferes with plant defense response, gene activation, and plant metabolism are described below.

Salmonella INTERFERES WITH PLANT BASAL DEFENSE RESPONSE AND GENE ACTIVATION

A basic plant defense response mechanism is the induction of MAPK cascades as well as Ca^{2+} influx into the cell upon recognition of flg22 by the FLS2 (flagellin-sensing 2) receptor. Kinase activation results in phosphorylation of WRKY transcription factors and thus, in the induction of defense genes. Ca^{2+} leads to the activation of Ca^{2+}-dependent protein kinases (CDPKs) 4, 5, 6, 11 and the NADPH-oxidase RbohD (respiratory burst oxidase homolog protein D) that triggers a reactive oxygen species (ROS) burst (Segonzac and Zipfel, 2011; Gao and He, 2013). MAPK-dependent activation of the plant defense response against *S.* Typhimurium was demonstrated to be important because MPK3, MPK4, and MPK6 are rapidly activated upon *Salmonella* infection, and *mpk3* and *mpk6* mutants reveal accelerated susceptibility toward *S.* Typhimurium (Schikora et al., 2008). Further, genes coding for calcium-binding proteins have higher expression levels upon *Arabidopsis* infection with a *Salmonella prgH* deficiency mutant compared to the WT (Schikora et al., 2011). Several CDPKs, calcium-binding proteins and (putative) WRKYs are predicted to be targeted by *Salmonella* with the KMM–SVM modeling approach, for instance, CDPK4 and WRKY28. It has been shown that WRKY28 contributes to the induction of oxidative burst as well as the activation of SA-, JA-, and ET-dependent defense signaling pathways (Chen et al., 2013). All three hormone dependent pathways were shown to play a role in defense against *Salmonella* (Iniguez et al., 2005; Schikora et al., 2008).

Mitogen-activated protein kinases are known targets of bacterial effectors. For instance, MPK4 is phosphorylated by the *P. syringae* effector AvrB leading to the induction of the JA signaling pathway. This mechanism has been demonstrated to positively influence the growth of *P. syringae* (Cui et al., 2010). *P. syringae* effector HopF2 which exerts mono-ADP ribosyltransferase activity inhibits MKK5 activity (Wang et al., 2010). The KMM–SVM model predicts an interaction between *Salmonella* effectors and *Arabidopsis* mitogen-activated protein kinase kinase kinase ANP1 (Kshirsagar et al., 2015). ANP1 can be activated by H_2O_2 and induces the MPK3 and MPK6 signaling cascades thereby leading to the expression of defense-related genes (Kovtun et al., 2000).

Other transcription factors predicted to be targeted by *Salmonella* effectors are WRKY46, 53, and 70. Thilmony et al. (2006) found that the pathogen-associated molecular patterns (PAMP)-inducible transcription factor WRKY53 is significantly downregulated in *P. syringae* infected *Arabidopsis* plants thereby interfering with WRKY53-dependent cellular mechanisms. WRKY53 plays an important role in regulation of plant senescence and defense response as the transcription factor can be directly phosphorylated by MEKK1 (Zentgraf et al., 2010). *Arabidopsis* mutants in *wrky*46, 53, and 70 revealed increased susceptibility toward *P. syringae* leading the authors to the conclusion that these three transcription factors have an overlapping or synergistic role in regulating defense response against *P. syringae* (Hu et al., 2012).

Salmonella IMPACT ON PLANT METABOLISM

The KMM–SVM predictions indicate that *S.* Typhimurium effectors heavily target *Arabidopsis* metabolic and biosynthetic processes (Kshirsagar et al., 2015). For visualization, we utilized MapMan (Thimm et al., 2004), a software tool that assigns *Arabidopsis* proteins to specific plant processes and pathways, to see what metabolic pathways are putatively interfered with by *S.* Typhimurium effectors. A comparison with available transcriptomic data revealed a clear overlap in the pathways predicted to be targeted by *S.* Typhimurium effectors (**Figure 1A**) and those identified to involve genes that are upregulated upon *S.* Typhimurium *prgH*$^-$ vs. WT infection (**Figure 1B**; Schikora et al., 2011). In **Figure 1A**, every small blue square displays one *Arabidopsis* protein predicted by KMM–SVM to be targeted by one or more *S.* Typhimurium effectors. In **Figure 1B**, *Arabidopsis* genes known to be upregulated during *S.* Typhimurium *prgH*$^-$ vs. WT infection are visualized by white to blue small squares depending on the degree of upregulation. Thus, the darker blue the square is, the more efficiently this gene is suppressed by *S.* Typhimurium TTSS-1 effectors. In conclusion, the metabolic processes *S.* Typhimurium most intensively interferes with include

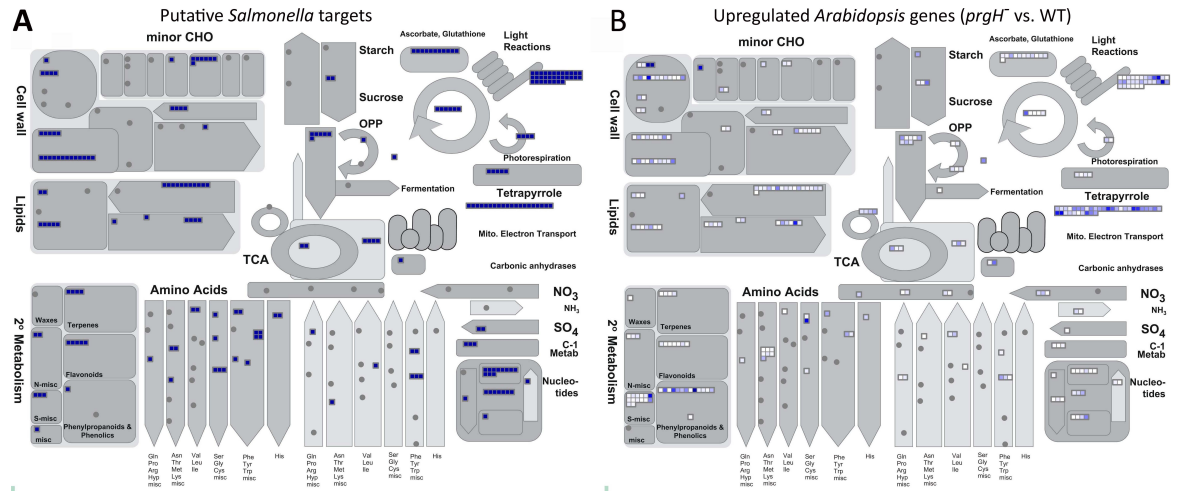

FIGURE 1 | Overviewof metabolic processes putatively targeted and known to be repressed by *S.* Typhimurium effectors. MapMan (Thimm et al., 2004) analysis providing a metabolism overview of **(A)** predicted *Arabidopsis* targets of *S.* Typhimurium effectors predicted with cut-offs of 1 (voting score) and 0.98 (probability aggregated score) by the KMM–SVM model (Kshirsagar et al., 2015) and **(B)** *Arabidopsis* genes experimentally identified to be upregulated upon infection with *S.* Typhimurium *prgH*⁻ vs. WT (Schikora et al., 2011). Each small square displays a predicted *Arabidopsis* target of *S.* Typhimurium **(A)** or an upregulated *Arabidopsis* gene **(B)**. In **(B)**, the color intensity visualizes the degree of upregulation.

those related to the cell wall, lipids, light reactions, tetrapyrrole, and secondary metabolism of, e.g., terpenes (**Figure 1B**).

The impact of the pathogen on plant metabolism may be general as parallels can also be found in available data for another pathogen, *P. syringae*. Thilmony et al. (2006) analyzed the transcriptional response of *Arabidopsis* infected with *P. syringae* WT and mutants with deficiencies in expressing COR (coronatine) toxin and/or *hrp*-dependent TTSS effectors. Many of the identified TTSS effector regulated *Arabidopsis* genes are involved in metabolic processes similar to those described for *Salmonella* above. Especially cell wall genes, genes involved in photosynthesis and the Calvin cycle are repressed but also genes involved in secondary metabolism related to phenylpropanoids and terpenes.

COMPARISON OF PLANT AND HUMAN HOST RESPONSES TO *Salmonella* CHALLENGE

One major difference between humans and plants is that plant cells have a cell wall and mammalian cells do not. Thus, the question arises, how *Salmonella* can overcome this barrier and enter the cytoplasm. Schikora et al. (2008) describe the presence of green fluorescent protein (GFP)-labeled *S.* Typhimurium inside *Arabidopsis* root cells and intact protoplasts 3 and 20 h after infection, thereby demonstrating that *Salmonella* can enter the plant cytoplasm and proliferate there. Plant pathogenic bacteria are known to target the plant cell wall by a variety of enzymes with hydrolytic activity. For example, *Pseudomonas viridiflava* secretes a pectate lyase (Jakob et al., 2007) and *Xanthomonas oryzae* produces a xylanase to degrade the cell wall (Rajeshwari et al., 2005). Both are necrotrophic bacterial pathogens. On the other hand, there are reports showing that bacteria can be found inside intact plant cells. For instance, Quadt-Hallmann et al. (1997) detected the endophytic bacterium *Enterobacter asburiae* inside cotton root cells 6 h after inoculation and hydrolysis of cellulose at these sites. Another report demonstrated the colonization of banana periplasm and cytoplasm of intact cells by endophytic bacteria (Thomas and Sekhar, 2014). Endosymbiotic bacteria, e.g., Rhizobacteria, are taken up into the plant cytoplasm and are engulfed inside a membrane of plant origin, called the symbiosome membrane. There is evidence that the engulfed organism actively inhibits its degradation through plant cellular mechanisms (Parniske, 2000). This procedure of bacterial internalization and active suppression of degradation reveals similarities to human bacterial pathogens like *Salmonella*. For the human host, the best described process of how *Salmonella* enters the cell is driven by the TTSS-1 effectors SipA, SipC, SopA, SopB, SopD, SopE2, and SptP. These effectors induce modification of the actin cytoskeleton, promote membrane ruffling, recruitment of vesicles to the site of *Salmonella* internalization and rearrangement of the cytoskeleton to its normal shape after engulfment of the pathogen in the SCV (Schleker et al., 2012b). To our knowledge, it is unknown, how *Salmonella* overcomes the cell wall barrier. The pathogen may secrete cell wall hydrolyzing enzymes and/or interfere with cell wall biogenesis, or it enters a necrotrophic phase and thus kills the plant cells. On the other hand it could as well be that the invaded plant cell stays intact or preferential infection occurs when plant material decays through other processes (Schikora et al., 2011). In favor of the presence of cell wall-modifying mechanisms is evidence from the KMM–SVM modeling approach. It predicts the interaction of *Salmonella* effectors with *Arabidopsis* proteins involved in cell wall organization, e.g., xyloglucan endotransglucosylase proteins that are involved in cell wall construction of growing cells. Moreover, *Arabidopsis* proteins of the Arp2/3 complex that mediates actin polymerization and Actin-11, for instance, are putative *Salmonella* effector targets involved in cytoskeleton organization (Kshirsagar et al., 2015).

Salmonella EFFECTORS INTERFERE WITH HOST UBIQUITIN PROTEASOME MECHANISMS IN BOTH ORGANISMS

As can be seen in the high-scored KMM–SVM predictions, experimental data and the known human targets of *Salmonella* effectors, host ubiquitin-related cellular processes seem to be targeted by *Salmonella* in both, human and plant hosts.

One central eukaryotic regulatory cellular mechanism involves the attachment of ubiquitin or ubiquitin-like proteins (Ub) to target molecules generally through an enzyme cascade comprising Ub activation by E1, Ub conjugation by E2 and Ub-substrate ligation by E3. In *Arabidopsis* over 5% of the proteome are proteins involved in the ubiquitination machinery demonstrating its importance (Smalle and Vierstra, 2004). Hundreds of human and plant E3 ligases are proposed to exist which belong to several different known types of E3 ligases which include RING-finger, HECT, and U-box type single protein E3 ligases as well as multi subunit RING-finger type E3 ligases comprising Cullin-RING and APC/C (anaphase promoting complex/cyclosome) E3 ligases (Zeng et al., 2006; Vierstra, 2009). E3 ligases comprise an E2 binding domain (HECT, U-box, RING-finger) and a target recognition subunit, e.g., F-box. In case of multi subunit E3 ligases additionally a Cullin, an adapter protein and for APC/C other subunits are present. Substrates can be mono- or polyubiquitinated with diverse linkage types between the Ub proteins. The modified proteins are either targeted for proteasomal degradation or play a functional role in diverse processes, e.g., endocytosis, gene expression, DNA repair or NF-κB activation in mammalian cells and, e.g., hormone signaling and defense response in plants.

It is known that pathogens exploit the mammalian and plant Ub system to enable their invasion and survival but so far only a very limited number of host–pathogen interactions within this highly important regulatory process have been described (Groll et al., 2008; Duplan and Rivas, 2014). All of these known interactions of *Salmonella* are with the mammalian Ub machinery whereas nothing is known on the plant side. There is a large space of putative interactions that remains to be elucidated based on the fact that already four *Salmonella* effectors are experimentally confirmed E3 ligases (SopA, SlrP, and SspH1 and 2). SopA, a HECT-like E3 ligase, which has been shown to interact with the E2 UbcH7, is known to induce polymorphonuclear neutrophil migration, a process attributed to its Ub ligase activity. However, so far the host targets are unknown (Zhang et al., 2006). The effector itself is ubiquitinated by the host E3 ligase RMA1. Monoubiquitinated SopA is assumed to be involved in *Salmonella* escape from the SCV and polyubiquitinated SopA is degraded by the host proteasome (Zhang et al., 2005). The E3 ligase SlrP has been shown to ubiquitinate thioredoxin 1 (Bernal-Bayard and Ramos-Morales, 2009) and to interact with ERdj3 (endoplasmic reticulum DNA J domain-containing protein 3; Bernal-Bayard et al., 2010). Both interactions are supposed to induce cell death. It is speculated that the interaction of SlrP with ERdj3 contributes to the inhibition of antigen presentation by MHC-I (Granados et al., 2009; Bernal-Bayard et al., 2010). The interaction of SspH1, an effector possessing E3 ligase activity, with PKN1 (serine/threonine-protein kinase N1) is proposed to inhibit NF-κB and thus IL-8 (interleukin-8) secretion (Haraga and Miller, 2006). SspH2 has been shown to interact with the E2 UbcH5 and to synthesize K48-linked Ub chains and thus likely targets its substrates for proteasomal degradation (Levin et al., 2010). SspH2 binds filamin A and profilin-1 thereby preventing cross-linking of F-actin and polymerization (Miao et al., 2003). Moreover, SspH2 has been reported to interact with Sgt1 (suppressor of G2 allele of SKP1 homolog), AIP (AH receptor-interacting protein), Bub3 (mitotic checkpoint protein BUB3), 14-3-3γ (protein kinase C inhibitor protein 1), and BAG2 (BAG family molecular chaperone regulator 2) but functions of these interactions are not known (Auweter et al., 2011). In addition to this, AvrA and SseL are deubiquitinating *Salmonella* enzymes that cleave Ub from IκBα and thereby inhibit NF-κB-mediated gene expression (Ye et al., 2007; Le Negrate et al., 2008). Recently, GogB, an effector mimicking a eukaryotic F-box, has been shown to inhibit NF-κB activation through its interaction with a host SCF (Skp-Cullin-F-box) E3 ligase (Pilar et al., 2012). As, for instance, it has been demonstrated that *Salmonella* enhances the internalization of MHC-II antigens by a ubiquitination event through a so far not identified effector it is possible that other *Salmonella* E3 ligases remain to be discovered (Lapaque et al., 2009). Furthermore, it can be presumed that many substrates of the known *Salmonella* E3 ligases are unknown. Moreover, host Ub mechanisms targeting *Salmonella* effectors as well as *Salmonella* effectors that mimic other proteins of the host Ub system, e.g., F- or U-box proteins, remain to be found.

Although to date there have not yet been any experimentally confirmed interactions of *Salmonella* effectors with the plant Ub system, several reports indicate an important role of the plant Ub proteasome system in pathogen defense response in general. About 50 genes involved in the Ub-dependent degradation pathway have been found to be upregulated upon inoculation of *Arabidopsis* with a *S.* Typhimurium *prgH*$^{-}$ mutant compared to the WT control. Thus, *Salmonella* TTSS-1 effectors hinder the expression of these genes which mainly code for E2 and RING ligases (Schikora et al., 2011). It has been demonstrated that TTSS effectors of other bacterial pathogens also interact with the plant Ub proteasome machinery. One example is the group of *Ralstonia solanacearum* GALA effectors which are LRR (leucine-rich repeat) F-box proteins that interact with SKP1-like proteins (Angot et al., 2006). Secondly, *P. syringae* HopM1 targets *Arabidopsis* proteins and induces their proteasomal degradation. One of these proteins is the guanine nucleotide exchange factor MIN7, which plays a role in vesicle trafficking (Nomura et al., 2006).

The KMM–SVM modeling approach by Kshirsagar et al. (2015) predicts *Salmonella* effectors to interact with E3 ligases, RING proteins, F- and U-box domain containing proteins. The *Salmonella* E3 ligase SspH2 and the deubiquitinase SseL are predicted to interact with a variety of *Arabidopsis* proteins including those, e.g., involved in transcriptional regulation, stimulus and defense response, biosynthetic and metabolic processes, RNA processing, protein localization and transport.

CONCLUSIONS AND FUTURE WORK

Due to the lack of direct experimental data on *Salmonella*–*Arabidopsis* and limited data on the *Salmonella*–human interactions, we here utilized published predictions of these interactomes. This is of course a limitation as we cannot

ascertain the reliability of the predictions without further testing. However, the predictions for human were statistically evaluated quantitatively using the gold standard data, giving us some confidence. One popular measure for evaluation is the F1 score, which can be interpreted as a weighted average of the precision and recall, where an F1 score reaches its best value at 1 and worst score at 0. For our *Salmonella*–human predictions the F1 score was 74 (Kshirsagar et al., 2012). This value compares favorably with other performances, such as for HIV-human interactions reported at 54 (Dyer et al., 2011). We cannot do this for *Arabidopsis*, but the overlap between our predictions and previous experimental studies especially using transcriptomics and cell signaling support the biological relevance of the predictions. However, often the same interactions are predicted for several *Salmonella* effectors, hinting at the functional importance of the host protein targeted, but the actual pairs of interacting proteins may not be true. Furthermore, missing information for many proteins makes it challenging to generate predictions for such proteins. This introduces the bias of predicting interactions for relatively better studied proteins and pathways. Ultimately, only the experiments can verify if a predicted interaction is real or not. The benefit in the prediction approach lies in the generation of biological hypotheses that are experimentally testable, vastly narrowing the search space for new interactions.

From the current state of knowledge it is also difficult to judge how similar or different *Salmonella* is compared to a bona fide plant pathogen. Nevertheless, *Salmonella* triggers plant processes typical for plant pathogens as reviewed above. While interpreting the *Salmonella–Arabidopsis* predictions, we in some cases made comparisons with the plant pathogen *P. syringae* and its interaction with *Arabidopsis*. This is mainly due to the fact that the interaction between *P. syringae* and *Arabidopsis* is a well-studied model system for which data is available. Looking at the lifestyle of both bacteria, *Salmonella* as well as *P. syringae* colonize the plant apoplast and make use of TTSS and effectors. As we focus here on the interaction of *Salmonella* effectors with plant proteins, we think that comparing the interplay of both bacteria with *Arabidopsis* on this level is possible. One obvious difference between the two organisms in plants is that *Salmonella* has been found inside root cells and *P. syringae* only in the intercellular space. This may point at a difference in infection strategies. This is supported by a transcriptomic analysis where a small set of *Arabidopsis* genes is only differentially regulated in response to *Salmonella* but not *P. syringae* (Schikora et al., 2011). Interestingly, *Pseudomonas* bacteria (most likely non-pathogenic) have been detected in the plant cytosol (Pirttilä et al., 2000) indicating that bacteria of this genus are capable of entering the plant cell. Casadevall (2008) proposes that it might well be that intracellular survival of microbes is an ancient and common mechanism of microbes rather than an exception. Thus, it may be speculated that *P. syringae* lost the ability to invade the plant cytosol and/or the plant evolved effective means to prevent internalization.

The predictions, known interactions and experimental data reviewed here indicate that *Salmonella* partly targets the same cellular processes in both hosts. For example, *Salmonella* effectors interfere with proteins of the ubiquitin degradation pathway in *Arabidopsis* and human and moreover, *Salmonella* effectors are E3 ligases, deubiquitinases, and F-box mimicking enzymes with specific functions in both hosts. Although the same type of cellular machinery is targeted and utilized, *Salmonella* seems to do it differently in human compared to *Arabidopsis*. For example, as reviewed above, in the human host *Salmonella* inhibits the immune response, e.g., by interfering with transcription factor NF-κB mediated gene expression, whereas in *Arabidopsis* expression of defense-related genes may be suppressed by targeting plant transcription factors like ERF2 and ERF094 (ET responsive transcription factor 2 and ERF094) as obtained from the predictions (also predicted by high class skew model). Differences in the way of interfering with the same cellular processes or achieving similar functions in both hosts may be due to several possibilities: (i) the bacterium has adapted to target those pathways differently in each host type, (ii) the bacterium acts elsewhere in the host, and the catabolic response to those changes is common to both hosts, (iii) the catabolic response is generic, and independent of bacterial action, (iv) the bacterium targets those pathways in one host, but not the other, but the second host's response looks similar to the result of targeting that pathway in the first host (either as a result of targeting elsewhere, or a generic response).

When comparing interaction mechanisms of *Salmonella* with its human, animal, and plant hosts, one obvious question is, whether one of these was the primary host or whether a co-evolution occurred. It has been proposed that *Salmonella* evolution took place in five phases starting from a common ancestor about 25–40 million years ago (Bäumler et al., 1998; Porwollik et al., 2002). First, *Salmonella* separated from *E. coli*. This was accompanied by the acquisition of possibly about 500 genes including SPI-1 genes. Next, *S. enterica* diverged from *S. bongori* and gained—among others—the SPI-2 genes. In a third phase, diphasic *S. enterica* strains occurred and in phase four, *S. enterica* spp. I, which includes *S.* Typhi and *S.* Paratyphi, separated from the other spp. recruiting genes for adaptation to warm-blooded hosts. In a last phase, *S.* Typhimurium evolved and recruited about 140 genes the functions of which are mostly unknown. In total, more than 900 *S.* Typhimurium LT2 genes were identified that are not present in the genomes of the enterobacteria *E. coli* K12 and O157:H7, *K. pneumoniae* MGH 78578 and *Y. pestis* CO92 (Porwollik et al., 2002). Despite this evolutionary development, beside *S. enterica* other human-pathogenic enterobacteria like *E. coli* can colonize plants and thus use plants as vectors for transmission to its animal and human hosts (Jayaraman et al., 2014). It has been shown that during interaction of *S. enterica* and *E. coli* O157:H7 with *Medicago truncatula* about 30% of the plant genes are commonly regulated by both pathogens (Jayaraman et al., 2014) indicating that both pathogens to some extent provoke the same plant response. Barak et al. (2009) identified two *S. enterica* genes that play a role in swarming and biofilm formation and are important for plant colonization. Homologous genes have been found in plant-associated bacteria, e.g., *Agrobacterium tumefaciens*, *Ralstonia solanacearum*, and *P. syringae*, but not in *E. coli* (Barak et al., 2009). From a practical point of view it may be favorable for enteropathogenic bacteria like *Salmonella* to be able to use plants as secondary host for survival and transmission to its animal host. Thus, a co-evolution with both hosts would be

likely. Interestingly, it has been reported that there is only a 5% overlap between the *Salmonella* promoters induced during infection of tomato compared to infection of macrophages (Teplitski et al., 2009) demonstrating that the majority of genes *Salmonella* utilizes in its animal and plant hosts are different. Moreover, there is evidence that the response of plants varies dependent on the *Salmonella* serovar that infects the plant (Berger et al., 2011) possibly indicating that genetic differences are the reason. Last but not least, it has been demonstrated that *Salmonella* is recognized by its plant hosts and the acquired TTSS-1 and -2 play an important role in suppressing plant defense response. Thus, it is obvious that *Salmonella* utilizes the same TTSS to interfere with defense response in its animal and plant hosts but so far it is uncertain which effectors *Salmonella* utilizes in the plant host. Further investigations will help to gain more insight into the evolution of *Salmonella* with respect to the pathogen's ability to infect plants.

Beside the above-discussed difference that plants have and animal cells do not have a cell wall, another difference *Salmonella* has to face is the temperature. While the human body temperature is nearly constant around 37°C, the temperature of plants varies greatly according to the temperature of the environment. It is known that *Salmonella* is able to adapt to different environmental conditions by, e.g., sensing changes in pH and temperature and adjusting gene regulation accordingly. A variety of these regulatory mechanisms involve the expression of virulence genes. For instance, the promoters of *Salmonella* virulence genes *orgA, pagC, prgH,* and *spvA* reveal related sequence motifs to the *tlpA* promoter which is specifically activated upon temperature increase (Clements et al., 2001).

In conclusion, plants play an important role in the transmission of *Salmonella* infections and mounting evidence supports the notion that they constitute a bona fide host for *Salmonella*. As such, *Salmonella* can infect plants and initiates a two-way communication of plant response to invasion and *Salmonella* defense against this response and exploitation of resources. Communication is clearly via PPIs, but to date, the only known (experimentally confirmed) interactions involve mammalian proteins, especially human, and even those are very small in number due to the limited number of studies carried out in this field to date. We therefore focused this review on current approaches to prediction of the full interactome between human and *Salmonella* proteins and its extension to prediction of the interactome with *Arabidopsis* as a plant host. We find that there is significant overlap in the pathways predicted to be targeted by *Salmonella* in both hosts despite their evolutionary distance, while there are also distinct and host-specific responses. These involve in particular plant biosynthetic pathways not available in the human host and the complex human immune system response not available in plants. It is likely that the fundamental mechanisms of interference are highly related and particularly striking is the prevalence of predicted binding partners relating to the ubiquitin degradation system in both hosts. These predictions provide a rich source of experimentally testable hypotheses that can speed up scientific discovery of both the main-stream human host response as well as the niche host–pathogen interaction pair involving the newly discovered plant host for *Salmonella*.

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An optimized method for the extraction of bacterial *m*RNA from plant roots infected with *Escherichia coli* O157:H7

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Analysis of microbial gene expression during host colonization provides valuable information on the nature of interaction, beneficial or pathogenic, and the adaptive processes involved. Isolation of bacterial *m*RNA for *in planta* analysis can be challenging where host nucleic acid may dominate the preparation, or inhibitory compounds affect downstream analysis, e.g., quantitative reverse transcriptase PCR (qPCR), microarray, or RNA-seq. The goal of this work was to optimize the isolation of bacterial *m*RNA of food-borne pathogens from living plants. Reported methods for recovery of phytopathogen-infected plant material, using hot phenol extraction and high concentration of bacterial inoculation or large amounts of infected tissues, were found to be inappropriate for plant roots inoculated with *Escherichia coli* O157:H7. The bacterial RNA yields were too low and increased plant material resulted in a dominance of plant RNA in the sample. To improve the yield of bacterial RNA and reduce the number of plants required, an optimized method was developed which combines bead beating with directed bacterial lysis using SDS and lysozyme. Inhibitory plant compounds, such as phenolics and polysaccharides, were counteracted with the addition of high-molecular-weight polyethylene glycol and hexadecyltrimethyl ammonium bromide. The new method increased the total yield of bacterial *m*RNA substantially and allowed assessment of gene expression by qPCR. This method can be applied to other bacterial species associated with plant roots, and also in the wider context of food safety.

Keywords: food-borne pathogens, lettuce, spinach, rhizosphere, *m*RNA isolation

INTRODUCTION

The analysis of bacterial gene expression is important in the determination of how adaptation to different environments develops and informs on the roles of different genes during this process. Human pathogens are now recognized to interact with plants and use them as hosts, as a result of recent high-profile outbreaks from contaminated fruit and vegetables (Cooley et al., 2007; Buchholz et al., 2011). Adaptation of food-borne pathogens to secondary hosts has opened up new areas of research and investigation. Current research suggests that the interaction of human pathogens is more complex than previously perceived in that they can persist for long periods of time (reviewed in Brandl, 2006; Holden et al., 2009; Barak and Schroeder, 2012) and invoke an immune response from the plant (Thilmony et al., 2006; Schikora et al., 2008; Roy et al., 2013). Furthermore, the interactions include quite specific and targeted recognition of the plant host cells by the bacteria (Rossez et al., 2013). Important questions remain as to how the bacteria adapt to secondary hosts, for which analysis of bacterial gene expression is fundamental.

Bacterial gene expression is typically assessed using quantitative reverse transcriptase PCR (qPCR) or microarray techniques, and more recently RNA-seq (An et al., 2013), which require the isolation of high quality and quantity of *m*RNA. Bacterial *m*RNA has a short half-life and is less stable than eukaryotic *m*RNA transcripts, which have capped and polyadenylated RNA tails. Therefore, appropriate measures need to be in place to capture *m*RNA transcripts that may be inherently unstable, but nevertheless important for bacterial adaptation. In addition to challenges with bacterial *m*RNA stability, extractions from mixed samples bring additional considerations, not least for inhibitory compounds that may affect downstream analysis. Although there are a number of published techniques for the isolation of total RNA from bacteria-infected plant leaves (Schenk et al., 2008; Soto-Suarez et al., 2010; Fink et al., 2012; Goudeau et al., 2013), samples can still be dominated by plant RNA making it challenging to assess bacterial *m*RNA. In published reports, leaves were typically infected via infiltration, introducing a high bacterial inoculum into the sample. In other studies, total RNA was extracted from inoculated plant extracts (Mark et al., 2005; Hernandez-Morales et al., 2009; Kyle et al., 2010; Shidore et al., 2012), such as leaf lysates, which helped to reduce the dominance of plant RNA in the sample.

There are limited studies where bacterial expression has been assessed directly from the plant root system (roots and rhizosphere). However, roots are known to support relatively high densities of bacteria, providing a more favorable habitat than the phyllosphere. In general, published reports describe that a high number of plant roots is required to retrieve sufficient bacterial RNA (Matilla et al., 2007; Hou et al., 2012, 2013; Zyśko et al., 2012). Further to this, the application of techniques described for RNA purification from infected leaves cannot always be successfully applied to roots. Therefore, optimization of the RNA extraction methods is required to obtain sufficient quantity and quality of bacterial *m*RNA, coupled with a reduction in the amount of accompanying plant root RNA. We optimized the method for

the food-borne pathogen *Escherichia coli* O157:H7, frequently associated food-borne outbreaks from consumption of contaminated spinach and lettuce. Although roots of these plants are not consumed, the pathogen can colonize this habitat successfully (Wright et al., 2013), from where it can contaminate the edible portion, either directly or through cross-contamination during processing.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

E. coli O157:H7 isolate Sakai *stx*-negative (Hayashi et al., 2001) was routinely cultured overnight in Luria broth at 37°C, with aeration. For plant-infection assays, the bacteria were sub-cultured at 1:50 dilution into 15 ml rich-defined MOPs media supplemented with 0.2% glucose (RD MOPS glucose; Neidhardt et al., 1974), in a 200 ml Erlenmeyer flask and incubated at 18°C, with aeration for ∼18 h. Bacterial cultures were diluted to OD_{600} of 0.2 (equivalent to $\sim 2 \times 10^8$ cfu ml^{-1}) in sterile phosphate buffered saline (PBS) prior to infecting plant roots.

PLANT PROPAGATION AND INFECTION

Lettuce (*Lactuca sativa*) cultivar All Year Round or spinach (*Spinacia oleracea*) cultivar Amazon seeds (Sutton Seeds, UK) were soaked in sterile distilled water for 2 h before being surface sterilized in 2% calcium hypochlorite solution (10 ml) for 10 min. The seeds were then washed vigorously six times with sterile distilled water and germinated on distilled water agar (0.5% w/v) in the dark for 3–5 days, at ∼22°C. Seedlings were transplanted into 175 ml hydroponic tubes (Greiner, Frickenhausen, Germany) containing autoclaved perlite and sterile 0.5× Murashige and Skoog (MS) medium (Sigma Aldrich, USA). Seedlings were grown in a cabinet with a light intensity of 150 μmol m^2 s^{-1} (16 h photoperiod) for a further 21 days at 22°C. To assess bacterial numbers from infected plant material, the roots were washed with PBS to remove excess, non-adherent bacteria. The root sample was homogenized using mortar and pestle and serially diluted for viable counts on selective agar plates.

RNA EXTRACTION AND PURIFICATION

Total RNA was extracted from infected root samples either by the BPEX method (Schenk et al., 2008) or by the Bead/SDS/phenol method (**Figure 1**): a method which was adapted and optimized from Jahn et al. (2008) and Schenk et al. (2008).

Sample preparation

Sample preparation was common to both methods. Whole plants were gently removed from the hydroponic tubes and washed with sterile PBS to remove as much perlite as possible before bacterial infection. Plants were pooled into groups of 5 and the roots were submerged into 20–25 ml bacterial suspension ($OD_{600} = 0.2$ equivalent to $\sim 5 \times 10^7$–1×10^8 cfu ml^{-1}), and incubated at 18°C for 2 h. After incubation, the plants were removed from the bacterial suspension and all the tissue above the crown (shoots, leaves, and petioles) were aseptically removed with a sterile scalpel and the roots immediately immersed into 20 ml ice-cold, 95% ethanol: 5% phenol (pH 4.0). The sample was incubated on ice for 5 min and gently agitated on a vortex mixer to remove any excess and loosely attached bacteria. The root sample was placed into a foil packet and immediately stored in liquid nitrogen until all samples had been processed.

BPEX-Schenk method for extraction

The BPEX method was originally developed to recover *Pseudomonas syringae* phytopathogen *m*RNA from infected plant material (Schenk et al., 2008), and tested for recovery of *E. coli* *m*RNA from infected roots. The main change from the published method was in the tissue type (root vs leaf disks), although the protocol is provided to allow a direct comparison with the optimized method. Infected plant tissue was ground to a fine powder in liquid nitrogen with a pre-cooled pestle and mortar. The sample (∼1 g) was transferred to an Eppendorf containing 750 μl of *b*acteria *p*lant *ex*traction (BPEX) buffer [0.35 M glycine, 0.7 M NaCl, 2% (w/v) polyethylene glycol (PEG) 20000, 40 mM EDTA, 50 mM NaOH, 4% (w/v) SDS] supplemented with 100 mM β-mercaptoethanol just prior to use. The sample was mixed on a vortex mixer in the buffer prior to incubation at 95°C for 90 s with shaking. An equal volume (750 μl) of phenol/chloroform mix (5:1, pH 4.0) (Sigma, St. Louis, USA) was added and the sample shaken for 5 min to form an emulsion before centrifugation at 16,000 × *g* for 7 min. The upper phase was collected and added to an equal volume of phenol/chloroform mix (5:1, pH 4.0), shaken, and centrifuged again as the previous step. The upper phase was collected and added to an equal volume of phenol/chloroform/isoamyl alcohol (IAA) mix (25:24:1, pH 4.0) (Sigma, St. Louis, USA) shaken and centrifuged again as before. A volume of 495 μl of the upper phase was added to 550 μl chloroform/IAA mix (24:1) and overlayed with 55 μl pre-warmed (55°C) hexadecyltrimethyl ammonium bromide (CTAB)/NaCl (10% CTAB, 0.7 M NaCl) solution. The suspension was shaken and centrifuged as before. The upper phase was added to 1/4 vol 8 M LiCl solution, mixed by inversion and the RNA precipitated at –20°C for 30 min. To collect the RNA, the sample was centrifuged at 16,000 × *g* for 20 min at 4°C and the RNA pellet resuspended in 100 μl RNase-free water. The RNA was cleaned and DNase treated using RNeasy Plant Mini kit (Qiagen, Limburg, Netherlands) as per the manufacturer's guidelines.

Bead/SDS/phenol method for extraction

The optimized method is represented in **Figure 1**. The samples were removed from the liquid nitrogen, beaten with a spatula to break up the root into smaller pieces, and transferred into an 1.5 ml micro-centrifuge tube (DNase, RNase free: Ambion, Austin, USA) preloaded with ∼250 mg mixture of 1 mm glass and 0.1 mm silica beads (ThermoScientific Ltd., Waltham, USA), from which the weight was determined. Cooled, sterile equipment was used throughout the process. The micro-centrifuge tube was then returned to liquid nitrogen for processing subsequent samples until all of the samples were collected. For the lysis step, 800 μl of Tris-EDTA (TE) buffer (10 mM Tris-HCl; 1 mM EDTA) supplemented with 500 μg ml^{-1} lysozyme, 0.1% SDS, and 100 mM β-mercaptoethanol was added to each root sample. The tubes were placed into TissueLyser (Qiagen, Limburg, Netherlands) and agitated for 30 s with a 30 s interval on ice for three cycles. After the last cycle, the samples were

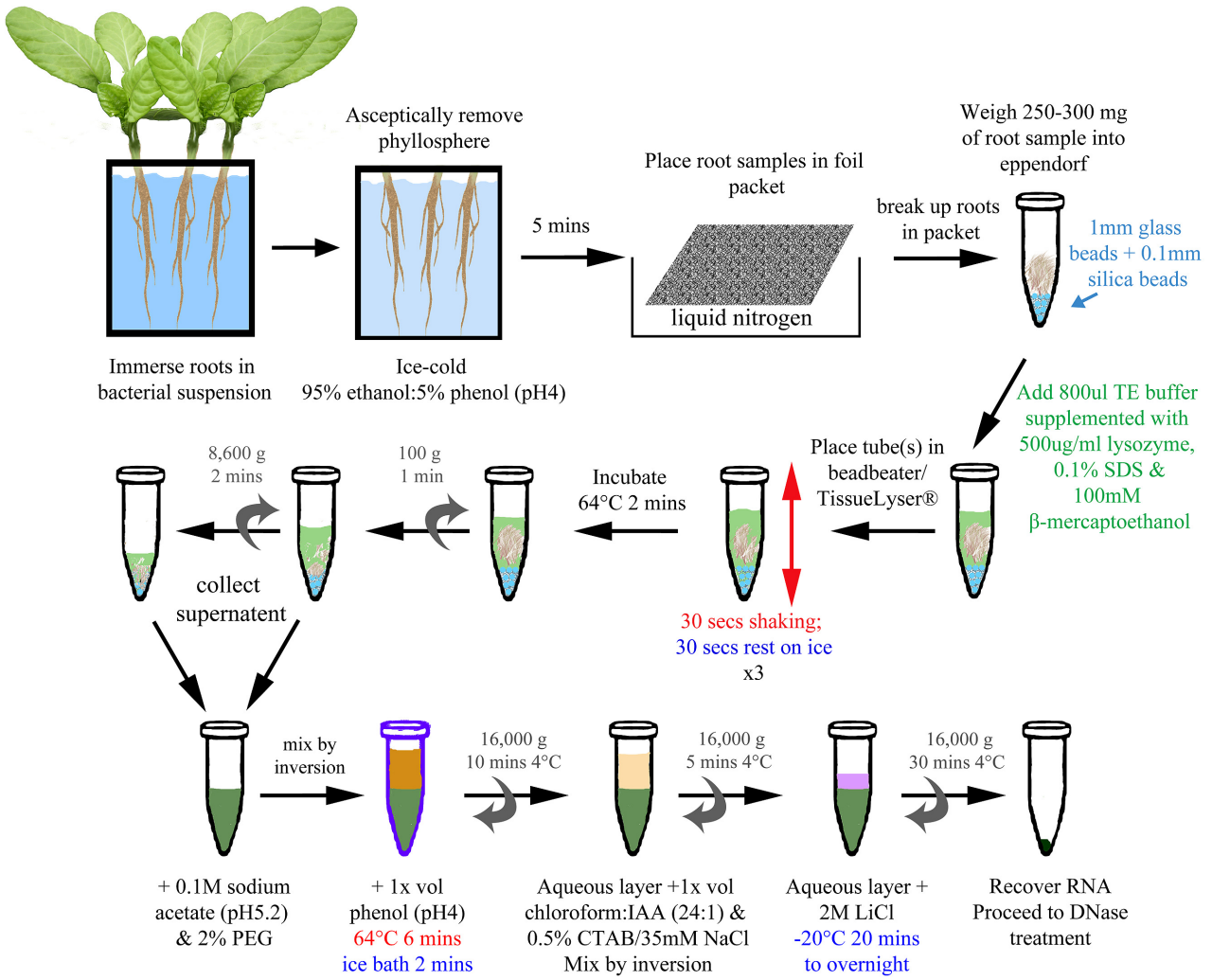

FIGURE 1 | Bead/SDS/phenol method of RNA extraction from infected plant roots. A schematic of the optimized method (described in detail within Section "Material and Methods"). The final concentrations of reagents are indicated in the figure. Colored text has been used to highlight different stages and treatments (e.g., blue for addition of beads/cold; green for lysis; red for shaking/heat).

returned to ice before transfer to a heatblock set at 64° C for 2 min. To extract nucleic acids, the supernatant was collected and pooled after two centrifugation steps: first at 100 × *g* for 1 min to pellet the beads and large fragments of root, followed by a second at 8,600 × *g* for 2 min to compact the debris further, yielding more supernatant. One hundred millimolar sodium acetate (pH 5.2) and 2% (w/v) PEG 20000 was added to the supernatant and inverted to mix. An equal volume (~1 ml) of phenol (pH 4.0) was then added, mixed by inversion, and the sample incubated at 64°C for 6 min, with the tubes inverted every 40 s. The sample was transferred to an ice bath for 2 min before centrifugation at 16,000 × *g* for 10 min at 4°C. The upper aqueous layer was added to an equal volume of chloroform/IAA mix (24:1) and 1/20 volume of pre-warmed (55°C) CTAB/NaCl solution in a fresh micro-centrifuge tube. The sample was mixed by inversion and then centrifuged at 16,000 × *g* for 5 min at 4°C. The upper aqueous layer was added to 1/4 vol 8 M LiCl, mixed by inversion, and then incubated at –20°C for 20 min to overnight to precipitate the nucleic acid. The nucleic acid was recovered by centrifugation at 16,000 × *g* for 30 min at 4°C. The pellet was resuspended in 100 µl RNase-free water and the sample cleaned and DNase treated using the RNeasy Plant Mini kit (Qiagen, Limburg, Netherlands) as per the manufacturer's instructions.

Total RNA concentration was determined using a NanoDrop (Wilmington, USA) spectrophotometer and the relative proportions of ribosomal RNA determined using a BioAnalyser 2100 (Agilent Technologies, Santa Clara, USA), for both methods.

*c*DNA SYNTHESIS AND qPCR CONDITIONS

*c*DNA was transcribed from 1 µg total RNA using Superscript II (Invitrogen, Carlsbad, USA) following the random primer protocol. A mixture of ten 11-mer oligonucleotide primers (Ea1 ± Ea10)

at 100 nM (Fislage et al., 1997) designed specifically for *Enterobacteriaceae* *m*RNA was used. Quantitative reverse transcriptase PCR was carried out using specific primers for *E. coli* O157:H7 *gyrB* housekeeping gene (*gyrB*.RT.F = CATCAGAGAGGTCGGCTTCC; *gyrB*.RT.R = CATGGAGCGTCGTTATCCGA) using StepOnePlus™ real-time PCR system (Applied Biosystems, Life Technologies, Carlsbad, USA) and iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Hercules, USA). Each 20 µl reaction volume was composed of iTaq SuperMix, 300 nM of forward and reverse primers and 1 µl of *c*DNA (diluted 1:4). The PCR program consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. Melt curve analysis was also performed with an initial denaturation at 95°C for 15 s, followed by annealing at 60°C for 1 min with an 0.3°C increase (step and hold) and final step of 95°C for 15 s. Data were collected from three technical replicates and from two biological replicates.

RESULTS

EXTRACTION OF TOTAL RNA FROM PLANT ROOTS INFECTED WITH BACTERIA

An optimized method was previously reported for the extraction of *P. syringae* *m*RNA from infiltrated plant leaves (Schenk et al., 2008). A buffer system was developed that yielded 75–125 µg of total RNA per 150–200 mg sample, from the equivalent of 20 infiltrated leaf disks (7 mm). We tested this protocol (termed the "BPEX" method) to determine if it could also be used to recover *m*RNA of a food-borne pathogen from infected roots. Our work focuses on the bacterial genes induced during the stages of colonization of lettuce and spinach roots, and as such the roots in our experiments are not infected via infiltration; rather the bacteria colonize the outer surface. This necessitated processing the entire root to recover sufficient RNA. Also, to obtain equivalent bacterial numbers to those reported, where each leaf disk was infiltrated with 1×10^8 cfu bacteria, at least 20 root samples were pooled for each extraction. In this method, the samples were ground to a fine powder in liquid nitrogen, prior to incubation in an extraction buffer (BPEX) coupled with cell lysis. RNA was purified using traditional acidic phenol extraction and LiCl-mediated precipitation.

Use of the BPEX method typically yielded 3.6–9.6 µg of total RNA from 250 mg *E. coli* O157:H7-infected lettuce roots. Analysis of the total RNA showed strong bands corresponding with 18S and 28S *r*RNA of lettuce (**Figure 2A**: Lanes 1, 2, 3) with only very faint bacterial RNA corresponding to 16S and 23S *r*RNA in mixed samples (**Figure 2A**; Lanes 1, 2, 3). Examination of the electropherogram trace showed that the bacterial *r*RNA peaks were considerably smaller than the plant *r*RNA peaks, e.g., BPEX sample 1 (**Figure 2B**). The pre-dominance of plant *r*RNA in the BPEX-prepared samples indicated that plant *m*RNA would also dominate over bacterial transcripts. Therefore, to increase the levels of bacterial *m*RNA with a concomitant decrease in plant-derived RNA, a modified method was designed. The aim was threefold: (i) to reduce the number of plants required per extraction; (ii) to reduce the carry-over of plant tissue in the sample; and (iii) incorporate a lysis step which targeted bacterial cells.

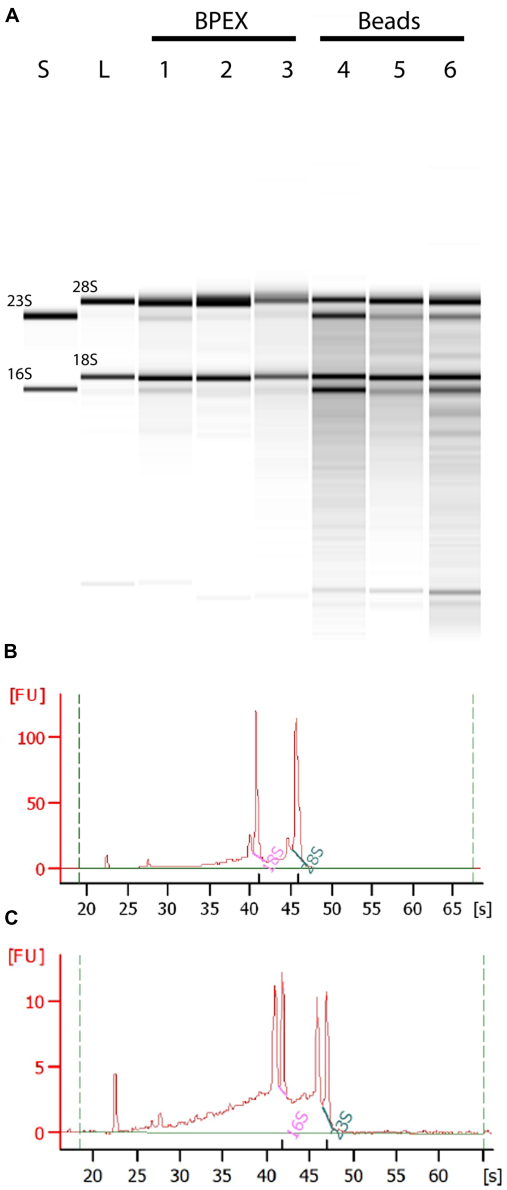

FIGURE 2 | Total RNA from plant root extractions by BPEX and Bead/SDS/phenol methods. Intact plant roots were suspended in 20 ml of bacterial inoculum (OD_{600} of 0.2) for 2 h at 18°C. Total RNA was extracted using either the BPEX or the Bead/SDS/phenol method and RNA quality was assessed by spectroscopy on a Bioanalyser 2100 machine (Agilent). **(A)** A montage of an electropherograph of the total RNA levels following extraction of inoculated plant material. The lanes show RNA from an *in vitro* culture of *E. coli* O157:H7 isolate Sakai only (lane S); uninfected lettuce roots (lane L); *E. coli* O157:H7 Sakai infected lettuce roots (Lane 1, 306 ng μl^{-1}; Lane 4, 119 ng μl^{-1}; Lane 5, 195 ng μl^{-1}); and *E. coli* O157:H7 Sakai infected spinach roots (Lane 2, 248 ng μl^{-1}; Lane 3, 301 ng μl^{-1}; Lane 6, 141 ng μl^{-1}). Lanes 1, 2, and 3 show extraction using the BPEX method, and Lanes 4, 5, and 6 using the Bead method. The samples were run alongside a commercial RNA transcript ladder (0.2, 0.5, 1.0, 2.0, 4.0, and 6.0 kb, Agilent); the 16S and 23S (bacterial), and 18S and 28S (plant) *r*RNA bands are indicated. Electropherogram of *E. coli* O157:H7 Sakai infected lettuce roots extracted with **(B)** the BPEX method (sample derived from lane 1) or with **(C)** the novel Bead/SDS/phenol method (sample derived from lane 4). The uninfected spinach control was similar to lettuce (lane L) (not shown).

DEVELOPMENT OF THE BEAD/SDS/PHENOL PROTOCOL

Physical disruption of tissues and cells using inert beads has been used for nucleic acid extraction from filamentous fungi (Leite et al., 2012), microalgae (Kim et al., 2012), soil and sludge samples (Griffiths et al., 2000), and in commercial kits. We postulated that this method of disruption may also aid in bacterial RNA extraction and used a combination of 1 mm glass beads and 0.1 mm silica beads for sample lysis. Since tissue can become over-heated during agitation, which leads to nucleic acid degradation, the samples were "rested" for 30 s on ice between the three treatment cycles.

Bacterial cells were specifically targeted for lysis in an attempt to reduce plant-derived nucleic acid contamination, with the inclusion of lysozyme in the extraction buffer. However, a combination of the hot SDS/phenol extraction protocol described previously (Jahn et al., 2008) with the bead beating step resulted in very low RNA yields; not greater than 0.5 μg. It was possible that contaminants or inhibitors from the plant material, such as polysaccharides, phenolic compounds, and secondary metabolites deleteriously affected RNA recovery. Therefore, the protocol was modified to include additional steps after the initial lysis and the incorporation of PEG and CTAB/NaCl treatments, followed by LiCl precipitation (**Figure 1**). The inclusion of high-molecular-weight PEG in the extraction protocol promotes the removal of polysaccharides and phenolic compounds from plant tissues that bind to or co-precipitate with RNA (Gehrig et al., 2000). CTAB is a detergent that acts to separate polysaccharides from nucleic acids (Chang et al., 1993; Jaakola et al., 2001). Lithium chloride is inefficient at precipitating DNA, proteins, or carbohydrates, and thus improves RNA yield and purity compared to other nucleic acid precipitation methods, i.e., ethanol and sodium acetate (Barlow et al., 1963; Cathala et al., 1983).

ANALYSIS OF RNA YIELDS

A combination of physical disruption using beads together with the chemical treatments (**Figure 1**) resulted in total RNA concentrations that averaged 5.1 μg (range from 1.5 to 8.9 μg) from 250 mg of infected root tissue. Assessment of the RNA showed a substantial increase in bacterial-derived 16S and 23S *r*RNA in bead-treated samples compared to BPEX samples (**Figure 2A** – compare lanes 4–6 with lanes 1–3). The optimized method increased the bacterial *r*RNA to levels that were equivalent or close to plant *r*RNA (**Figure 2C**).

QUANTITATIVE REVERSE TRANSCRIPTASE PCR (qPCR) ANALYSIS

Bacterial gene expression was quantified from the samples obtained using the optimized method, by carrying out qPCR. The *gyrB* gene was selected as a housekeeping gene that was expected to be expressed under the conditions tested and expression from the root-derived samples was compared to samples obtained from bacteria grown under *in vitro* conditions. The amount of *gyrB* transcript was found to be similar between both lettuce roots and *in vitro* samples (**Table 1**), indicating similar copy numbers and stable *gyrB* expression under *in vitro* conditions and in plant extracts. It is of note that the amount of detectable *gyrB* was lower in the infected spinach root extracts compared to lettuce roots (higher *Ct* value for *gyrB*).

Table 1 | Comparison of Ct values from *E. coli* O157:H7 isolate Sakai for an *in vitro* culture and for infected plant roots.

Sample	Average Ct ± SD
Sakai culture	23.435 ± 0.415
Sakai + lettuce roots	23.525 ± 0.295
Sakai + spinach roots	26.997 ± 0.527

In brief, plant roots were suspended in 20 ml bacterial inoculum (OD_{600} of 0.2) for 2 h at 18° C, washed vigorously, and RNA prepared. The bacteria-only culture was suspended in 20 ml 1× PBS for 2 h at 18° C. The gyrB gene was quantified using a StepOnePlus PCR machine (Applied Biosystems). The values show the average of six samples from two independent experiments.

QUANTIFICATION OF BACTERIA

To quantify the bacteria present in the samples, the average number of *E. coli* O157:H7 (Sakai) cells present in a 250 mg root sample (typically from 7 to 8 roots) was determined. The experimental set-up was as described for RNA extraction, although the roots were washed with PBS instead of the initial step of *m*RNA preservation in the ice-cold phenol/ethanol mixture. On average, 5.6×10^7 cfu of *E. coli* O157:H7 (Sakai) were present in each lettuce sample. The values for spinach roots were similar, which represent between 10 and 15% of the initial inoculum used for infection. These figures are equivalent to the limit of bacterial gene detection reported by Schenk et al. (2008) of $\sim 5\ 10^7$ cfu/sample derived from 20 leaf disks.

DISCUSSION

The isolation of total RNA from plant material is well defined for leaves, but less so for studies involving the roots or rhizosphere. The majority of reports focus on analysis of gene expression for phytopathogens and the number of examples describing the extraction of food-borne pathogen RNA from plants is limited. Here, we describe optimization of a method that resulted in high-quality bacterial *m*RNA from the infected roots of fresh produce plants: lettuce and spinach.

Reported investigations of gene expression of enteric bacteria, such as *Salmonella enterica* (Goudeau et al., 2013), *E. coli* K-12 and *E. coli* O157:H7 (Kyle et al., 2010; Fink et al., 2012) used large quantities of plant material, e.g., 100 g of leaf tissue coupled with high concentrations of bacteria, approx. 10^8 cfu per sample. Samples were processed to separate bacteria from plant tissue using physical methods, such as a Stomacher (Kyle et al., 2010; Goudeau et al., 2013) or by shaking (Fink et al., 2012) followed by filtration to remove plant debris. RNA was prepared either using commercial kits (Kyle et al., 2010; Goudeau et al., 2013) or a hot-phenol method (Fink et al., 2012). Some of these studies investigated gene expression on post-harvest material, using pre-packaged commercial leaves rather than propagating plants, where it is possible to obtain the large amount of material required. In contrast, investigation of bacteria gene expression on living plant roots presents challenges in obtaining similar weights of material. A hydroponic system for high-throughput propagation of lettuce plants was described recently (Hou et al., 2012, 2013), where sterilized seeds were germinated in 96-well pipette tip boxes. Whole transcriptome analysis of *E. coli* was examined three days after

inoculation with the plant roots, although the report does not state the weight of material required for RNA extraction. The main difference in our method was the amount of plant material required and the time at which the samples were taken. For example, our method allows for gene expression analysis after a short time of interaction, rather than after several days of colonization where the bacterial numbers are likely to have increased, dependent on the inoculation time and plant-bacterial system investigated.

The optimized method uses a combination of bead-beating, SDS lysis, phenol extraction, and CTAB purification to extract high-quality bacterial RNA from as little as 250–300 mg infected roots. Optimization has been carried out for two fresh produce plant species that have previously been associated with large-scale outbreaks of food-borne pathogens. It was interesting to note that the efficiency of recovery was lower for spinach root compared to lettuce, although the numbers of bacteria recovered from the plant tissue were similar. Since *E. coli* O157:H7 Sakai adheres to lettuce and spinach roots in comparable numbers (Wright et al., 2013), we anticipated similar levels of *gyrB* transcript. The reduction in level may be as a result of plant-associated inhibitors in spinach roots, or because of different plant-derived environmental cues acting on *gyrB* expression. Therefore, we suggest that the method needs to be validated if it is adopted for other plant species. Verification of the technique using qPCR shows that this method could be applied to food safety settings, e.g., for detection of food-borne pathogens in fresh produce. It may be possible to couple this technique to standard methods, which would enable examination of viability and expression of genes of interest, e.g., toxin genes. Furthermore, it facilitates analysis of bacterial gene expression *in planta* for not only food-borne pathogens but also other plant-associated bacteria, providing an insight into the adaptive processes that underpin host–microbe interactions.

AUTHOR CONTRIBUTIONS

Al authors were involved in the design of the work and interpretation of data; Ashleigh Holmes and Louise Birse were involved in data acquisition and analysis; Ashleigh Holmes, Louise Birse, and Nicola J. Holden drafted the work; all authors approved the final version.

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Pyrosequencing detects human and animal pathogenic taxa in the grapevine endosphere

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Generally, plants are not considered as hosts for human and animal pathogens (HAP). The recent produce-associated outbreaks of food-borne diseases have drawn attention toward significant deficiencies in our understanding of the ecology of HAP, and their potential for interkingdom transfer. To examine the association of microorganisms classified as HAP with plants, we surveyed the presence and distribution of HAP bacterial taxa (henceforth HAPT, for brevity's sake) in the endosphere of grapevine (*Vitis vinifera* L.) both in the plant stems and leaves. An enrichment protocol was used on leaves to detect taxa with very low abundance in undisturbed tissues. We used pyrosequencing and phylogenetic analyses of the 16S rDNA gene. We identified several HAPT, and focused on four genera (*Propionibacterium, Staphylococcus, Clostridium*, and *Burkholderia*). The majority of the bacterial sequences in the genus *Propionibacterium,* from grapevine leaf and stem, were identified as *P. acnes*. Clostridia were detected in leaves and stems, but their number was much higher in leaves after enrichment. HAPT were indentified both in leaves and wood of grapevines. This depicts the ability of these taxa to be internalized within plant tissues and maintain their population levels in a variety of environments. Our analysis highlighted the presence of HAPT in the grapevine endosphere and unexpected occurrence of these bacterial taxa in this atypical environment.

Keywords: endosphere, grapevine, pathogens, bacteria, pyrosequencing

INTRODUCTION

Endophytes (non-pathogenic microorganisms living inside plant tissues and cells) are common inhabitants of interior plant parts (the endosphere) universally present and found in all the species of plants studied up till now (Schulz and Boyle, 2006). Some endophytes are able to promote plant growth and to suppress plant diseases (Compant et al., 2005; Lugtenberg and Kamilova, 2009).

Intriguingly, among these endosphere-dwellers, we can occasionally find some human and animal pathogens (HAP) (Holden et al., 2009; Kirzinger et al., 2011). HAP not only contaminate plant surfaces, but also actively interact with plants and can colonize them as alternative hosts (Holden et al., 2009). Some human bacterial pathogens are capable to colonize inner plant tissues (Tyler and Triplett, 2008), a phenomenon that in most cases can be considered as an opportunistic exploitation of a short-term habitat (Campisano et al., 2014). Generally, pathogens are studied solely for their harmful impact on human and animal health, causing disease and epidemics. On the other hand, the regular interaction of human and animal carriers with their environment puts these pathogens in contact with alternative niches, including additional hosts (Enders et al., 1993; Lenz et al., 2003). It is then unsurprising that previous works found out well known and potential HAP undergoing an endophytitc stage in their lifestyle (Kirzinger et al., 2011). Scientific literature often reports that members of the family *Enterobactericeae*, including pathogenic *Salmonella* and *Shigella* genus strains, *Vibrio cholerae* strains, and the human opportunistic pathogen *Pseudomonas aeruginosa* were found on plants or inside plants (Akhtyamova, 2013). *Salmonella enterica* strains have been isolated as endophytic colonizers of barley roots, spreading to the rhizodermis layers (Kutter et al., 2006).

Enteric bacterial pathogens, usually transmitted through foods, are well adapted to vertebrate hosts and generally colonize the gut (Wagenaar, 2008). Some have humans as their principal host, while many others are persistent in animal populations, adapted to a particular reservoir or environment, and affect humans only incidentally (Lynch et al., 2009). HAP on plants are generally thought of as having a reservoir in the intestines of a vertebrate host and, once discarded in manure, coming into direct or indirect contact with epigeous or hypogeous plant tissues in a variety of ways. Traditionally, they were considered to be fleeting on plant surfaces, persisting inertly in cracks, wounds, and stomatal openings. They were considered unable to aggressively modify or to communicate with the plant. However, it is now apparent that enteric bacteria do not just land on and reside in plants. These pathogens can stick tightly

to produce, multiply, and enter into the tissues of leaves or fruits, in some cases even moving into inner plant parts (Berger et al., 2010; Erickson, 2012). Whether endophytic growth may actually be part of HAP life cycle is under debate. Findings such as those mentioned before, reporting an endophytic stage for HAP, would explain why existing surface decontamination procedures may be inefficient in removing contaminants from plant produce (Rosenblueth and Martinez-Romero, 2006; Teplitski et al., 2009; Saldaña et al., 2011; Barak and Schroeder, 2012; Olaimat and Holley, 2012). The diffusion of HAP in ecosystems are of scrupulous importance from the perspectives of both biology and evolution of cross-kingdom pathogenesis and/or adaptation (Lenz et al., 2003; Kirzinger et al., 2011). While the majority of published research has focused on describing the enteric HAP, there is no doubt that other HAP can also interact with plants as part of their life-cycle. Here, we surveyed the presence and distribution of HAP taxa (HAPT) in the endosphere of grapevine (*Vitis vinifera* L.). We used pyrosequencing of the bacterial 16S rDNA gene to identify sequences belonging to genera where HAP are abundant. We present the first (to the authors' best knowledge) report of the occurrence of bacteria in taxa potentially pathogenic to human and animals in the grapevine endosphere.

MATERIALS AND METHODS

SAMPLE COLLECTION AND DNA EXTRACTION

Grapevines samples were taken in Northern Italy. For stem endophytes analysis, 12 plants (7 plants cv Chardonnay and 5 plants cv Merlot) were sampled from vineyards in Trentino, Italy with farmers permission (sites are mapped here: http://goo.gl/maps/7AI7j) during the fall of 2010, from October 27th to November 11th. One lateral shoot per plant was removed and stored briefly at 5–10°C. Plant material was pre-processed as described previously (Pancher et al., 2012) and DNA was extracted from surface-disinfected and aseptically peeled grapevine stems. Briefly, plant material was pulverized in sterile steel jars using liquid nitrogen and a mixer-mill. DNA was extracted from each sample using FastDNA spin kit for soil and a FastPrep-24 mixer (MP Biomedical, USA) according to standard manufacturer protocols. Plants used for leaf endophytes analysis (cv Barbera) were sampled from a vineyard in Lombardia, Italy (site is mapped here: http://goo.gl/5Nfh9R) on October 15th, 2007. Plant leaves were surface-sterilized as previously described (Bulgari et al., 2009) and aseptically prepared for DNA extraction. In a subset of leaf samples DNA isolation was preceded by a microbe enrichment strategy as described in previous studies (Jiao et al., 2006; Bulgari et al., 2009, 2011). Briefly, plant tissues were sterilized, grounded in liquid nitrogen, and aseptically incubated at 28°C for 12 h in gentle agitation in an enzymatic solution (0.1% macerozyme, 1% cellulase, 0.7M mannitol, 5 mM N-morpholinoethanesulfonic acid, 9 mM $CaCl_2$, and 65 μM KH_2PO_4). In another subsample, DNA isolation was performed directly after surface sterilization. DNA was extracted for each sample according to the protocol described by Prince et al. (1993) modified by the addition of lysozyme (3 mg/ml), L-lysine (0.15 mol/l), EGTA (6 mmol/l, pH 8.0), and by the incubation at 37°C for 30 min, before the lysis step.

PYROSEQUENCING OF ENDOPHYTIC COMMUNITIES

To obtain amplicons for pyrosequencing, we amplified the 16S rDNA gene from each sample using High Fidelity FastStart DNA polymerase (Roche, USA) and the universal primers 799f/1520r with 454 adaptors and a sample-specific 10-mer barcode (designed following the instructions for Roche 454 technology[1]). These primers allow selective amplification of bacterial DNA, targeting 16S rDNA hypervariable regions v5-v9 (Chelius and Triplett, 2001) and minimize the chance of amplification of plastid DNA (Ghyselinck et al., 2013). The PCR product was separated on 1% agarose gel and gel-purified using Invitrogen PureLink (Invitrogen, USA). DNA was quantified via quantitative PCR using the Library quantification kit—Roche 454 titanium (KAPA Biosystems, USA) and pooled in a final amplicon library. The 454 pyrosequencing was carried out at the Sequencing Platform facility in Fondazione Edmund Mach, on the GS FLX+ system using the new XL+ chemistry dedicated to long reads of up to 800 bp, following the manufacturer's recommendations. The new XL+ chemistry coupled with the unidirectional sequencing strategy led to the sequencing of multiple variable regions on a single read and to overcome the bottleneck associated with a short read approach.

DATA ANALYSIS

We generated 16S rDNA gene sequences relative to endophytes from three batches of samples: leaf endophytes with bacterial enrichment, leaf endophytes without enrichment, and stem endophytes. We used Roche 454 GS FLX+ sequencing as described above and analyzed the sequencing output using a standard Qiime pipeline (Caporaso et al., 2010). As a first step we demultiplexed and filtered sequences on the basis of quality score and read length (only sequences with a minimum average score of 20 and length between 250 and 1000 bp were retained), in order to remove short and low quality reads. Moreover, in this step we removed sequences with more than 6 ambiguous bases or with homopolymers longer than 6 bases. We performed chimera identification and filtering using usearch61 (Edgar, 2010). After removing chimeras, we used uclust (Edgar, 2010) for clustering all the sequences into Operational Taxonomic Units (OTUs), by applying a similarity threshold of 97%. This is commonly used to represent species level similarity (although this threshold does not necessarily match with what is regarded as species for many microorganisms) (Crawford et al., 2009). For each OTU we picked a representative sequence using Qiime and we used these sequences to assign a taxonomical identity to each OTU using the RDP classifier (Wang et al., 2007). In this step we used a confidence threshold of 0.8 and *e*-value $\leq$ 0.001 against the Greengenes 97% reference data set. Based on taxonomical identity, we manually selected OTUs corresponding to HAPT from all three datasets. To understand the distance between these OTUs and database reference strains, we downloaded 16S rDNA sequences of HAP and non-pathogenic microorganisms from NCBI (www.ncbi.nlm.nih.gov) (Altschul et al., 1997) and aligned them to the representative sequences assigned to the

[1]http://www.liv.ac.uk/media/livacuk/centreforgenomicresearch/The_GS_FLX_Titanium_Chemistry_Extended_MID_Set.pdf

same taxon in our datasets. For the alignment we used MUSCLE (Edgar, 2004; Caporaso et al., 2010) and filtered the alignment using Qiime. Then, we built the phylogenetic trees shown in **Figures 1–4** from these alignments using DNAML (Maximum Likelihood) in BioEdit (Hall, 1999) and rendered them using Itol (Letunic and Bork, 2006, 2011). Information on OTU type and abundance was co-displayed with the generated trees. OTUs were colored according to the origin of samples (types: stem, leaf enriched, leaf non-enriched, HAP reference strain, non-pathogenic reference strain). Abundance was displayed as circles for every OTU (abundance is proportional to the circle radius).

The 16S rDNA gene nucleotide sequences, representative of the OTUs identified in this study, are available with NCBI GenBank accession numbers KJ851800-KJ851922 (Supplementary Table 1).

RESULTS

We investigated the composition of bacterial endophytic communities in the grapevine endosphere (both in the leaf and the stem) by pyrosequencing the bacterial 16S rDNA gene. Sequence analysis revealed the presence of several OTUs (70 from enriched leaves, 13 from non-enriched leaves, and 40 from stems, see **Table 1**) classified as HAPT or non-pathogenic taxa closely related to HAPT. We focused our subsequent analysis on four genera containing potential pathogens: *Propionibacterium, Staphylococcus, Clostridium*, and *Burkholderia*.

We inferred phylogenetic trees from the alignment of 16S rDNA nucleotide sequences. OTUs assigned to these taxa were identified, with varying abundance, in enriched and non-enriched leaves and in stems of grapevine. Most OTUs from grapevine leaves were assigned to genera *Clostridium* (44 and 4 OTUs from enriched- and not-enriched leaves, respectively) and *Staphylococcus* (17 and 6 OTUs from enriched- and not-enriched leaves, respectively), albeit the most abundant OTU from enriched leaf samples (denovo115) was associated with non-pathogenic species of the genus *Burkholderia* (**Figure 2**). The most abundant OTU from non-enriched leaves (denovo703) was assigned to a cluster including both pathogenic and non-pathogenic species of the genus *Staphylococcus*. On the other hand, most OTUs from endophytic bacteria in grapevine stems were assigned to genera *Burkholderia* (19 OTUs) and *Propionibacterium* (10 OTUs), although the most abundant OTU

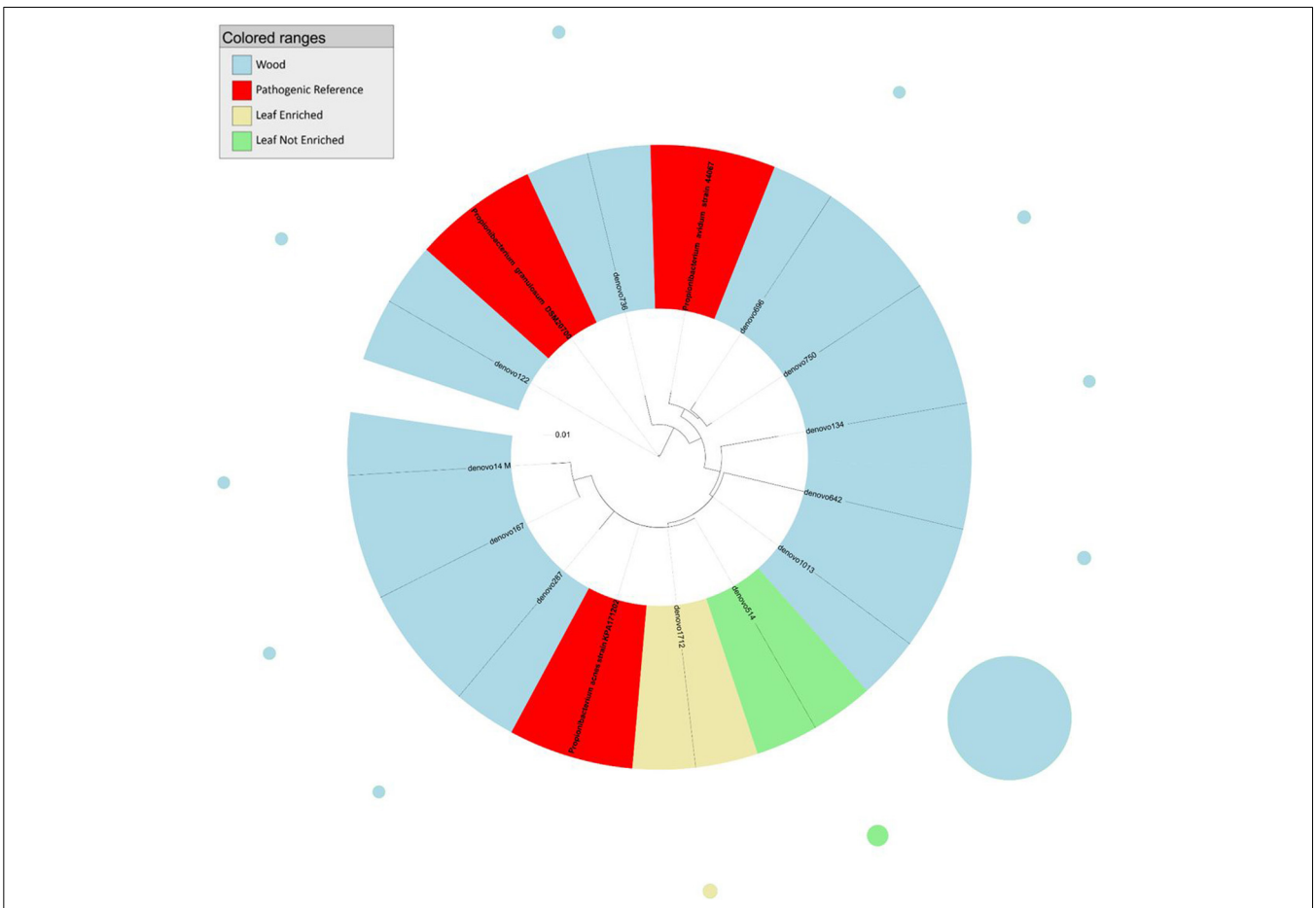

FIGURE 1 | Phylogenetic relationships based on partial 16S rDNA gene sequences obtained from pyrosequencing (this work) and closely related *Propionibacterium* sequences, retrieved from GenBank (Pathogenic and non-pathogenic Reference). The tree was built using a maximum likelihood method and rendered using iTOL. The relative abundance of each OTU is reported as circles and it is proportional to circle radius. Sequences tag and relative abundance circles were colored according to the source dataset.

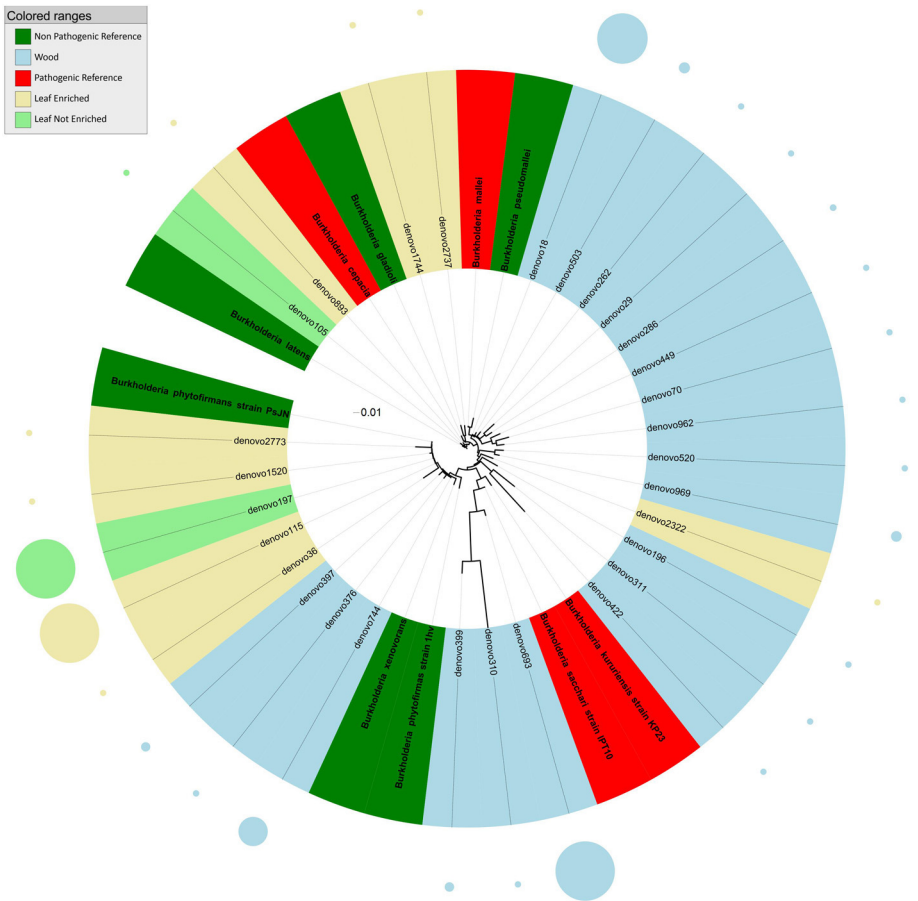

FIGURE 2 | Phylogenetic relationships based on partial 16S rDNA gene sequences obtained from pyrosequencing (this work) and closely related *Burkholderia* sequences, retrieved from GenBank (Pathogenic and non-pathogenic Reference). The tree was built using a maximum likelihood method and rendered using iTOL. The relative abundance of each OTU is reported as circles and it is proportional to circle radius. Sequences tag and relative abundance circles were colored according to the source dataset.

(denovo979) was taxonomically closer to pathogenic species *Staphylococcus epidermidis* (**Figure 3**).

The analysis of the phylogenetic tree of propionibacteria showed three main clades within the *Propionibacterium* genus (**Figure 1**). In detail, all sequences from grapevine leaves and most sequences from the stem clustered (cluster 1) with *Propionibacterium acnes* (which we previously demonstrated to be endocelluarly associated with grapevine stems, Campisano et al., 2014), while other sequences from the stem clustered with the species *P. granulosum* (cluster 2) and *P. avidum* (cluster 3).

Phylogenetic analysis indicated that sequences from grapevine endosphere assigned to the genus *Burkholderia* formed four distinct clusters (**Figure 2**). The first cluster included endophytic bacteria from grapevine leaves grouping with HAP and plant pathogens such as *B. latens*, *B. cepacia*, and *B. gladioli*. The second cluster included bacteria, identified exclusively in stems, grouping with *Burkholderia mallei* (HAP) and *B. pseudomallei* (non-pathogenic endophyte). The third cluster included endophytic bacteria from grapevine leaves and stem, grouping with environmental and endophytic bacteria such as *Burkholderia phytofirmans* and *B. xenovorans*. The forth cluster included HAP species of the genus *Burkholderia* (*B. kururiensis* and *B. sacchari*), and consisted of endophytic bacteria identified exclusively in grapevine wood tissues.

The tree obtained by analysis of 16S rDNA sequences assigned to the genus *Staphylococcus* (**Figure 3**) revealed that endophytes were distributed in six main clusters. Cluster 1 included sequences from grapevine leaf endophytes strictly related to the HAP species *S. saprophyticus* and *S. haemolyticus*, and to the non-pathogenic species *S. succinus*, *S. xylosus*, *S. lugdunensis*, and *S. warneri*. Cluster 2 included sequences from grapevine leaves and stems grouping with the pathogenic *S. aureus*, and one OTU from leaf strictly related to non-pathogenic species *S. sciuri*. The majority of OTUs from grapevine leaf and wood endophytic bacteria clustered together with the pathogenic species *S. epidermidis* (cluster 3). Cluster 4 included one OTU representing sequences from plant stems grouping closely to non-pathogenic species *S. caprae* and *S. urealyticus*. The remaining grapevine sequences, from both leaf and stem, grouped in two unassigned clusters (these OTUs shared sequence identity <97% in comparison with

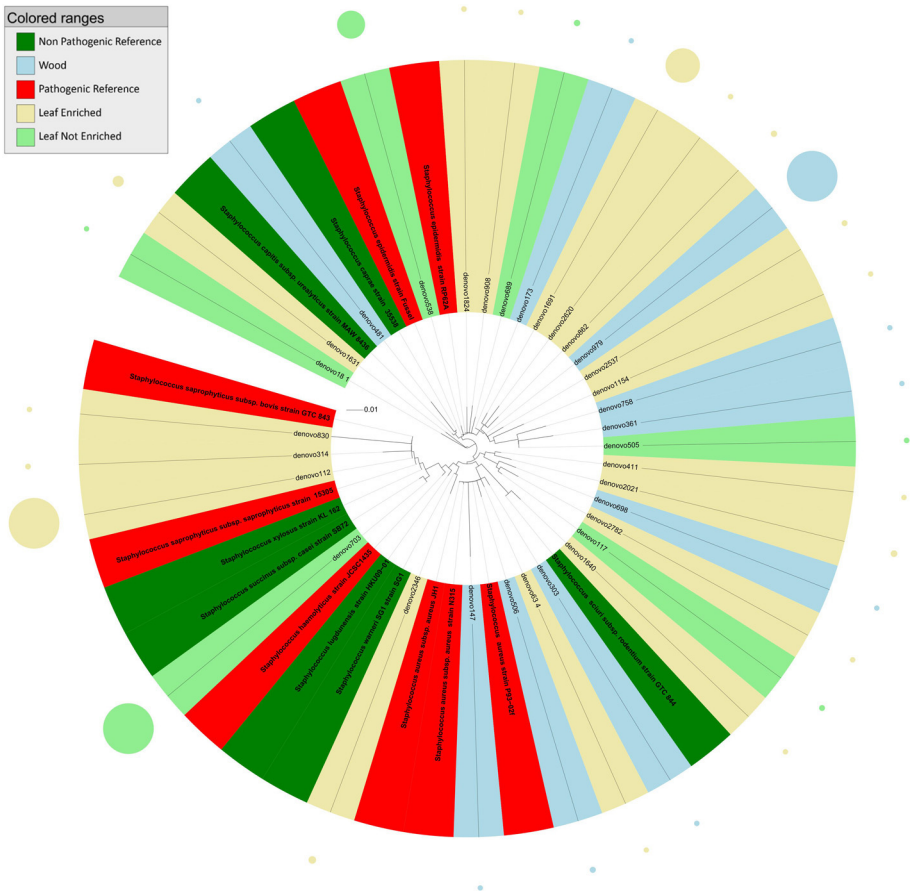

FIGURE 3 | Phylogenetic relationships based on partial 16S rDNA gene sequences obtained from pyrosequencing (this work) and closely related *Staphylococcus* sequences, retrieved from GenBank (Pathogenic and non-pathogenic Reference). The tree was built using a maximum likelihood method and rendered using iTOL. The relative abundance of each OTU is reported as circles and it is proportional to circle radius. Sequences tag and relative abundance circles were colored according to the source dataset.

16S rDNA gene nucleotide sequences previously deposited in GenBank). Blastn (best-hit) of these sequences indicated that the cluster 5 (represented by denovo411 and denovo2021) is related to *S. warneri*, while the cluster 6 (represented by denovo698, denovo2782, and denovo117) to *S. auricularis*.

Sequences identified as genus *Clostridium* (**Figure 4**) clustered into three groups relative to the tree inferred from the 16S rDNA sequence alignment. Most endophyte sequences in this genus were obtained from grapevine leaf. In detail, the first cluster included endophytic bacteria grouping with HAP such as *Clostridium difficile*, *C. tetani*, and with non-pathogenic environmental species (*C. vincentii* and *C. bijerinckii*). The second cluster included grapevine endophytic bacteria closely related to HAP *Clostridium botulinum* and *C. perfrigerans*, and non-pathogenic bacteria *C. drakei* and *C. ghonii*. The third cluster contained grapevine endophytic bacteria of the genus *Clostridium* related to unassigned species.

DISCUSSION

In the present study, we investigated microbial communities in the endosphere of grapevine leaves and stems by pyrosequencing and phylogenetic analyses of the 16S rDNA gene. Several endophytic OTUs belonging to four genera (*Propionibacterium, Staphylococcus, Clostridium*, and *Burkholderia*), known to include some species recognized as human- and animal-pathogens (HAP), were identified within the characterized microbial communities. Such OTUs were identified in enriched and not-enriched leaves and in grapevine stems. As reported in literature, the specific treatment of leaf samples with cell hydrolytic enzymes (microbe enrichment strategy) releases all microbes living in association with plant tissues (Jiao et al., 2006; Bulgari et al., 2009, 2011), improving the bacterial display and identification. The majority of the bacterial sequences in the genus *Propionibacterium,* from grapevine leaf and stem, were identified as *P. acnes.* This species is among the causing agents of acnes and its members are generally associated with human skin, where they feed on fatty acids secreted by sebaceous glands (Webster et al., 1981; Zouboulis, 2004). It also colonizes the human gut (Perry and Lambert, 2011), and is reported as an opportunistic pathogen in post-surgical infections (Nisbet et al., 2007). We previously identified genomic changes in *P. acnes* type Zappae, tightly associated with grapevines (Campisano et al., 2014). The

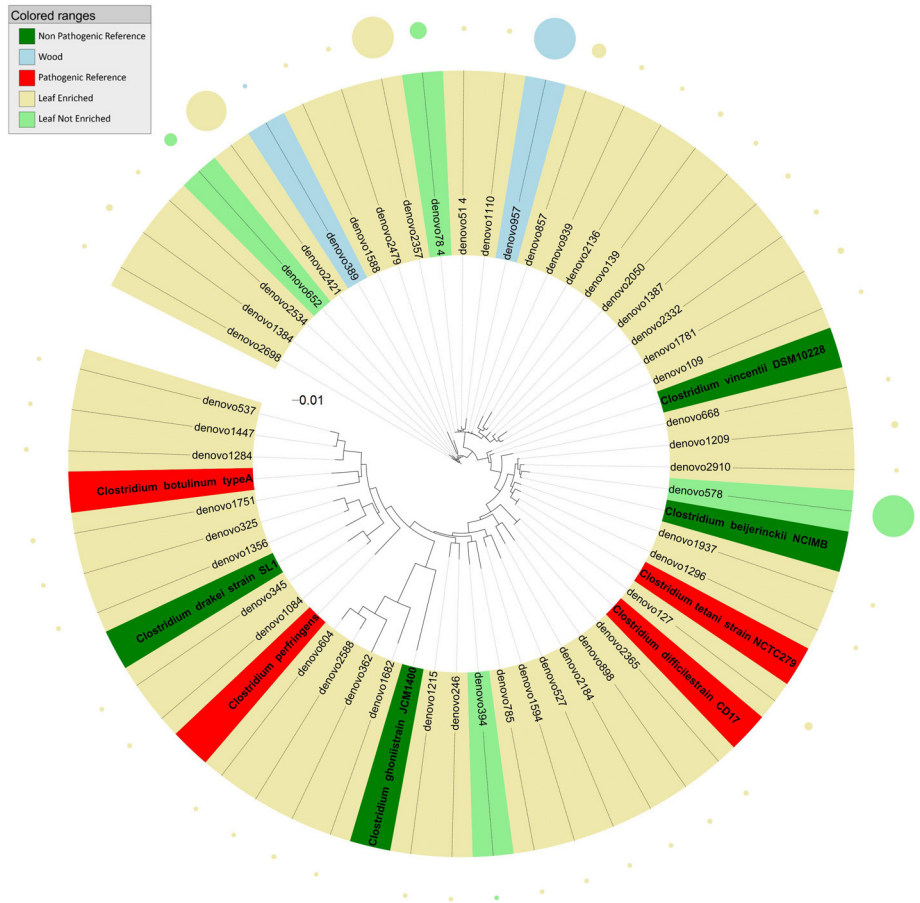

FIGURE 4 | Phylogenetic relationships based on partial 16S rDNA gene sequences obtained from pyrosequencing (this work) and closely related *Clostridium* sequences, retrieved from GenBank (Pathogenic and non-pathogenic Reference). The tree was built using a maximum likelihood method and rendered using iTOL. The relative abundance of each OTU is reported as circles and it is proportional to circle radius. Sequences tag and relative abundance circles were colored according to the source dataset.

wide diversity in sequences assigned to genus *Propionibacterium* shows that these endophytes are mostly present in the stem woody tissues (where the majority of sequences is amplified) and only marginally present in leaves, reinforcing the notion of a tight symbiosis of these bacteria with the plant.

The genus *Burkholderia* includes more than 60 species, some of which are known plant-dwellers (endophytes and epiphytes, Compant et al., 2008). Some species of *Burkholderia,* such as *B. mallei* and *B. cepacia* (*Burkholderia cepacia* complex, BCC), are recognized as HAP and plant pathogens (Govan et al., 1996; LiPuma, 1998; Coenye and Vandamme, 2003). Recently, *Burkholderia* species had gained considerable importance owing to their pathogenicity, but two findings had a strong impact on their ecological perception: (i) the identification of nitrogen fixation in *Burkholderia* species other than *B. vietnamiensis* (which belongs to the BCC), such as *B. brasilensis* M130 and *B. kururiensis*, and (ii) the description of legume-nodulating *Burkholderia* and their subsequent characterization as genuine endosymbionts (Suarez-Moreno et al., 2012). We found sequences clustering with such pathogenic *Burkholderia* in grapevine leaves. Stem-associated *Burkholderia* OTUs either grouped with non-pathogenic (or plant beneficial) species such as *B. phytofirmans* (Sessitsch et al., 2005) and *B. xenovorans* (Caballero-Mellado et al., 2007), or formed a separate phylogenetic group very similar to *B. kururiensis* and *B. sacchari*, by blastn analysis of best hit.

We can speculate that the presence of potentially pathogenic *Burkholderia* associated sequences in leaf green tissues but not in the woody stems represents a difference in the ecology of the plant-beneficial burkholderias from the HAP ones. All tissues in the leaf are in close proximity of the plant surface, while stem-associated tissues are possibly more difficult to colonize by transient colonizers. This may explain why plant-beneficial burkholderias were found mainly in the plant woody stem, while HAP ones only survive in leaves. We also noted that highly represented *Burkholderia* OTUs belong to the cluster including plant-associated species *B. phytofirmans* and *B. xenovorans* or to the clusters including *B. kururiensis* and *B. sacchari*, while the potentially pathogenic ones are in much lower numbers. *B. phytofirmans* is one of most studied endophytes. This strain was visualized colonizing the grapevine root surface, entering the endorhiza and spreading to grape inflorescence stalks, pedicels

Table 1 | Specifics for each datasets: number of samples collected, number of reads retained after quality filtering, OTUs retained after singleton removal, OTUs assigned to HAPTs and number of reads clustered in these OTUs.

Dataset	Plant samples	Total reads	Reads after filtering	total OTUs	OTUs of HAPT				Read of HAPT			
					Burkholderia	*Clostridium*	*Propionibacterium*	*Staphylococcus*	*Burkholderia*	*Clostridium*	*Propionibacterium*	*Staphylococcus*
Leaf enriched	56	201517	175578	2937	8	44	1	17	3095	3394	92	2139
Leaf not enriched	42	214376	186114	724	2	4	1	6	248	41	150	1652
Wood	12	97399	84750	1069	19	2	10	9	2922	42	2371	2748

and then to immature berries through xylem vessels (Compant et al., 2008). We also reported in this work as one of the most abundant burkholderias in grapevine.

OTUs from grapevine leaves were mostly identified as genera *Clostridium* and *Staphylococcus*. These taxa include endophytic OTUs closely related to the pathogenic species *Staphylococcus saprophyticus, S. aureus*, and *S. epidermidis*. Quite interestingly, most *Staphylococcus* sequences appeared to associate taxonomically with *S. epidermidis* (**Figure 3**). *S. epidermidis* colonizes the epithelial surfaces of every human being. Furthermore, it is one of the most common causes of nosocomial infections. In addition to the abundant prevalence of *S. epidermidis* on the human skin, this high incidence is mainly due to the exceptional capacity of *S. epidermidis* to stick to the surfaces of indwelling medical devices during device insertion (Otto, 2008, 2009). At our knowledge this is the first report of pathogenic *Staphylococcus* associated sequences in plants.

Almost all the endophytic sequences assigned to the genus *Clostridium* were amplified from grapevine leaf DNA. This group included sequences grouping with HAP such as *Clostridium difficile*, *C. perfrigens, C. botulinum*, and *C. tetani*. A major factor important to the colonization of plants is how long bacteria persist in the soil before dying. *Clostridium* spores persisted in soil for 16 months and were found on the leaves of parlsey grown in the contaminated soil (Girardin et al., 2005). We can speculate that the presence of *Clostridium,* human and animal pathogenic taxa (HAPT) in grapevine leaves could be related to long–lasting spore contamination. Honey sometimes contains spores of *C. botulinum*, which may cause infant botulism in humans 1 year old and younger. The toxin eventually paralyzes the infant's breathing muscles (Tanzi and Gabay, 2002). *C. difficile* can flourish when other bacteria in the gut are killed during antibiotic therapy, leading to pseudomembranous colitis (a cause of antibiotic-associated diarrhea).

HAPT were indentified both in leaves and wood of grapevines. This depicts the ability of these pathogens to be internalized within plant tissues. Isolation of human pathogenic enterobacteria from within the tissue of fresh and minimally prepared produce has been reported (Eblen et al., 2004; Shi et al., 2007; Soto et al., 2007; Holden et al., 2009). Furthermore, it appears that once a plant has been colonized by bacteria there is the potential for vertical transmission to successive generations, as demonstrated for *S. typhimurium* on tomatoes (Guo et al., 2001). The exploration of endophytic communities, using metagenome-based community analyses (Bulgarelli et al., 2012; Lundberg et al., 2012) coupled with the exploration of the pathogenic potential of pathogens, are beginning to reveal that many HAP are capable of exploiting plant hosts. This means the apparent pathogens may have adapted to the plants and have become plant symbionts, for at least one stage of their life cycle. This also shows the ability and potential of HAP to persist on multiple hosts, with plants serving as intermediate hosts or reservoirs for them. The ability of these pathogens to maintain their population levels in a variety of environments likely increases their pan genome and evolutionary potential (Campisano et al., 2014). Although we focused on four genera (*Propionibacterium, Staphylococcus, Clostridium*, and *Burkholderia*) only, we identified several other taxa known

for harboring HAP bacteria. Our analysis highlighted the presence of potential HAPT in the grapevine endosphere and, to the authors' knowledge, represents the first report of the unexpected occurrence of these bacterial taxa in this atypical (yet crucially important for agriculture) environment.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00327/abstract

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Colonization of plants by human pathogenic bacteria in the course of organic vegetable production

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In recent years, increasing numbers of outbreaks caused by the consumption of vegetables contaminated with human pathogenic bacteria were reported. The application of organic fertilizers during vegetable production is one of the possible reasons for contamination with those pathogens. In this study laboratory experiments in axenic and soil systems following common practices in organic farming were conducted to identify the minimal dose needed for bacterial colonization of plants and to identify possible factors like bacterial species or serovariation, plant species or organic fertilizer types used, influencing the success of plant colonization by human pathogenic bacteria. Spinach and corn salad were chosen as model plants and were inoculated with different concentrations of *Salmonella enterica* sv. Weltevreden, *Listeria monocytogenes* sv. 4b and EGD-E sv. 1/2a either directly (axenic system) or via agricultural soil amended with spiked organic fertilizers (soil system). In addition to PCR- and culture-based detection methods, fluorescence *in situ* hybridization (FISH) was applied in order to localize bacteria on or in plant tissues. Our results demonstrate that shoots were colonized by the pathogenic bacteria at inoculation doses as low as 4×10 CFU/ml in the axenic system or 4×10^5 CFU/g in the soil system. In addition, plant species dependent effects were observed. Spinach was colonized more often and at lower inoculation doses compared to corn salad. Differential colonization sites on roots, depending on the plant species could be detected using FISH-CLSM analysis. Furthermore, the transfer of pathogenic bacteria to plants via organic fertilizers was observed more often and at lower initial inoculation doses when fertilization was performed with inoculated slurry compared to inoculated manure. Finally, it could be shown that by introducing a simple washing step, the bacterial contamination was reduced in most cases or even was removed completely in some cases.

Keywords: organic food, vegetable, organic fertilizer, *Salmonella enterica*, *Listeria monocytogenes*

INTRODUCTION

The consumption of vegetables is essential for a healthy nutrition and is recommended by different health organization in order to provide minerals and vitamins as well as for the prevention of cardiovascular diseases (World Health Organization, 2003; United States Department of Agriculture, 2011). In the years 1997–1999 an increase of the consumption of fresh vegetables was recorded in the USA, staying on this high level for the following years (Blanck et al., 2008; Berger et al., 2010). Most of this food is consumed raw or after minimal processing. Therefore, it is crucial to avoid its contamination with human pathogenic bacteria, viruses or health threatening substances throughout the production chain. Nevertheless, in the last years an increasing number of outbreaks caused by the consumption of vegetables contaminated with human pathogenic bacteria were reported (Sivapalasingam et al., 2004; Heaton and Jones, 2008). For example, in 2007 lettuce was the one of the three most frequent sources of foodborne disease outbreaks in the USA (Center of Disease Control and Prevention, 2010).

Salmonella enterica and *Listeria monocytogenes* are two foodborne human pathogens involved in many outbreaks. In the years 2002–2007 *S. enterica* was the most frequent causative agent of foodborne diseases in the USA. The largest outbreak in this time period with 802 documented patients was traced back to the consumption of "hummus" (mashed chickpeas) contaminated with this bacterium (Center of Disease Control and Prevention, 2010). Hanning et al. (2009) summed up *S. enterica* outbreaks in the USA based on the consumption of fresh produce showing that many different vegetable plant species were involved in those outbreaks. Due to its importance as produce contaminant much research has been done in this direction proving the high potential of *S. enterica* to colonize surfaces as well as interior of various plants (Brandl, 2006; Berger et al., 2010; Krtinić et al., 2010). *Listeria monocytogenes* outbreaks based on the consumption of fresh produce were reported in lower numbers compared to those derived from other food sources (US Food and Drug Administration, 2003). Nevertheless, a recent outbreak of listeriosis in the USA in 2011 was caused by the consumption of contaminated cantaloupes (Center of Disease Control and Prevention, 2011). Also in sporadic cases of infections with *L. monocytogenes*, vegetables were identified as causative food source (Farber and Peterkin, 1991). However, in a high number of outbreaks or

single cases, no specific food source could be identified, because of the very long incubation time until appearance of listeriosis symptoms (McLauchlin, 1996). Therefore, the US food and drug administration (FDA) as well as the EU dictate a zero tolerance policy for *S. enterica* and *L. moncytogenes* in ready to eat food throughout the production chain, although the EU-legislation allows 100 CFU of *L. moncytogenes* in 25 g of sample material, for food already placed on the marked.

Various possibilities of contamination with human pathogenic bacteria exist within the vegetable production chain. For example, bacteria could be transferred to the plants by contaminated irrigation water, via soil or direct contact (Wachtel et al., 2002). Different wild animals were also proven to be possible carrier of various human pathogenic bacteria (Palmgren et al., 1997; Makino et al., 2000; Handeland et al., 2002; Millan et al., 2004; Renter et al., 2006; Hellström et al., 2008; Sánchez et al., 2010; Wacheck et al., 2010; Vieira-Pinto et al., 2011). It cannot be excluded that those animals can access the agricultural fields and directly contact the plants grown there. The fertilization with slurry or manure is an additional way of contamination (Chen and Jiang, 2014). It has been shown that organic fertilizers can contain various human pathogenic bacteria (Hutchison et al., 2004, 2005). A transfer via direct contact with the plants or indirectly via the fertilized soil is therefore possible. In order to enable a reduction of the pathogen load in the soil the US government dictates that organic fertilizer has to be incorporated not less than 120 days before harvest of plants. However, regulations regarding the time point of organic fertilizer application strongly differ between countries. Furthermore, contamination of produce may also occur in postharvest processing steps. For example *L. monocytogenes* was detected in samples of the food processing environment like door handles, floors and walls (O'Connor et al., 2010). Similar results for *S. enterica* were obtained in a comparable study (Lettini et al., 2012). Those bacteria can also form biofilms on stainless steel surfaces and by that be protected from disinfection (Sinde and Carballo, 2000).

Based on the hypothesis that most of the contaminations of vegetable plants by human pathogenic bacteria can effectively be reduced already at farm level, this study focuses on the first steps of the organic vegetable production chain at the farm level until harvest. In order to demonstrate the influence of plant host species and bacterial species on plant colonization success, the minimal bacterial infection doses of the selected bacterial strains *S. enterica* sv. Weltevreden, *L. monocytogenes* sv. 4b and *L. monocytogenes* EGD-E sv.1/2a needed for the colonization of spinach or corn salad plants were determined in inoculation experiments. The *S. enterica* sv. Weltevreden strain used in this study was originally isolated from an outbreak of salmonellosis caused by the consumption of contaminated alfalfa sprouts (Emberland et al., 2007). The *L. monocytogenes* serotypes 1/2a and 4b were selected because of their high clinical relevance as causative strains of a large number of listeriosis cases. To meet the zero tolerance policy in ready to eat foods throughout the production chain for the contamination with *L. monocytogenes* and *S. enterica*, another objective was to develop and apply a most sensitive and reliable combination of cultivation enrichment and PCR-detection approach for the studied pathogenic bacteria. Furthermore, the colonization sites of the inoculated bacterial species on plant roots were identified by combining fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy (CLSM). The growth conditions in the axenic system as well as the absence of competition by soil bacteria might enhance bacterial colonization of plants (Klerks et al., 2007). Therefore, and in order to verify the results of the inoculation experiments, regarding the influence of plant- as well as bacterial species, in a more natural system, experiments in a soil system using spiked manure or slurry for fertilization of the plants were applied. By this, the influence of the type of organic fertilizer used, on plant colonization by the selected human pathogenic bacteria was analyzed. The results presented identified factors influencing the frequency of colonization of vegetable plants by human pathogenic bacteria. This may help to minimize the risk of produce contamination already at the farm level and thus may increase the safety for the consumer.

MATERIALS AND METHODS

PLANT SPECIES AND BACTERIAL STRAINS

Spinach (*Spinacia oleracea*) variety "Butterflay" and corn salad (*Vallerianella locusta*) variety "Verte á ceour plein 2" (Bingenheimer Saatgut AG, Echzell-Bingenheim, Germany) and three bacterial strains were used for inoculation experiments: *L. monocytogenes* EGD-E sv. 1/2a (DSM20600, Deutsche Stammsammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ, Germany), *L. monocytogenes* sv. 4b (SLCC4013, special *Listeria* culture collection, Würzburg, Germany) and *S. enterica* sv. Weltevreden (2007-60-3289-, culture collection, zoonosis laboratory, DTU-FOOD, Denmark).

PREPARATION OF PLANT SEEDLINGS

Plant seeds were surface sterilized according to the protocol published by Rothballer et al. (2003) with slight modifications. The seeds were washed in 1% Tween80 (2 min) and subsequent in 70% ethanol (2 min) followed by three washing steps in sterile deionized water. After an incubation step in 13% sodium hypochlorite solution (Sigma-Aldrich® Co., St. Louis, USA) (20 min) seeds were again washed three times in sterile deionized water, incubated in sterile deionized water for 4 h followed by a second incubation step in 13% sodium hypochlorite solution for 10 min. Five washing steps in sterile deionized water completed the surface sterilization. Seeds were then placed on NB (Nutrient Broth) agar plates and incubated for 3 days at room temperature in the dark to allow germination and to control the success of surface sterilization. Only germinated seedling showing no visible contamination were used for further experiments.

INOCULATION EXPERIMENTS IN AN AXENIC MODEL SYSTEM

Listeria monocytogenes strains were cultivated in Brain-Heart-Infusion (BHI) liquid media at 30°C and *S. enterica* sv. Weltevreden in buffered peptone water (BPW) at 37°C over night. The bacterial cells were washed and afterwards diluted in 1 × PBS to a final density of 4×10^8 CFU/ml. The germinated seedlings were inoculated for 1 h in different dilutions ranging from 4×10 to 4×10^6 CFU/ml of the different bacterial strains. Non-inoculated seedlings were used as control. After inoculation seedlings were planted to sterile "Phytatray 2"-boxes

(Sigma-Aldrich® Co., St. Louis, USA) filled with 20 ml sterile quartz sand and 10 ml MS (Murashige and Skoog) medium (Sigma-Aldrich® Co., St. Louis, USA). Three boxes with four seedlings per box were prepared for each inoculation dose. Plants were grown for 3 weeks in a phytochamber (humidity: 50%, day length: 14 h, day temperature: 23°C, night temperature: 18°C, light intensity: 360 μmol/m^{2}s) until harvest.

SPIKING EXPERIMENTS IN THE SOIL SYSTEM

Fresh, not processed organic bovine slurry and manure as well as organically managed agricultural soil (Ap horizon) obtained from the "Versuchsgut Scheyern" of the Helmholtz Zentrum München used in this study were initially tested for the presence of *L. monocytogenes* and *S. enterica* using enrichment as well as PCR-methods. Only organic fertilizer and soil considered free of those bacteria were used for further experiments. Bacterial strains were prepared as decribed above and added to slurry and manure at concentrations of 4×10^5, 4×10^6, 4×10^7, and 4×10^8 CFU/ml slurry or CFU/mg manure. Five hundred grams of fresh weight of agricultural soil were mixed with 100 ml deionized water and 20 g of spiked manure or 30 ml of spiked slurry and filled into planting pots. The amount of organic fertilizer used is in accordance to usual farming practice (2–4 kg/m^2 for manure, 3–6 l/m^2 for slurry). The final bacterial concentrations in the soil were 2.4×10^4, 2.4×10^5, 2.4×10^6, and 2.4×10^7 CFU/g for slurry setups and 1.6×10^4, 1.6×10^5, 1.6×10^6, and 1.6×10^7 CFU/g for manure. After a preincubation of the pots for 3 days at room temperature in a mini greenhouse (Edm. Romberg & Sohn GmbH & Co. KG, Ellerau, Germany), the sterile seedlings were planted in the pots. Three pots with four seedlings each were used per dilution step and incubated for 4 weeks in a phytochamber with the settings described above. Pots prepared with non-spiked organic fertilizer served as negative control.

SAMPLING AND SAMPLE PREPARATION

Plant samples of the axenic system were taken in triplicates whereas five samples per treatment were taken from the soil system. After harvest, root and shoot of the plants were separated aseptically. The plant parts were washed in 30 ml of 1 × PBS and afterwards ground in 1 ml 1 × PBS. One milliliter of the resulting cell suspension was used for enrichment cultures. Also the 1 × PBS used for washing was further processed. After a cell harvest for 10 min at 6000 rpm the resulting pellet was resuspended in 2 ml of 1 × PBS. Again 1 ml of the cell suspension was used for further enrichment. Additionally, fixed samples of plant parts were prepared after cutting off parts of roots and shoots. *Salmonella enterica* inoculated or spiked samples were fixed in 4% paraformaldehyde (PFA) according to Amann et al. (1990), whereas *L. monocytogenes* inoculated or spiked samples were fixed in 1:1 Ethanol/PBS (Roller et al., 1994). Fixed plant samples were stored at −20°C in 1:1 Ethanol/PBS until further processing.

ENRICHMENT OF THE BACTERIA

For the selective enrichment of *Listeria* spp. 1 ml of cell suspension was added to 9 ml Buffered-*Listeria*-Enrichment-Broth (BLEB) (Merck KGaA, Darmstadt, Germany). After 2 h of preincubation at 30°C *Listeria*-Selective-Enrichment supplement (Merck KGaA, Darmstadt, Germany) was added and the culture was further incubated at 30°C. After 24 h, 48 h, and 7 days 0.1 ml of the enrichment culture were transferred to 10 ml Half-Fraser-Bouillon (Merck KGaA, Darmstadt, Germany) and incubated aerobically at 37°C. Enrichment culture dependent detection of *Listeria* spp. was conducted after 24 and 48 h using Oxford-Agarplates (Merck KGaA, Darmstadt, Germany) and Palcam-Agarplates (Merck KGaA, Darmstadt, Germany) inoculated with the second enrichment culture. In case of a detection of *Listeria* spp. Rapid'L.mono medium (Bio-Rad Laboratories GmbH, Munich, Germany) plates were used to identify *L. monocytogenes*. After inoculation, the identification plates were incubated at 37°C for 48 h.

In order to enrich *S. enterica* cells, 1 ml of cell suspension was added to 9 ml of BPW and incubated aerobically 37°C for 24 h. Afterwards cultures were streaked out on Xylose-Lysine-Deoxycholat (XLD) agar plates (Fluka, Buchs, Switzerland) and incubated at 37°C for 24 h in order to identify *Salmonella* spp.

DNA-EXTRACTION

DNA-extraction was conducted for *L. monocytogenes* after 48 h of enrichment in BLEB and for *S. enterica* after 24 h of enrichment in BPW. Cells of 2 ml enrichment culture were harvested at 6000 rpm for 10 min. *Salmonella enterica* samples were processed directly whereas the pellet *L. monocytogenes* enrichment cultures was resuspended in 50 μl of 300 U/ml Mutanolysin (Sigma-Aldrich® Co., St. Louis, USA) and incubated for 30 min at 37°C in order to increase the DNA-yield (Fliss et al., 1991). DNA-extraction was conducted using the "Bio 101 FastDNA® SPIN Kit for Soil" and the "Fast-Prep-Instrument" (MP-Biomedicals LLC., Solon, USA) following the manufacturers' instructions. Quality and quantity of the extracted DNA was analyzed using a ND-1000 Nanodrop-photometer (Thermo Fisher Scientific, Waltham, USA) and stored at −20°C until further processing.

PCR-BASED DETECTION OF *L. MONOCYTOGENES* AND *S. ENTERICA*

The specific PCR-detection of *L. monocytogenes* was conducted using the *iap* targeted primerset MonoA/MonoB described by Bubert et al. (1992) and the detection of *S. enterica* by using the *inv-A* specific Primerset inv-Af/inv-Ar described by Rahn et al. (1992). The "Top Taq Polymerase" system (QIAGEN, Hilden, Germany) was applied for all PCR reactions following the manufacturers protocols. In order to increase specificity a "Touchdown-PCR" program (Don et al., 1991) was used. Therefore, the annealing temperature was lowered by 1°C every cycle starting from 70°C down to 63°C followed by 35 cycles with 63°C annealing temperature. The initial denaturation of 94°C for 5 min was followed by cycles comprised of a denaturation of 94°C for 30 s, an annealing for 30 s and an elongation of 72°C for 1 min. A final elongation step at 72°C for 10 min was integrated before samples were stored at 4°C. Success of the PCR-reaction and length of the amplification product was controlled by applying horizontal gelelctrophoresis. To verify the correct amplification of a positive PCR-signal the resulting fragments were purified using the NucleoSpin® Extract II Kit (Macherey & Nagel, Düren, Deutschland) and sequenced with a capillary sequencer ABI 3730

(Applied Biosystems) following the chain termination method (Sanger et al., 1977).

FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) AND CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

FISH was conducted on root samples of plants grown in the axenic system, initially inoculated with 4×10^6 CFU/ml of the respective bacteria. Since the plants were free of contamination, no species specific detection for *L. monocytogenes* or *S. enterica* was necessary and the application of bacteria-specific 16S-rRNA targeted EUB-338 probe mix (EUB-338 I Amann et al., 1990, EUB-338 II, and EUB-338 III Daims et al., 1999) was sufficient. FISH was conducted based on Manz et al. (1992) and Amann et al. (1992) with modifications. Hybridization was performed in a 2 ml tube as described by Grube et al. (2009). After dehydration in an ethanol-series of 50, 80, and 100% ethanol for 3 min each, 100 µl of hybridization buffer [360 µl of 5 M NaCl, 40 µl of 1 M Tris/HCl pH 8.0, 2 µl of 10% (m/v) SDS, 1.6 ml of deionized sterile water] as well as 10 µl of a 20 ng/µl probe solution was added to the fixed sample. The hybridization at 46°C for 2 h was followed by a stringent washing step for 15 min at 48°C in washing buffer [9 ml of 5 M NaCl, 1 ml of 1 M Tris/HCl pH 8.0, 50 µl of 10% (m/v) SDS, add 50 ml with sterile deionized water]. Afterwards, samples were placed on a microscope slide, embedded in Citifluor-A F1 (Citifluor Ltd., London, Großbritannien) and covered with a cover slid. CLSM analysis was performed using a LSM 510.meta (ZEISS, Oberkochen, Germany) equipped with a C-Apochromat® 63x/1.2 W Korr. objective. The "Zeiss LSM Image Browser version 4.2" software was used for image processing.

RESULTS

INOCULATION EXPERIMENTS IN THE AXENIC SYSTEM

PCR and enrichment based detection of plant colonization

The detection of the inoculated bacteria was based on selective enrichment and specific PCR. In all samples qualified as "positive" the inoculated bacteria were detected with at least one of the applied methods. The results are summarized in **Table 1**.

In spinach and corn salad, *S. enterica* sv. Weltevreden was detected in all root samples and all samples of the root washing liquid. In spinach shoot and shoot washing liquid samples the bacteria also were found at initial inoculation doses as low as 4×10 CFU/ml. Nevertheless, in samples inoculated with 4×10 and 4×10^2 CFU/ml, *S. enterica* sv. Weltevreden was detected more often in shoot plant samples compared to washing liquid samples. In contrast, corn salad shoots and the according washing liquids were found to be positive for the bacteria at inoculation doses of 4×10^3 and 4×10^2 CFU/ml, respectively.

In spinach plants, both of the used *L. monocytogenes* strains were found in root and root washing liquid samples at all inoculation doses. In samples inoculated with *L. monocytogenes* sv. 4b the lowest inoculation dose which resulted in a contamination of spinach shoot but not the according washing liquid could be observed was 4×10^2 CFU/ml. Positive detection was possible in nearly all shoot and shoot washing liquid samples inoculated with higher bacterial doses. This was also found for *L. monocytogenes* EGD-E sv. 1/2a in samples inoculated with 4×10^2 CFU/ml or more. In corn salad plants inoculated with *L. monocytogenes* sv. 4b only few root and root washing liquid samples were positive at inoculation doses of less than 4×10^3 CFU/ml and only few shoots were colonized at inoculation doses below

Table 1 | Results of the inoculation experiment in the axenic system.

		S. enterica sv. Weltevreden				*L. monocytogenes* sv. 4b				*L. monocytogenes* EGD-E sv. 1/2a			
Plant	Inoculation (CFU/ml)	Root	Root washing liquid	Shoot	Shoot washing liquid	Root	Root washing liquid	Shoot	Shoot washing liquid	Root	Root washing liquid	Shoot	Shoot washing liquid
Spinach	control	0	0	0	0	0	0	0	0	0	0	0	0
	4.00E+01	3	3	2	1	2	2	0	0	3	3	0	0
	4.00E+02	3	3	3	1	3	3	2	0	3	3	2	3
	4.00E+03	3	3	3	3	3	3	2	3	3	3	2	1
	4.00E+04	3	3	3	3	3	3	3	3	3	2	3	3
	4.00E+05	3	3	3	3	3	3	3	2	3	3	3	3
	4.00E+06	3	3	3	3	3	3	3	3	3	3	3	3
Corn salad	control	0	0	0	0	0	0	0	0	0	0	0	0
	4.00E+01	3	3	0	0	1	1	0	0	0	0	0	0
	4.00E+02	3	3	0	1	0	0	0	0	2	2	0	0
	4.00E+03	3	3	3	3	2	3	1	1	2	1	1	0
	4.00E+04	3	3	2	2	2	3	0	1	3	2	1	1
	4.00E+05	3	3	3	3	3	3	0	0	3	3	0	0
	4.00E+06	3	3	2	3	3	3	2	2	3	3	2	1

Numbers and colors in the table indicate the numbers of positive detections of the bacterial strains of interest with at least one of the applied detection methods. For each dilution step three plants were analyzed.

4×10^6 CFU/ml. The same was true for corn salad inoculated with *L. monocytogenes* EGD-E sv. 1/2a, with the exception that root colonization took already place at an inoculation dose of 4×10^2 CFU/ml.

Combined FISH/CLSM analysis of inoculated roots

In order to localize the inoculated bacteria on the roots of spinach and corn salad and to identify preferential colonization sites, a combined FISH/CLSM analysis was conducted. *Salmonella enterica* sv. Weltevreden was found to colonize spinach roots preferentially in the root hair zone. It was detected both, on the surface of the root hairs (**Figure 1**) as well as in cell interspaces of the main root in this zone (**Figure 2**). On corn salad roots the bacteria were mainly detected on the surface of root tip cells (**Figure 3A**) and rarely in the root hair zone (data not shown). On the root tip even a colonization of the glycocalix was observed (**Figure 3B**). *Listeria monocytogenes* sv. 4b colonized spinach roots in the root hair zone in cell interspaces of the root (**Figure 4**). It was not detected on root hairs or on the root tips (data not shown). Corn salad plants were mainly colonized by *L. monocytogenes* sv. 4b shortly behind the root tip but were not detected at the root tip (**Figure 5**). They were also found in cell interspaces of older root parts, but only in rare cases and small numbers (**Figure 5**).

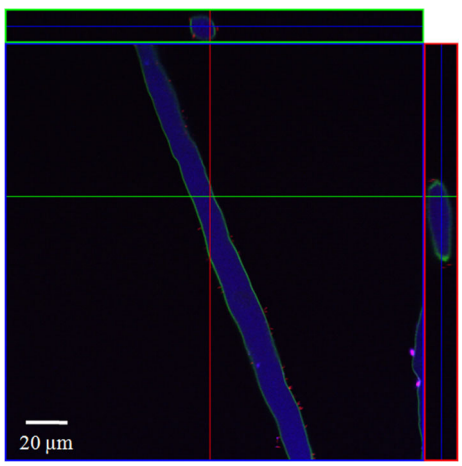

FIGURE 1 | CLSM image of a spinach root hair. The plant seedling was inoculated for 1 h with *S. enterica* sv. Weltevreden at an inoculation density of 4×10^6 CFU/ml. The plant was harvested after 3 weeks of growth in an axenic system. Probe Cy3 marked EUB-338, I, II, III was applied during FISH procedure (red signal).

SPIKING EXPERIMENTS IN THE SOIL SYSTEM

The detection of *S. enterica* sv. Weltevreden was performed using *invA* gene specific PCR after enrichment in BPW, whereas colonization by the *L. monocytogenes* strains was analyzed using the *iap* gen targeted PCR as well as a selective enrichment. All obtained PCR-fragments were sequenced to avoid false positive results. In the samples qualified as "positive" at least one of the used methods of analysis was successful. The results are summarized in **Table 2**.

Colonization by S. enterica sv. Weltevreden

Spinach roots were colonized by *S. enterica* sv. Weltevreden at all investigated spiking densities independent from the organic fertilizer used. Shoot plant samples of spinach were exclusively tested "positive" when fertilized with spiked slurry even at the lowest spiking density of 4×10^5 CFU/ml. In contrast, in shoot washing liquid samples the bacteria were only detected at high spiking doses of at least 4×10^7 CFU/ml, independent of the organic fertilizer used.

Corn salad roots were found to be colonized by the tested bacteria in samples of all spiking doses. More frequent positive detection events were observed in root washing liquid samples compared to the root samples. *Salmonella enterica* sv. Weltevreden was detected in three shoot samples fertilized with spiked slurry at spiking densities of 4×10^6 and 4×10^7 CFU/ml

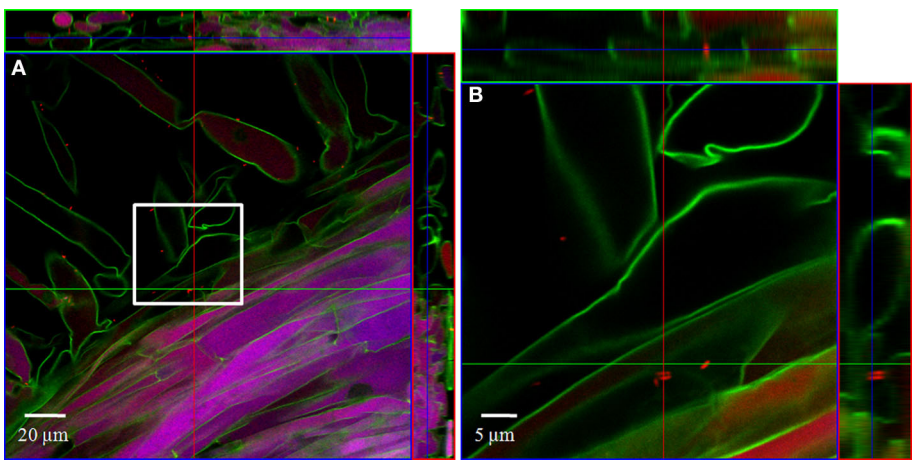

FIGURE 2 | CLSM images of a spinach root. The plant seedling was inoculated for 1 h with *S. enterica* sv. Weltevreden at an inoculation density of 4×10^6 CFU/ml. The plant was harvested after 3 weeks of growth in an axenic system. Probe Cy3 marked 338, I, II, III was applied during FISH procedure (red signal). **(B)** is an enlarged capture of the area marked in **(A)**.

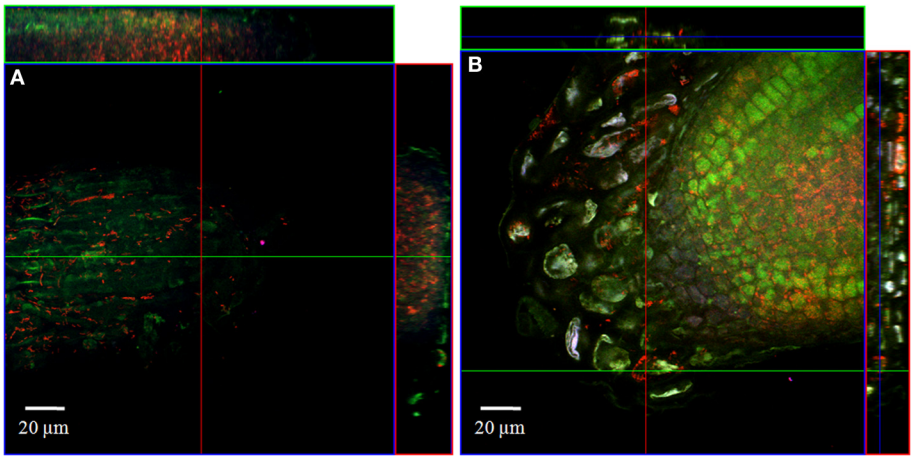

FIGURE 3 | CLSM images of corn salad root tips. The plant seedlings were inoculated for 1 h with *S. enterica* sv. Weltevreden at an inoculation density of 4×10^6 CFU/ml. The plants were harvested after 3 weeks of growth in an axenic system. Probe Cy3 marked EUB-338, I, II, III was applied during FISH procedures (red signal). The images show **(A)** surface colonization and **(B)** glycocalix colonization of the root tips.

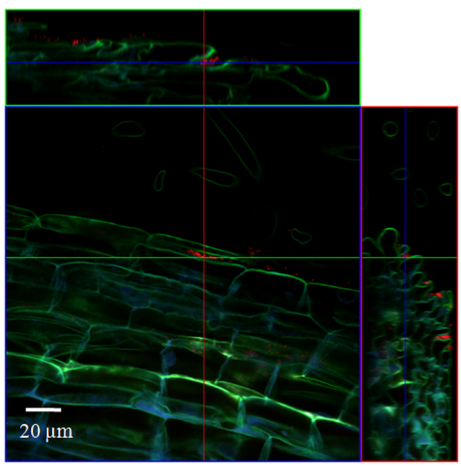

FIGURE 4 | CLSM image of a spinach root. The plant seedling was inoculated for 1 h with *L. monocytogenes* sv. 4b at an inoculation density of 4×10^6 CFU/ml. The plant was harvested after 3 weeks of growth in an axenic system. Probe Cy3 marked EUB-338, I, II, III was applied during FISH procedure (red signal).

whereas only one washing liquid sample at a spiking density of 4×10^7 CFU/ml was found to be positive. Corn salad shoots of plants fertilized with spiked manure were exclusively colonized by the bacteria of interest at the highest spiking dose of 4×10^8 CFU/ml. Washing liquid samples also here were more often tested positive compared to plant samples.

Colonization by the L. monocytogenes serovariations

No bacterial colonization was found in plants when *L. monocytogenes* sv. 4b spiked slurry was used and for corn salad amended with *L. monocytogenes* EGD-E sv. 1/2a spiked organic fertilizer. In spinach samples, fertilized with *L. monocytogenes* sv. 4b spiked manure, the bacteria of interest were only detected in root and root washing samples of the two highest spiking doses. For corn salad fertilized with spiked manure, only few samples independent of the spiking doses were tested positive. In this treatment even a contamination of a control plant was observed, which might be due to the fact that a contamination of soil or manure with *L. monocytogenes* cannot be excluded as those bacteria are very commonly found in this environments. Spinach fertilized with *L. monocytogenes* EGD-E sv. 1/2a spiked organic fertilizer was also only very rarely colonized.

DISCUSSION

The experiments presented in this study were conducted to identify the minimal infection doses of *S. enterica* sv. Weltevreden, *L. monocytogenes* sv. 4b, and *L. monocytogenes* EGD-E sv. 1/2 a needed for colonization of spinach or corn salad plants at axenic or at soil conditions. In order to achieve the highest sensitivity for the detection of a pathogen contamination, an enrichment step was used before DNA extraction and PCR analysis were performed. However, due to this enrichment step no complete quantitative analysis of the contamination was possible. In a similar experimental approach Arthurson et al. (2011) stated that the detection limit for *S. enterica* sv. Weltevreden is approximately 10^4 CFU/g soil or plant material when quantitative PCR is applied. The same detection limit was reached for *L. monocytogenes* by Chen et al. (2011) when using direct PCR detection with different food matrices. In contrast, the authors were able to lower the detection limit down to 3 CFU/ml by introducing an enrichment step of 48 h in BLEB. Danyluk and Schaffner (2011) stated that on spinach plants *E. coli* O157:H7 cell numbers of 10^{-1} CFU/g on 0.1% of the plants at the time point of harvest are enough to possibly cause an outbreak. This assumption was made using a model, based on data, which revealed a strong growth of contaminant bacteria on harvested plant material if no cooling was applied (Abdul-Raouf et al., 1993; Chang and Fang, 2007; Lee and Baek, 2008). This demonstrated the need of a method with an extremely low detection limit, when analyzing human pathogenic bacteria on plants.

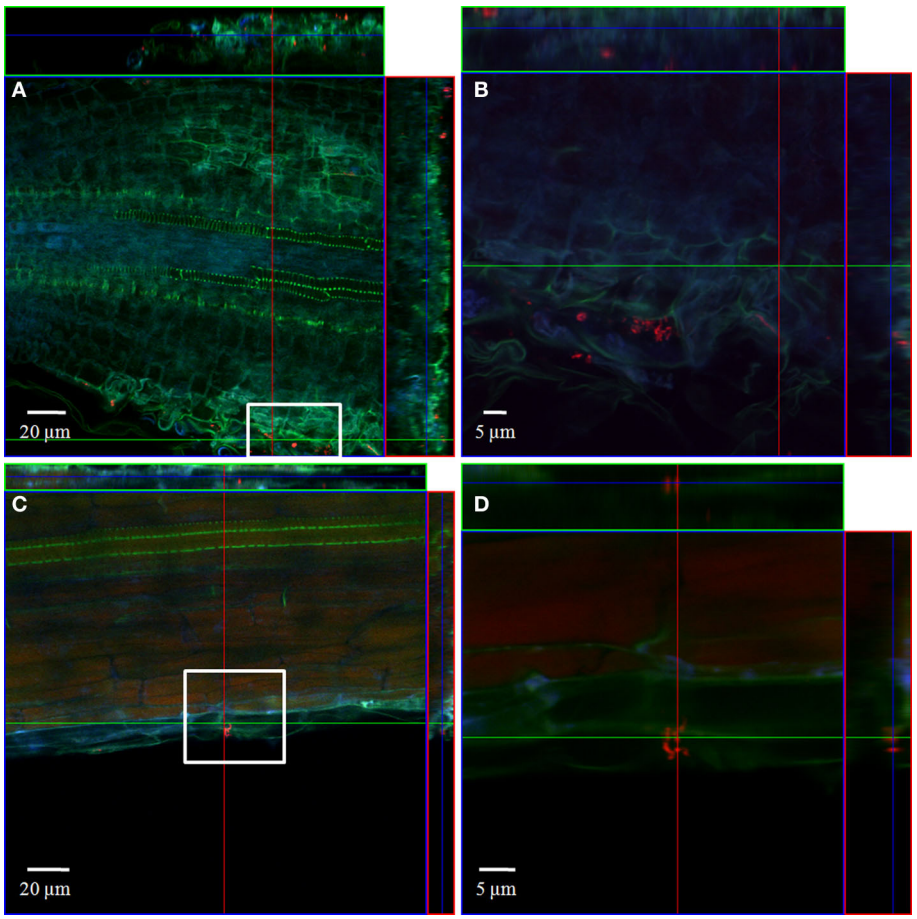

FIGURE 5 | CLSM images of a corn salad root tip (A,B) and a corn salad main root (C,D). The plant seedlings were inoculated for 1 h with *L. monocytogenes* sv. 4b at an inoculation density of 4×10^6 CFU/ml. The plants were harvested after 3 weeks of growth in an axenic system. Probe Cy3 marked EUB-338, I, II, III was applied during FISH procedure (red signal). **(B)** is an enlarged capture of the area marked in **(A)**, **(D)** is an enlarged capture of the area marked in **(C)**.

In addition to the highly sensitive detection, we also intended to localize the inoculated or spiked pathogens using fluorescence labeling of the bacteria by FISH and CLSM-analysis. Earlier studies have already shown that *S. enterica* can form biofilms on leaf surfaces (Kroupitski et al., 2009b) and even colonize the leaf interior via open stomata (Kroupitski et al., 2009a), which supports our findings in the axenic inoculation experiments. Especially in spinach, *S. enterica* sv. Wletevreden and *L. monocytogenes* sv. 4b were often detected in shoot samples, but could not be washed off. Although, it was not possible with FISH/CLSM combined analyses of plant shoot material to clearly identify bacterial cells (data not shown) due to the strong auto-fluorescent properties of the plant material (Amann et al., 1990; Cheng et al., 2001; Ongeng et al., 2013), a strong colonization of spinach shoots could be demonstrated with the PCR-technique. In contrast, in all our experiments using corn salad or *L. monocytogenes* EGD-E sv. 1/2a as inoculant, the bacteria were detected more often in washing liquid samples compared to the plant samples. In those cases it was even possible to remove the bacteria completely by the simple washing step. FISH/CLSM combined analysis of the root samples from the axenic system nevertheless revealed differential colonization sites depending on bacterial species as well as on plant species used for inoculation. Although no endophytic colonization which was observed for *S. enterica* and *E. coli* by Jablasone et al. (2005) was detected, the presence of the bacteria in cell interspaces of the root surface show a quite strong and persistent colonization of the plant roots.

The species or serovar of human pathogenic bacteria used for inoculation or spiking has a major influence on the extend of plant colonization. *Salmonella enterica* sv. Weltevreden was able to colonize the plants more efficiently and at lower initial inoculation or spiking dose compared to both serovariations of *Listeria monocytogenes*. This may be due to the fact that the strain used in this study was originally isolated from an outbreak traced back to the consumption of alfalfa sprouts (Emberland et al., 2007) and therefore was well adapted to colonize plants, in contrast to *L. monocytogenes* sv. 1/2a EGD-E which was isolated from rabbits (Murray et al., 1926) and *L. monocytogenes* sv. 4b isolated from a listeriosis patient (according to the Special *Listeria* Culture Collection, SLCC). Furthermore, it is known that *S. enterica* can be transferred to plants via contaminated organic fertilizer and soil (Klerks et al., 2007; Arthurson et al., 2011; Barak et al., 2011).

Table 2 | Results of the spiking experiments in the soil system.

Plant	Fertilizer	Spiking density (CFU/ml)	*S. enterica* sv. Weltevreden				*L. monocytogenes* sv. 4b				*L. monocytogenes* EGD-E sv. 1/2a			
			Root	Root washing liquid	Shoot	Shoot washing liquid	Root	Root washing liquid	Shoot	Shoot washing liquid	Root	Root washing liquid	Shoot	Shoot washing liquid
Spinach	Slurry	control	0	0	0	0	0	0	0	0	0	0	0	0
		4.0E+05	4	4	1	0	0	0	0	0	0	0	0	0
		4.0E+06	5	5	2	0	0	0	0	0	1	0	1	0
		4.0E+07	5	5	1	1	0	0	0	0	0	0	0	1
		4.0E+08	5	3	2	1	0	0	0	0	1	1	0	1
	Manure	control	0	0	0	0	0	0	0	0	0	0	0	0
		4.0E+05	2	4	0	0	0	0	0	0	1	0	0	0
		4.0E+06	5	5	0	0	0	0	0	0	0	0	0	0
		4.0E+07	5	5	0	1	2	0	0	0	0	0	0	0
		4.0E+08	5	5	0	2	1	1	0	0	0	0	0	0
Corn salad	Slurry	control	0	0	0	0	0	0	0	0	0	0	0	0
		4.0E+05	1	4	0	0	0	0	0	0	0	0	0	0
		4.0E+06	2	4	1	0	0	0	0	0	0	0	0	0
		4.0E+07	3	4	2	1	0	0	0	0	0	0	0	0
		4.0E+08	5	5	0	0	0	0	0	0	0	0	0	0
	Manure	control	0	0	0	0	0	0	1	0	0	0	0	0
		4.0E+05	0	2	0	0	0	0	0	0	0	0	0	0
		4.0E+06	1	5	0	0	1	0	0	1	0	0	0	0
		4.0E+07	4	5	0	0	1	0	0	0	0	0	0	0
		4.0E+08	4	5	1	3	0	1	0	1	0	0	0	0

Numbers and colors indicate the number of positive detection events. For each dilution step five plants were analyzed.

In studies proving an endophytic colonization potential of *S. enterica*, *L. monocytogenes* was, in contrast, only detected on the plant surface (Jablasone et al., 2005; Kutter et al., 2006). Although being able to survive in soil (Botzler et al., 1974; Gorski et al., 2011) or manure amended soil (Jiang et al., 2004), *L. monocytogenes* was found to be inhibited by resident soil microbiota which can lead to a reduction of the cell numbers (McLaughlin et al., 2011). All this support our findings, that *L. monocytogenes* was only in few cases able to colonize plants in the soil system whereas colonization of plants grown in the axenic system was observed at even very low inoculation doses. Although differences in colonization success and interaction of some *Salmonella* spp. strains with lettuce cultivars have already been identified (Franz et al., 2007; Klerks et al., 2007), more research on the mechanisms of plant contamination by human pathogenic bacteria, including a higher number of bacterial strains is needed to clearly identify the reasons for the observed differences in the colonization success.

Furthermore, the plant species plays a crucial role in the colonization success by human pathogenic bacteria. *Salmonella enterica* and both *L. monocytogenes* serovariations were detected more frequently and at lower initial inoculation dose in spinach samples grown in the axenic system compared to corn salad plants. The same was true for the spiking experiments with *S. enterica*. Literature supports those findings. Yadav et al. (2005) for example showed that bacterial colonization of leaf surfaces strongly depends on the properties of those leaves, like water and phosphorus content or leafs and mesophyll thickness. Zhang et al. (2010) detected significantly different bacterial communities on leafs of different vegetable plant species. Even a plant cultivar based effect on the colonization of lettuce by *S. enterica* was proven by Klerks et al. (2007).

The type of organic fertilizer used also influences the colonization of the plants by the human pathogenic bacteria used. *Salmonella enterica* was detected more often and at lower spiking doses if the plants were grown in slurry amended soil. The reduction or removal of the plant contamination with simple washing of the vegetables was also less successful in those samples. Hutchison et al. (2004) found a direct influence of humidity and severity of pathogen contamination in a large sampling campaign of organic fertilizers. The higher humidity of the slurry compared to manure might therefore enhance survival of the pathogenic bacteria first in the organic fertilizer and later in soil. This is supported by findings of Semenov et al. (2009) showing that *S. enterica* as well as *E. coli* display an enhanced survival rate in slurry amended soil compared to manure amended soil due to

the higher content of dissolved organic nitrogen and carbon in the slurry.

CONCLUSIONS

De Roever (1998) formulated research needs to improve safety of fresh produce for the consumer. One of the key issues, which were also addressed in our study, is to improve farming practice in order to decrease the potential for produce contamination. We were able to show that this potential is strongly depending on the plant species under consideration. Therefore, "high risk plants," in our case spinach, need to be treated with more care during production, harvest and processing. Furthermore, the type of organic fertilizer used also influences the colonization success. In order to minimize the risk of transfer of human pathogenic bacteria to produce, manure instead of slurry should preferably be used. Fermentation or composting increased the safety of the fertilizer by minimizing the number of pathogenic bacteria present (Vinnerås, 2007; Abdel-Mohsein et al., 2010). Plant roots were usually colonized more frequently compared to shoots. Therefore, co-harvesting of roots should be avoided as much as possible. Although the pathogenic bacteria were shown to colonize the plant surface, the possibility of an endophytic colonization, especially for *S. enterica* sv. Weltevreden cannot be excluded. We demonstrated that washing was suitable to reduce or even remove the bacterial contamination in most cases. Since the bacteria were frequently detectable in the washing liquid, it should not be reused. Washing of vegetable plants is therefore useful but should be performed under flowing water. By this simple means, the risk of contamination of produce with human pathogenic bacteria can already be reduced on farming level, which increases safety for the final consumer.

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