Interactions of *Salmonella* with lettuce stomata

Thesis Submitted to the Robert H. Smith Faculty of Agriculture, Food and Environment

Hebrew University of Jerusalem

For The Degree of "Master of Agriculture Science"

By

Alexandra Polyansky

June 2013

Interactions of *Salmonella* with lettuce stomata

Thesis Submitted to the Robert H. Smith Faculty of Agriculture, Food and Environment

Hebrew University of Jerusalem

For The Degree of "Master of Agriculture Science"

By

Alexandra Polyansky

June 2013

This work was supervised by Prof. Shlomo Sela

Institute of Postharvest and Food Sciences,

Department of Food Quality and Safety,

Agriculture Research Organization,

The Volcani center, Bet Dagan

Acknowledgment

I would like to thank Prof. Shlomo Sela for the guiding and inspiration. To all our lab members for enjoyable work environment and help: Riky Pinto, Yulia Kroupitsky, Nadia Gruzdev, Eti Keynan, Sharon Herzberg and Tami Hazin. To Eddy Belausov for the microscopy help and to my husband Ilia for the support.

Abstract

Enteric diseases linked to consumption of fresh produce have dramatically increased in the last several decades. Fresh produce-associated outbreaks result in high economical losses to farmers, distributors and the food industry. Food-borne pathogens can contaminate fresh produce in the field, during harvest, or during postharvest handling, processing and storage. Unlike animal-derived foods, fresh produce cannot undergo intensive decontamination procedures as they are highly sensitive to both chemical and physical treatments; consequently, the industry urgently requires alternative treatments or agro-technology to be applied throughout the production chain. The current lack of controlled, measured and effective sanitization methods prompt the need for new technologies to reduce contamination of produce. Our ability to assess the risk associated with produce contamination and to devise innovative control strategies, depends on the identification of critical determinants that affect the fitness of human pathogens on plants. *Salmonella* outbreaks underline the challenges to the agro-industrial sector as well as to the public health authorities.

Salmonella enterica is a Gram-negative enteric bacterium that can successfully colonize animals and humans. Recent reports indicated that *Salmonell*a are also able to infect plants. Our laboratory has previously reported that *Salmonella* can attach to and penetrate lettuce stomata.
The aim of this study was to further investigate the interaction between *Salmonella* and the plant leaf, with emphasize on the *Salmonella*-stomata interactions.

We initially investigated whether *Arabidopsis* can serve as a plant model to study *Salmonella*-leaf interactions. We found that *Salmonella* attached very well to *Arabidopsis* leaves but there was no affinity toward stomata. Although, *Salmonella* has induced stomatal closure after two hours of inoculation, no internalization was observed.
Consequently, we decided to further investigate *Salmonella* and lettuce leaf interactions.

We found that *Salmonella* induced the closure of lettuce stomata after one hour of inoculation and that a concentration of 10^3 CFU/ ml was enough for the induction of closure. Using dead bacteria, chloramphenicol-treated bacteria and external lipopolysaccharide (LPS), it was found that bacterial viability, de-novo synthesis of proteins, and LPS were required for the induction of stomatal closure.
Furthermore, *Salmonella* actively reopened the stomata at two hours of inoculation and a 10^5 CFU/ml were needed for the reopening. Exogenous abscisic acid (ABA) resulted in stomata closure at two hours in spite of the presence of *Salmonella*, suggesting that *Salmonella* is activating a stomatal response signaling at a stage earlier than ABA-induced signaling.

Using mutants it was found that Type III secretion system (TTSS) is required for stomatal reopening. The

TTSS mutants also attached and internalized less efficiently compared to the wt.

Reactive oxygen species (ROS) plays a major role in cellular signaling in plants and in the immune defense against intruders. We found that *Salmonella* induces ROS production, specifically in the guard cells. ROS formation was observed only in stomata containing internalized *Salmonella*, suggesting a correlation between ROS production and *Salmonella* internalization. Treatment of bacteria with protein synthesis inhibitor (chloramphenicol) inhibited both *Salmonella* internalization and ROS production, supporting the notion that the plant can respond to the pathogen only following internalization.

In conclusion, our data suggest that *Salmonella* triggers plant immune system by inducing stomatal closure and the production of ROS within the guard cells following internalization. Similar to plant pathogens, *Salmonella* can force stomatal opening using its TTSS. Further studies are required to gain a better understanding of the mechanism that enable *Salmonella* to re-open lettuce stomata.

Abbreviation list

ABA- Abscisic acid

Amp- Ampicillin

CDC- Center for Disease Control and Prevention

CFU- Colony forming unit

CLSM- Confocal laser scanning microscope

Cm- Chloramphenicol

Col- Columbia

COR- Coronatine

DAMPs- Danger-associated molecular patterns

DCF- 2′,7′-Dichlorofluorescin diacetate

DIC- Differential interference contrast

DMSO- Dimethyl sulfoxide

E. coli - *Escherichia coli*

ETI- Effector-triggered immunity

ETS- Effector-triggered susceptibility

GFP- Green fluorescent protein

Gm- Gentamicin

HR- Hypersensitive response

Km- Kanamycin

LB- Luria Bertani

Ler- Landsberg erecta

LPS- lipopolysaccharide

MAMPs- Microbe-associated molecular patterns

Nal- Nalidixic acid

NCBI- National Center for Biotechnology Information

O.N- Over Night

O.D- Optical Density

PAMP- Pathogen-associate molecular patterns

PCR- Polymerase chain reaction

Pst DC3000- *Pseudomonas syringae* pv. Tomato

PR genes- Pathogenesis-related genes

PRRs- Pattern recognition receptors

PTI- PAMP-triggered immunity

ROS- Reactive oxygen species

RPM- Rounds per minute

SAR- Systemic acquired resistance

SDW- Sterile Distilled Water

SEM- Scanning electron microscope

STm- *Salmonella* Typhimurium

Sm- Streptomycin

TAE- Tris-acetic acid -EDTA buffer

TTSS- Type III secretion system

Ws- Wassilewskija

Wt- Wild type

1. Introduction

1.1 *Salmonella* and salmonellosis

Salmonella is a Gram-negative bacterium, facultative anaerobic, a genus of the family Enterobacteriaceae. According to contemporary classification, the genus *Salmonella* contains only two species, *Salmonella* bongori and *Salmonella* enterica (Cianflone., 2008), *Salmonella* can infect both humans and animals. Most pathogenic species of *Salmonella* affecting humans are within the species *S. enterica*. There are more than 2,500 serovars of *S. enterica* (Andrews et al., 2010; Cianflone., 2008). *Salmonella* have long been associated with a wide spectrum of infectious diseases, including typhoid fever and nontyphoid salmonellosis. Salmonellosis usually involves gastroenteritis, accompanies by stomach ache, diarrhea, headaches and vomiting. The infectious dose is estimated at approximately 10^6 organisms (Cianflone., 2008). Most cases of salmonellosis are due to ingestion of contaminated food items, such as eggs, dairy products, and meats. *Salmonella* can be transferred also by contact with human and animal feces, or contaminated water (Angulo et al., 1997). Risk factors for salmonellosis include gastric hypoacidity, recent use of antibiotics, extremes of age, immunosuppressive conditions, with infants and children (<5years) being at highest risk (Cianflone., 2008). *Salmonella* causes approximately 1.03 million human infections each year in the United States, result in 19,588 hospitalizations and 378 deaths (Scallen et al., 2011). There are also substantial financial costs associated with *Salmonella* infection. There are clear public health benefits and economic gain by being able to control the spread of this pathogen in the food chain.

1.2 Contamination of fresh produce

In the last decade, outbreaks after consuming fruits and vegetables were reported in U.S.A, Europe and Israel (CSPI, 2008; Doyle & Erickson, 2008; Sivapalasingam et al., 2004; Burnett & Beuchat, 2001; Brandl, 2006; Anonymous, 2003). Fruits and vegetables, and in particular leafy greens, that are consumed raw, were recognized as vehicles for transmission of human pathogens (Barak and Schroeder, 2013). Outbreaks related to fresh produce result in a higher number of infection cases than outbreaks associated with consumption of beef, poultry, eggs and sea food (Jacobsen and Bech, 2012).

Fruits and vegetables which were involved in these outbreaks, included: lettuce (Horby, 2003; Gillespie,2004), tomatoes (Greene et al., 2008; Barak & Liang, 2008a), sprouts (CDC, 2002a, Mohle-Boetani et al., 2008), basil (Pezzoli et al., 2007), spinach (CDC, 2006a), melons (CDC, 2002b) and watermelons (CDC, 2006b).

Salmonella enterica is the most commonly identified pathogen acquired from fresh produce in the United States, people there are more likely to encounter *Salmonella enterica* from eating fresh produce than animal products (Sivaplasingham et al., 2004; Buck et al., 2003; Warriner et al., 2009, Barak and Schroeder, 2013**).** *Salmonella* outbreaks were linked to contaminated tomatoes, seed sprouts, cantaloupes, serrano and jalapeno peppers and unpasteurized fruit juices (Fatica & Scheider, 2011).

Lettuce, especially the iceberg cultivar, has been related to many outbreaks (Anon, 2005; Horby et al., 2003; Gillespie, 2004). There are several potential reasons for the increased number of outbreaks associated with fresh produce, including 1) increase in the consumption of fruits and vegetables for healthy diet; 2) Changes in agricultural, processing and distribution practices that have enhanced both the supply and range of products, For example, increased shipping distances, might influence the risk of exposure to contaminants, and enables time for bacterial multiplication, and The increased demand for fresh produce has resulted in growing fruit and vegetable near other agricultural areas, such as animal farms, which can increase the likelihood of contamination (Berger et al., 2009; Deering et al., 2012); 3) Increase in the demand for rinsed (sometimes cut) ready-to-eat vegetables (Burnett & Beuchat, 2001; Hanning et al., 2009).

1.2.1 Sources of contamination

Human pathogens can contaminate produce at any point of the food chain, in the field, during or after harvesting, as well as during sorting, processing and packing. Contamination sources in the field include irrigation water, organic fertilizers, soil, and animal / human feces (Brandl, 2006). *Salmonella* can be transferred from the soil to the plant via sprinkling irrigation and water splashes during rain events. Fresh produce can be contaminated by pathogen attachment to the plant surface or by internal colonization (Jacobson & Bech, 2012). Contamination can also occur by direct contact with feces, contaminated bioaerosol, insects, workers hands or processing surfaces and contaminated wash water. Improper storage conditions, such as high temperature, can induce pathogen reproduction and contribute to the degree of contamination (Heaton & Jones, 2008). Harvesting iceberg lettuce for bagged salads involves not only cutting the head from the plant but also coring the head in the field. Because of the close proximity between the soil and cutting the heads from the plants, cross-contamination may occur (Ericson 2012).

1.3 Interaction between *Salmonella* and plant

1.3.1 Attachment

Bacteria can attach to different parts of the plant, such as roots (Rodríguez-Navarro et al., 2007) and leaves (Beattie & Lindow, 1995). On leaf surface, bacteria can attach to cuticle, trichomes and stomata (Beattie & Lindow, 1995; Lindow &Brandl, 2003). In recent years, there are studies that shows that Enteropathogens can adapt to the phyllosphere environment, where they might interact with epiphytic bacteria and gain a foothold (Beuchat., 2002; Brandl., 2006; Heaton et al., 2008). It was found that enteropathogenic bacteria such as *Escherichia coli* and *Salmonella*, can attach to different parts of plant. *E.coli* O157:H7 attached to romaine lettuce roots (Wachtel et al., 2002). The pathogen attached to spinach leaves and roots (Mitra et al., 2009), it also showed attachment to arugula leaves, and the bacteria were found on guars cells (Berger et al., 2009). Takeuchi and colleague found that *E.coli* O157:H7 and *L. monocytogenes* attached preferentially to cut edges than to surfaces of lettuce

(Takeuchi et al., 2000). *Salmonella* Typhimurium attached equally to cut edges and surfaces on iceberg lettuce leaves (Takeuchi et al., 2000), Recent work had shown a similar attachment of different *S. enterica* serovars (Tennessee, Negev, Braenderup, Thompson, Newport) to surfaces and cut edges on iceberg lettuce, romaine lettuce and cabbage. *Salmonella* attached better to romaine lettuce than to iceberg lettuce (Patel & Sharma, 2010). Opposite results showed that in romaine lettuce, *Salmonella* Typhimurium attached preferentially to cut edges then to surfaces (Kroupitski et al, 2009a). *Salmonella* Chester also attached to injured tissue better than to surface of green pepper (Liao & Cooke, 2001). *S. enterica* adhered better to red tommato fruit of cultivar WVa700 than to green fruit (Barak et al., 2011). *S. enterica* serovars show different attachment to leafy vegetables. Typhimurium, Enteritidis and Senftenberg attached efficiently, while Arizona, Heidelberg and Agona did not (Berger et al., 2009). Barak and colleagues showed difference in attachment of *Salmonella* to different vegetables, as well as among different tomato species (Barak et al., 2008b). The attachment efficiencies of *S.* Montevideo to tomatoes was influenced by environmental conditions, such as temperature and humidity (Iturriaga et al., 2007; Zhuang et al., 1995).

Attachment of foodborne pathogens to injured tissue has a safety implication, because the attached bacteria are more tolerant to disinfection (Frank &Takeuchi, 2000; Kroupitski et al., 2009a). Furthermore, it enables the survival of the pathogen during the shelf life of the product (Fatica & Scheider, 2011).

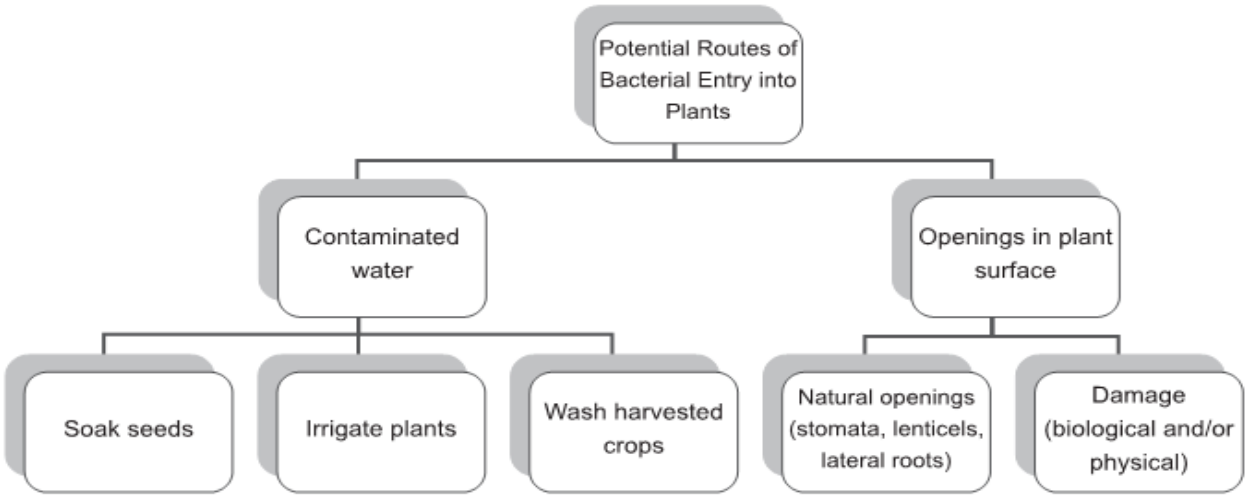


Fig 1.1 Potential routs of bacteria entry (Deering et al., 2012)

1.3.2 Internalization

Plant pathogens can internalize plant through roots, natural openings, like stomata or an injured tissue **(**Beattie & Lindow, 1995; Brencic & Winans, 2005; Antunez-Lamas et al., 2009; Vailleau et al., 2007; Czajkowski et al., 2010). Potential routs of bacterial entry into plants are described in Fig 1.1. Similar routes of entry were also reported for enteric pathogens (Erickson, 2012).

1.3.2.1 Roots internalization

Studies showed internalization of human pathogens through roots following by bacterial spreading to all plant parts. The first study reported on *E. coli* O157:H7 root internalization to romaine lettuce. The plants were irrigated with polluted water and fertilized with cows manure. After internalization, the bacteria were found in the edible parts of the lettuce (leaves) (Solomon et al., 2002). Bernstein and colleagues showed entry of *S*. Newport into romaine lettuce via roots (Bernstein at al., 2007b). Klerks and colleagues shows similar phenomenon with *S*. Dublin in romaine lettuce cultivar Tamburo (Klerks et al., 2007a; Klerks et al., 2007b). Internalization of *E. coli* through roots was shown also in corn seedlings, 48 hours after soil contamination (Bernstein et al, 2007a). Dong and colleagues showed *E. coli* K-12 and *S*. Thyphimurium internalization to seedlings of alfalfa (*Medicago sativa*) via root cracks (Dong et al., 2003). Cooley and colleagues showed internalization of *S*. Newport and *E. coli* O157:H7 through *Arabidopsis thaliana* roots. Furthermore, root inoculation led to contamination of the entire plant, indicating that the pathogens are capable of moving on or within the plant (Cooley et al., 2003). Schikora and colleagues showed that *S*. Thyphimurium can infect *Arabidopsis* leaf tissue and seedlings. In opposite to other reports, they showed that the infection resulted in wilting, chlorosis and eventually death of the infected organs (Schikora et al., 2008). Recent findings showed that in sweet basil roots, *S*. Newport internalization depends on the plant's age, however the survival of the bacteria inside the plant was limited to 30 hours, compared to at least 8 days following attachment to leaf surface (Gorbatsevich et al., 2012). The internalization of human pathogen through roots is plant-pathogen specific and does not directly correlate with the presence of bacteria in the edible part of the plant (Hirneisen et al., 2012; Velasco et al., 2012).

1.3.2.2 Internalization through leaves

Structurally, the leaf is composed of an upper and a lower epidermis (Fig 1.2). The epidermis is covered with cuticle, which composed of polymers (cutin, cutan) that are covered with wax. Light can get through the cuticle but gas and water cannot. The mesophyll is located between the upper and lower epidermis and is composed of parenchyma cells. There are two types of parenchymal cells: palisade parenchyma (closer to upper epidermis) and spongy parenchyma (closer to lower epidermis). The mesophyll contains the chloroplasts, which are responsible for photosynthesis. The Vascular system located in the mesophyll, includes the phloem (transport nutrients) and xylem (transport water and

minerals) (Hopkins, 1999). Stomata, which regulate the flow of gases (carbon dioxide and oxygen) into and out of the leaf, also regulate the water loss.

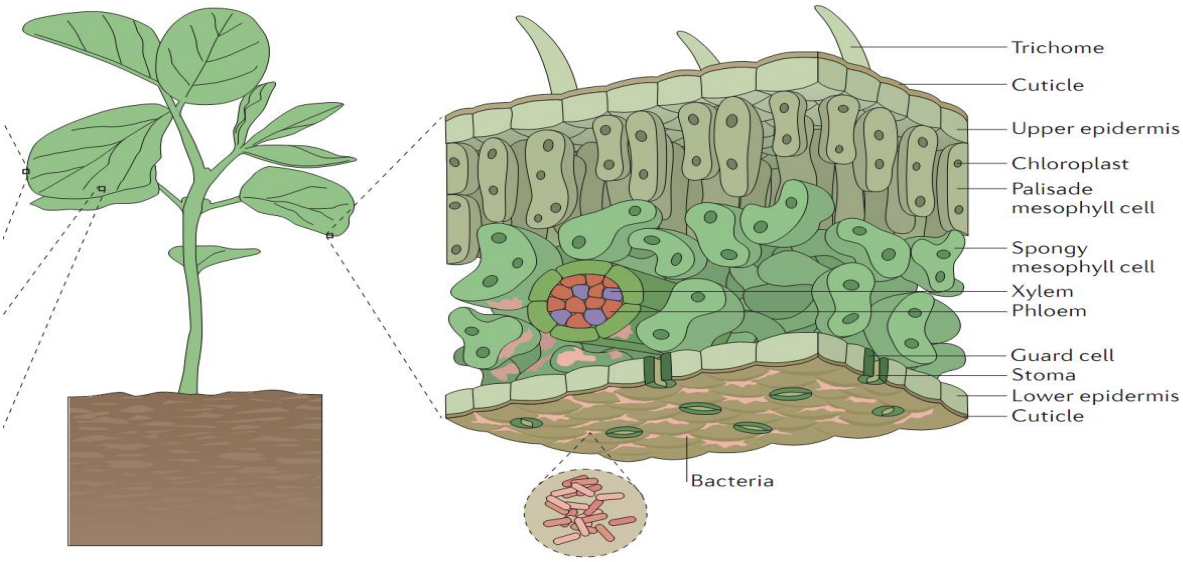


Fig 1.2 Schematic leaf structure (Vorholt, 2012)

Stomata are located in the epidermis, surrounded by two guard cells. Plants can regulate the opening and closure of stomata by plant hormones in response to environmental cues, such as, light intensity, relative humidity and CO_2 concentration (Melotto et al., 2008; Hetherington & Woodward, 2003). Plant pathogens can penetrate the leaf tissue through stomata. For example, *Xantomonas campertis* and *Pseudomonas syringae* pv. tomato DC3000 (Pst) internalize *Arabidopsis* leaves (Gudesblat et al., 2009; Melotto et al., 2008). Human pathogens can also internalize the leaf tissue through stomata. It was reported that *Pseudomonas aeruginosa* and *Staphylococcus aureus* can enter through stomata in unattached *Arabidopsis* leaves (Plotnikova et al, 2000; Prithiviraj et al, 2005). In iceberg lettuce, Takeuchi and colleagues reported that *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes* can colonize the apoplast under the stomata (Takeuchi & Frank, 2000; Takeuchi et al., 2000), Kroupitski and colleagues showed the presence of *Salmonella* in injured leaf tissue and inside stomata (Kroupitski *et al*, 2009a), and Seo & Frank demonstrated the presence of *E. coli* O157:H7 under stomata (20-100 micron depth) in unattached iceberg lettuce leaves (Seo & Frank., 1999). In contrast to these studies, no internalization of *E. coli* O157:H7 in iceberg lettuce was found after soil and leaves inoculation (Zhang et al., 2009). Mootian and colleagues showed that *E. coli* O157:H7 was found in the protected locations of lettuce tissue following exposure to low concentration of the pathogen in soil or irrigation water (Mootian et al., 2009). Franz and colleagues showed *S.* Thyphimurium presence under stomata of unattached romaine lettuce leaves (Franz et al., 2007). Li and colleagues showed that *E. coli* O157:H7 penetration to romaine lettuce may occur also during leaf vacuum cooling, after harvest, due to physical force (Li et al., 2008).

In a recent study in our laboratory, it was found that *S.* Typhimurium can penetrate through open stomata of iceberg lettuce in a mechanism that involved light dependent-chemotaxis (Kroupitsky et al., 2009b). Stomatal internalization seems to be plant species-specific; lettuce and arugula displayed high internalization efficiency by *Salmonella,* while parsley and tomato leaves showed no internalization (Goldberg et al., 2011). Interestingly, Roy and colleagues showed that *Salmonella* (SL1344) and *E. coli* (0157:H7) cannot proliferate inside *Arabidopsis* and lettuce apoplasts after dip inoculation, but the reduction in bacterial numbers during 15 days was higher with *E. coli* (100 fold) than with *Salmonella* (10 fold) (Roy et al., 2013).

1.4. Plant innate immunity

1.4.1 Response of plants to bacterial pathogens

Plants have developed an innate immune system that protect the plant from unwelcome pathogens. Plants can recognize the pathogens by sensing conserved microbial elicitors, collectively term pathogen-associated molecular patterns (PAMP), or more generally, microbe-associated molecular patterns (MAMP). Plant have evolved two main strategies to sense and react against pathogens, one is PAMP-triggered immunity (PTI), and the other is effector-triggered immunity (ETI). PTI recognizes, PAMPs by following binding to receptor proteins called pattern recognition receptors (PRRs). PAMPs include various components of bacterial surface, such as flagellin or lipopolysaccharide (LPS), as well as cellular components, which might be released by the invading pathogen, such as cell wall or cuticular fragments (Dodds & Rathjen, 2010).

Once the plant recognizes PAMPs it can respond by activating defense-related genes, resulting in expression of antimicrobial molecules and induction of cell death (Underwood et al, 2007; Boller & He, 2009; Melotto et al., 2008). In order to overcome PTI, some pathogens secrete proteins called effectors which suppress the innate immunity system. This process is called effector-triggered susceptibility (ETS). In order to overcome ETS, the plant activates a second strategy, termed effector-triggered immunity (ETI). ETI is based on gene-for-gene hypothesis, which genetically describes the recognition of pathogens by plants through the interaction of pathogen-derived avirulence (Avr) genes and host-borne resistance (R) genes. PTI and ETI give rise to similar responses, these include a rapid influx of calcium ions from external stores, a burst of active oxygen species, activation of mitogen-activated protein kinases (MAPKs), reprogramming of gene expression, deposition of cal-losic cell wall appositions at sites of attempted infection and, often, localized cell death (HR). Although ETI is qualitatively stronger and faster and often involves a form of localized cell death called the hypersensitive response (HR) (Dodds & Rathjen, 2010). Plants are also protected by a mechanism called systemic acquired resistance (SAR), which occurs at sites distant from the primary and secondary immune responses and may protect the plants against subsequent pathogen attacks (Wit, 2007) SAR requires signal molecules activating the expression of pathogenesis-related (PR) genes (Durrant & Dong, 2004). SAR is effective against a broad range of pathogens and is dependent on different plant

hormones including salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA) or combinations of them (Bari & Jones, 2009).

1.4.1.1 Stomatal response to pathogens

Stomata are microscopic pores formed by pairs of guard cells in the epidermis of terrestrial plants, they are essential for gas exchange with the environment and for controlling water loss. Plants regulate stomatal aperture in response to environmental conditions; stomata open in response to high humidity and low CO_2 in order to maintain CO_2 intake (Zeng et al., 2010; Kim et al., 2010) and are induce to close by dark. Drought causes production of the plant hormone abscisic acid (ABA), which promotes stomatal closure and thereby reduces transpirational water loss (Kim et al., 2010). Stomatal openings are also serving as a major route of pathogen entry into the plant and plants have evolved mechanisms to close stomata as an immune response against bacterial invasion (Zeng et al., 2010). Melotto and colleagues found that plant stomata close in response to a plant pathogen, *Pseudomonas syringae* pv. tomato (Pst) DC3000, and to a human pathogen, *E. coli* O157:H7. This response can also be triggered by well-characterized MAMPs, such as flg22 (a peptide derived from bacterial flagellin) and LPS. This observation suggests that bacterium-triggered stomatal closure is an outcome of PAMP-triggered immunity (Melotto et al. 2006). MAMPs were shown to induce stomatal closure in several plants, including tomato (Melotto et al. 2006), *Commelina communis* (Lee et al., 1999), grape (Allegre et al., 2009), *Pisum sativum* (Srivastava et al., 2009), and *Arabidopsis* (Melotto et al. 2006; Zeng & He, 2010). In addition to triggering stomatal closure, flg22 also prevents stomatal opening in response to light (Zeng et al., 2008). Studies using purified MAMPs have shown that stomatal closure in response to biotic signals requires the phytohormone abscisic acid (ABA), the guard cell-specific OPEN-STOMATA 1 (OST1) kinase, the production of reactive oxygen species (ROS) and nitric oxide (NO), the heterotrimeric G protein, and the regulation of K^+ channels, all of which are hallmarks of abiotic signal-induced stomatal closure (Melotto et al., 2006; Zeng et al., 2008; Neil et al., 2008). Some pathogens might not overcome stomatal based immunity and may survive as epiphytes on the plant surface until environmental conditions, like high humidity, or heavy rain will induce opening of the stomata and enable the pathogens to enter. Indeed, many bacterial diseases outbreaks occur in these periods (Zeng et al., 2010).

Some pathogens apparently have evolved virulence factors to actively counteract stomatal closure. For example, Pst DC3000 induces stomatal closure at two hours post infection, but later can express coronatine (COR), a phytotoxin that can reverse stomatal closure and reopen stomata as early as four hours following infection (Melotto et al., 2006). Similarilty, *Xanthomonas campestris pv campestris* (Xcc) is capable of manipulating stomatal closure of *Arabidopsis* through a secreted small molecule; the bacteria induce stomatal closure after one hour, but force reopening of stomata after two hours (Gudesblat et al., 2009). The human pathogens, *E. coli* O157:H7 apparently cannot overcome stomatal closure in *Arabidopsis* leaves, resulting in constitutive reactivation of the stomatal immune response

(Melotto et al., 2006). Kroupitski and colleagues showed that *S.* Typhimurium can induce partial stomatal closure, compared to Pst, after two hours of inoculation with iceberg lettuce leaves. They suggested two possible explanations for the poor stomatal closure; the first one is that lettuce cannot recognize the bacterial MAMPs, and the second is that *Salmonella* is unable to overcome the stomatal-based immune system. (Kroupitski et al., 2009). Recently, Roy and colleagues found that *Salmonella* (SL1344) has induced stomatal closure both in *Arabidopsis* and in lettuce, after two hours of inoculation, however, the stomata were reopened after four hours, in the presence of 60% and 95% relative humidity (RH), while after inoculation with *E. coli* (O157:H7) the stomata remained closed under the same conditions. They also showed that *Salmonella* was not able to reopen the *Arabidopsis* stomata in dark conditions and that *ost1-2* mutants' plants (unable to close stomata in response to PAMPs) did not close stomata in response to *Salmonella* and *E. coli* (Roy et al., 2013).

1.4.1.2 Reactive oxygen species (ROS) production

One of the most rapid defense reactions to pathogen attack (part of PTI) is the so-called oxidative burst, which constitutes the production of reactive oxygen species (ROS) at the site of invasion (Apostol et al., 1989). Several biotic and abiotic stresses can induce ROS production in plants. These include salinity, UV radiation, drought, heavy metals, extreme temperatures, nutrient deficiency, air pollution, herbicides and pathogen attacks (Gill & Tuteja, 2010).

ROS comprise hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical ($HO^{\cdot}$), and singlet oxygen ($^1O_2$), all of which are toxic and can cause cellular damage (Miller et al., 2008). ROS can also damage plant pathogens directly by the oxidation of important biomolecules (Adam et al., 1989; Keppler & Baker, 1989) and induce programmed cell death (PCD) (Apel and Hirt, 2004). Guard cells can also generate ROS in response to pathogen, and consequently reduce stomatal aperture (Gudesblat et al., 2009; Apel and Hirt, 2004). ROS are produced in response to ABA and mediate the ABA-induced stomatal closure via activation of plasma membrane calcium channels (Pei et al., 2000). Among the different components of ROS, only H_2O_2 can cross plant membranes and can therefore directly function in cell to cell signaling (Pitzschke et al., 2006). During plant-pathogen interaction, superoxide anions are produced enzymatically outside the cell and are rapidly converted to hydrogen peroxide that can cross the plasma membrane (Apel and Hirt, 2004). H_2O_2 might act as a secondary signal for the induction of gene expression, as a toxic antimicrobial effector, or as a substrate for oxidative cross-linking of wall components (Imaly, 2003; Delledonne et al., 2002, Mittler, 2002; Neill, 2002).

1.4.2 *Salmonella* TTSS and the plant immune response

Salmonella virulence in animals is mediated by a number of protein effectors that are injected into the cytosol of the host cells by a syringe-like organelle on the surface of the bacterial cell. This secretion system is termed Type III Secretion System (TTSS) and it is common in Gram-negative bacteria. *Salmonella* possesses two TTSS, each encoded by a distinct pathogenicity island: *Salmonella*

pathogenicity island (SPIs) 1 and 2. (Galan, 2001; Waterman & Holden 2003; Srikanth et al., 2011). The effectors injected by TTSS ultimately manipulate the cellular functions of the infected host and facilitate the progression of the infection. SPI1 and SPI2 play distinct roles in different organs within the host. SPI1 primarily promotes the invasion of non-phagocytic intestinal epithelial cells and the initiation of the inflammatory responses in the intestines (Coombers et al., 2005; Hapfelmeier ey al., 2004; Srikanth et al., 2011), while SPI-2 mainly involved in the intracellular survival (Brawn et al., 2007; Lawley et al., 2006; Steele et al., 2002; Srikanth et al., 2011).

Gram-negative plant pathogens also use their TTSS during infection to deliver protein effectors into the host plant. These effectors are involved in delaying or inhibiting the plant's defense responses, including the production of ROS (Grant et al., 2006). Recent studies have shown that *Salmonella* uses its TTSS also in the plant host, although *Salmonella* is not considered a plant pathogen. Iniguez and colleagues showed that *Salmonella* mutants in the structural component of the SPI-1 TTSS needle apparatus (encoded by *sipB* and *spaS*), required for translocating the effectors, and had increased bacterial populations in the roots of *Medicago truncatula* compared to the wt. Moreover, they found that *Salmonella* SPI-1 is required for the recognition of the pathogen by activating the promoter of the plant pathogenesis-related protein 1 (PR1) gene, (Iniguez et al., 2005). Schikora and colleagues demonstrated that *Salmonella* mutants in TTSS1 and 2 (TTSS1: *invA, prgH*, TTSS2: *ssaV, ssaF*) unable to deliver effectors are also compromised in infection of *A. thaliana* plants. Transcriptome analysis revealed a higher gene expression in plant infected with a TTSS mutant (*prgH*) than after infection with wt *S*. Typhimurium 14028. The authors explain it by the ability of wild type bacteria to repress the plant defense immune response. Moreover, there was a stronger HR after infection with the TTSS mutant. The authors suggest that the mutant was unable to restrain the induced HR response. They also found that *Salmonella* originating from infected plants are highly virulent for human cells and mice compared to *Salmonella* grown in laboraotry medium (LB) (Schikora et al., 2011). Shirron and Yaron found that wild type *S*. Typhimurium but not a TTSS mutant (*invA*) was able to suppress the oxidative burst and the increase of extracellular pH after inoculation of tobacco cells. They have concluded that *Salmonella* actively suppresses the immune system of tobacco plants and that TTSS is required for this suppression (Shirron & Yaron, 2011). Ustun and colleagues showed that *Salmonella* TTSS effector SseF is recognized by the plant immune system in *Nicotiana benthamiana*, resulting in effector-triggered immunity (Ustun et al., 2012). The involvement of TTSS in *Salmonella* interactions with plants suggest that there is a high degree of conservation in the defense and infection mechanism of animal and plant hosts during *Salmonella* infection (Schikora et al., 2011).

2. Objectives of the study

Recent studies suggest that *Salmonella* has adapted to the plant environment and evolved mechanisms to manipulate stomata closure and gain entry into sub-stomatal tissues (Kroupitski et al., 2009b). Therefore, the general objective of this study is to further understand the mechanisms involved in *Salmonella*-stomata interactions.

The specific objectives of the study are:

1. To characterize stomatal response to *Salmonella* infection.
2. To identify bacterial genes involved in *Salmonella*-leaf interaction.

3. Material and methods

3.1 Materials

Table 1. Reagents used in this study

Agarose I / TBE Blend 1.0%	Amresco, Solon, OH, USA
Arabinose	Sigma chemicals, St. Louis, MO, USA
2′,7′-Dichlorofluorescin diacetate	Sigma chemicals, St. Louis, MO, USA
Dimethyl sulfoxide	Sigma chemicals, St. Louis, MO, USA
Ethanol, Absolute, AR	Gadot, Netanya, Israel
Ethidium Bromide	Sigma chemicals, St. Louis, MO, USA
Glucose	Merck, Darmstadt, Germany
Glutaraldehyde	Sigma chemicals, St. Louis, MO, USA
Glycerine AR	Gadot, Netanya, Israel
Lipopolysaccharide (1mg/ml in water)	Sigma chemicals, St. Louis, MO, USA
Luria Bertani (LB) Broth	Difco, Sparks, MD, USA
Luria Bertani (LB) Agar, Lennox	Difco, Sparks, MD, USA
Phosphate buffer	In 1 L: K_2HPO_4-1M-71.7ml, KH_2PO_4 -1M-28.3ml, pH=7.2
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
Potassium hydrogen phosphate	Merck, Darmstadt, Germany
Tris Acetate	Sigma chemicals , St. Louis, MO, USA
Water (ultra-pure sterilized water)	Beit-Haemek, Israel

Table 2. Antibiotics[a]

Ampicillin (100 µg/ml in water)	Sigma chemicals, St. Louis, MO, USA
Chloramphenicol (30 µg/ml in 70% ethanol)	Sigma chemicals, St. Louis, MO, USA
Gentamicin (20 µg/ml in water)	BDH, Poole, England
Kanamycin (50 µg/ml in water)	Sigma chemicals, St. Louis, MO, USA
Naldixic acid (30 µg/ml in water)	Sigma chemicals, St. Louis, MO, USA

Rifamycin (100 µg/ml in methanol ABS)	Sigma chemicals , St. Louis, MO, USA
Streptomycin (100 µg/ml in water)	Sigma chemicals , St. Louis, MO, USA
Tetracyclin (5 µg/ml in 70% ethanol)	Sigma chemicals , St. Louis, MO, USA

[a]All antibiotics were filtered through 0.22 µm Low protein binding durapore (PVDF) membrane.

Table 3. Molecular biology kits and DNA markers

Clean & Concentration DNA kit	Zymo, Suite, Orange, CA
HiSpeed plasmid MidiKit	QIAGEN, Hilden, Germany
Zyppy Plasmid Miniprep	Zymo, Suite, Orange, CA
Go Taq Green Master Mix	Promega, Madison, USA
ΦX174 DNA/*Hae*III Marker	Fermentas Inc., Hanover MD, USA
Lambda DNA/*Hind*III Marker	Fermentas Inc., Hanover MD, USA

Table 4. Bacterial strains

Strain	**Characteristic**	**Reference/source**
S. Typhimurium SL1344 (STm)	Sm^r, Nal^r	M. Brandl (ARS, USDA)
STm GFP	*att*::miniTn7T (Sm^r, Nal^r, Gm^r, *gfp*)	Our lab
STm pCherry	Amp^r ,	Our lab
STm, pKD46	Sm^r, Nal^r, Amp^r,	Our lab
STm M913	*fliGHI*::Tn*10* (Sm^r, Tet^r)	W.D Hardt (Institute of microbiology, Switzerland)
STm M913 pCherry	*fliGHI*::Tn*10* (Sm^r , Tet^r, Amp^r)	This study
STm M935	*cheY*::Tn*10* (Sm^r , Tet^r)	W.D Hardt (Institute of microbiology, Switzerland)
STm SL1344 *invA*$^-$	Δ*invA* (Sm^r, Nal^r, Kan^r)	This study
STm *invA*$^-$, pCherry	Δ*invA* (Sm^r, Nal^r, Kan^r, Amp^r)	This study
STm *avrA*$^-$	Δ*avrA* (Sm^r, Nal^r, Kan^r)	This study
STm *avrA*$^-$, pCherry	Δ*avrA* (Sm^r, Nal^r, Kan^r, Amp^r)	This study
STm SPI-1 mutant	Δ*spi1* (Sm^r, Nal^r, Cm^r)	This study
STm 4 SPI-1 mutant, pCherry	Δ*spi1:* deletion mutant (Sm^r, Nal^r, Cm^r, Amp^r)	This study
STm SPI-2 mutant	Δ*spi2* (Sm^r, Nal^r, Kan^r)	This study
STm SPI2 mutant, pCherry	Δ*spi2* (Sm^r, Nal^r, Cm^r, Kan^r)	This study
STm SPI-2/SPI-2 double	Δ*spi1*/Δ*spi2* (Sm^r, Nal^r, Cm^r,	This study

mutant	Kan^r)	
STm SPI-2/SPI-2 double mutant, pCherry	Δ*spi1*/Δ*spi2* (Sm^r, Nal^r, Cm^r, Kan^r)	This study
E. coli BW25141/pKD4	Km^r, containing template plasmid pKD4	Datsenko & Wanner, 2000
E. coli BW pKD3	Cm^r	Datsenko & Wanner, 2000
Pseudomonas syringae pv. Tomato	Rif^r	S. Manulis (The Volcani Center)

Table 5. Plasmids

Plasmid Name	**Characteristic**	**Reference/source**
pKD46	Recombinase expression plasmid, Amp^r	Datsenko & Wanner, 2000
pKD4	template plasmid for mutagenesis, Km^r	Datsenko & Wanner, 2000
pKD3	template plasmid for mutagenesis, Cm^r	Datsenko & Wanner, 2000
pCherry	pKB2690 expressing Amp^r	Our lab

Bacteria storage

Stock cultures were stored in 40% glycerol at -80ºC.

Growth condition

For each experiment, a fresh culture was made. A single colony was picked from a fresh plate and transferred to 5 ml LB with proper antibiotics. Bacteria were grown in LB broth for 22± 2 h at 37°C (*S. enterica*) or 30°C (*P. syringae*) with shaking (150 rpm) to obtain stationary phase cultures.

Plant material

Arabidopsis seeds of ecotypes *Columbia* (Col), *Landsberg erecta* (Ler) and *Wassilewskija* (Ws) were a gift from Amnon Lers (Volcani center). The seeds were kept at 4°C and were seeded directly in to pots, containing growth medium mix "Shaham-Givat Ada", which included: peat, tuff, ventilating materials, water adsorption materials and slow releasing of nutrients materials. The pots were covered (to keep high humidity) and kept for two days at 4°C. Then they were transferred to a controlled long day growth chamber (16 h of light and 8 h of dark), at 22ºC and were watered twice a week. Two to six week-old plants were used.

Fresh iceberg lettuce (*Lactuca sativa*) was purchased at a local retail store.

3.2 Methods

3.2.1 Standard molecular-biology methods

3.2.1.1 Plasmid extraction and purification

Plasmids were extracted from stationary phase bacteria, with Zyppy Plasmid Miniprep kit (Zymo, Suite, Orange, CA), according to the mnaufacturer's instructions. 3.2.1.2 Competent cells preparation

A single colony was selected from a fresh agar plate of STm/pKD46 and transferred to 4 ml LB with 100 µg/ml Amp. Bacteria were grown O.N with shaking (150 RPM) at 30ºC. Fifty ml LB were inoculated with 500 µl of the O.N culture and grown with shaking at 30ºC for 2.5 h. Arabinose (50 µl from a stock of 500 mM) was added to the culture to induce the production of the λ red-recombinase, which is encoded by the pKD46 plasmid. The culture was grown for another 1-1.5 h under the same conditions until it reached $O.D_{595nm}$=0.9. Bacteria were incubated on ice for 15 min and then centrifuged at 4,300 rounds per minute (RPM) for 15 min in a cold (4ºC) centrifuge (Heraeus biofuge primo R. Thermo Electron Corporation, Langenselbold, Germany). The supernatant was removed and the pellet was washed twice with cold glycerol 15%. The pellet was then resuspended with 1 ml of glycerol 15% and the competent bacteria were centrifuged (4500 RPM) for 5 min. The supernatant was removed and the pellet was resuspended in 80 µl of cold glycerol (15%). The competent cells were used immediately for electroporation.

3.2.1.3 Electroporation

Competent cells (80 µl) were transfer to a pre-cooled (4ºC) electroporation cuvette, and 8 µl of a cleaned PCR product were added and mixed by gentle pipettation. The cuvette was put in the electroporator (Electroporator II, Invitrogen, Carlsbad, CA) and the electroporation conditions were as follow: pulse lenght-7 ms, power-15 kW/cm, capacity-50 µF, resistance-1500 Ohm, voltage-1500 V. Immediately after electroporation the bacterial cells were resuspended in 700 µl of 'Super Optimal broth' with catabolite repression (SOC) solution containing glucose (20 mM) and the bacteria were incubated for 1.5 h with shaking (225 RPM) at 37ºC. After the incubation, 150 µl of the cells were spread on LB agar containing Km (20 µg/ml) or Cm (5 µg/ml) and incubated O.N at 37ºC. The remaining cells were transferred to a 50 ml Falcon tube with 10 ml LB plus the appropriate antibiotic (Km or Cm) and grown O.N at 37 ºC in shaking. Large colonies that appeared after 14-16 h were selected. If colonies didn't appear, a 150 µl of the O.N culture were spread on Km (50 µg/ml) or Cm (10 µg/ml) supplemented LB plate and incubated for another O.N under the same conditions. Suspected colonies were taken for further analysis.

3.2.1.4 Crude extraction of chromosomal DNA from a colony

Single colony was picked and suspended in 100 µl of 50 mM NaOH in an eppendorf tube by vortex (Mini-Gennie, Biofan, Latvia). The suspension was incubated at 95ºC for 1 min, cooled to 4ºC on ice and neutralized with 16 µl of 1M tris-HCL (pH 8.0). Then the suspension was centrifuged in

microcentrifuge for 2 min at 14,000 RPM (Eppendorf centrifuge 5147C, Eppendorf, Engelsdorf, Germany) and the supernatant containing the DNA was collected used immediately, or stored at -20 ºC.

3.2.1.5 DNA fragment separation

Separation of DNA fragments was done by electrophoresis in agarose gel 1% (w\v) in TAE buffer, at 90V.3.2.1.6 Labeling bacteria with the Cherry fluorescent protein

Bacterial labeling with the Cherry-fluorecent protein was done by transformation of pCherry into the cell, as described before (Choi et al, 2006). Briefly, six ml of stationary culture grown in LB medium were equally distributed into 4 eppendorf tubes. The cells were harvested by centrifugation at room temperature for 2 min at 16,000 g. The pellet in each tube was washed twice with 1 ml of room temperature 300 mM sucrose and the 4 pellets were then combined in a 100 µl sucrose. One µl of the pCherry plasmid (~100 ng DNA) was electroporated into competent cells, as described above. After electroporation the cells were transferred to 1 ml warm LB (room temperature), and incubated at 37ºC for 1.5 h. After the incubation, 150 µl of the solution were plated on LB-Amp plate and incubated O.N at 37ºC. Visible red-fluorescent colonies usually appeared after 24 h.

3.2.2 Genetic methods

3.2.2.1 Site-specific mutagenesis

Mutations in STm SL1344 genome were performed by allelic-replacement, as described by Datsenko and Wanner (2000). Briefly, for each mutation, two primers were designed with 20 nucleotides from the beginning and from the end of the antibiotic-resistance cassette (Km/Cm), and 50 nucleotides from the chromosomal DNA sequence to be replaced. The primers (Table 6) were designed using the Gene Runner program (Version 3.02; Hastings Software, Inc., Westwood, NJ, USA). PCR reaction were done using pKD4 or pKD3 plasmid as template, with program 1 (see, Table 7).

Table 6. Primers list

Sequence	Gene\region	Primer designation
TTACGGTTTAAGTAAAGACTTATATTCAGCTATCCTTTTTTTATGAGCGG **TGTAGGCTGGAGCTGCTTCG**	*Spi1*	Spi1_for
GTCATGAGTTGCTCTTCATCTTCTTTCGAACGCATGTATTGTGGATGTTC**CATATGAATATCCTCCTTAG**	*Spi1*	Spi1_rev
AGGCCAGGCCATTAATGACAAAATGAATGGTAATGATTTGCTCAACCCAG**TGTAGGCTGGAGCTGCTTCG**	*Spi2*	Spi2_for
TTCGGTAGAATGCGCATAATCTATCTTCATCACCATACGTAACAAGGCTG**CATATGAATATCCTCCTTAG**	*Spi2*	Spi2_rev
GTGCTGCTTTCTCTACTTAACAGTGCTCGTTTACGACCTGAATTACTGAT **CATATGAATATCCTCCTTAG**	*invA*	invA_for
ATTCACTGACTTGCTATCTGCTATCTCACCGAAAGATAAAACCTCCAGAT **TGTAGGCTGGAGCTGCTTCG**	*invA*	invA_rev
GTATGCTAAGTCCTACGACTCGTAATATGGGGGCGAGTTTATCGCCTCAG **CATATGAATATCCTCCTTAG**	*avrA*	avrA_for
TTACGGTTTAAGTAAAGACTTATATTCAGCTATCCTTTTTTTATGAGCGG **TGTAGGCTGGAGCTGCTTCG**	*avrA*	avrA_rev

GACAGGCGTAATGAAATCGT	*Spi1*	vspi1_for
AACGTAAGAGACAAATGGCC	*Spi1*	vspi1_rev
TGGGACTACAGCCTCATTTA	*Spi2*	vspi2_for
AGCGATTATTTTCAGTCTCT	*Spi2*	vspi2_rev
TCGTGGTTACTACTGATGCT	*invA*	vinvA_for
GTAATACCACCATGCTGTCA	*invA*	vinvA_rev
TCATCCGGAGGTGGTTGGTA	*avrA*	vavrA_for
GAGTAGTCTTATGGCGCTGG	*avrA*	vavrA_rev

*Vector-derived nucleotides are marked by bold letters

Table 7. PCR conditions

Program number	Reaction conditions
1	95°C, 4'- 35 (94°C, 30"- 55°C, 30"- 72°C, 1'30")- 72°C, 10'
2	95°C, 3'- 35 (95°C, 30"- 58°C, 1'- 72°C, 2'30")- 72°C, 5'

3.2.2.2 PCR ptoducts cleaning

PCR products were cleaned with Clean & Concentration DNA kit (Zymo), according to the manufacturer's instructions.

3.2.3 Attachment and internalization assays

3.2.3.1 Inoculum preparation

Bacteria were grown in LB with the appropriate antibiotics (when necessary) O.N. Bacteria were washed by centrifugation (5000 RPM for 10 min), and resuspended in 5 ml SDW. Following additional washing step, the pellet was finally resuspended in 5 ml SDW to yield 10^9 CFU/ml (OD_{600}= 1.0).

3.2.3.2 Inoculation of *Arabidopsis* leaves

Arabidopsis leaves were detached from the plant and submerged in 30 ml sterilized-distilled water (SDW) in a 50-ml sterile polypropylene tube (Labcon, Petaluma, CA). Leaves were preconditioned for 20 min under illumination (100-150 μE^{-2} s^{-1}) at 30°C. The water was then removed and replaced with 10 ml of GFP-tagged *Salmonella* at a concentration of ~10^9 CFU/ml SDW. The tubes were incubated for 2 h (or longer, when indictaed) under illumination at same temperature. Following incubation, the leaves were rinsed twice with SDW for 1 min each to remove unattached bacteria, and visualized immediately under a confocal laser scanning microscopy (CLSM).

3.2.3.3 Inoculation of lettuce leaves

Two layers of outer leaves were removed and the inner leaves were used. The leaves were cut with a clean razor blade to 1 cm x 1 cm pieces, excluding the main veins so that the pieces lay flat on a microscopic slide. The leaf pieces were placed with the abaxial (lower) side facing down onto a microscopic slide. P.cherry STm (10^9 CFU/ml) was gently added underneath the leaf and the slides were incubated in a petri dish (miniplast 90x15, Ein-Shemer, Israel) in a climate-controlled growth chamber (Conviron Adaptis, CNP6010, Winnipeg, Canada) under illumination (100-150 μE^{-2} s^{-1}) at 30ºC for 2 h. Samples were then washed by dipping the leaf pieces 10 times gently in SDW in a petri dish to remove unattached bacteria and taken immediately for CLSM.

3.2.3.4 Determination of *Salmonella* attachment and internalization

Quantification of attachment and internalization was performed by determining bacterial localization on or within the leaf tissue using CLSM. The localization of fluorescent-tagged bacteria on the leaf surface (attachment) and underneath the epidermis (internalization) was determined in 30 randomly chosen microscopic fields (at magnification of X60 for *Arabidopsis* and X40 for lettuce). Bacterial numbers were scored as follows: 0, 1 to 10, 10 to 50, 50 to 100, and ≥100 *Salmonella* cells per one microscopic field. In order to simplify the quantification of surface-associated and internalized bacteria, the data are presented, in most cases, as the incidence of *Salmonella*, i.e., percentage of fields containing one or more surface attached or internal bacteria in 30 microscopic fields of the same leaf. Each experiment included three different *Arabidopsis* leaves or lettuce three leaf pieces (a total of 90 microscopic fields were examined per experiment), each experiment was repeated independently at least three times on different days.

3.2.4 Measuring stomatal aperture

Lettuce or *Arabidopsis* leaves were prepared and inoculated with bacteria (10^8 CFU/ml) or SDW alone (control), as described above using different inoculation times (depending on the experiment). At the pre-determined times the leaves were taken for CLSM, without washing to minimize any further modulation of stomatal aperture.

Random images of the inoculated side were taken using an Olympus camera (Tokyo, Japan) and the stomatal aperture was measured by ImageJ, an open source Java-written program (Version 1.4 10; Bethesda, MD). An example of stomatal aperture measurement is shown in Fig. 3.1. For each experimental condition, images of at least 15 stomata were analyzed. Each experiment included three different leaves *(Arabidopsis)* or three leaf pieces (lettuce) and was repeated at least three times on different days. In total, at least 45 stomata were measured per treatment.

Each experiment included a water control in order to confirm that the stomata were responsive (at least 70% of stomata opened).

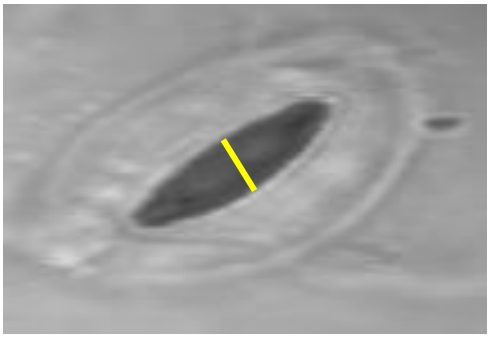


Figure 3.1 Measurement of stomatal aperture. An image of *Arabidopsis* stomate stomata)

3.2.5 Detection of ROS

The production of H_2O_2 in leaves was tested by fluorescence microscopy following staining with 2´7´-dichlorofluorescin diacetate (H_2DCF-DA). This nonfluorescent dye can cross the plasma membrane freely, and is then cleaved to its impermeable counterpart, dichlorofluorescin (H_2DCF), by endogenous esterases. H_2DCF, which accumulates in the cell, functions as a reporter of cytoplasmic H_2O_2 by converting upon oxidation to its fluorescent form, DCF. Stock of 20 mM H_2DCF-DA in DMSO was prepared and stored in -20 ºC. Following incubation of the leaf with bacteria or water (control), the leaf was placed with the abaxial side down, in a working solution of H_2DCF-DA (1:1000 dilution of stock solution in SDW) for 10 min in the dark, at room temperature. The leaf samples were washed by dipping in SDW as described before, and immediately photographed under a fluorescence microscope. Each experiment included three different lettuce leaf pieces and was repeated at least three times on different days. In total, at least 45 stomata were evaluated for a given treatment. The fluorescence level was measured by FLUOVIEW 500 software (Olympus, Tokyo, Japan).

3.2.6 Microscopy

3.2.6.1 Confocal laser scanning microscope (CLSM)

All microscopic observations and image acquisitions were performed with an Olympus IX-81 laser scanning confocal microscope (FV 500, Olympus Optical Co., Tokyo, Japan) equipped with argon-ion and HeNe lasers. A water immersion objective magnification of X40 (40X0.9 NA PlanApo) was used for lettuce and X60 (60X1.0 NA PlanApo) for *Arabidopsis*. For ROS detection, samples were excited by 488-nm light and the emission was collected through a BA 505-525 filter. For Cherry-labeled bacteria detection, samples were excited by a 543-nm HeNe laser and image acquisition was accomplished using a BA560 IF emission filter. To detect chlorophyll autofluorescence, a BA 660 IF emission filter was used. When ROS and cherry-labeled bacteria were detected in the same sample, we used DM (dichroic mirror) 488/543. In all cases, where more than one color was monitored, sequential acquisition was performed. Transmitted light images were obtained using Nomarski differential interference contrast (DIC) microscopy. Confocal optical sections were obtained at 1 µm increment. 3D images were obtained using the FLUOVIEW 500 software supplied with the microscope.

3.2.6.2 Scanning electron microscopy (SEM)

Arabidopsis and lettuce leaves inoculated with STm for 2 h, were washed twice in water and fixed with 5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for 2 h at room temperature. The pieces were washed five times (10 min each) in phosphate-buffered, and then were processed by washing with increasing concentrations of ethanol (25%, 50%, 75%, 95% for 20 min each, and twice at 100% for 30 min). The pieces were dried with CO_2 in a critical-point drier (CPD 030; Bal-Tec AG, Balzers, Liechtenstein), coated with gold (Polaron Equipment Ltd., Watford, United Kingdom), and taken for observation under a JEOL JSM 35C scanning electron microscope (Tokyo, Japan).

3.2.7 Light intensity measurements

Illumination intensity was measured by a photometer (model LI-185B; Li-COR, Inc., Lincoln, NE).

3.2.8 Bioinformatics methods

Sequence analysis was performed at the NCBI site (www.ncbi.nlm.nih.gov) using the *S*. Typhimurium genome as reference.

3.2.9 Statistical methods

Statistical analysis was performed using the Instat program, version 3.0.6 (GraphPad Software, Inc., La Jolla, CA). Analysis of variance was performed by the Kruskal-Wallis (Nonparametric ANOVA) comparison test. $P < 0.05$ indicated significant differences.

4. Results

4.1 *Arabidopsis thaliana* as a model plant to study *Salmonella*-leaf interaction

Arabidopsis thaliana is a small plant in the mustard family that has become the model system of choice for research in plant biology. Its entire genome is known and sequenced (Meinke et al., 1998), its life cycle is short (about six weeks) and it grows under supervised conditions (temperature, light and humidity) in growth rooms (Somerville and Koornneef, 2002)**.** These features make *Arabidopsis* a potential candidate to serve as a model plant for studying the interactions of *Salmonella* with leafy greens at the molecular level.

4.1.1 Attachment and internalization of *Salmonella* to *Arabidopsis* leaves

In order to examine the ability of *Salmonella* to attach to and internalize *Arabidopsis* leaves taken from two to six weeks old plants were taken from three *Arabidopsis* ecotypes ('col', 'ws', 'ler'). The localization of *Salmonella* on the leaf surface or internally following co-inoculation for two hours is presented in Table 8. The Incidence (%) of *Salmonella* inside *Arabidopsis* leaf (internalization) was very low in all three ecotypes (Table 8, and Fig. 4.1).

Table 8: Incidence (%) ± SD and localization of *Salmonella* on *Arabidopsis* leaves.

Plant age (Weeks)	Location in the leaf	Ecotype col	Ecotype ws	Ecotype ler
2	Surface	100±0	100±0	100±0
	Internal	0±0	1.82±0.6	3.4±1.1
3	Surface	100±0	100±0	100±0
	internal	0±0	0±0	7.5±4.1
4	Surface	100±0	100±0	100±0
	Internal	1.92±0.5	0±0	0.96±0.2
5	Surface	100±0	100±0	100±0
	Internal	1.3±0.5	7.3±3.7	0±0
6	Surface	100±0	100±0	100±0
	Internal	0±0	0±0	4.8±3.55

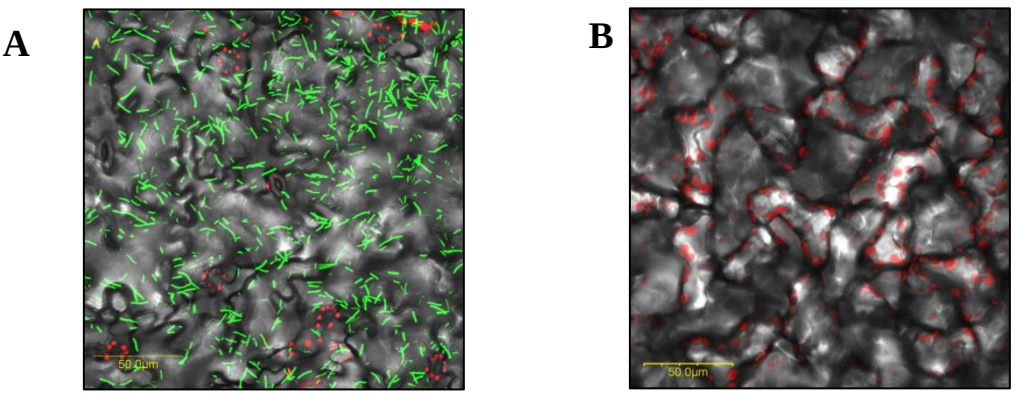


Figure 4.1. Confocal microscopy images showing GFP-tagged bacteria residing on the surface of the leaf (A) but not in internal leaf tissues (B) following 2-h internalization assay. Internal leaf tissue image (B) are composed of a stack of fluorescent images taken every 1.2 µm to a depth of 40 µm along a z-section of the same field. All images were overlaid with DIC images. Bars 50 µm.

A possible effect of longer incubation time (up to 4 h) on internalization was examined in ecotype 'col' at 4 weeks of age. No internalization was observed even following 4 h of co-incubation (Data not shown). The attachment of *Salmonella* to *Arabidopsis* leaves was high (more than 100 bacteria per field in all experiments). In some cases, the bacteria formed aggregates along the junctions of the epidermal cells (Fig 4.2A). Attachment of *Salmonella* to cut edges of the leaf was also observed, but no internalization of bacteria was seen further than the cutting site (Fig 4.2B).

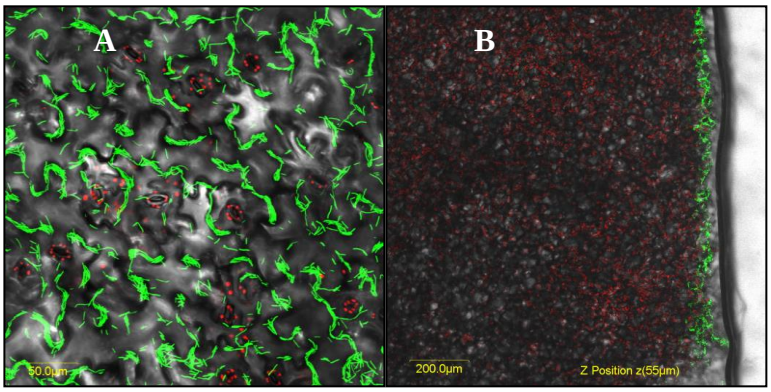


Figure 4.2. Confocal microscopy images showing GFP-tagged bacteria on *Arabidopsis* leaf surface. (A) Bacteria (green) reside on the surface of the leaf in aggregates between the junctions of the epidermal cells. The images were overlaid on a DIC image. Bars 50µm. (B) Bacteria (green) in a cut leaf showing no further penetration beyond the injured tissue at 2 h following inoculation. Bar 200 µm. The image was taken at a depth of 55 µm. No internalized bacteria were observed at other depths below the surface.

It was previously shown that *Salmonella* had an affinity toward stomata in iceberg lettuce (Kroupitsky et al., 2009b). Unlike lettuce, no attraction of *Salmonella* to stomata was observed in *Arabidopsis* in all three ecotypes (ws, col, ler) (Fig 4.3). Although, the attachment of the bacteria to *Arabidopsis* leaves was better than to lettuce leaves, more numbers of bacteria were seen per field in *Arabidopsis* leaf tissue (Fig 4.3).

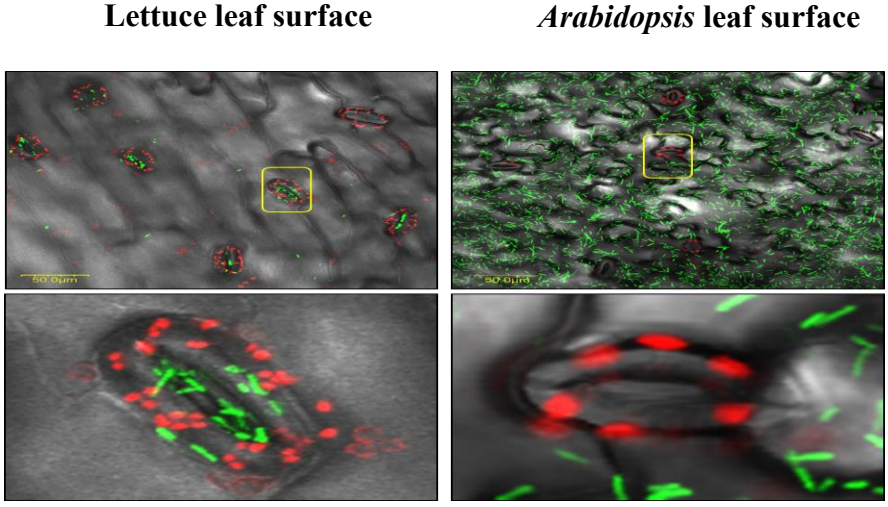


Figure 4.3 Confocal microscopy images showing GFP-tagged bacteria (green), plastid (red). Bacteria residing on the surface of iceberg lettuce and *Arabidopsis* leaf, showing affinity of *Salmonella* to stomata in lettuce but not in *Arabidopsis*, and better attachment to *Arabidopsis* after two hour of inoculation. Lower images show a higher magnification of the marked stomata from the upper images (yellow squares). Bars 50 µm.

It was reported that leaf topography can influence bacterial attachment (Lindow & Brandl, 2003). In order to test if the different attachment pattern in the two plants is related to such differences, we compared the topography of the types of leaves by SEM (Fig 4.4).

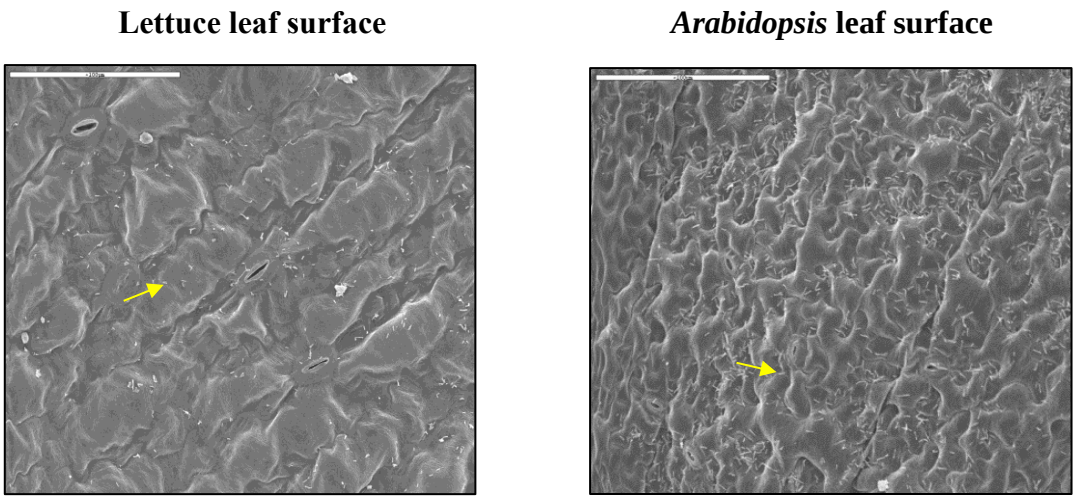


Figure 4.4. SEM microscopy images of *Arabidopsis* and lettuce leaf after two hours of inoculation with *Salmonella* showing the topography of the two leaves. Yellow arrows pointing at the location of bacteria. Bar 100 µm.

The difference in *Salmonella* attachment to the leaves might at least partially be linked to the difference in the topography of the leaves. In *Arabidopsis* more bacteria are found along the junctions of the epidermal cells. This might reflect the "mounty" characteristics of the *Arabidopsis* surface, which results in the capture of bacteria in the lower "valleys" which are more protected from washing than higher sites. Nevertheless, there is no linkage between the leaf topography and the differential attachment to stomata.

4.1.2 Effect of *Salmonella* on *Arabidopsis* stomata

A previous study showed that *Arabidopsis* stomata close within two hours, in the presence of *E. coli* O157:H7 and *Pseudomonas syringae* pv. Tomato (Pst DC3000) (Melotto et al., 2006). Kroupitsky and colleagues found that Pst DC3000 also induced stomatal closure in lettuce after two hours, while *S.* Typhimurium does not (Kroupitsky et al., 2009b). In order to check if *Salmonella* also induces stomatal closure in *Arabidopsis*, we examined stomatal aperture in *Arabidopsis* (ecotype 'col' at 4 weeks of age) in the presence of STm and Pst DC3000 after 2 h (Fig 4.5).

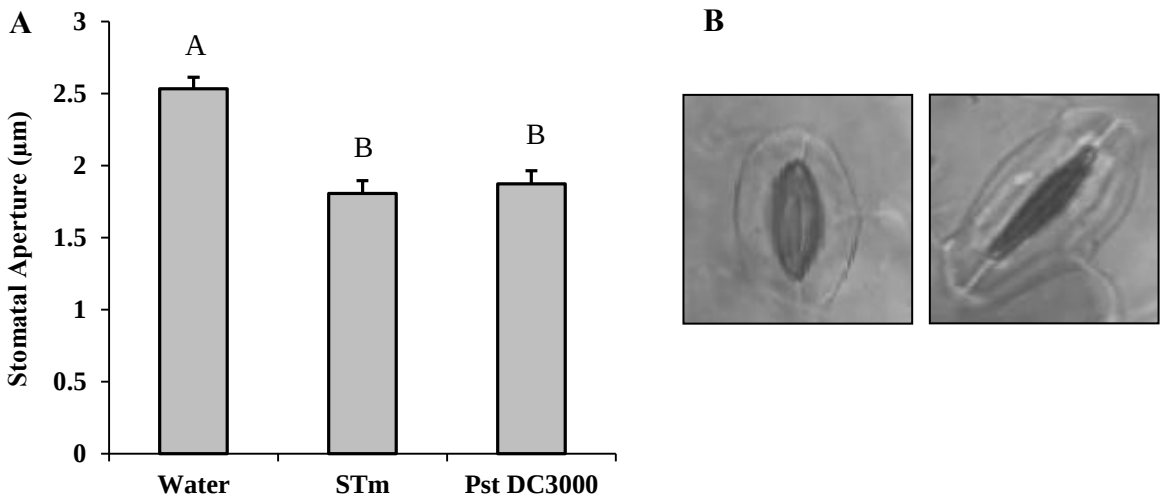


Figure 4.5. Stomatal aperture in *Arabidopsis* (col) leaves exposed for 2 hours to water (negative control), STm, and Pst DC3000 (positive control) (A). Results are shown as means and standard errors. Different letters indicate significant differences ($P < 0.05$) between the means, Statistical analysis was performed using Nonparametric ANOVA (Kruskal-Wallis Test). Stomata at different closure stages are shown (B). Open stomata (left) and closed stomate (right).

Similar to the phytopathogen Pst DC3000, STm induced stomatal closure in *Arabidopsis* after two hours of incubation. Although *Salmonella* attached very well to *Arabidopsis* leaves and induces stomata closure, there was no internalization of the pathogen through the leaf stomata. Therefore, we decided not to continue with this model plant, and continued the study of *Salmonella*-leaf interactions using the iceberg lettuce model.

4.2 *Salmonella*- leaf interactions in iceberg lettuce

4.2.1 Involvement of TTSS in *Salmonella* attachment and internalization

It was recently found that TTSS is involved in colonization of Shiga-toxigenic *E. coli* (STEC) O157:H7 in baby spinach leaves (Saldana et al., 2011). Schikora and colleagues demonstrated that *Salmonella* mutants that were unable to deliver TTSS effectors were also compromised in infection of *Arabidopsis thaliana* plants (Schikora et al., 2011). In order to check whether TTSS is also involved in the attachment of *Salmonella* to lettuce leaves and in stomatal internalization, we have constructed the following TTSS mutants in STm SL1344 strain: spi1$^-$ (defective in TTSS-1), spi2$^-$ (defective in TTSS-2), spi1$^-$/spi2$^-$, *invA*$^-$ (defective in a component of the export apparatus of TTSS-1) and *avrA*$^-$ (defective in one of the effectors of TTSS-1) (Kimbrough and Miller, 2002).

The level of attachment of wt STm and the five TTSS mutant to the lettuce leaf was comparable, the mutants attached less efficiently to lettuce leaf (see supplementary data-Table S1). Although the incidence of internalization in the five TTSS mutants was lower than that of the wt strain, only the internalization of the *spi1*$^-$ mutant was significantly lower than that of the wt (Fig. 4.6).

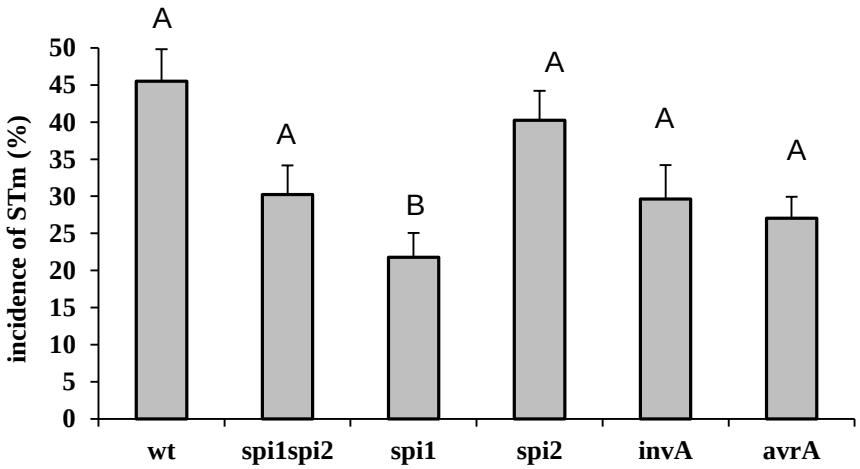


Figure 4.6. Incidence of *Salmonella* and its TTSS mutants inside lettuce leaf tissue after two hours of co-incubation. Results are shown as means plus standard errors. Different letters indicate significant differences (P <0.05) between the mean, Statistical analysis was performed using Nonparametric ANOVA (Kruskal-Wallis Test).

4.2.2 Effect of *Salmonella* on stomatal aperture in iceberg lettuce

4.2.2.1 Effect of *Salmonella* and *Pseudomonas* on stomatal aperture

Melotto and colleagues showed that after inoculating *Arabidopsis* with Pst DC3000 for one hour the plant closed its stomata and reopened them after three hours in response to secretion of a bacterial phytotoxin named coronatin. *E. coli* O157:H7 on the other hand couldn't reopen the stomata but did induced it closure (Melotto et al., 2006). In a previous study in our lab it was found that the plant pathogen Pst DC3000 induced stomatal closure in iceberg lettuce after two hours of incubation, as oppose to *Salmonella* which only slightly affected stomatal closure. It was suggested that either lettuce does not recognize *Salmonella* as a pathogen, or *Salmonella* actively induces opening of stomata (Kroupitsky et al., 2009b). In order to refine the analysis of stomatal response to *Salmonella* we have followed stomatal response in the presence of of *Salmonalla* for up to 4 hours (Fig. 4.7).

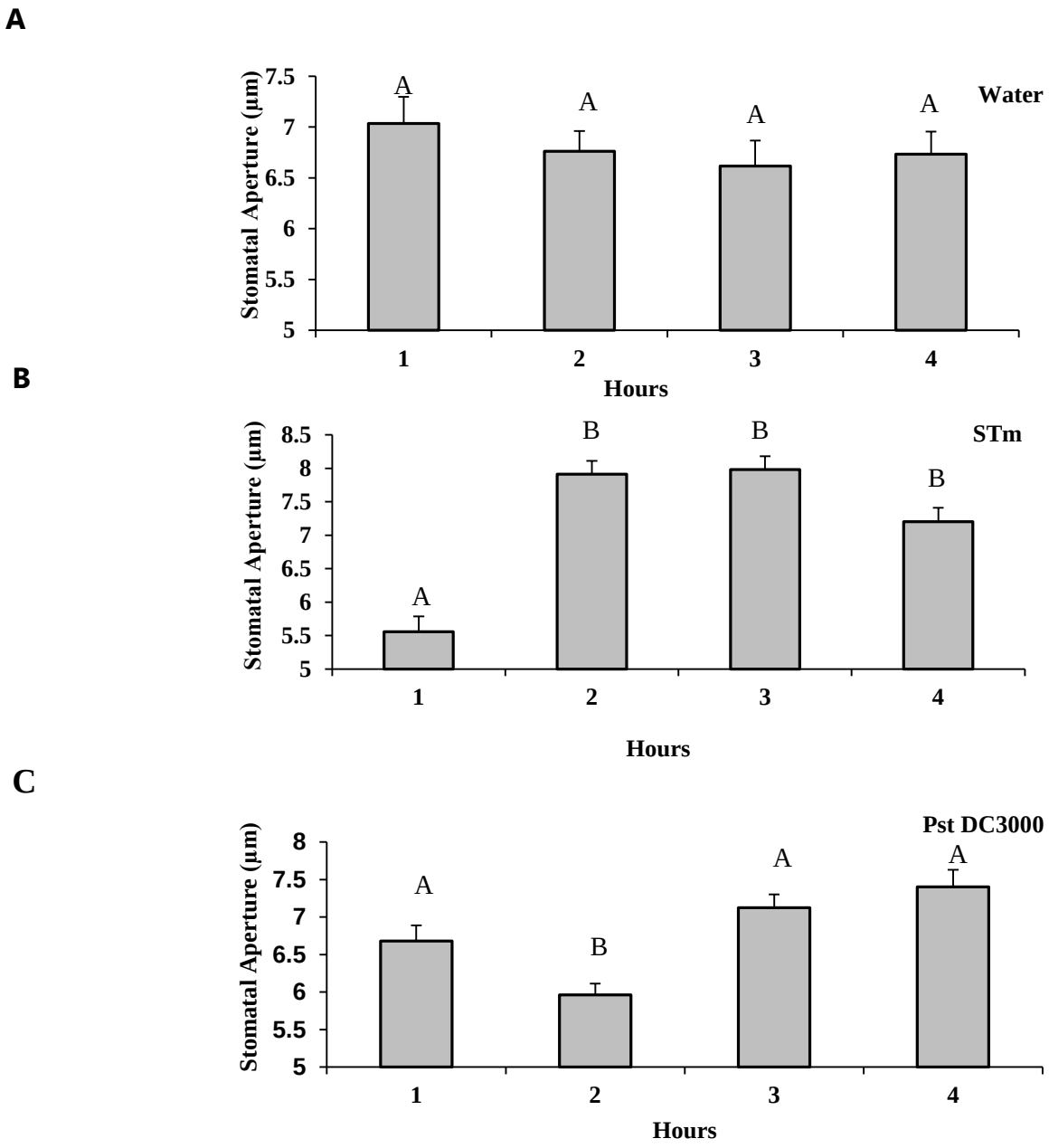


Figure 4.7. Stomatal response in the presence of *Salmonella* and Pst DC3000 in iceberg lettuce leaves. (A) Stomatal aperture in lettuce leaves exposed for 1 to 4 hours in water, (B) or in the presence of STm (C) or Pst DC3000. Results are shown as means and standard errors. Different letters indicate significant differences ($P < 0.05$) between the means, Statistical analysis was performed using Nonparametric ANOVA (Kruskal-Wallis Test).

Lettuce stomata exposed to water remained open during four hours, however, Pst DC3000 and STm, induced stomatal closure. Stomatal closure occurred at one hour in the presence of STm and after two hours in the presence of Pst. Lettuce reopened its stomata after two hours of incubation with STm and after three hours of incubation with Pst.

4.2.2.2 Effect of *Salmonella* concentration on stomatal aperture

In order to find the minimal concentration of STm that can induce stomatal closure, we repeated the experiment with different bacterial concentrations (Fig. 4.8A, B). All the tested concentrations of STm induced stomatal closure after one hour of inoculation. After two hours the minimum bacterial concentration that reopened stomata was 10^5 CFU/ml.

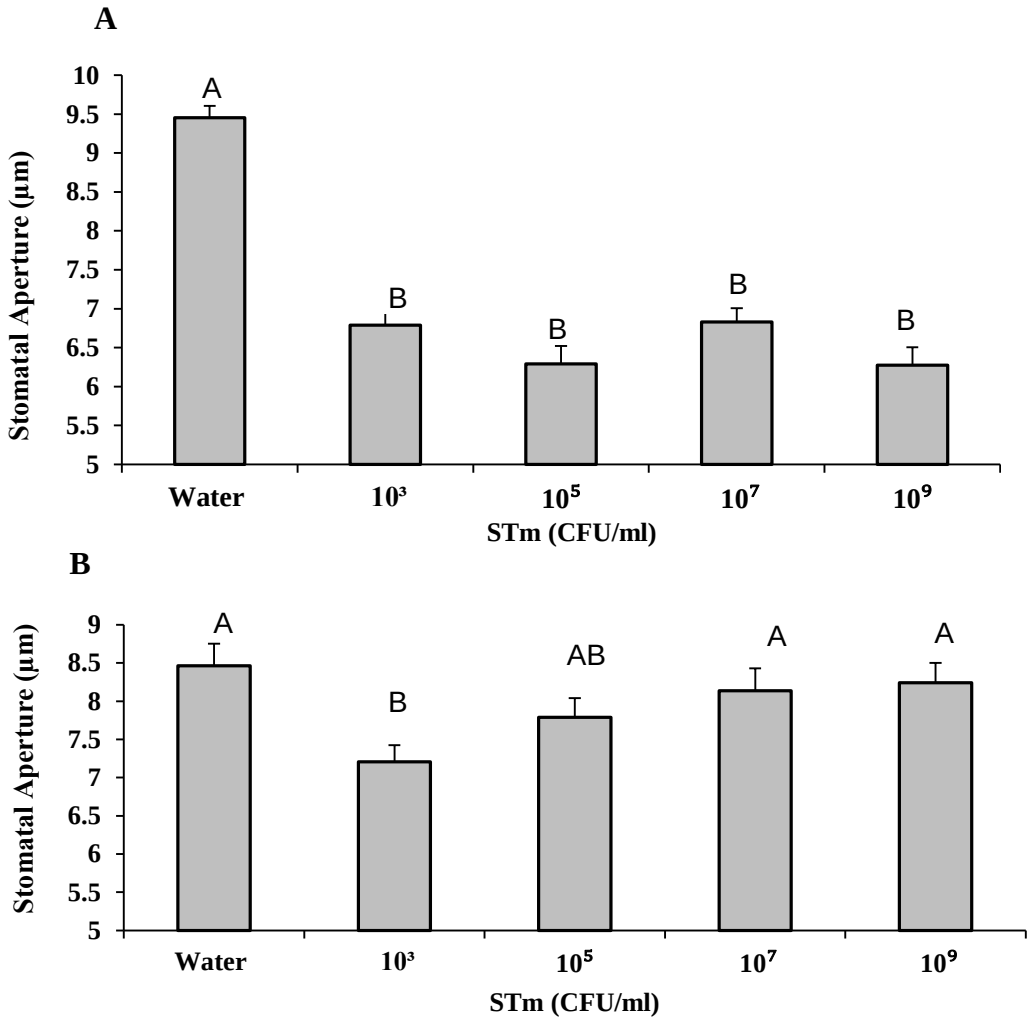


Figure 4.8. Stomatal aperture of lettuce leaves in the presence of increasing STm concentrations (CFU/ml) at one (A) and two (B) hours. Water served as control. Results are shown as means and standard errors. Different letters indicate significant differences ($P < 0.05$) between the means, Statistical analysis was performed using nonparametric ANOVA (Kruskal-Wallis Test).

4.2.2.3 Involvement of bacterial surface components in triggering stomatal closure

Plants can sense the presence of microorganisms by interacting with conserved MAMPs, such as flagella and LPS **(**Dodds & Rathjen, 2010) In order to check if stomatal closure is induces by flagella and LPS, we tested the effect of a *fliGHI* mutant, which does not express flagella, and purified *Salmonella* LPS. The *fliGHI* mutant and exogenous LPS induced closure of stomata at 1 h similar to the wt strain. However, in the presence of LPS lettuce could not reopen the stomata (Fig. 4.9).

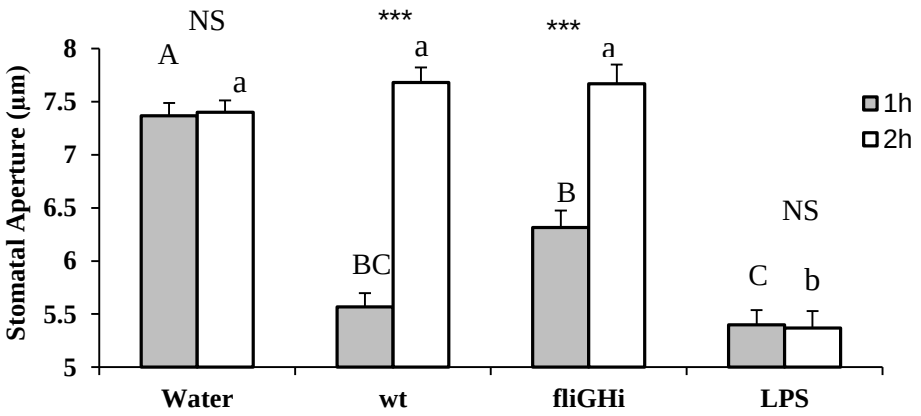


Figure 4.9. Stomatal aperture in leaves exposed for 1 and 2 hours to wt STm, *fliGHI* mutant and LPS extract. Exposure to water served as control. Results are shown as means plus standard errors. Different letters indicate significant differences ($P < 0.05$) between the means at 1 h (capital letters) and 2 h (lowercase letters). Differences between stomatal aperture at 1 and 2 h are denoted as follow: NS-Not significant, *** correspond to $P<0.001$. Statistical analysis was performed using nonparametric ANOVA (Kruskal-Wallis Test).

4.2.2.4 Effect of *Salmonella* viability on the closure of stomata

We next wanted to know if stomatal response to STm requires live bacteria or perhaps dead bacteria can also induce closing of stomata at 1 h following by opening at 2 h post inoculation. Lettuce leaves were incubated with heat-killed STm (boiled for 10 min), and stomatal aperture was measured (Fig. 4.10). Unlike live bacteria, dead bacteria did not induce stomatal closure.

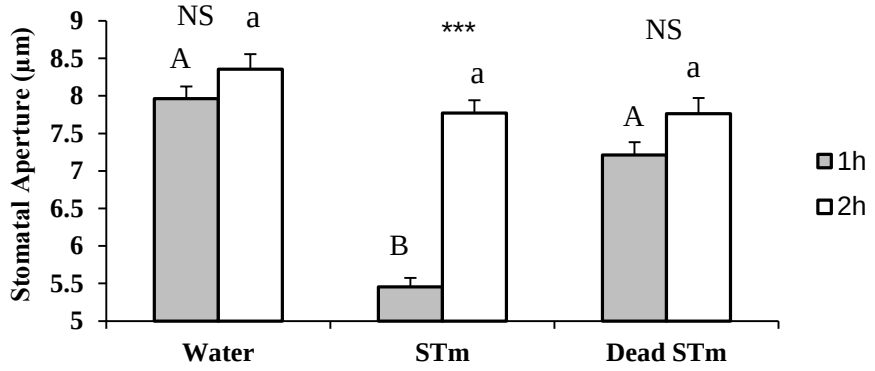


Figure 4.10. Stomatal aperture in lettuce leaves exposed to live and heat-killed STm. Exposure to water served as control. Results are shown as means plus standard errors. Different letters indicate significant differences ($P < 0.05$) between the means at 1 h (capital letters) and at 2 h (lowercase letters). Differences between stomatal aperture at 1 and 2 h are denoted as follow: NS-Not significant, *** corresponds to $P<0.001$. Statistical analysis was performed using nonparametric ANOVA (Kruskal-Wallis Test).

4.2.2.5 *De novo* protein synthesis and stomatal closure

In order to know whether live bacteria require new protein synthesis to induce stomatal closure, we added chloramphenicol (Cm), which inhibits protein synthesis. Cm was added to the washed bacteria at 30 min before the inoculation. Cm-treated *Salmonella* did not induced stomatal closure (Fig. 4.11), indicating that de novo synthesis is required for this process.

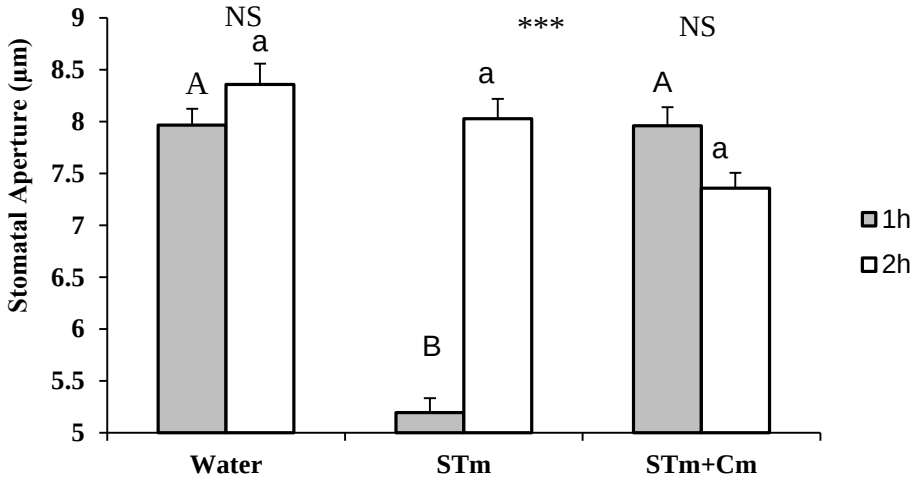


Figure 4.11. Effect of chloramphenicol on stomatal closure. Stomatal aperture in leaves in the presence of water (negative control), STm (positive control) and STm treated with Cm. Results are shown as means plus standard errors. Different letters indicate significant differences ($P < 0.05$) between the means at 1 h (capital letters) and 2 h (lowercase letters). Differences between stomatal aperture at 1 and 2 h are denoted as follow: NS-Not significant and *** corresponse to $P<0.001$, Statistical analysis was performed using nonparametric ANOVA (Kruskal-Wallis Test).

4.2.2.6 *Salmonella* TTSS role in stomatal response

Recently, it was reported that *Salmonella* suppresses the plant immune system by the TTSS (Shirron & Yaron, 2011; Schikora et al., 2011), and that the plant can recognize the presence of *Salmonella* by sensing its TTSS-SPI1 and expressing the PR1 protein (Iniguez et al., 2005). Furthermore, in this study we found the TTSS mutants were less able to internalize lettuce stomata than the wt (Fig. 4.6). In order to examine whether TTSS is also involved in stomatal response to *Salmonella*, lettuce leaves were inoculated with the wt strain and the following TTSS mutants: *spi1*, *spi2*, *spi1/spi2*, *invA*, *avrA*. Incubation with water served as a negative control (Fig. 4.12). All TTSS mutants induced stomatal closure after one hour similar to the wt strain; however, all the mutants couldn't reopen the stomata at two hours post-inoculation.

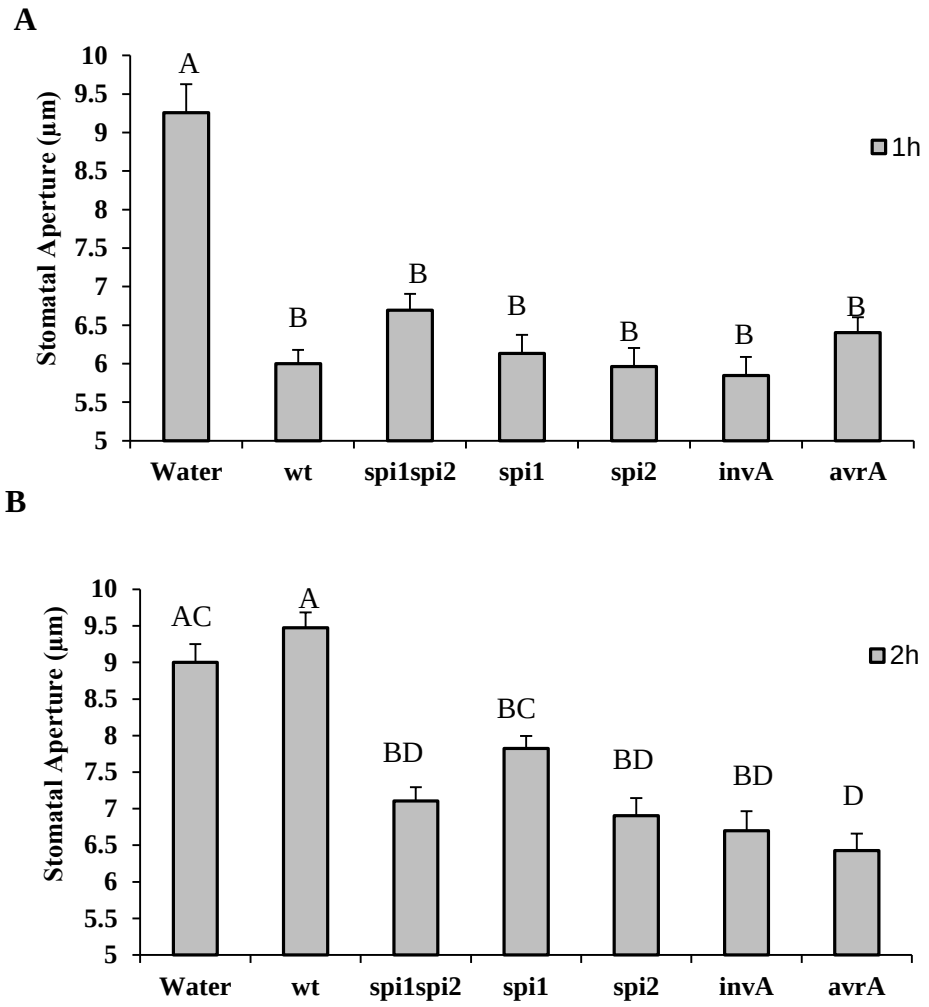


Figure 4.12. Stomatal aperture in lettuce leaves inoculated TTSS mutants. stomatal aperture of STm wt strain and the TTSS mutants (*spi1*, *spi2*, *spi1/spi2*, *invA*, *avrA*) was tested at 1 h (A) and 2 h (B). Exposure to water served as control. Results are shown as means plus standard errors. Different letters indicate significant differences ($P < 0.05$) between the means. Statistical analysis was performed using nonparametric ANOVA (Kruskal-Wallis Test).

4.2.2.7 Effect of abscisic acid on *Salmonella*-mediated signaling

Abscisic acid (ABA) is a plant hormone that regulates among other functions the closure of stomata (Kim et al., 2010). In order to check if STm-mediated reopening of stomata (at 2 h) occurs after ABA-mediated closure, we first tested the minimal concentration of exogenous ABA that can induce stomatal closure. It was found that the minimal concentration was 0.5-1 ppm. Next, stomatal aperture at 2 h post inoculation with STm alone or in the presence of ABA was determined (Fig. 4.13). Unlike STm alone, STm with ABA was not able to reopen the stomata at two hours post-inoculation. This may suggest that *Salmonella* cannot overcome ABA-mediated closure of stomata.

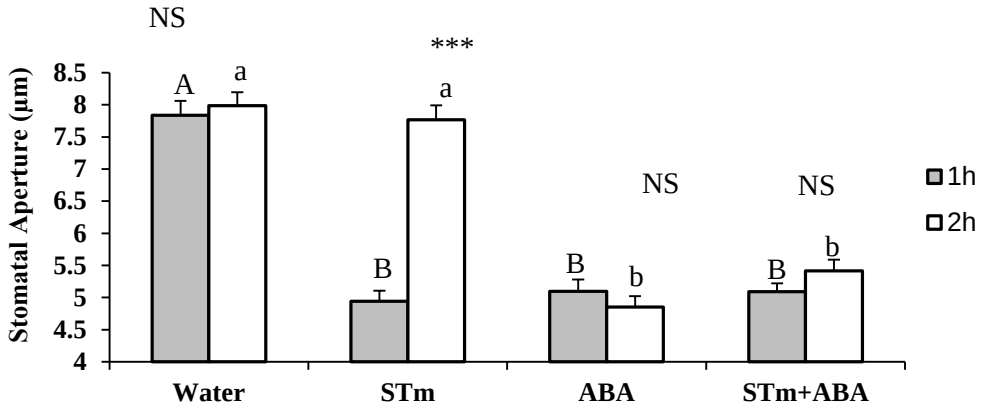
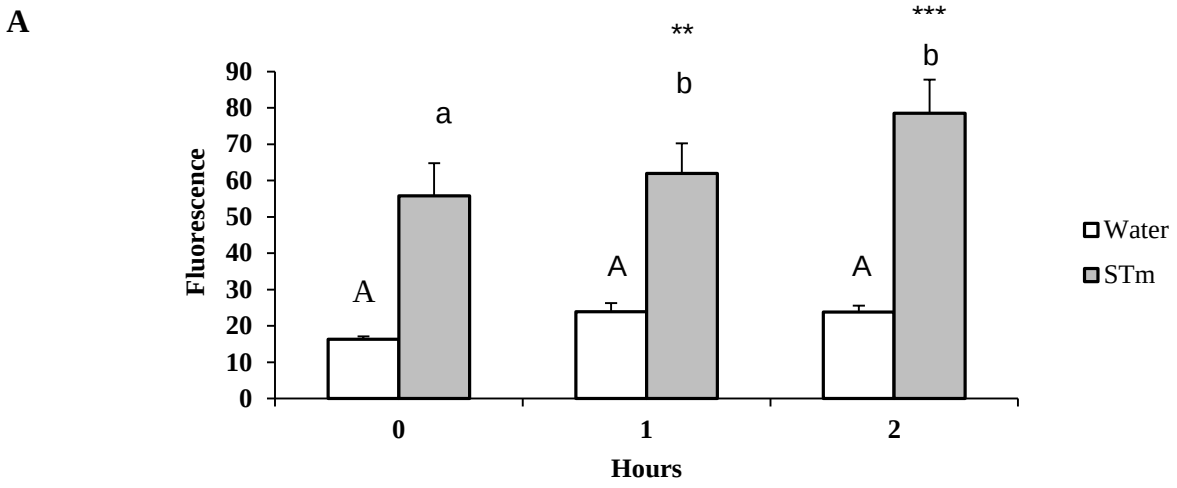


Figure 4.13. Effect of ABA on STm-mediated opening of stomata. Stomatal aperture in leaves was tested in the presence of water, STm, ABA, and STm supplemented with ABA. Results are shown as means plus standard errors. Different letters indicate significant differences ($P < 0.05$) between the mean apperture at 1 h (capital letters) and at 2 h (lowercase letters). Differences between the means of the same treatment are designated as follow: NS-Not significant, *** corresponds to $P<0.001$. Statistical analysis was performed using nonparametric ANOVA (Kruskal-Wallis Test).

4.2.3 Effect of *Salmonella* on the production of reactive oxygen species in the guard cells

4.2.3.1 *Salmonella* induces production of reactive oxygen species

Reactive oxygen species (ROS) are produced in the guard cells in response to ABA-mediated signaling and induced stomatal closure (Pitzschke et al., 2006). Since, *Salmonella* also induces stomatal closure, we wanted to check if ROS production is also involved in this process. ROS production was determined in the guard cells by quantification of the ROS-mediated fluorescence following staining with H_2DCF-DA (Fig. 4.14). Confocal microscopy images were taken immediately after staining, at different time point after inoculation, 0, 1 and 2 hours post inoculation. STm induced ROS production in the guard cells, and the amount of ROS is significantly higher after *Salmonella* inoculation.



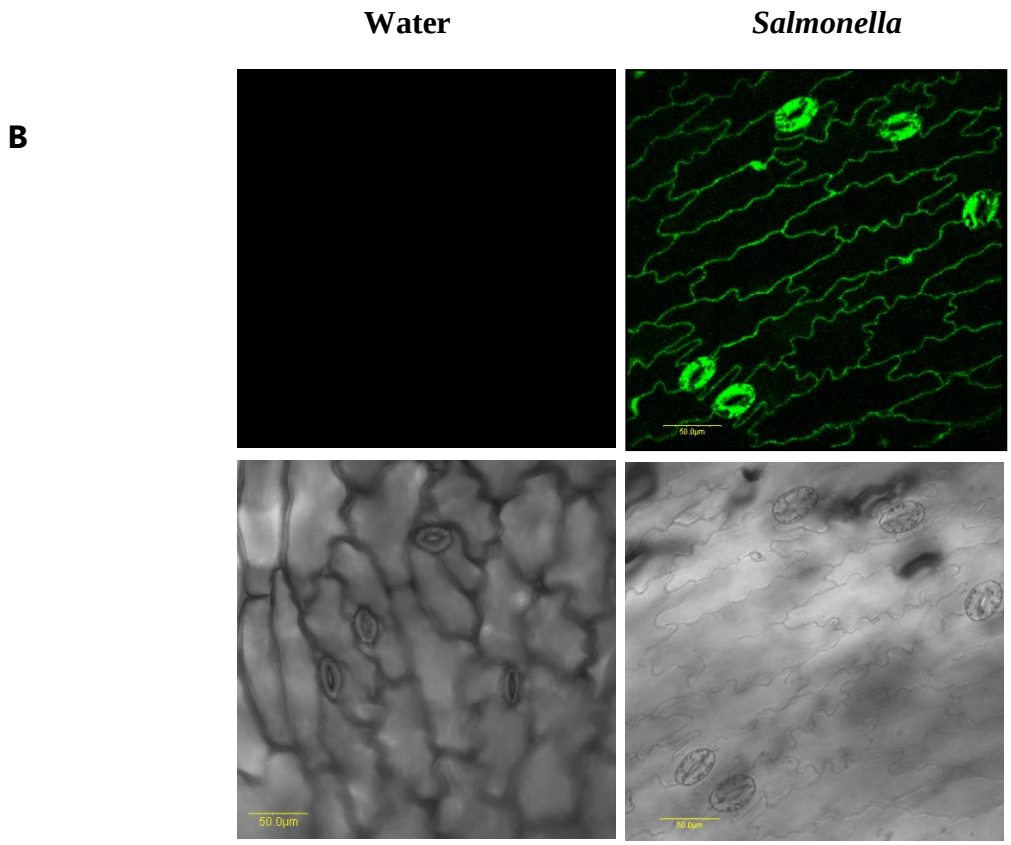


Figure 4.14. ROS expression in guard cells in response to STm. (A) Quantification of ROS in guard cells by fluorescence measurements after H_2DCF-DA staining. Time zero refers to dip inoculation for a second. (B) Representative confocal microscopy images showing the production of ROS (green), in guard cells after two hours of inoculation with *Salmonella*. Upper panels show Nomarski images and lower panels show fluorescent images of the same fields. Bar 50 µm. Results in (A) are shown as means plus standard errors. Different letters indicate significant differences ($P < 0.05$) between the means of the same treatment, differences between the means of the same time point are denoted as follow: *, **, *** correspond to $P<0.05$, $P<0.01$ and $P<0.001$, respectively. The data were analyzed using nonparametric ANOVA by the Kruskal-Wallis Test.

4.2.3.2 Involvement of flagella and LPS in ROS production

We found that STm induces ROS production in guard cells. In order to know if ROS production is induced by flagella or LPS, lettuce leaf pieces were inoculated with the STm wt strain, the *fliGHI* mutant, which does not express flagella, and with purified *Salmonella* LPS. Confocal images were taken and the presence of ROS-mediated fluorescence in the guard cells was measured (Fig. 4.15). The *fliGHI* mutant showed significantly less ROS than the wt after two hours of inoculation. The LPS extract induced ROS production similarly to wt after one and two hours of inoculation. The *fliGHI* mutant does not induce guard cells ROS production at two hours post-inoculation, suggesting that flagella have a role in triggering the ROS resposne. LPS alone can also induce ROS production, suggesting that the guard cells respond to both flagella and LPS.

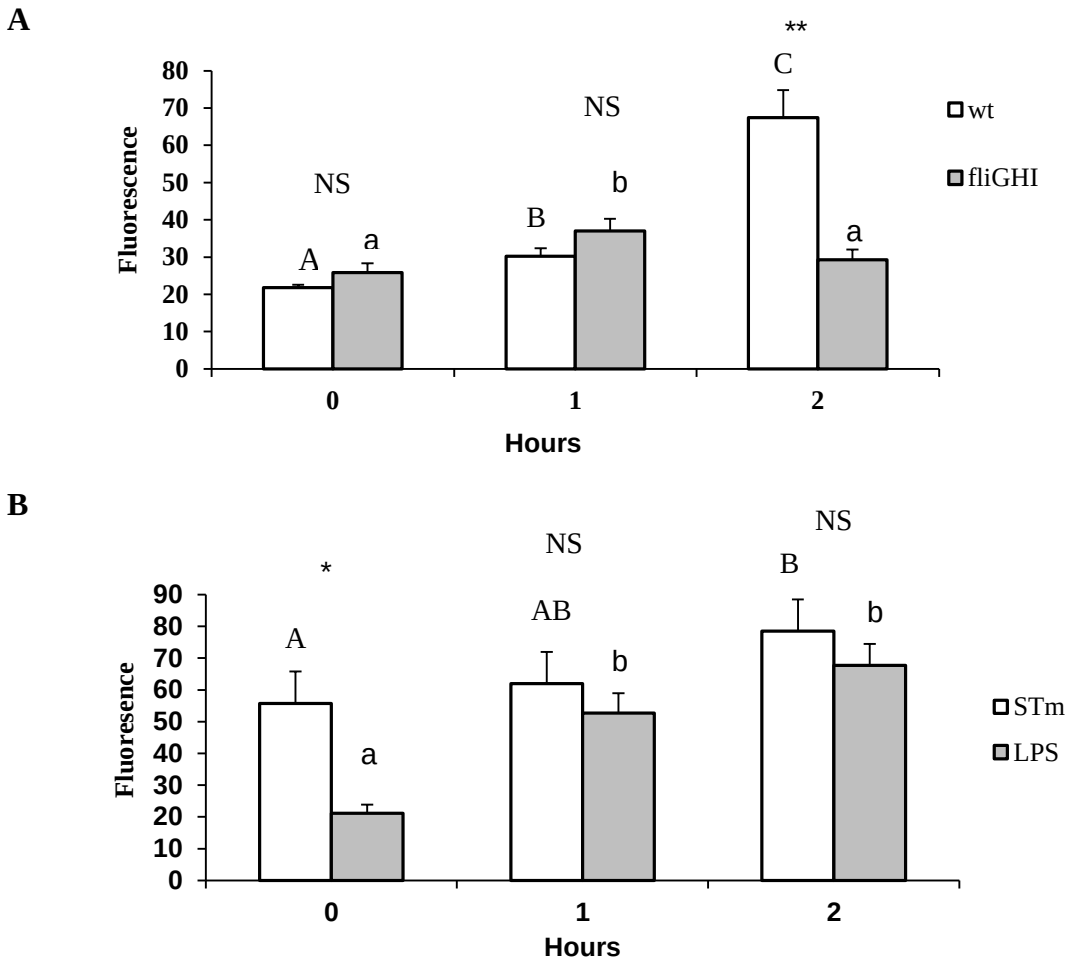


Figure 4.15. Effect of flagella (A) and LPS (B) on ROS production in guard cells. Leaves were inoculated with the wt and *fliGHI* strains (A), or in the presence of LPS (B). ROS production was measured as fluorescence in the guard cells after H_2DCF-DA staining, water served as a negative control. Pictures were taken by confocal microscopy. Results are shown as means plus standard errors. Different letters indicate significant differences ($P < 0.05$) between the means of the same treatment, differences between the means of the same time point are denoted as follow: NS-Not significant; *, ** correspond to $P<0.05$, $P<0.01$, respectively. The data were analyzed using nonparametric ANOVA by the Kruskal-Wallis Test.

4.2.3.3 Effect of *Salmonella's* viability on ROS production by guard cells

Since both flagella and LPS apparently involved in the induction of ROS in the guard cells, it is obvious that this plant response does not require live STm cells. To confirm this notion, we tested ROS production in the presence of live and heat-killed bacteria (Fig. 4.16). Surprisingly, dead *Salmonella* did not induced ROS production in the guard cells at two hours post-inoculation.

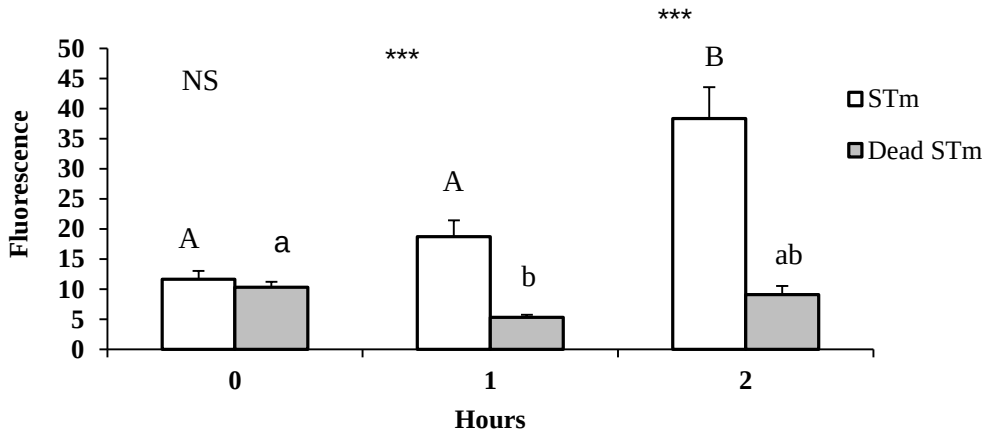


Figure 4.16. Effect of STm viability on the production of ROS in the guard cells. ROS was measured in the guard cells as fluorescence after H_2DCF-DA staining, water served as a negative control. Pictures were taken by confocal microscopy. Results are shown as mean fluorescence plus standard errors. Results are shown as means plus standard errors. Different letters indicate significant differences (P < 0.05) between the means of the same treatment, differences between the means of the same time point are denoted as follow: NS-Not significant; *** correspond to P<0.001. The data were analyzed using nonparametric ANOVA by the Kruskal-Wallis Test.

4.2.3.4 D*e novo* protein synthesis in *Salmonella* is required to induced ROS production in guard cells

We have previously shown that chloramphenicol-treated *Salmonella* was not able to induce stomatal closure and that heat killed *Salmonella* could not induce ROS production in guard cells. Because of these findings it was interesting to test if ROS production in the lettuce guard cells also requires *de novo* synthesis of proteins.

Unlike STm alone, the addition of chloramphenicol inhibited ROS production at two hours post-inoculation. (Fig. 4.17).

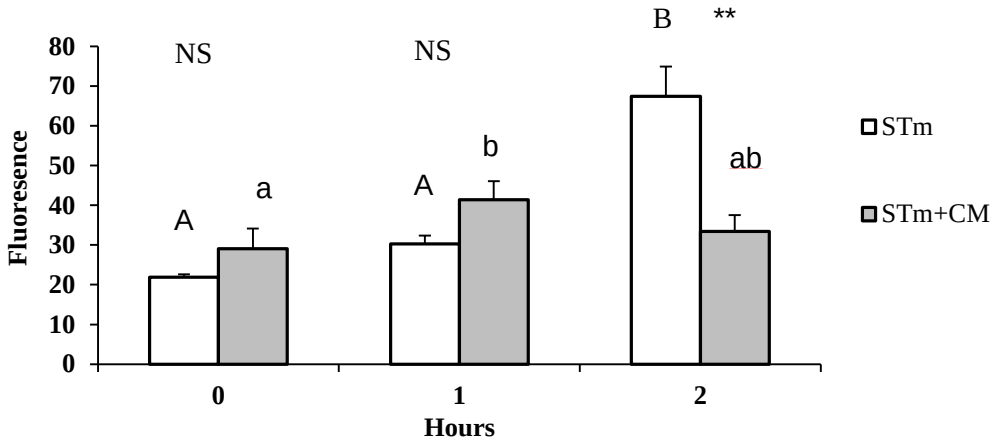
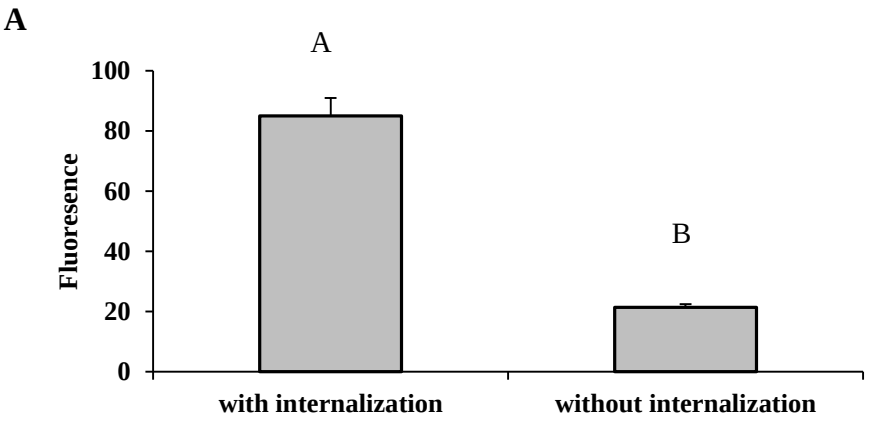
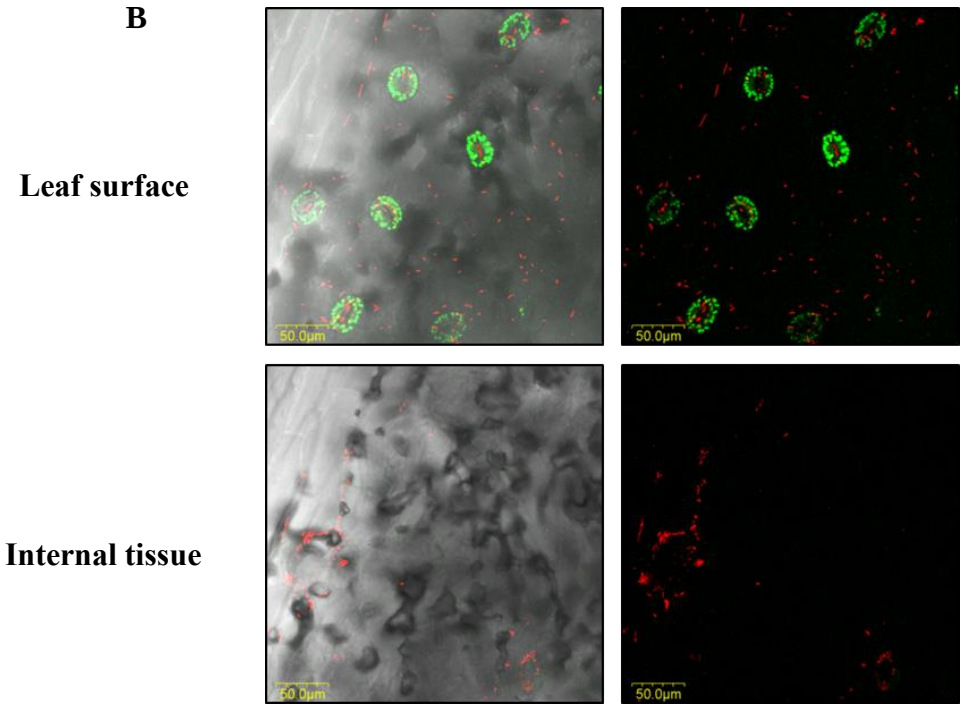


Figure 4.17. ROS determination in guard cells in response to Cm treated *Salmonella*. ROS level is represented by Fluorescence units after H_2DCF-DA staining, water served as a negative control. Results are shown as means plus standard errors. Different letters indicate significant differences (P < 0.05) between the means of the same treatment, differences between the means of the same time point are denoted as follow: NS-Not significant ** correspond to P<0.01. The data were analyzed using nonparametric ANOVA by the Kruskal-Wallis Test.

4.2.3.5 Stomatal internalization is required for ROS production in guard cells

ROS production by guard cells increased after two hours in the presence of *Salmonella* (Figs. 4.14, 4.15, 4.16, and 4.17). Previously, it was reported that internalization of *Salmonella* in lettuce stomata also increased at two hours (Kroupitsky et al., 2009). We therefore decided to check if there is a connection between the two processes. Lettuce pieces were inoculated with Cherry-fluorescent protein labeled bacteria for two hours, and ROS production was tested. Using confocal microscopy, both internalization and ROS production in guard cells were determined. Stomata were divided into two groups, those that contain internalized bacteria (bacteria were found underneath the stomata) and those with no internalization (no bacteria found underneath stomata). Fluorescence (ROS production) in the two groups of stomata is presented in Fig. 4.18. ROS production in guard cells was significantly higher when internalized *Salmonella* were observed underneath the stomata compared to stomata with no internalization.





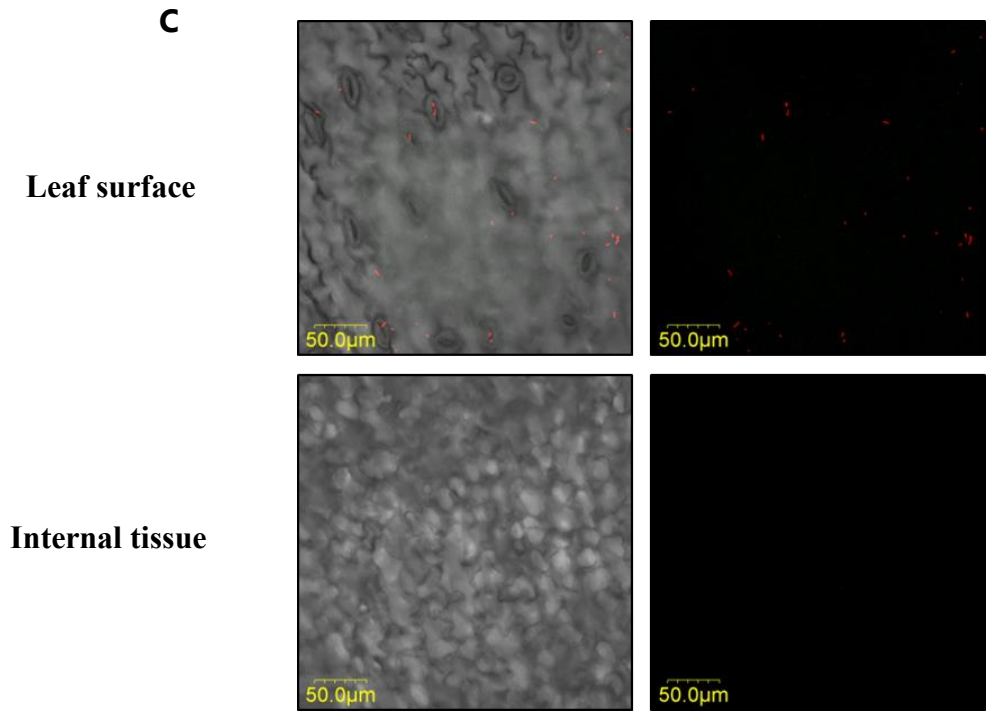


Figure 4.18. ROS determination in guard cells in response *Salmonella*. ROS was measured in guard cells as fluorescence after H_2DCF-DA staining, water served as a negative control (A). Results are shown as means plus standard errors. Different letters indicate significant differences ($P < 0.05$) between the means, Statistical analysis was performed using nonparametric two tailed Mann-Whitney test. Stomata having internalized bacteria display strong green fluorescence following H_2DCF-DA staining, whereas stomata without internalization do not fluoresce in green. Confocal microscopy images, showing leaf tissue with stomatal STm internalization (B), or without internalization (C). STm cells expressing the Cherry-fluorescent protein are shown in red. Images of internal leaf sections were taken every 1.2 µm up to a depth of 40 µm along a z section of the same field. All images were overlaid on a DIC image. Bars 50 µm.

4.2.4 Protein synthesis is required for *Salmonella* in internalization

There seem to be a connection between *Salmonella* internalization and ROS production in the lettuce guard cells. In order to test if the lack of ROS production in Cm-treated bacteria, is related to lack of internalization, the effect of Cm on STm internalization was also studied (Fig. 4.19). Indeed, Cm-treated *Salmonella* were also compromised in their ability to penetrate through stomata.

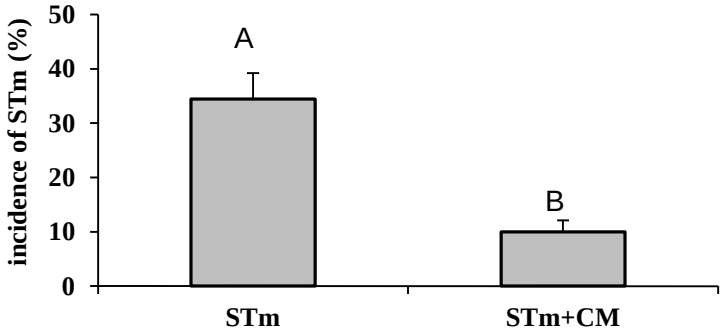


Figure 4.19. Internalization of STm in lettuce stomata. Internalization is presented as the incidence of STm in internal leaf tissue, after two hours of inoculation. Results are shown as means plus standard errors, Different letters indicate significant differences (P <0.05) between the means. Statistical analysis was performed using nonparametric two tailed Mann-Whitney test.

5. Discussion

5.1 *Arabidopsis* as a model plant to study *Salmonella*-leaf interactions

Our lab has previously shown that *S.* Typhimurium can penetrate through open stomata of iceberg lettuce in an active way (Kroupitsky et al., 2009b). Yet, further studies to understand the plant response at the molecular level are hampered because of the lack of genetic manipulation system in this plant. *Arabidopsis* is considered a good plant model due to its short life cycle, its known genome and the ability to grow the plant under supervised conditions (Somerville and Koornneef, 2002)**.**

Indeed, several studies on *Salmonella*-plant interactions have utilized *Arabidopsis* as a model plant (Schikora et al., 2008; Roy et al., 2013). Schikora and colleagues reported that *Salmonella* can infect various *Arabidopsis* tissues and proliferate in intracellular compartments, following seedlings or rossetes inoculation. *Salmonella* infection resulted in wilting, chlorosis and death of the infected organs (Schikora et al., 2008)**.** In contrast, our experiments failed to demonstrate any stomatal internalization in *Arabidopsis* plant. The difference between the findings of Schikora et al. (2008) and ours might be related to the inoculation method, dip-inoculation (in our study) versus vacuum infiltration or physical introduction of the bacteria by a syringe injection. While the infection model of Schikora et al. might perhaps be relevant to infection of an injured tissue, our infection model, suggest that *Salmonella* cannot enter through *Arabidopsis* stomata and infect the plant even when the pathogen is present in high concentration. Nevertheless, *Salmonella* was capable of inducing stomatal closure after one hour of inoculation. Stomatal closure might occur in response to sensing bacterial MAMPs, such as flagella and LPS. Roy and colleagues have recently reported similar results regarding stomatal response to *Salmonella* in *Arabidopsis* (Roy et al., 2013). However, they showed that both *Salmonella* and *E. coli* can be found within *Arabidopsis* leaves at 1 day post dip inoculation. Both bacterial species survived internally for 22 days, and that high relative humidity favored both bacterial penetration and survival (Roy et al., 2013). Our different result can be due to the different inoculation periods, or the duration of the experiments, days vs. two hours (in ours). Additionally, the results may reflect the method used for assessing bacterial localization. While, we have determined internalization by confocal microscopy, Roy et al. have utilized a bacteriological approach, i.e. surface disinfection to eliminate surface attached bacteria followed by grinding of the plant and plating the disinfected plant's extract. We have previously found that *Salmonella* cannot be eradicated from the surface of lettuce leaf using the same disinfection protocol probably because residual bacteria are entrapped in protected micro-sites, such as cracks on the leaf surface. Thus, it is not clear whether the internalized *Salmonella* were indeed localized to the apoplast or has merely evaded eradication on the leaf surface.

We have observed that *Salmonella* attaches to *Arabidopsis* leaves at high level and a large portion of the Bacteria were present as aggregates between the junctions of the epidermal cells. Bacteria residing in these locations may be protected from disinfection. Similar aggregates were not seen in the lettuce model. Based on confocal microscopy observations, it seems that more *Salmonella* cells were attached to *Arabidopsis*- compared to lettuce-leaves. There are several possible explanations to the different

attachment level of bacteria on the phylloplane. These include difference in the leaf morphology, age and chemical composition of the surface (Lindow & Brandl, 2003). Although *Salmonella* shows affinity toward stomata in lettuce (Kroupitsky et al., 2009b), it didn't show any affinity toward stomata on the *Arabidopsis* leaves. It is possible that this finding is related to differences in the leaf morphology and the nature of chemical attractants present in the two plants, or perhaps to the presence of anti-STm substances on *Arabidopsis* leaves.

5.2 *Salmonella* and lettuce leaf interaction

Since, STm did not internalize *Arabidopsis* stomata, further studies on *Salmonella* internalization were performed in iceberg lettuce.

5.2.1 Involvement of *Salmonella's* TTSS in attachment and internalization

Salmonella virulence in animals depends on effectors injected by Type III Secretion Systems (TTSSs). The system is encoded by two pathogenicity islands: *Salmonella* pathogenicity island (SPIs) 1 and 2. TTSS composed of syringe-like organelles on the surface of gram-negative bacteria, which enable the injection of effector proteins directly from the bacterial pathogen into the cytosol of eukaryotic cells (Galan, 2001; Waterman & Holden 2003). These effectors ultimately manipulate the cellular functions of the infected host and facilitate the progression of the infection. SPI1 and SPI2 play several roles in different organs within the mammalian host. SPI1 primarily promotes the invasion of non-phagocytic intestinal epithelial cells and the initiation of the inflammatory responses in the intestines **(**Coombers et al., 2005; Hapfelmeier ey al., 2004). SPI-2 is involved in the survival and persistence of *Salmonella* in the systemic compartment of the host (Brawn et al., 2007; Lawley et al., 2006; Steele et al., 2002). Plant Pathogens also use their TTSS during infection**.** The TTSS allows the phytopathogenic bacteria to deliver effector proteins, some of which delay or inhibit the plant's defense responses (Grant et al., 2006). TTSSs are conserved across the plant and animal pathogens, although the nature of the injected effectors may differ. Several effector proteins of STm, such as AvrA are highly homologous to effector proteins from phytopathogens, suggesting perhaps similar host-pathogen interactions in plants (Hardet & Galan, 1997). Recent studies have shown that *Salmonella* uses its TTSS also in plants, although *Salmonella* is not considered a plant pathogen. Schikora and colleagues demonstrated that *Salmonella* mutants that are unable to deliver effectors are also compromised in infection of *Arabidopsis thaliana* plants (Schikora et al., 2011). Ustun and colleagues showed that *S. enterica* TTSS effector SseF is recognized by the immune system of *N. benthamiana* plants, and induces effector-triggered immunity (Ustun et al., 2012).

Shiron and Yaron found that *Salmonella's* TTSS is also capable of suppressing the plant immune system. STm *invA* mutant increased the extracellular pH and ROS production of tobacco cells as oppose to the wt strain (Shiron and Yaron, 2011). The *invA* null mutant carries a deletion in the translocon gene *invA*, rendering it unable to translocate any effector protein of TTSS-1 (Galan & Curtis, 1991)**.** Schikora and colleagues found that STm TTSS mutant *prgH* (also encoded by the SPI1)

expressed more genes during infection of *Arabidopsis* and enhanced a stronger HR than wt (Schikora et al., 2011). Inguez and collegues reported that the removal of *Salmonella* TTSS-1 and some effector proteins by deletion of the pathogenicity island 1 (SPI1), reduced the bacterial colonization of *Medicago truncatula* roots (Iniguez et al., 2005)**.** In the present study we show that SPI1 is also involved in stomata internalization in lettuce leaves. We found that deletion of SPI1, encoding TTSS-1 resulted in lower STm internalization (Fig 4.6). Altogether, it seems that *Salmonella* exploits TTSS-1 not only during its interaction with the mammalian host, but also during colonization of the plant. More studies are needed to elucidate the specifc mechanisms involved.

5.2.2 *Salmonella*-stomata interaction

Stomata serve as a major route of entry into the host plant by many phytopathogens and symbionts **(**Zeng et al., 2010). In order to limit bacterial entry, plants have evolved mechanisms to close stomata as an early immune response against bacterial invasion (Mellotto et al., 2006; Zeng et al., 2010). Melotto and colleagues found that the plant close stomata in response to contact with the phytopathogen, *P. syringae* pv. tomato (Pst) DC3000, and the human pathogen, *E. coli* O157:H7 (Melotto et al. 2006). Kroupitski and colleagues showed that Pst DC3000 also triggered stomatal closure in lettuce. However, *Salmonella* did not triggered a similar response after two hours of inoculation (Kroupitski et al., 2009b). A finer analysis of stomatal response to STm at different time points revealed that STm induces stomata closure at 1 h post-inoculation, but later at 2 h the pathogen is able to force stomatal opening. A concentration of 10^3 STm CFUs were enough to trigger the closure response. Melloto and colleagues found that 10^7 Pst DC3000 CFUs were the minimal concentration required to induce stomatal closure in *Arabidopsis* (Melloto et al., 2006). It is possible that this difference is related to the different virulence of each pathogen in the two plant models. In any case, these findings suggest that lettuce recognizes *Salmonella* as an unwelcomed intruder and responds by trying to limit its entry into internal tissues.

Stomatal closure occurs in response to various MAMPs. For example, MAMPs were shown to induce stomatal closure in tomato (LPS) (Melotto et al. 2006), *Commelina communis* (oligogalacturonic acid and chitosan) (Lee et al., 1999), grape (oligogalacturonic acid, laminarin of dp13) (Allegre et al., 2009), *Pisum sativum* (Chitosan) (Srivastava et al., 2009), and *Arabidopsis* (LPS, flg22) (Melotto et al. 2006; Zeng et al., 2008; Zeng & He, 2010). Roy and colleagues have recently shown that flg22 can trigger stomatal closure also in lettuce leaves (Roy et al., 2013). Our results also support a role for flagella and LPS in lettuce stomatal response against STm. However, only live bacteria induced stomatal closure, and this process was dependent on *de novo* protein synthesis. These findings are apparently in contrast to our previous result showing that LPS alone can induce stomatal closure. A possible explanation is that we have used purified LPS in a much higher concentration compared to the actual concentration that the leaves perceive during infection. Alternatively, the lack of stomatal response in heat-killed bacteria might be due to changes in the 3-dimentional structure of the *Salmonella* MAMPs, which has affected their recognition by the cognate plants receptors. Still, this explanation cannot clarify the linkage between stomatal closure and the requirement of *de novo* protein synthesis. After induction of

stomatal closure at 1 h post-inoculation, STm can manipulate the guard cells and force stomatal re-opening. This phenomenon is reminiscent of microbial phytopathogens that have evolved virulence factors to actively counteract stomatal closure. For example, *P. syringae* pathovars express coronatine (COR), a phytotoxin that can reverse stomatal closure (Melotto et al., 2006). *Xanthomonas campestris pv campestris* (Xcc) is capable of manipulating stomatal closure of *Arabidopsis* through a secreted small molecule, whose nature is still not known (Grimmer et al., 2012). Both live Xcc and an extract derived from Xcc culture supernatant can inhibit PAMP- and ABA-induced stomatal closure in *Arabidopsis* (Gudesblat et al., 2009). The finding that STm can reopen lettuce stomata only at high bacterial concentrations and that protein synthesis is required for this activity may support the notion that upon contact with the plant STm expresses a molecule, which counteracts stomatal closure. We found that *spi1, spi2, invA* and *avrA* mutants could not overcome stomatal closure. This may suggest that effectors expressed by each of the two pathogenicity islands are capable of reversal of stomata closure. One such effector is AvrA, whose knockout mutation rendered STm stomata close at 2 h post inoculation. Further studies are required to confirm the role of AvrA in stomata modulation and to identify potential TTSS-2 effectors with similar activity.

Stomata closure is regulated by complex signal-transduction pathways involving the expression of the phytohormone, abscisic acid (ABA) and ABA-meidated ROS production (Melotto et al., 2006; Zeng et al., 2008; Neil et al., 2008). In order to gain knowledge regarding the specific signaling stage at which *Salmonella* interferes with stomatal regulation, we tested the ability of STm to reopen stomata after the addition of exogenous ABA. Our finding that *Salmonella* could not overcome ABA-mediated stomatal closure indicates that *Salmonella* interferes with a signaling stage prior to ABA-mediated signal-transduction. This is opposed to the case of Pst DC3000, in which coronatin forces stomatal re-opening by acting subsequently to the ABA-mediated signaling pathway in the *Arabidopsis* model (Melotto et al., 2006). Additional studies are needed to identify the mechanism by which STm induce stomatal opening. One of the earliest defense reactions to pathogen attack is the oxidative burst, which constitutes the production of reactive oxygen species (ROS), primarily superoxide and H_2O_2 at the site of attempted invasion (Apostol et al., 1989). ROS can damage the pathogens directly by the oxidation of important biomolecules (Adam et al., 1989; Keppler & Baker, 1989). ROS also induce programmed cell death (PCD), possibly to limit the spread of the pathogen from the site of infection to other plant's tissues. During incompatible reactions, when a pathogen is detected as an enemy and defense responses including PCD are induced, the production of H_2O_2 occurs in a biphasic manner (Apel & Hirt 2004). The initial and very rapid accumulation of H_2O_2 is followed by a second and prolonged burst of H_2O_2 production. During compatible interactions, when a pathogen overcomes the defense lines and systemically infects the host plant, only the first peak of H_2O_2 accumulation occurs (Baker & Orlandi, 1995). Beside their direct antimicrobial effect, ROS play a role in stomatal closure by an ABA-mediated signaling pathway (Pitzschke et al., 2006) that activates plasma membrane calcium channels (Pei et al., 2000). We showed that *Salmonella* induces ROS production in lettuce guard cells

immediately after inoculation and that the production of ROS increased after 2 h (Fig. 4.14). Although ROS production is usually accompanied by stomatal closure (Lee, 1999), STm apparently is able to overcome this process. Lee and colleagues, showed that oligogalacturonic acid (OGA) and chitosan induced the production of ROS in guard cells and reduced stomatal aperture, either by inhibiting stomatal opening or by inducing stomatal closing in tomato (*Lycopersicon esculentum* L.) and *Commelina communis* (Lee et al., 1999). ROS formation in guard cells was induced by chitosan in *Pisum sativum* (Srivastava et al., 2009), and by beta-glucan (Lam13) in grape epidermal peels (Allegre at al., 2009)**.** *Salmonella*-derived LPS can also trigger ROS formation in Tobacco plant cells **(**Shirron &Yaron, 2011) and in guard cells of lettuce leaves (this work). We report here for the first time that *Salmonella* internalization is required for the production of ROS in the guard cells**.** This finding may also explain the failure of the *fli*GHI mutant to induce production of ROS, since this mutant is also impaired in internalization (Kroupitsky et al., 2009b). Other lines of evidences also support the need of internalization to trigger ROS production in lettuce. Cm-treated bacteria and heat-killed bacteria were unable to internalize stomata and also failed to trigger guard cells ROS production.

In contrast to our findings, Shirron and Yaron have reported that live STm triggered only a low level oxidative burst in Tobacco epidermis guard cells. Using Tobacco cell culture, it was demonstrated that heat-killed or chloramphenicol-treated STm induced a higher oxidative burst compared to live bacteria, suggesting that live but not dead bacteria are capable of evading the ROS-mediated immune response (Shirron &Yaron, 2011). The apparent contradictory results can be due to differences in the models or bacterial strains used in the two studies, as well as to differences in the procedures used for bacterial inoculation and ROS quantification. An oxidative burst is one of the earliest plant responses to many invasive pathogens (Delledonne et al., 2002). ROS production associated with a local cell death was reported to be essential for intercellular bacterial invasion during lateral root base nodulation of the nitrogen-fixing symbiont *Azorhizobium caulinodans* during its interaction with the semi-aquatic tropical legume *Sesbania rostrata* (D'Haeze et al., 2003). ROS are also produced massively during the early stages of invasion of *Medicago truncatula* by *Sinorhizobium. meliloti*, (Ramu et al., 2002). Although the generation of ROS usually correlates with successful disease resistance responses, some pathogens may also induce ROS production to their own advantage. For example, necrotrophs appear to stimulate ROS production in the infected tissue to induce cell death that facilitates subsequent infection (Govrin & Levine, 2000). Invading microbes can produce antioxidants (e.g. ascorbic acid and glutathione) and enzymes (e.g. catalase and super oxide dismutase (SOD) to scavenge or detoxify toxic ROS (He´rouart et al., 2002; Imlay, 2003). Whether *Salmonella* utilizes some of these mechanisms during its interactions with the lettuce leaf remained to be explored.

5.2.3 A proposed model for *Salmonella*-leaf interactions

Based on our study, a proposed model regarding *Salmonella* interactions with iceberg lettuce leaf is depicted (Figure 5.1 A, B). Briefly, after one hour of inoculation with *Salmonella*, most stomata

respond by closing. In order to induce stomatal closure the bacteria needs to be viable, and at a density of at least 10^3 CFU/ml. Stomatal response is mediated apparently by LPS and certain *de novo* synthesized proteins collectively known as MAMPs, which are recognized by plants receptors located in the guard cells.

After two hours, *Salmonella* induces stomata re-opening via TTSS and this requires a minimum of 10^5 CFU/ml. The effect of TTSS occurs upstream to ABA-mediated signaling. ROS are produced only in stomata containing internalized *Salmonella.*

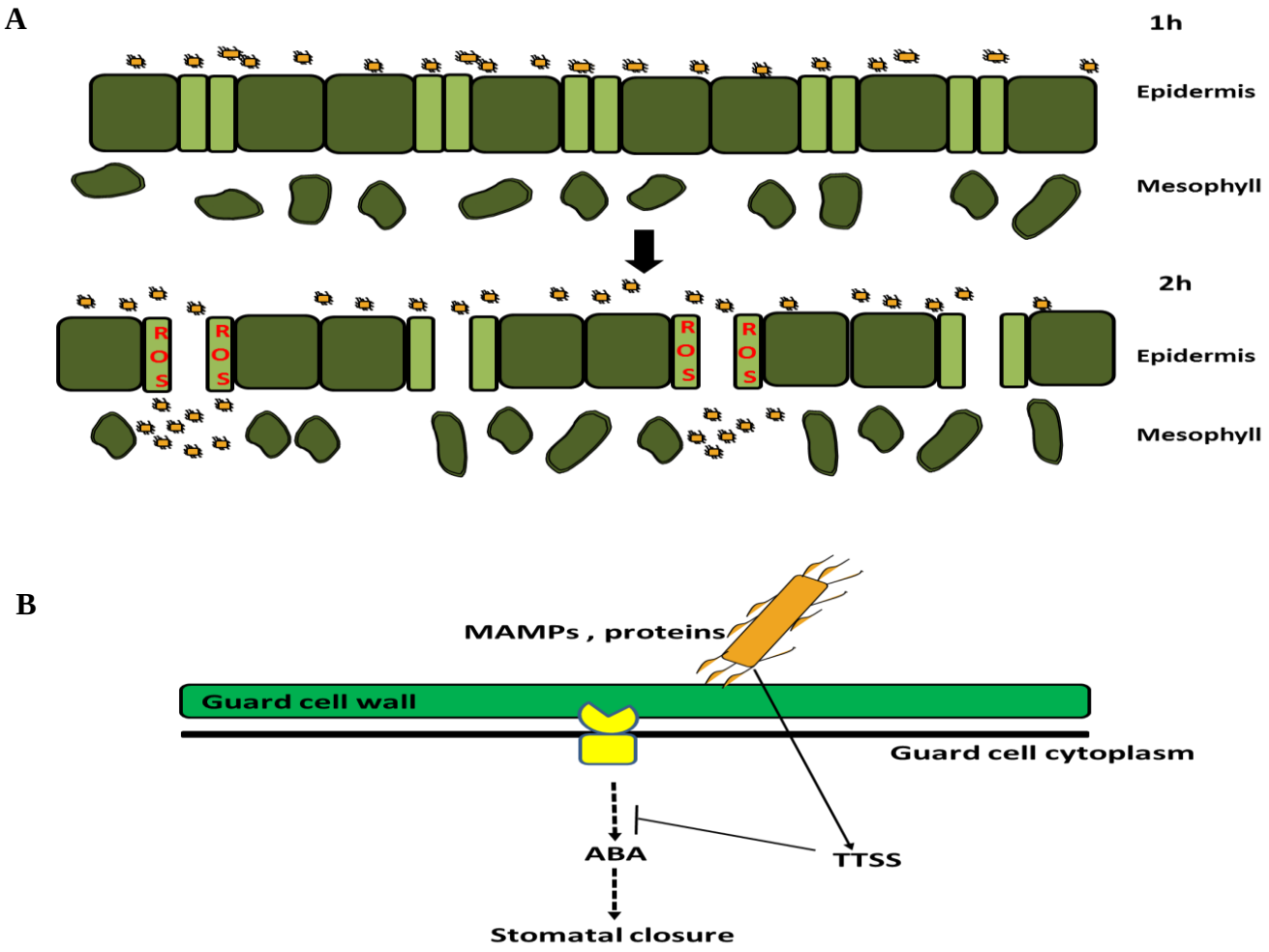


Figure 5.1. A suggested model for STm interactions with lettuce stomata. (A) A model depicting stomatal response and ROS production in response to *Salmonella*. After one hour of inoculation, most stomata are closed (guard cells in light green). After two hours of inoculation most stomata are open, and ROS production is detected only in stomata containing internalized *Salmonella.* (B) A model depicting *Salmonella* and MAMPs induced stomatal closure. *Salmonella* MAMPs are recognized by receptors (a hypothetical receptor is shown in yellow), which transmit a signal via ABA that mediates stomatal closure. *Salmonella* can interfere with the signaling pathway via secretion of protein effectors (TTSS), which act upstream to ABA signaling.

5.3 Summary

In the past few decades, the emergence of food-borne outbreaks linked to contaminated fruits and vegetables is of a great concern in industrialized countries. Food-borne pathogens can contaminate fresh produce in the field, during harvest, or during postharvest handling, processing and storage. Recently, it was found that *Salmonella enterica* has the ability to attach to and internalize leaves. This new route of contamination poses a risk to the fresh produce industry because internalized bacteria are resistant to surface decontamination. Hence, it is important to understand the mechanisms by which *Salmonella* interacts with the plant in order to prevent contamination and enhance the safety of fresh leafy vegetables

Initially, we have investigated whether *Arabidopsis* can serve as a plant model to study *Salmonella*-leaf interactions. *Arabidopsis* is the most studied model plant and its usage for studying *Salmonella*-plant interactions might therefore be advantageous. Although, *Salmonella* can adhere to *Arabidopsis* leaves and induced stomatal closure, no stomatal internalization has been evident. Consequently, the study was focused on the interactions of *Salmonella* with iceberg lettuce leaves. We found that *Salmonella* induces stomatal closure in lettuce after one hour of inoculation, and that a relatively small concentration of bacteria (10^3 CFU/ml) was enough for the induction. While exogenous LPS could induce stomatal closure by itself, only live bacteria were able to trigger a comparable effect. *De novo* protein synthesis was required for this effect. Unlike the enteric pathogen *E. coli* O157:H7, *Salmonella* actively reopened stomata after two hours. This process was dependent on the presence of intact SPI-1, suggesting perhaps that effectors delivered by TTSS-1 play a role in this process. A knockout mutant in the TTSS-1 protein effector AvrA lost the ability to reopen stomata, indicating that this virulent factor can manipulate stomatal closure mechanism. A relatively large concentration of bacteria was needed for stomatal reopening (10^5 CFU/ml), implying perhaps that the effector must achieve a minimal threshold concentration to exert it activity. ROS production is one of the earliest plant defense responses against intruders. We found that *Salmonella* induced ROS formation in lettuce guard cells, and that ROS production necessitated bacterial internalization. ABA-mediated ROS formation is one of the signals that induce stomatal closure. Yet, *Salmonella* was able to counteract stomatal closure after two hours of inoculation, in spite of the increase in ROS. Exogenous ABA resulted in stomatal closure even in the presence of *Salmonella*, suggesting that the pathogen interferes with the closure mechanism at an earlier stage. Further studies are required to identify the mechanism by which *Salmonella* manipulate stomata.

6. Reference

Adam, A., Farkas, T., Somlyai, G., Hevesi, M., Kiraly, Z. 1989. Consequence of O_2 generation during a bacterially induced hypersensitive reaction in tobacco: deterioration of membrane lipids. Physiol Mol Pathol 34: 13-26.

Allegre, M., He´loir M.C., Trouvelot, S., Daire, X., Pugin, A., Wendehenne, D., Adrian, M. 2009. Are grapevine stomata involved in the elicitor-induced protection against downy mildew? Mol Plant-Microbe Interact 22: 977-986.

Andrews, P.H.L., Bäumler, A.J., McCormick, B.A., Fang, F.C. 2010. Taming the elephant: *Salmonella* biology, pathogenesis and prevention. Infect Immun 78: 2356-2369.

Angulo, F.J., Tippen, S., Sharp, D.J., et al. 1997. A community waterborne outbreak of salmonellosis and the effectiveness of a boil water order. Am J Public Health 87: 580-584.

Anonymous. 2003. 8th Report of WHO surveillance programme for control of foodborne infections and intoxications in Europe. The FAO/WHO Collaborating Centre for Research and Training in Food Hygiene and Zoonoses.

Antunez-Lamas, M., Cabrera, E., Lopez-Solanilla, E., Solano, R., González-Melendi, P., Chico, J.M., Toth, I., Birch, P., Prichard, L., Liu, H., Rodriguez-Palenzuela, P. 2009. Bacterial chemoattraction towards jasmonate plays a role in the entry of *Dickeya dadanti*i through wounded tissues. Mol Microbiol 74: 662-671.

Apel, K., Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55: 373-399.

Apostol, I., Heinstein, P.F., Low, P.S. 1989. Rapid stimulation of an oxidative burst during elicidation of cultured plant cells. Role in defense and signal transduction. Plant Physiol 90:106-16.

Barak, J.D., Liang, A.S. 2008a. Role of soil, crop debris, and a plant pathogen in *Salmonella enterica* contamination of tomato plants. PLoS ONE 3: e1657.

Barak, J.D., Liang, A.S., Narm, K.E. 2008b. Differential attachment to and subsequent contamination of agricultural crops by *Salmonella enterica*. Appl Environ Microbiol 74: 5568-5570.

Barak, J.D., Kramer, L.C., Hao, L.Y. 2011. Colonization of tomato plants by *Salmonella enterica* is cultivar dependent, and type 1 trichomes are preferred colonization sites. Appl Environ Microbiol 77: 498-504.

Barak, J.D., Schroeder, B.K. 2013. Interrelationships of food safety and plant pathology: The life cycle of human pathogens on plants. Annu Rev Phytopathol 50: 241-266.

Baker M.A, Orlandi E.W. 1995. Active oxygen in plant pathogenesis. Annu Rev Phytopathol 33: 299-321.

Bari, R., Jones, J.D. 2009. Role of plant hormones in plant defense responses. Plant Mol Biol 69: 473-488.

Beattie, G.A., Lindow, S.E. 1995. The Secret life of foliar bacterial pathogens on leaves. Annu Rev Phytopathol 33: 145-172.

Beckers, H.J. 1987. Public health aspects of microbial contaminants in food. Vet Quarterly 9: 342-347.

Berger, C.N., Shaw, R.K., Brown, D.J., Mather, H., Clare, S., Dougan, G., Pallen. M., Frankel. G. 2009. Interaction of *Salmonella enterica* with basil and other salad leaves. ISME J 3: 261-265.

Bernstein, N., Sela, S., Neder-Lavon, S. 2007a. Effect of irrigation regimes on persistence of *Salmonella enterica* serovar Newport in small experimental pots designed for plant cultivation. Irrig Sci 26: 1-8.

Bernstein, N., Sela, S., Neder-Lavon, S. 2007b. Assessment of contamination potential of lettuce by *Salmonella enterica* serovar Newport added to the plant growing medium. J Food Prot 70: 1717-1722.

Beuchat, L. R. 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. Microbes Infect 4: 413-423.

Boller, T., He, S.Y. 2009. Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. Science 5928: 742-744.

Brandl, M. T. 2006. Fitness of human enteric pathogens on plants and implications for food safety. Annu Rev Phytopathol 44:367–392.

Brawn, L.C., Hayward, RD., Koronakis, V. 2007. *Salmonella* SPI1 effector SipA persists after entry and cooperates with a SPI2 effector to regulate phagosome maturation and intracellular replication. Cell Host Microbe 1: 63-75.

Brencic, A., Winans, S.C. 2005. Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. Microbiol Mol Biol Rev 69: 155-194.

Buck, J.W., Walcott, R.R., Beuchat, L.R., 2003. Recent trends in microbiological safety of fruits and vegetables. Plant Health Prog doi:10.1094/PHP-2003-0121-01-RV.

Burch, G., Sarathchandra, U. 2006. Activities and survival of endophytic bacteria in white clover (*Trifolium repens* L.). Can J Microbiol 9: 848-856.

Burnett, S.L., Beuchat, L.R. 2001. Human pathogens associated with raw produce and unpasteurized juices, and difficulties in decontamination. J Ind Microbiol Biotechnol 27: 104-110.

CDC, Centers for Disease Control and Prevention. 2002a. Outbreak of *Salmonella* serotype Kottbus infections associated with eating alfalfa sprouts – Arizona, California, Colorado, and New Mexico, February–April 2001. Morb Mortal Wkly Rep 51: 7-9.

CDC, Centers for Disease Control and Prevention. 2002b. Multistate outbreaks of *Salmonella* serotype Poona infections associated with eating cantaloupe from Mexico – United States and Canada, 2000–2002. Morb Mortal Wkly Rep 51: 1044-1047.

CDC, Centers for Disease Control and Prevention. 2006a. Multi-state outbreak of *E. coli* O157:H7 infections from spinach. http://www.cdc.gov/foodborne/ecolispinach/100606.htm.

CDC, Centers for Disease Control and Prevention. 2006b. *Salmonella* Oranienburg infections associated with fruit salad served in health-care facilities Northeastern United States and Canada. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5639a3.htm.

Choi, K.H., Kumar, A., Schweizer, H.P. 2006. A 10-min method for preparation of highly electrocompetent Pseudomonas aeruginosa cells: application for DNA fragment transfer between chromosomes and plasmid transformation. J Microbiol Methods. 64: 391–397.

Cianflone, N.F.C. 2008. Salmonellosis and the GI Tract: More than just peanut butter. Curr Gastroenterol Rep 10: 424–431.

Cooley, M.B., Miller, W.G., Mandrell, R.E. 2003. Colonization of *Arabidopsis thaliana* with *Salmonella enterica* and enterohemorrhagic *Escherichia coli* O157:H7 and competition by *Enterobacter asburiae*. Appl Environ Microbiol 69: 4915-4926.

CSPI, Centre for Science in the Public Interest. 2008. Outbreaks linked to fresh produce. http://cspinet .org/new/pdf/cspi_outbreak_alert.pdf. Last accessed Dec. 2010.

Czajkowski, R., de Boer, W.J., Velvis, H., van der Wolf, J.M. 2010. Systemic colonization of potato plants by a soilborne, green fluorescent protein-tagged strain of *Dickeya* sp. Biovar 3. Phytopathol 100: 134-142.

Datsenko, K.A., Wanner, B.L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97: 6640-6645.

Deering, A.J., Mauera, L.J., Pruitt, R.E. 2012. Internalization of *E. coli* O157:H7 and *Salmonella* spp. in plants: A review. Food Res Int 45: 567-575.

Delledonne, M., Murgia, I., Ederle, D., Sbicego, P.F., Biondani, A., Polverari, A, Lamb., C. 2002. Reactive oxygen intermediates modulate nitric oxide signaling in the plant hypersensitive disease-resistance response. Plant Physiol. Biochem 40: 605-610.

D'Haeze, W., Rycke, R., Mathis, R., Goormachting, S., Pagnotta, S., Verplancke, C., Capoen. W., Holsters, M.2003. Reactive oxygen species and ethylene play a positive role in lateral root base nodulation of a semiaquatic legume. Proc. Natl. Acad. Sci. U. S. A. 100: 11789-11794.

Dodds, P.N., Rathjen, J.P. 2010. Plant immunity: Towards an integrated view of plant-pathogen interactions. Nat. Rev. Genet. 11: 539–548.

Dong, Y.M., Iniguez, A.L., Ahmer, B.M.M., Triplett, E.W. 2003. Kinetics and strain specificity of rhizosphere and endophytic colonization by enteric bacteria on seedlings of *Medicago sativa* and *Medicago truncatula*. Appl Environ Microbiol 69: 1783-1790.

Doyle, M.P., Erickson, M.C. 2008. Summer meeting 2007- the problems with fresh produce: an overview. J Appl Microbiol 105: 317-330.

Durrant, W.E., Dong, X. 2004. Systemic acquired resistance. Annu Rev Phytopathol 42: 185-209.

Erickson, M.C. 2012. Internalization of fresh produce by foodborne pathogens. Annu. Rev. Food Sci. Technol 3: 283–310.

Fatica, M.K., Schneider, K.R. 2011. *Salmonella* and produce, survival in the plant environment and implication in food safety. Virulence 2: 573-579.

Franz, E., Visser, A.A., Van Diepeningen, A.D., Klerks, M.M., Termorshuizen, A.J., van Bruggen, A.H. 2007. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium. Food Microbiol 24: 106-112.

Freeman, B.C., Beattie, G.A. 2009. Bacterial growth restriction during host resistance to *Pseudomonas syringae* is associated with leaf water loss and localized cessation of vascular activity in Arabidopsis thaliana. Mol Plant-Microbe Interact 22: 857-867.

Galan, J.E., Curtiss, R.1991. 3rd Distribution of the invA, -B, -C, and -D genes of *Salmonella* typhimurium among other Salmonella serovars: invA mutants of *Salmonella* typhi are deficient for entry into mammalian cells. Infect Immun 59: 2901-2908.

Gill, S.S. and Tuteja, N. 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem 48: 909-930.

Gillespie, I.A. 2004. Outbreak of *Salmonella* Newport infection associated with lettuce in the UK. Euro Surveill 8: 2562.

Golberg, D., Kroupitski, Y., Belausov., E., Pinto,R., Sela, S. 2011. *Salmonella* Typhimurium internalization is variable in leafy vegetables and fresh herbs. Int J Food Microbiol 145: 250-257.

Gorbatsevich, E., Sela, S., Pinto, R., Bernstein, N. 2012. Root internalization, transport and *in-planta* survival of *Salmonella enterica* serovar Newport in sweet-basil. Environ Microbiol Rep 5: 151–159.

Govrin, E., Levine, A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr Biol 10: 751-757.

Grant, S.R., Fisher, E.J., Chang, J.H., Mole, B.M., Dangl J.L. 2006. Subterfuge and manipulation: type III effector proteins of phytopathogenic bacteria. Annu Rev Microbiol 60: 425-449.

Greene, S.K., Daly, E.R., Talbot, E.A., Demma, L.J., Holzbauer, S., Patel, N.J, Hill, T.A., Walderhaug, M.O., Hoekstra, R.M., Lynch, M.F., Painter, J.A. 2008. Recurrent multistate outbreak of *Salmonella* Newport associated with tomatoes from contaminated fields. Epidemiol Infect 136: 157-165.

Grimmer, M.K., Foulkes, M.J., Paveley, N.D. 2012. Foliar pathogenesis and plant water relations: a review.
J. Exp. Bot 63: 4321-4331.

Gudesblat, G.E., Torres, P.S., Vojnov, A.A. 2009. *Xanthomonas campestris* overcomes *Arabidopsis* innate immunity through a DSF cell-to-cell signal-regulated virulence factor. Plant Physiol 149: 1017-1027.
Gudesblat, G.E. 2009. Warfare at the gates. Plant Signal Behav 4: 1114-1116.

Hapfelmeier, S., Ehrbar, K., Stecher, B., Barthel, M., Kremer, M., Hardt, W.D. 2004. Role of the *Salmonella* pathogenicity island 1 effector proteins SipA, SopB, SopE, and SopE2 in *Salmonella enterica* subspecies 1 serovar Typhimurium colitis in streptomycin- pretreated mice. Infect Immun 72: 795-809.

Hardt, W.D., Galan, J.E. 1997. A secreted *Salmonella* protein with homology to an avirulence determinant of plant pathogenic bacteria. Proc Natl Acad Sci USA 94: 9887-9892.

Heaton, J.C., Jones, K. 2008. Microbial contamination of fruit and vegetables and the behaviour of enteropathogens in the phyllosphere: a review. J Appl Microbiol 104: 613-626.

He´rouart, D., Baudouin. E., Frendo, P., Harrison, J., Santos, R., Jamet. A., Van de Sype, G., Touati, D., Puppo, A. 2002. Reactive oxygen species, nitric oxide and glutathione: a key role in the establishment of the legume–*Rhizobium* symbiosis? Plant Physiol. Biochem 40: 619-624.

Hetherington, A.M., Woodward, F.I. 2003. The role of stomata in sensing and driving environmental change. Nature 424: 901-908.

Hirneisen, A., Sharma, M., Kalima, E. 2012. Human enteric pathogen internalization by root uptake into food crops. Foodborne Pathog Dis 9: 396-405.

Hopkins, W.G. 1999. Introduction to Plant Physiology, second ed., Wiley, New York.

Horby, P.W., O'Brien, S.J., Adak, G.K., Graham, C., Hawker, J.I., Hunter, P., Lane, C., Lawson, A.J. 2003. A national outbreak of multi-resistant *Salmonella* enteric serovar Typhimurium definitive phage type (DT) 104 associated with consumption of lettuce. Epidemiol Infect 130: 169-178.

Imlay, J.A. 2003. Pathways of oxidative damage. Annu Rev Microbiol 57: 395-418.

Iniguez, A.L., Dong, Y., Carter, H.D., Ahmer, B.M., Stone, J.M. 2005. Regulation of enteric endophytic bacterial colonization by plant defenses. Mol Plant- Microbe Interact 18: 169-178.

Iturriaga, M.H., Tamplin, M.L., Escartín, E.F. 2007. Colonization of tomatoes by *Salmonella* Montevideo is affected by relative humidity and storage temperature. J Food Prot 70: 30-34.

Jacobsen, C.S., Bech, T,B . 2012. Soil survival of *Salmonella* and transfer to freshwater and fresh produce. Food Res Int 45: 557-566.

Kim, T.H, Ohmer, M.B., Hu, H., Nishimura, N., Schroeder, J.I. 2010. Guard cell signal transduction network: advances in understanding Abscisic Acid, CO^2, and Ca^{2+} Signaling. Annu Rev Plant Biol 61:561-91.

Kimbrough, T.G., Miller, S.I. 2002. Assembly of the type III secretion needle complex of *Salmonella typhimurium*. Microbes Infect 4:75-82.

Klerks, M.M., Franz, E., van Gent-Pelzer M., Zijlstra, C., van Bruggen, A.H. 2007a. Physiological and molecular responses of *Lactuca sativa* to colonization by *Salmonella enterica* serovar Dublin. Appl Environ Microbiol 73: 4905-4914.

Klerks, M.M., Franz, E., van Gent-Pelzer M., Zijlstra, C., van Bruggen, A.H. 2007b. Differential interaction of *Salmonella enterica* serovars with lettuce cultivars and plant-microbe factors influencing the colonization efficiency. ISME J 1: 620-631.

Kroupitski, Y., Pinto, R., Brandl, M.T., Belausov, E., Sela, S. 2009a. Interactions of *Salmonella enterica* with lettuce leaves. J Appl Microbiol 106: 1876-1885.

Kroupitski, Y., Golberg, D., Belausov, E., Pinto, R., Swartzberg, D., Granot, D., Sela, S. 2009b. Internalization of *Salmonella enterica* in leaves is induced by light and involves chemotaxis and penetration through open stomata. Appl Environ Microbiol 75: 6076-6086.

Lawley, T.D., Chan, K., Thompson, L.J., Kim, C.C., Govoni, G.R., Monack, D.M. 2006. Genome-wide screen for *Salmonella* genes required for long term systemic infection of the mouse. PLoS Pathog. 2:e11.

Lee, S., Choi, H., Suh, S., Doo, I.S., Oh, K.Y., Choi, E.J., Taylor, ATS., Low, P.S., Lee, Y. 1999. Oligogalacturonic acid and chitosan reduce stomatal aperture by inducing the evolution of reactive oxygen species from guard cells of tomato and *Commelina communis*. Plant Physiol 121:147-152.

Lindow, S.E., Brandl, M.T. 2003. Microbiology of the phyllosphere. Appl Environ Microbiol 69: 1875-1883.

Liao, C.H., Cooke, P.H. 2001. Response to trisodium phosphate treatment of *Salmonella* Chester attached to fresh-cut green pepper slices. Can J Microbiol 47: 25-32.

Meinke, D.W., Cherry, J.M., Dean, C., Rounsley, S.D., Koornneef, M.1998. Arabidopsis thaliana: a model plant for genome analysis. Science 282: 662-682.

Melotto, M., Underwood, W., He, S.Y. 2008. Role of stomata in plant innate immunity and foliar bacterial diseases. Annu Rev Phytopathol 46: 101-122.

Melotto, M., Underwood, W., Koczan, J., Nomura, K., He, S.Y. 2006. Plant stomata function in innate immunity against bacterial invasion. Cell 126: 969-980.

Miller, G., Shulaev, V., Mittler, R. 2008. Reactive oxygen signaling and abiotic stress. Plant Physiol 133: 481-489.

Mitra, R., Cuesta-Alonso, E., Wayadande, A., Talley, J., Gilliland, S., Fletcher, J. 2009. Effect of route of introduction and host cultivar on the colonization, internalization, and movement of the human pathogen *Escherichia coli* O157:H7 in spinach. J Food Prot 72: 1521-1530.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7: 405-410.

Mohle-Boetani, J.C., Farrar, J., Bradley, P., Barak, J.D, Miller, M., Mandrell, R., Mead, P., Keene, W.E., Cummings, K., Abbott, S., Werner, S.B., Barak, J.D. 2008. *Salmonella* infections associated with mung bean sprouts: Epidemiological and environmental investigations. Epidemiol Infect 137: 357–366.

Mootian, G., Wu, W.H., Matthews K.R. 2009. Transfer of *Escherichia coli* O157:H7 from soil, water, and manure contaminated with low numbers of the pathogen to lettuce plants. J Food Prot 72: 2308-2312.

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

Neill, S.J., Desikan, R., Clarke, A., Hurst, R.D., Hancock, J.T. 2002. Hydrogen peroxide and nitric oxide as signaling molecules in plants. J Exp Bot. 53:1237-1247.

Patel, J., Sharma, M. 2010. Differences in attachment of *Salmonella enterica* serovars to cabbage and lettuce leaves. Int J Food Microbiol 139: 41-47.

Pezzoli, L., Elson, R., Little, C., Fisher, I., Yip, H., Peters, T., Hampton, M., Pinna, E. De. 2007. International outbreaks of Salmonella Seftenberg in 2007.
http://www.eurosurveillance.org/ew/2007/070614.asp.

Pitzschke, A., Foranzi, C., Hirt, H . 2006. Reactive oxygen species signaling in plants. Antioxid redox sign 8: 9-10.

Plotnikova, J.M., Rahme, L.G., Ausubel, F.M. 2000. Pathogenesis of the human opportunistic pathogen *Pseudomonas aeruginosa* PA14 in *Arabidopsis*. Plant Physiol 124:1766-1774.

Prithiviraj, B., Bais, H.P., Jha, A.K., Vivanco, J.M. 2005. *Staphylococcus aureus* pathogenicity on *Arabidopsis thaliana* is mediated either by a direct effect of salicylic acid on the pathogen or by SA-dependent, NPR1-independent host responses. Plant J 42: 417-432.

Ramu, S.K., Peng, H.M., Cook, D.R. 2002. Nod factor induction of reactive oxygen species production is correlated with expression of the early nodulin gene *rip1* in *Medicago truncatula*. Mol. Plant-Microbe Interact. 15: 522-528.

Rodríguez-Navarro, D.N., Dardanelli, M.S., Ruíz-Saínz, J.E. 2007. Attachment of bacteria to the roots of higher plants. FEMS Microbiol Lett 272: 127-136.

Roy, D., Panchal, S., Rosa, B., Melotto, M. 2013. *Escherichia coli* O157:H7 induces stronger plant immunity than *Salmonella enterica* Typhimurium SL1344. Phytopathol 103:326-332.

Saldaña, Z., Sánchez, E., Xicohtencatl-Cortes, J., Puente, J.L., Girón, J.A. 2011. Surface structures involved in plant stomata and leaf colonization by Shiga-toxigenic Escherichia coli O157:H7. Front Microbiol 119: 1-9.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M.A., Roy, S.L. 2011. Foodborne illness acquired in the United States major pathogens. Emerg Infect Dis 17:7–15.

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

Schikora, A., Virlogeux-Payant, I., Bueso, E., V. Garcia. A., Nilau Ame, T., Charrier, L., Pelletier, S., Menanteau, P., Baccarini, M., Velge, P., Hirt, H. 2011. Conservation of *Salmonella* infection mechanisms in plants and animals. PLoS ONE 6: e24112.

Seo, K.H., Frank, J.F. 1999. Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. J Food Prot 62: 3-9.

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

Sivapalasingam, S., Friedman, C.R., Cohen, L., Tauxe, R.V. 2004. Fresh produce: growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. J Food Protect: 2342-2353.

Solomon, E.B., Yaron, S., Matthews, K.R. 2002. Transmission of *Escherichia coli* O157:H7 from contaminated manure and irrigation water to lettuce plant tissue and its subsequent internalization. Appl Environ Microbiol 68: 397-400.

Solomon, E.B., Matthews, K.R. 2005. Use of fluorescent microspheres as a tool to investigate bacterial interactions with growing plants. J Food Prot 68: 870-873.

Somerville, C., and Koornneef, M. 2002. A fortunate choice: The history of *Arabidopsis* as a model plant. Nat. Rev. Genet 3**:** 883-889.

Srikanth, M.L., Hallstrom, R.K., McCormick, B. A. 2011. *Salmonella* effector proteins and host-cell responses. Cell Mol Life Sci 68: 3687-3697.

Srivastava, N., Gonugunta, V.K., Puli, M.R., Raghavendra, A.S. 2009. Nitricoxide production occurs downstream of reactive oxygen species in guard cells during stomatal closure induced by chitosan in abaxial epidermis of *Pisum sativum*. Planta 229: 757-765.

Steele-Mortimer, O., Brumell, J.H., Knodler, LA., Meresse, S., Lopez, A., Finlay, B.B. 2002. The invasion-associated type III secretion system of *Salmonella enterica* serovar Typhimurium is necessary for intracellular proliferation and vacuole biogenesis in epithelial cells. Cell Microbiol 4: 43-54.

Takeuchi, K., Matute, C.M., Hassan, A.N., Frank, J.F. 2000. Comparison of the attachment of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Pseudomonas fluorescens* to lettuce leaves. J Food Prot 63: 1433-1437.

Takeuchi, K., Frank, J.F. 2000. Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. J Food Prot 63: 434-440.

Tauxe, R., Kruse, H., Hedberg, C., Potter, M., Madden, J., Wachsmuth, K. 1997. Microbial hazards and emerging issues associated with produce; a preliminary report to the national advisory committee on microbiologic criteria for foods. J Food Prot 60: 1400-1408.

Ton, J., Flors, V., Mauch-Mani, B. 2009. The multifaceted role of ABA in disease resistance. Trends Plant Sci 14: 310-317.

Torres, M.A., Jones, J.D.G., Dangl, J.L. 2006. Reactive oxygen species signaling in response to pathogens. Plant Physiol 141: 373-378.

Underwood, W., Melotto, M., He, S.Y. 2007. Role of plant stomata in bacterial invasion. Cell Microbiol 9: 1621-1629.

Ustun, S.U., Mu¨ller, P., Palmisano, R., Michael Hensel., Bo¨rnke, F. 2012. SseF, a type III effector protein from the mammalian pathogen *Salmonella enterica*, requires resistance-gene-mediated signalling to activate cell death in the model plant *Nicotiana Benthamiana*. New Phytol 194:1046-1060.

Vailleau, F., Sartorel, E., Jardinaud, M.F., Chardon, F., Genin, S., Huguet, T., Gentzbittel, L., Petitprez, M. 2007. Characterization of the interaction between the bacterial wilt pathogen *Ralstonia solanacearum* and the model legume plant *Medicago truncatula*. Mol Plant- Microbe Interact 20: 159-167.

Lopez-Velasco, G., Sbodio, A., Tomás-Callejas, A., Wei, P., Tan, K. H., & Suslow, T. V. 2012. Assessment of root uptake and systemic vine-transport of *Salmonella enterica* sv. Typhimurium by melon *Cucumis melo* during field production. Int J Food Microbiol 158: 65-72.

Votholt, J.A. 2012. Microbial life in the phyllosphere. Nature Rev 10: 828-840.

Wachtel, M.R., Whitehand, L.C., Mandrell, R.E. 2002. Association of *Escherichia coli* O157:H7 with pre-harvest leaf lettuce upon exposure to contaminated irrigation water. J Food Prot 65: 18-25.

Waterman, S.R., Holden, D.W. 2003. Functions and effectors of the *Salmonella* pathogenicity island 2 type III secretion system. Cell Microbiol 5: 501-511.

Zhang, G., Ma. L. Beuchat, L.R., Erickson, M.C., Phelan, V.H., &, Doyle. M.P. 2009. Lack of internalization of *Escherichia coli* O157: H7 in lettuce (*Lactuca sativa* L.) after leaf surface and soil inoculation. J Food Prot 72: 2028-2037.

Zeng, W., He, S.Y. 2010. A prominent role of the flagellin receptor FLS2 in mediating stomatal response to *Pseudomonas syringae* pv. tomato DC3000 in *Arabidopsis*. Plant Physiol 153:1188-1198.

Zeng, W., Melotto, M., He, S.Y. 2010. Plant stomata: a checkpoint of host immunity and pathogen virulence. Current Opinion Biotech 21: 1-5.

Zeng, W., He, S.Y., Assmann ,S.M. 2008. The plant innate immunity response in stomatal guard cells invokes G-protein-dependent ion channel regulation. Plant 56: 984-996.

Zhuang, R.Y., Beuchat, L.R., and Angulo, F.J. 1995. Fate of *Salmonella* Montevideo on and in raw tomatoes as affected by temperature and treatment with chlorine. Appl Environ Microbiol 61: 2127-2131.

Supplementary data:

Table S1: Effect of TTSS on *Salmonella* localization on and below the surface of lettuce leaves

No. of bacteria per field[(1)]	**Average of microscopic fields harboring STm cells (±SE)**					
Surface Localization	**wt**	***spi1/spi2***	***spi1***	***spi2***	***invA***	***avrA***
0	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a	0±0 a
1-10	1.11±0.61 a	3.89±1.75 a	5.67±1 a	7.78±1.43 b	7.44±1.28 b	7.22±1.87 a
10-50	7.89±2.73 a	14.11±1.64 a	17±1.33 a	12±1.02 a	14.67±1.48 a	14.67±1.36 a
50-100	12.11±3.14 a	5.89±1.05 a	5.33±0.89 a	5.44±1.3 a	4.56±1.2 a	4.89±1.75 a
≥100	8.89±3.03 a	6.11±1.81 a	2±0.88 a	4.78±1.1 a	3.33±1.28 a	3.22±2.17 a
Internal localization						
0	14.78±2.08 A	18.33±1.41 A	22±0.42 A	15±1.43 A	19±2.27 A	20.22±1.49 A
1-10	6.44±0.74 A	8±1.13 A	5.89±0.18 A	9.11±1.08 A	7.22±1.16 A	0.89±0.89 A
10-50	6.56±1.84 A	2.89±0.53 AB	1.44±0.37 B	5.44±0.64 A	3.33±1.2 AB	1.78±0.57 AB
50-100	1.56±0.86 A	0.67±0.44 A	0.11±0.11 A	0.33±0.16 A	0.33±0.16 A	0.56±0.24 A
≥100	0.67±0.44 A	0.22±0.14 A	0±0 A	0.22±0.14 A	0.11±0.11 A	0±0 A

(1) Thirty microscopic fields (magnification of X 40) were examined per treatment in triplicate (30X3). Each experiment was repeated three times on different days with different pieces of lettuce (*n = 270).* Different letters indicate significant differences ($P < 0.05$) between the mean percentages of surface and internal fields, respectively, in the same row. Statistical analysis was performed using nonparametric ANOVA (Kruskal-Wallis Test).

תקציר

בשנים האחרונות עלה בעולם מספר ההתפרצויות של מחלות הקשורות לאכילה של תוצרת חקלאית טרייה. התפרצויות אלו גורמות לתחלואה ואף לתמותה ובנוסף לנזק כלכלי רב לחקלאים, למשווקים ולתעשיית המזון כולה. פתוגנים הגורמים לזיהומי מזון יכולים לזהם תוצרת חקלאית בשדה, בזמן הקציר, לאחר הקציר, בשלב הטיפול ועיבוד התוצרת ובעת האיחסון. בניגוד למזונות מן החי, תוצרת חקלאית טרייה אינה יכולה לעבור תהליכי חיטוי אינטנסיביים מכיוון שהיא עדינה ורגישה לטיפולים כימיים ופיסיקאליים. עקב כך, דרושים לתעשייה בדחיפות טיפולים אלטרנטיביים ליישום לאורך כל שרשרת הייצור. הבנת דרכי האילוח של התוצרת וגורל הפתוגנים בצמח הינה חיונית לצורך פיתוח גישות חדשות להקטנת הזיהומים.

Salmonella enterica הוא חיידק מעיים גרם שלילי היכול להדביק ביעילות בעלי חיים ובני אדם. בשנים האחרונות, נמצא כי סלמונלה יכולה לזהם גם צמחים. במעבדתנו נמצא שסלמונלה יכולה להיצמד ולחדור דרך פיוניות של חסה אייסברג. מטרת עבודה זו הייתה להרחיב את הממצאים הקודמים בדגש על יחסי הגומלין בין סלמונלה לפיוניות.

מאחר שארבידופסיס משמש כצמח מודל במחקרים רבים, רצף הגנום שלו ידוע וקיימים מוטנטים רבים, בדקנו בתחילה האם ארבידופסיס יכול לשמש כצמח מודל גם לחקר יחסי הגומלין בין סלמונלה לעלה. מצאנו שסלמונלה נצמדה אמנם ביעילות רבה לעלי ארבידופסיס, אך לא הייתה זיקה של החיידק לפיוניות. למרות זאת, סלמונלה גרמה לסגירת פיוניות, שעתיים לאחר אילוח, אולם חדירה לתוך רקמת העלה דרך הפיוניות לא נצפתה. עקב כך, החלטנו להמשיך ולחקור את יחסי הגומלין שבין סלמונלה לעלי חסה. מצאנו שסלמונלה גרמה לסגירת פיוניות שעה לאחר האילוח, וכי ריכוז של 10^3 CFU/ ml הספיק לגרימת הסגירה. נמצא שחיות (viability) החיידק, סינטזת חלבונים בזמן האילוח וליפופוליסכריד דרושים לגרימת סגירת הפיוניות. סלמונלה גרמה לפתיחת הפיוניות באופן פעיל שעתיים לאחר אילוח בנוכחות ריכוז של לפחות 10^5 CFU/ml. הוספת חומצה אבציסית (ABA) גרמה לסגירת פיוניות שעתיים אחרי אילוח, למרות נוכחות של סלמונלה, תוצאה המרמזת שמנגנון ההשפעה של סלמונלה נמצא לפני ABA בשרשרת מעבר הסיגנלים לבקרת השליטה על מפתח הפיונית.

באמצעות שימוש במוטנטים, נמצא שמערכת Type III secretion system (TTSS) דרושה לפתיחה מחדש של הפיוניות. כמו כן, המוטנטים הפגומים ב TTSS היו פחות יעילים בחדירה דרך הפיוניות לעומת זן הבר.

ידוע כי Reactive oxygen species (ROS) משחקים תפקיד מרכזי במעבר הסיגנל התאי בצמחים ובמערכת החיסונית שלהם, הנלחמת בפולשים זרים. מצאנו שסלמונלה גורמת לייצור ROS בעיקר בתאי השמירה. ייצור ROS נצפה אך ורק בפיוניות אשר דרכן חדרה סלמונלה ונצפתה מתחתן, ממצא המרמז על קשר ישיר בין ייצור ROS לחדירת סלמונלה.

לסיכום חיידק הסלמונלה מעורר בצמח תגובה חיסונית המתבטאת בסגירת פיוניות ולאחר חדירה גם לייצור של ROS בתאי השמירה. בדומה לפתוגנים צמחיים, סלמונלה מפעילה מנגנון אשר יכול לגרום לפתיחת הפיוניות, בתהליך בו מעורבת מערכת הפרשה מסוג TTSS. נדרשים מחקרים נוספים על מנת להבין את מנגנון הפעילות של סלמונלה המאפשר לחיידק להתגבר על מערכת ההגנה הכללית של הצמח ולגרום לפתיחה של הפיוניות.

יחסי גומלין בין סלמונלה לפיוניות של חסה

עבודת-גמר

מוגשת לפקולטה לחקלאות, מזון וסביבה על שם רוברט ה. סמית

של האוניברסיטה העברית בירושלים

לשם קבלת תואר 'מוסמך למדעי החקלאות'

ע"י

אלכסנדרה פוליינסקי

יוני 2013