ROLES OF PLANT DEFENSE RESPONSE AND BACTERIAL SURFACE POLYSACCHARIDES IN SURVIVAL OF HUMAN ENTERIC PATHOGENS *ESCHERICHIA COLI* O157:H7 AND *ESCHERICHIA COLI* O104:H4 ON PLANTS

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ABSTRACT OF THE DISSERTATION

Roles of plant defense response and bacterial surface polysaccharides in survival of human enteric pathogens *Escherichia coli* O157:H7 and *Escherichia coli* O104:H4 on plants

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Dissertation Director:

Karl R. Matthews, Ph.D.

Human enteric pathogens are associated with numerous outbreaks by consumption of contaminated fresh produce, which indicates that plants or vegetable crops can be potential hosts for pathogens. In order to enhance safety of fresh produce, it is important to understand the interactions between human enteric pathogens and plants. However, little information is available about the behavior of human enteric pathogens on plants, such as mechanisms of survival and persistence.

In this study, we investigated survival and persistence of *Escherichia coli* O157:H7 and O104:H4 strains on *Arabidopsis thaliana* and romaine lettuce, as well as production of capsular polysaccharide (CPS) and induction of plant defense response. Colonization study with *E. coli* O157:H7 86-24 wild-type strain and its isogenic mutants of surface polysaccharides showed that colanic acid-deficient and lipopolysaccharide (LPS)-deficient mutants significantly less survived on *Arabidopsis* plant and lettuce on day 1 and 5 post-inoculation, compared to the wild-type. The two mutants of colanic acid

and LPS induced 2-fold greater *PR1* gene expression and produced significantly lower amount of CPS compared to wild-type strain ($P < 0.05$). The results may suggest that structures of colanic acid and LPS of *E. coli* O157:H7 influence the plant defense response, thereby resulting in different survival and colonization on plants.

To investigate fitness of an emerging Shiga toxin-producing *E. coli* (STEC), colonization of *E. coli* O104:H4 strains on plants were compared with of that of *E. coli* O157:H7 strains. Results showed that *E. coli* O104:H4 strains (RG1, C3493, and LpfA) significantly survived better than *E. coli* O157:H7 strains (7386 and sakai) on *Arabidopsis* plant and lettuce at day 5, with greater production of CPS and lower expression of *PR1* gene ($P < 0.05$). These results indicate that different level of plant defense response and CPS production may have an impact on survival or fitness of *E. coli* O104:H4 and O157:H7 on plants.

In order to develop control strategies in crop cultivation environments, it is essential to learn about the behavior of human enteric pathogens on plants, particularly factors influencing the ability of pathogen to overcome plant host immunity. The present study provides better understanding of roles of plant defense response and surface polysaccharides on the molecular interactions between human pathogens and plants. Interestingly, the similar trend of bacterial survival/persistence between *Arabidopsis* (model plant) and lettuce (plant crop) may suggest a potential use of *Arabidopsis* as an appropriate model plant for studying the mechanisms of plant responses to human enteric pathogens on leafy vegetables. This study also provides an insight into potential roles of CPS in the survival of human enteric pathogens.

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Dedication

This dissertation is dedicated to my parents,

Hyung Soo Jang and Byung Soo Cho,

and my sister,

Hae Won Jang

for their endless love, support and encouragement.

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List of Abbreviation

BGL2	β-1,3-glucanase
CPS	Capsular polysaccharide
CRI	Congo red indicator
EPS	Extracellular polysaccharides
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
GUS	β-glucuronidase
HC	Hemorrhagic colitis
HR	Hypersensitive response
HUS	Hemolytic uremic syndrome
ISR	Induced systemic resistance
JA	Jasmonic acid
LPS	Lipopolysaccharide
MAMPs	Microbe-associated molecular patterns
NB-LRR	Nucleotide binding-leucine rich repeat
PAMPs	Pathogen associated molecular patterns
PR	Pathogenesis-related
PRRs	Pattern recognition receptors
PTI	PAMP-triggered immunity
ROS	Reactive oxygen species

SA	Salicylic acid
SAR	Systemic acquired resistance
SPI	*Salmonella* pathogenicity island
Tafi	Thin aggregative fimbriae
TSA	Trypric soy agar
TSB	Tryptic soy broth
T3SS	Type three secretion system

CHAPTER 1. Introduction

1.1. Background and Significance

Numerous outbreaks of foodborne illness have been linked to consumption of contaminated fresh produce such as lettuce and spinach. This causes tremendous financial burden to the fresh produce industry and damages consumer confidence in produce safety (Scharff, 2012). Leafy green vegetables are considered as one of the most important vehicles of foodborne illness caused by human enteric pathogens including *Escherichia coli*O157:H7 (Herman et al., 2015; Lynch et al., 2009). An *E. coli* O157:H7 outbreak associated with fresh produce accounted for 21% of 183 foodborne outbreaks and 34% of 5,269 cases during 1982-2002 (Rangel et al., 2005). Moreover, foodborne illness associated with non-O157 pathogenic *E. coli* has been increasingly reported and posed a serious and emerging public health concern. In 2011, deadly outbreaks caused by Shiga toxin-producing *E. coli* (STEC) O104:H4 occurred in European countries, USA, and Canada; fenugreek sprouts were implicated as the food vehicle of the transmission (EFSA, 2011; WHO, 2011). A number of STEC serotypes can cause diarrhea, hemorrhagic colitis (HC), and life-threatening hemolytic uremic syndrome (HUS) (Karch et al., 2005). The increased outbreaks linked to the consumption of fresh produce have brought considerable public health and scientific researches in understanding interactions of human pathogens with plants (Brandl et al., 2013).

Recent findings have indicated that plants can be good alternate hosts for human enteric pathogens such as *E. coli* O157:H7 and *Salmonella* as a refuge for effective survival although plants are not traditionally considered as hosts for the human pathogens

(Brandl et al., 2013). Plants can be easily contaminated at any step in the pre-harvest environments such as contaminated water or fecal contamination of the edible parts (Barak and Schroeder, 2012; Lynch et al., 2009). In general, human pathogens are known to be able to internalize and colonize in internal plant tissues (Deering et al., 2012; Fletcher et al., 2013). While numerous reports have demonstrated circumstances that can bring contamination and persistence of the foodborne bacteria in plant and fresh produce, little is known about how the human enteric pathogens interact with plants and the influence on plant immunity. In recent years, areas of food safety and plant pathology integrated to effectively increase scientific information regarding the mechanisms of plant responses to human enteric pathogens compared to phytopathogens (Fletcher et al., 2013). Importantly, details about the plant defense responses to human enteric pathogens with molecular and genetic details needs to be elucidated. In other words, it is important to determine how plants including vegetable crops respond to human enteric pathogens and whether the plant-pathogen interactions influence an induction of plant defense responses that regulate bacterial fitness and survival.

1.2. Objectives

We hypothesized that structural factors of Shiga toxin-producing *E. coli* associated with adherence and virulence such as surface polysaccharides influence plant defense response and survival of the pathogen on plant or vegetable crop.

The objectives of this research are to provide a better understanding of molecular interactions between foodborne pathogens and plant defense response as well as an influence of bacterial factors involved in the survival of human enteric pathogens.

Specific objectives of this research are;

1. To investigate influence of surface polysaccharides of *E. coli* O157:H7 on plant defense response and survival of the human enteric pathogen on *Arabidopsis thaliana* and lettuce (*Lactuca sativa*).
2. To determine fitness of an emerging human enteric pathogen *E. coli* O104:H4 on plant-pathogen interactions compared to *E. coli* O157:H7 in terms of ability of survival/persistence and induction of plant defense response.

These two objectives are presented in Chapters three and four in this dissertation.

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CHAPTER 2. Literature Review

2.1. Safety issues of fresh produce: Incidence of foodborne infections associated with fresh produce

Fresh produce is a nutritious source of minerals, vitamins, and phytochemicals. Over the past two decades, consumption of fresh fruits and vegetables has increased due to the elevated awareness of health and nutrition benefits of fresh produce. At the same time, however, the number of outbreaks associated with fresh produce has increased. According to a report by the Center for Science in the Public Interest in 2015, produce was the top category of food vehicle with 643 outbreaks (19% of total outbreaks) and 20,456 illnesses (24% of total illnesses) between 2004 and 2013 in the United States (Figure 2.1) (CSPI, 2015). Using outbreak data during 1998- 2008, the Center for Disease Control and Prevention (CDC) also reported that produce (fruits-nuts and vegetables) accounted for 46% of illnesses, and 22% of the estimated illnesses were attributed to leafy vegetables. The leafy vegetables were responsible for the second-most hospitalizations (14%) and the fifth-most cause of death (6%) among the food commodities (Painter et al., 2013). In 2011, a very large outbreak caused by Shiga toxin-producing *E. coli* O104:H4 occurred in Europe and North America, which caused 4,075 cases including 908 hemolytic uremic syndrome (HUS) cases and 50 deaths (WHO, 2011). Trace back investigation of the outbreaks identified fenugreek sprouts as the food vehicle, and the fenugreek seeds imported from Egypt were the actual original source of the pathogen (EFSA, 2011). Foodborne outbreaks linked to the consumption of contaminated fresh produce such as lettuce, spinach, and alfalfa sprouts continue not only

in the United States, but also throughout the world (Heaton and Jones, 2008). Therefore, special attention to the safety of fresh produce has become significant with respect to public health.

2.2. Plant-Microbe interactions

2.2.1. Microbial attachment on plants

Microbial attachment is the first step in plant-microbe interactions required for subsequent colonization or survival of pathogens within or on plant tissue. Bacterial attachment is affected by physicochemical properties of attachment surface and bacterial cell surface structures, and surrounding medium (Frank, 2001). Investigation of factors affecting the bacterial attachment to plant tissue has been shown that two major stages may occur after bacteria get into contact with plant surfaces (Dunne, 2002). In the primary stage (docking phase), an initial adhesion occurs depending on physical factors such as electrostatic charge and hydrophobic interactions between microorganisms and surface, which is a weak and reversible binding. Therefore, bacterial cells attached at this stage can be easily removed by mild mechanical forces. In the secondary stage (locking phase), a strong and irreversible binding occurs by involving molecular factors of bacterial cells such as flagella, curli fimbriae, and polysaccharides. At this stage, the bacterial cells are able to firmly attached to plant tissue surface, making it difficult to remove them and requiring a strong force or use of chemical/biological reagents to detach the cells from the surface (Bower et al., 1996; Dunne, 2002).

2.2.2. Bacterial cell surface structures in plant-microbe interactions

Roles of bacterial cell surface structures such as flagella, curli fimbriae, exopolysaccharides, or capsular polysaccharides (CPS) have been studied to demonstrate the roles in bacterial adherence to abiotic or biological surfaces. It has been reported that the bacterial cell structures influence the attachment, colonization, or biofilm formation on plants or leafy vegetables (Barak et al., 2005, 2007; Patel et al., 2011; Xicohtencatl-Cortes et al., 2009).

Flagella are filamentous appendage of bacteria cell surface that provides function of bacterial motility. It is also shown that flagella contribute to bacterial attachment and biofilm formation on abiotic or biological surfaces. For example, isogenic mutation in flagella major subunit (*fliC*) of *E. coli* O157:H7 resulted in a significant reduction of adhesion of cells to baby spinach and lettuce (Xicohtencatl-Cortes et al., 2009). Flagella of enterotoxigenic *E. coli* also showed to affect the bacterial attachment to fresh salad leaves including lettuce, basil, and spinach (Shaw et al., 2011). Deletion of *fliC* gene encoding flagella of *Salmonella* Senftenberg showed reduction in bacterial adherence on basil leaves (Berger et al., 2009). Deletion of *SirA*, a gene involved in biofilm formation of *Salmonella enterica*, reduced the bacterial attachment to spinach leaves and tomato as well as food contact surfaces such as glass and polystyrene (Salazar et al., 2013).

Curli are long, thin, and coiled proteinaceous component of extracellular matrix produced by many *Enterobacteriaceae* (Olsén et al., 1993). Curli have been shown to be involved in bacterial attachment to surfaces, cell aggregation, biofilm formation, and contributed to increased resistance to chemical and physical treatments (Cookson et al., 2002; Ryu et al., 2004). Some studies have shown that curli have an impact on

attachment or persistence of *E. coli* on plant surfaces. For instance, Macarisin et al. (2012) reported that curli-expressing *E. coli* O157:H7 resulted in stronger attachment to spinach compared to curli-deficient mutant. Patel et al. (2011) also showed that strong curli-expressing *E. coli* O157:H7 strains were attached to iceberg lettuce and cabbage surfaces better than weak curli-expressing strains. Similarly, curli non-producing *E. coli* K12 strains showed a lack of attachment ability to plant surfaces such as tomato, alfalfa sprouts, and *Arabidopsis* compared to curli-producing *E. coli* K12 (Jeter and Matthysse, 2005). In addition, curli-producing *E. coli* O157:H7 showed greater persistence on spinach over 2 weeks than a noncurliated strain (Macarisin et al., 2013). S. Newport *agfB* mutant deficient in expression of curli and surface-bound protein also showed reduced attachment and colonization on alfalfa sprouts compared to the wild-type strain (Barak et al., 2005). In contrast, it is suggested that the expression of curli may not be essential for attachment because there was no significant difference in the attachment to lettuce surfaces between curli-producing and non curli-producing strains of *E. coli* O157:H7 (Boyer et al., 2007). Deletion of *S.* Typhimurium *CsgD* gene regulating expression of curli and cellulose did not affect binding ability to alfalfa sprouts or seed coats (Torres et al., 2005). These differences could be explained by different regulatory mechanisms of bacteria in expression of specific adhesins, recognition to the surface structures, or environmental factors (Barak et al., 2005; Torres et al., 2005). It has been demonstrated that curli are typically expressed under environmental stresses such as low growth temperature, low osmotic pressure, and limited nutrients (Olsen et al., 1993).

Diverse bacteria produce extracellular polymeric substances primarily consisting of polysaccharides, as well as proteins, nucleic acids, and lipids (Vu et al., 2009).

Bacterial surface polysaccharides generally include capsular polysaccharides (CPS), exopolysaccharides or extracellular polysaccharides (EPS), and as O-antigen component of lipopolysaccharide (LPS) (D'Haeze and holsters, 2004; Jann and Jann, 1997). CPS are cell-bound substances tightly associated with cell surface whereas EPS, known as slime or free EPS, are loosely bound to the cell surface (Dong et al., 2006; Roberts, 1996). CPS protect bacterial cells from hostile environment conditions such as desiccation, osmotic or oxidative stresses, and antimicrobial compounds, which can assist the evasion of host immune system (Roberts, 1996). CPS are also generally regarded as important virulence factor of many pathogenic bacteria and associated with biofilm formation (Campos et al., 2004; Vu et al., 2009). As a key function of EPS; it plays a role in bacterial attachment and colonization on biotic or abiotic surfaces. Mutations in genes required for production of polysaccharides of *E. coli* O157:H7, poly-β-1,6-*N*-acetylglucosamine (PGA), cellulose, and colanic acid reduced the bacterial binding to alfalfa sprouts, seed coats (Matthysse et al., 2008), lettuce cut ends, and pepper fruit (Mathews et al., 2014), which suggested that the polysaccharides are required for binding to plants. Colanic acid is a negatively charged polymer composed of glucose, galactose, fucose, which forms capsule outside of bacteria cell. It is shown that *Salmonella enterica* uses cellulose and O-antigen capsule in the bacterial attachment and colonization on alfalfa sprouts while colanic acid had no influence on the interactions, which may indicate that specific polysaccharides are required for optimal binding and colonization on plant (Barak et al., 2007).

2.3. Plant immune system: Interactions between phytopathogens and plant hosts

2.3.1. Innate immune system

In nature, plants are constantly exposed to various pathogens including bacteria, viruses, fungi, oomycetes, and insects, which can threaten the physiologies of plants (Pieterse et al., 2009). However, plants can protect themselves from pathogens through surface barriers such as thick cell walls and waxy cuticle, or induction of plant immune system (Abramovitch et al., 2006; Jones and Dangl, 2006).

Plants and mammals have differences in fundamental biology affecting the capacity to defend themselves against pathogenic microorganisms. While mammals protect themselves by both adaptive and innate immunity, plants mostly depend on innate immunity (Jones and Dangl, 2006). While mammals can deliver immune cells or molecules to infection sites using circulatory system, plants rely on cell-autonomous events since they lack a similar circulatory system. Plant cells produce cellulose-based cell wall that serves as an barrier to entry of microorganisms (Zhang and Zhou, 2010).

A number of bacteria including plant and human pathogens can attach, survive, and even proliferate on plant surfaces. To promote pathogenesis, pathogenic bacteria must first enter plant tissues. Unlike fungal pathogens, bacteria can enter a plant host through natural openings such as stomata or wounds because they lack the ability to penetrate the plant epidermis (Melotto et al., 2006). Damage such as cracks in the leaf cuticle can serve as sites that bacteria colonize since the epidermal cells are exposed. Several studies have shown that internalization and fitness of *Salmonella* spp. and *E. coli* O157:H7 are associated with stomata or naturally occurring lesions (Figure 2.2) (Brandl and Mandrell, 2002; Duffy et al., 2005; Itoh et al., 1998; Kroupitski et al., 2009). The stomata and wounds also provide protective niches for bacteria (Erickson, 2012).

To fight infection, plants respond to the bacterial attack with an innate immune system consisting of two branches, PAMP (pathogen-associated molecular patterns)-triggered immunity (PTI) and effector-triggered immunity (ETI) as shown in Figure 2.3 (Jones and Dangl, 2006). In PTI, a primary innate immunity is achieved by the interaction of PAMP molecules with plant receptors called pattern recognition receptors (PRRs) (Pel and Pieterse, 2013). PAMPs triggering innate immune response are shown to include flagellin, peptidoglycan, and chitins that are essential components of many plant and human bacteria (Nürnberger et al., 2004). The recognition of PAMPs by PRRs leads to the activation of defensive responses that prevent microbial growth and multiplication. For instance, the defensive responses typically include cell wall reinforcement through callose deposition, production of ionic fluxes (primary Ca^{2+}, K^{+}, and H^{+}), and reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Nicaise et al., 2009). The PTI provides the first line of defense against pathogen attack. For successful colonization, plant pathogens translocate virulence effectors into the plant host by using type three secretion system (T3SS) to overcome the PTI responses and promote pathogenesis, which results in effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). Once pathogens acquired the ability to overcome the primary defenses, plants apply more specific defense mechanisms to perceive and inhibit the evaded bacteria. In other words, plants have the capacity to detect the effectors released by pathogens and activate stronger defense responses. In the pathogen-plant interactions, the effectors secreted by the pathogen into the plant cells are recognized by specific NB (nucleotide binding)-LRR (leucine rich repeat) R protein encoded by resistance genes, which activates the secondary immune response called effector-triggered immunity (ETI)

(Chisholm et al., 2006). ETI is an accelerated PTI response that inhibits the activity of virulent effector proteins and microbial growth (Chisholm et al., 2006). The response of ETI results in hypersensitive response (HR) in plants, a rapid death of cells localized at the site of infection, which prevents the spread of infection by the bacteria to other parts of the plant tissues. During the HR, plant cells produce and secrete ROS, nitric oxide, and Ca^{2+} and H^{+} ions with molecular signals such as jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) (Jones and Dangl, 2006).

2.3.2. Systemically induced immune responses

Plants are also able to trigger induced defense responses at remote sites from initial infection to protect undamaged tissues against subsequent pathogen attack (Durant and Dong, 2004). These systemically induced defense responses are generally categorized as two types of resistance, systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Figure 2.4). SAR is induced by PTI- and ETI-mediated recognition of pathogen and dependent on accumulation of salicylic acid (Mishina and Zeier, 2007). Salicylic acid (SA) acts as an essential signal molecule for the establishment of SAR and is required for the expression of pathogenesis-related (*PR*) genes. Transgenic plants that are impaired in SA signaling are unable to develop SAR and to activate *PR* genes upon pathogen infection, demonstrating SA is necessary for SAR signaling pathway (Durant and Dong, 2004). Whereas SAR is dependent on an SA signaling, ISR is activated by root colonization of certain beneficial rhizosphere bacteria, which is commonly regulated by jasmonic acid (JA) and ethylene (ET) signaling

(Pieterse et al., 1998; Ton et al., 2002). Both SAR and ISR are effective to regulate virulent phytopathogens as a broad spectrum of plant immunity (Pieterse et al., 2009).

2.4. Interactions between human enteric pathogens and plants

2.4.1. Plants as an alternative host for human enteric pathogens

As described in archetypal plant immune system, much of work on plant defense responses against various plant pathogens has been conducted, such as *Arabidopsis thaliana* with *Pseudomonas syringae*, which provides considerable knowledge of plant-microbe interactions. However, little is known about whether human enteric pathogens colonize and manipulate the plant host defense response (Holden et al., 2009).

Recently, increased outbreaks linked to the consumption of fresh vegetables and fruits have brought considerable public health and scientific interest in understanding interactions of human pathogens with plants (Brandl et al., 2013). Traditionally, plants are not considered as hosts for human pathogens. Human enteric pathogens such as *Salmonella* spp. and *E. coli* O157:H7 which are usually thought of residing in intestines of animal and human hosts, can just enter the plant host tissue directly or indirectly after contacting plant tissue by various environmental routes. Thus, the human enteric pathogens were historically considered to temporarily last on plant surfaces, passively in natural openings (stomata), cracks, or wounds. In other words, it was thought that they were unable to actively modify the plant physiology or communicate with it (Brandl, 2006). However, research suggests that human enteric pathogens do not just land on plant surfaces and passively inhabit plants; they can tightly adhere to produce, actively

colonize, and enter into the plant tissues, possibly as a part of the life cycle of human pathogens (Figure 2.5) (Berger et al., 2010; Erickson, 2012; Fletcher et al., 2013). Recent studies support that plants may be alternate hosts for human enteric pathogens by providing a refuge, which can be an effective survival strategy for the human pathogens to colonize edible plants (Fletcher et al., 2013; Holden et al., 2009).

2.4.2. Plant defense response to human enteric pathogens

As discussed in plant immune system, plant response to bacterial phytopathogens, which can trigger plant defense response. With respect to PTI, plant hosts recognize bacterial components of cell surface structures called PAMPs and induce the basal innate immune response to limit the spread and growth of pathogen (Nürnberger et al., 2004). Recent studies indicate that PAMPs are associated not just with phytopathogens but with different types of human pathogens. For example, flagella-deficient and curli-deficient mutants of *E. coli* O157:H7 resulted in greater survival on *Arabidopsis thaliana* with less induction of plant defense response compared to wild-type *E. coli* O157:H7 (Seo and Matthews, 2012). *S.* Typhimurium lacking flagella also showed a better colonization on *Arabidopsis* and alfalfa (*Medicago sativa*) compared to wild-type strain, which demonstrates that *Salmonella* activates the innate immune response of the plants (Iniguez et al., 2005). Meng et al. (2013) reported that flagellin subunit of *S.* Typhimurium, FliC, activates the PTI in tobacco (*Nicotiana benthamiana*) and tomato plants based upon the recognition of flagellin as PAMP. Stomatal immunity, as one of the earliest PTI response, is shown to induced by *E. coli* O157:H7 and *S.* Typhimurium on *Arabidopsis* plant (Roy et al., 2013). These findings suggest that cell surface structures of the human enteric

pathogens are recognized by plant defense system, thereby impacting on the survival or persistence of pathogens on plants. Moreover, Seo and Matthews (2014) showed that capsular polysaccharides (CPS) expressed by *E. coli* O157:H7 impacts plant defense response, resulting in a variability in survival on *Arabidopsis*. In the study, a greater amount of CPS was produced when *E. coli* O157:H7 cells were cultured in or exposed to soil or manure; plants inoculated with those cells exhibited lower induction of defense-related gene and greater survival of the pathogen on plant tissues (Seo and Matthews, 2014). It is possible that bacterial EPS or CPS structures may mask the bacterial cell surface structures that might be recognized by the plant host, thereby limiting the defense response.

In order to establish successful colonization and infection, phytopathogens need to suppress the plant host defense system in the battle between pathogen and plant. Phytopathogens can utilize a T3SS to deliver virulence proteins (effectors) into the plant host to suppress PAMP-triggered defense responses (Li et al., 2005; Nomura et al., 2006). With respect to roles of T3SS of human pathogens in plant immunity, studies with *Salmonella* spp. have reported the influence of T3SS on the interactions with plant hosts, but there have been conflicting reports. Iniguez et al. (2005) reported that T3SS of *Salmonella* is considered to induce plant defense response because *Arabidopsis* plant inoculated with wild-type *Salmonella* showed higher GUS activity and *PR1* gene expression compared to T3SS mutant lacking a component of T3SS. Also, this study showed that deletion in *spaS* (encoding structural component of T3SS) and *sipB* (required for effectors translocation) results in higher colonization in roots of alfalfa and wheat compared to wild-type strain (Iniguez et al., 2005). By contrast, *S.* Typhimurium mutants

lacking pathogenicity islands 1 and 2 (SPI-1 and SPI-2) showed reduced proliferation in *Arabidopsis*, suggesting *Salmonella* T3SS are involved in suppression of plant defense responses (Schikora et al., 2011). Similarly, T3SS mutants of *S*. Typhimurium were shown to induce high oxidative burst, pH changes, and hypersensitive response (HR) in tobacco leaves compared to the wild-type strain (Shirron and Yaron, 2011). In contrast, Meng et al. (2013) reported that population on tobacco leaves between *S. enterica* wild-type and the T3SS mutant was not different, indicating that *Salmonella* SPI-1 and SPI-2 systems are not involved in suppression of plant defense. The discrepancies in the literature concerning the role of T3SS of *Salmonella* on plant interactions are possibly due to differences in experimental conditions, plant species, and *Salmonella* serotypes tested. Studies regarding the roles of T3SS in human enteric pathogens such as *E. coli* O157:H7 are important to understand molecular interactions between human pathogens and plants.

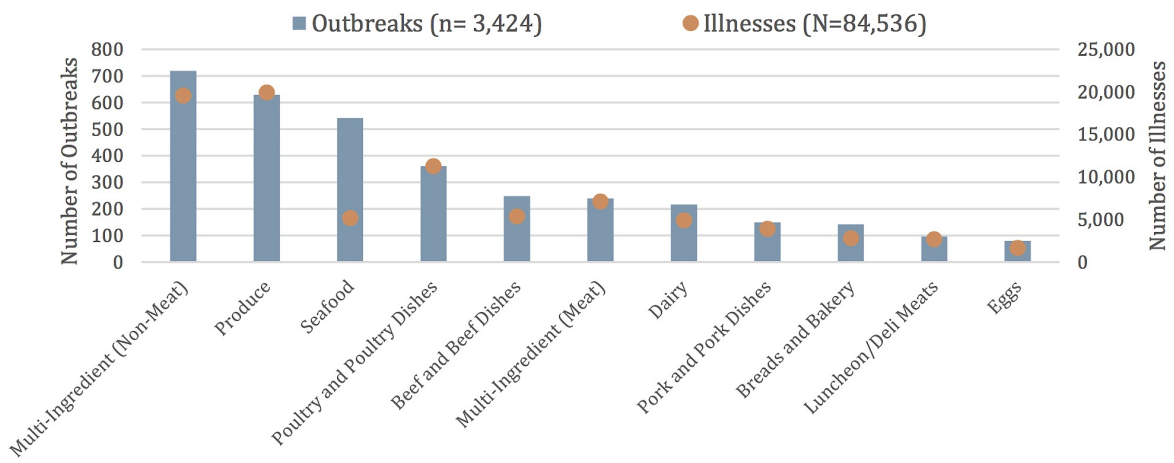

Figure 2.1. Numbers of solved outbreaks and illnesses by food categories, 2004-2013. Among the food categories, produce was associated with the greatest number of outbreaks and illnesses other than multi-ingredients. A category of produce includes fruits, vegetables, and produce dishes. Multi-ingredient food (non-meat) includes salads, sandwiches, sauces and dressings, rice and beans, nuts and dried spices. *Adapted from Center for Science in the Public Interest (CSPI), 2015*.

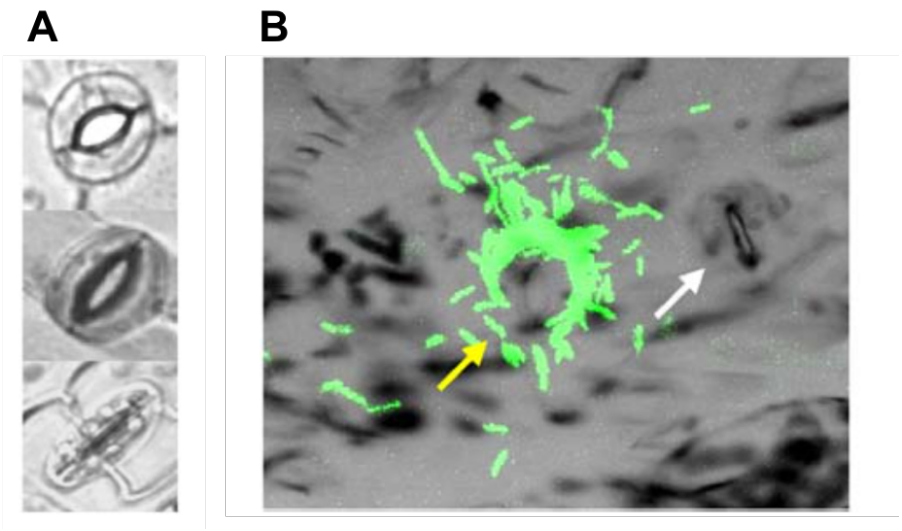

Figure 2.2. Plant stomatal function against bacterial invasion. (A) Stomatal aperture as open (top and middle panels) and closed (bottom panel). (B) A confocal microscopic image of GFP *Pseudomonas syringae* pv. tomato DC3000 cells (green) on *Arabidopsis* epidermal peel; bacteria localized adjacent an open stoma (yellow arrow) while not localized around a closed stoma (white arrow). *Adapted from Melotto et al., 2006.*

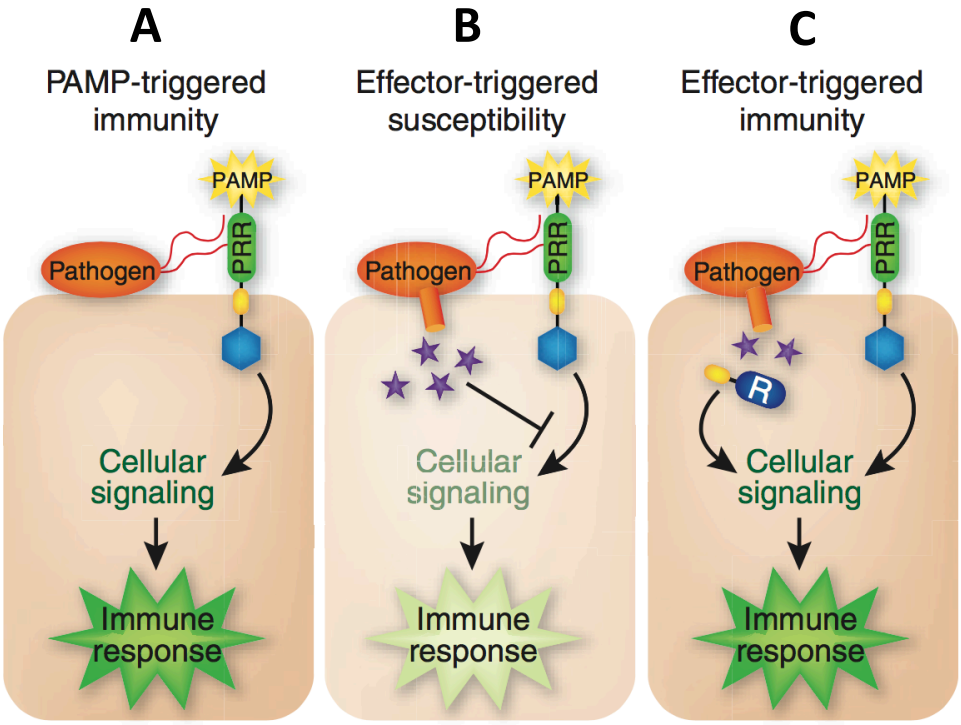

Figure 2.3. Schematic illustration of the plant immune system. (A) Upon pathogen attack, recognition of pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs) in plant host lead to cellular signaling for immune response, resulting in a PAMP-triggered immunity (PTI). (B) To counteract PTI, virulent pathogen secretes effectors (purple stars) into plant host cell to suppress the plant immune response; effector-triggered susceptibility (ETS). (C) In turn, plant recognize the effectors by resistance (R) proteins (dark blue), resulting in activation of a secondary plant immune response called effector-triggered immunity (ETI). *Adapted from Pieterse et al., 2009.*

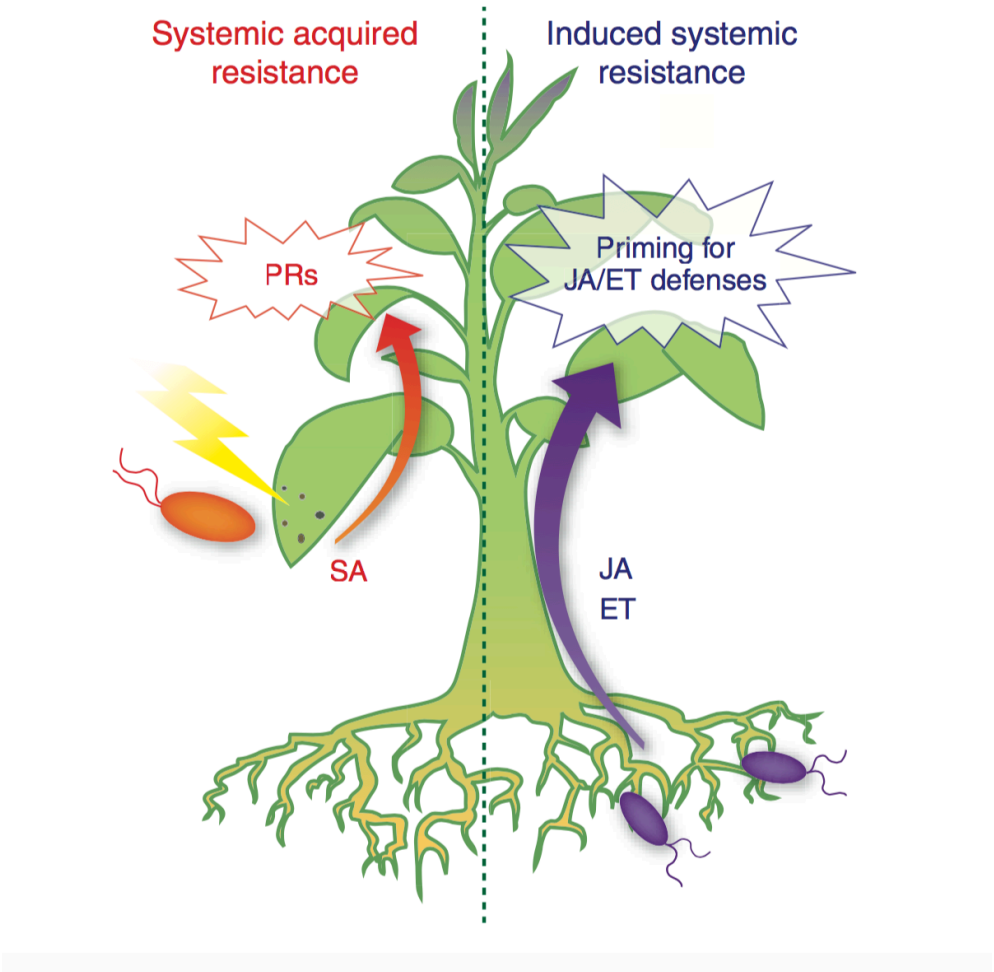

Figure 2.4. Systemically induced immune responses in plant. Systemic acquired resistance (SAR) is typically triggered in healthy tissues of local infection sites. Induced systemic resistance (ISR) is typically activated in roots by beneficial soil-borne microorganisms. Travel of signal molecules such as salicylic acid (SA; required for pathogenesis-related (*PR*) gene expression), jasmonic acid (JA), and ethylene (ET) through the vascular system enable an activation of defense responses in remote sites from initial infection. *Adapted from Pieterse et al., 2009.*

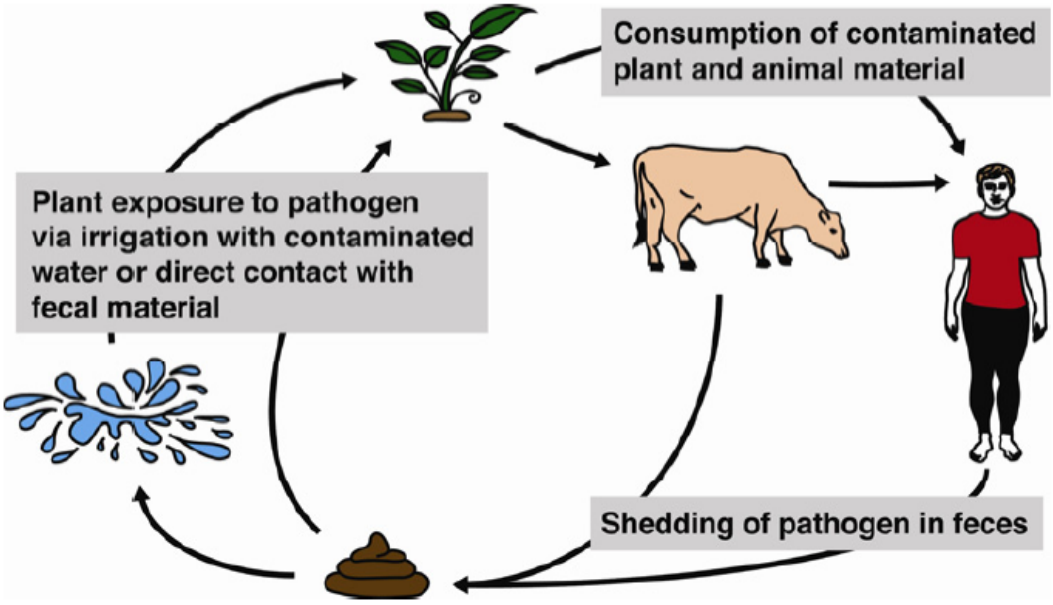

Figure 2.5. Concepts in the ecological cycle of human enteric pathogens between plants, animals, and humans. *Adapted from Fletcher et al., 2013.*

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CHAPTER 3.

Influence of surface polysaccharides of *Escherichia coli* O157:H7 on plant defense response and survival of the human enteric pathogen on *Arabidopsis thaliana* and lettuce (*Lactuca sativa*)

Hyein Jang and Karl R. Matthews

3.1. Abstract

This study aimed to determine the influence of bacterial surface polysaccharides (cellulose, colanic acid, and lipopolysaccharide; LPS) on the colonization or survival of *Escherichia coli* O157:H7 on plants and the plant defense response. Survival of *E. coli* O157:H7 were evaluated on *Arabidopsis thaliana* and romaine lettuce as a model plant and an edible crop (leafy vegetable), respectively. The population of the wild-type strain of *E. coli* O157:H7 on *Arabidopsis* plants and lettuce was significantly ($P < 0.05$) greater compared with the colanic acid-deficient and LPS-truncated mutants on day 1 and day 5 post-inoculation. This result indicates that colanic acid and LPS structures may contribute to the ability of bacterial survival or persistence on plants. The wild-type strain of *E. coli* O157:H7 produced approximately twice the amount ($P < 0.05$) of capsular polysaccharide (CPS) than the colanic acid and LPS-truncated mutants. The significantly lower production of CPS was associated with significantly greater (2-fold) expression of pathogenesis-related gene (*PR1*) compared with the wild-type and cellulose-deficient mutant ($P < 0.05$). Collectively, the results of this study may suggest that specific surface polysaccharides of *E. coli* O157:H7 differentially induce the plant defense response, consequently affecting the survival of the human pathogen on plants. The survival and persistence of *E. coli* O157:H7 was similar on *Arabidopsis* and lettuce regardless of day post-inoculation.

Key words: *E. coli* O157:H7, surface polysaccharides, produce, food safety

3.2. Introduction

Numerous outbreaks and foodborne illnesses are linked to consumption of fresh vegetables contaminated with human enteric pathogens, such as *Escherichia coli* O157:H7 and *Salmonella*. According to a report of the Center for Disease Control and Prevention (CDC), leafy vegetables are the most frequent cause (22%) of estimated annual foodborne illnesses in the United States, the second leading cause of hospitalizations (14%), and fifth in number of deaths (6%) (Painter et al., 2013). Lettuce and spinach contamination with *E. coli* O157:H7 caused a number of foodborne outbreaks in the United States and Europe, which represents a considerable public health concern (CDC, 2013; Friesema et al., 2008). *E. coli* O157:H7 infection can cause abdominal cramps, severe bloody diarrhea, and may progress to hemolytic uremic syndrome (Nataro and Kaper, 1998).

Human enteric pathogens have not been traditionally considered to be closely related to plant physiology. However, recent findings indicate that plants can be good alternate hosts for human enteric pathogens by providing a refuge (Melotto et al., 2014; Schikora et al., 2012). It has been reported that *E. coli* O157:H7 and *Salmonella* spp. can internalize into, colonize, and survive on plant tissues, so that plants may serve as potential vectors transferring the pathogens to a new host (Brandl, 2006; Deering et al., 2012). Therefore, better understanding on molecular interactions between human enteric pathogens and plants, including edible plants such as leafy vegetables, has become increasingly important as a new approach to improve food safety. In particular, molecular mechanisms of plant defense responses influencing survival and colonization of human enteric pathogens on plants have to be elucidated (García and Hirt, 2014).

Plants have an innate immunity system consisting of two main branches that they use to protect themselves from invading microbes; PAMPs (pathogen-associated molecular patterns)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006). PTI is activated upon recognition of conserved molecular signatures of microorganisms, referred to as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), by pattern recognition receptors (PRRs) (Pel and Pieterse, 2013). The PTI responses result in plant cell wall thickening, stomatal closure to prevent pathogen entry, production of reactive oxygen species and expression of defense-related genes or proteins to prevent microbial growth and colonization (Melotto et al., 2006; Nicaise et al., 2009). Successful virulent pathogens are able to counteract PTI by delivering virulence factors into plant host cell using different virulence strategies such as translocation of effectors via type 3 secretion system and production of phytotoxins (Melotto and Kunkel, 2013). However, plant nucleotide-binding leucine-rich repeat (NB-LRR) proteins encoded by resistance (R) genes recognize the virulence effectors and restore plant immunity through effector-triggered immunity (ETI) (Chisholm et al., 2006). The ETI results in localized cell death (necrosis) called hypersensitive response to limit the further spread of pathogens (Chisholm et al., 2006).

Research on plant defense responses precipitated by human enteric pathogens is limited compared to that of phytopathogens. Information about the interactions between human pathogens and plant defense responses has just begun to accumulate (Melotto et al., 2014). It is known that *E. coli* O157:H7 and *S.* Typhimurium induce salicylic acid-dependent immunity in *Arabidopsis thaliana* such as stomatal closure or pathogenesis-related (PR) gene expression (Iniguez et al., 2005; Melotto et al., 2006; Roy et al., 2013).

Cell surface structures of *E. coli* O157:H7 and *S.* Typhimurium such as flagella and curli (extracellular protein fibers; known as thin aggregative fimbriae (Tafi) in *Salmonella*) have been shown to activate plant defenses that may restrict bacterial colonization on *Arabidopsis* (García et al., 2014; Seo and Matthews, 2012), tomato, and tobacco plants (Meng et al., 2013). Nonetheless, bacterial surface structures of the human enteric pathogens contributing to plant defense responses are largely unknown.

Bacterial surface polysaccharides can play crucial roles in various plant-microbe interactions because they are the first point of contact with plant host cells and environments (D'Haeze and Holsters, 2004). Surface polysaccharides of *E. coli* isolates include capsular polysaccharide (CPS; K antigen-specific) and lipopolysaccharide (LPS; O antigen-specific) (Jann and Jann, 1997). CPS is tightly associated with the cell surface, which can act as a protective layer against hostile environments (e.g. desiccation, oxidative stresses, and antimicrobial peptide) or host immune responses (Campos et al., 2004; Whitfield, 2006). LPS is the cell surface component of outer membrane in Gram-negative bacteria, which may confer barrier function allowing bacterial growth or survival on plants (Lerouge and Vanderleyden, 2001). By contrast, LPS recognition by a lectin S-domain receptor kinase has been shown to induce plant immune responses (Ranf et al., 2015). Therefore, the influence of LPS on plant-microbe interactions needs to be fully elucidated (Lerouge and Vanderleyden, 2001). *E. coli* O157:H7 produces extracellular polysaccharides (EPS) such as cellulose and colanic acid, which are involved in bacterial attachment, biofilm formation, and stress tolerance (Chen et al., 2004; Mathews et al., 2014; Matthysse et al., 2008; Yoo and Chen, 2012).

Differential expressions of surface polysaccharides may have a significant impact on the survival of pathogens through protection against plant defense responses. However, very little is known about influences of bacterial surface polysaccharides of human enteric pathogens on plant-pathogen interactions, especially in terms of plant defense responses. The objectives of this study were to investigate roles of surface polysaccharides of *E. coli* O157:H7 (cellulose, colanic acid, and LPS) on plant defense responses and consequent effects on survival of the enteric pathogen on plants. Colonization of *E. coli* O157:H7 on lettuce was also examined to compare the survival trend between *Arabidopsis* (a model plant) and food commodity (leafy vegetable). Furthermore, a possible role of CPS produced by *E. coli* O157:H7 was evaluated as a means for evasion of plant defense responses.

3.3. Materials and methods

3.3.1. Bacterial strains and media

Escherichia coli O157:H7 86-24 wild-type strain and its isogenic mutants were used in this study (Table 1). Stock cultures were prepared in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) containing 20% glycerol and stored at –70°C after confirming the culture by streaking onto Sorbitol MacConkey agar (Difco). The bacteria were cultured in TSB or grown on tryptic soy agar (TSA; Difco) at 37°C for 20 h with the following concentrations of antibiotics; streptomycin (100 μg/mL), nalidixic acid (30 μg/mL), and kanamycin (50 μg/mL).

3.3.2. Curli production

Curli expression by *E. coli* O157:H7 strains was determined by Congo red indicator (CRI) agar method (Hammar et al., 1996). CRI agar was composed of 1 g/L yeast extract (Bacto, Becton Dickinson, Sparks, MD), 10 g/L casamino acids (Bacto), and 15 g/L Bacto agar (Difco) supplemented with 20 mg/L Congo red (MP Biomedicals, Solon, OH) and 10 mg/L Coomassie brilliant blue (Bio-Rad Laboratories, Hercules, CA). Briefly, overnight cultures were streaked onto the CRI agar and incubated at 25°C for 48 h. Colony color was examined as an indicator of curli production; colorless (white) colony indicates curli-negative while red represents curli-positive.

3.3.3. Plant materials and growth conditions

Arabidopsis thaliana and romaine lettuce (*Lactuca sativa* L. var. *longifolia*) were used to compare the colonization or survival trend of *E. coli* O157:H7. Seeds of *Arabidopsis thaliana* (Columbia ecotype; Col-0, CS 70000) and romaine lettuce (Parris Island Cos) were purchased from Arabidopsis Biological Resource Center (ABRC; Columbus, OH) and W. Atlee Burpee & Co. (Warminster, PA), respectively. Before planting, *Arabidopsis* seeds were placed in sterile distilled water at 4°C for 3 days to improve the germination rate and synchrony. Planting seeds and growth of *Arabidopsis* were performed according to the standard guideline of ABRC (ABRC, 2013). *Arabidopsis* and lettuce plants were grown in Sunshine Redi-earth professional growing mix soil (Sun Gro, Agawam, MA) and Fafard Canadian growing mix 2 (Sun Gro), respectively. The plants were cultivated in the climate-controlled Research Greenhouse at Rutgers University (New Brunswick, NJ) under natural light supplemented with artificial

greenhouse light to achieve a 16 h-photoperiod (21 ± 2°C during the day and 19 ± 2°C at night, relative humidity 65 ± 5%).

3.3.4. Preparation of inoculum and bacterial challenge of plants

Each *E. coli* O157:H7 strain was cultured in two 30 mL of TSB with appropriate antibiotics at 37°C for 20 h (shaking at 200 rpm). The bacterial cells were harvested by centrifugation at 4,200 × g for 8 min (Allegra^{TM} 21R, Beckman Coulter, Palo Alto, CA). After the supernatant was discarded, the pellet was washed twice in 30 mL of 0.1% sterile peptone water (Difco) and then resuspended into sterile distilled water (540 mL) to achieve approximately 1.5×10^8 CFU/mL. Bacterial levels in the inoculum were confirmed by plating serial 1:10 dilutions on TSA with appropriate antibiotics. In order to examine the survival of *E. coli* O157:H7 on plants, 4-week old whole plants of *Arabidopsis* and lettuce were dip-inoculated in the bacterial inoculum of 50 mL for 30 s and 600 mL 60 s, respectively. Population of *E. coli* O157:H7 was determined on day 0, 1, and 5 post-inoculation (Seo and Matthews, 2012). At each time point, whole (9 to 11) leaves of *Arabidopsis* were aseptically harvested, weighed, and placed in a sterile sample bag (Whirl-Pak, Nasco, Fort Atkinson, WI) with 5 mL 0.1% peptone water. Weighed lettuce samples (5 to 6 leaves) were diluted with 0.1% peptone water (1:10, w/v). The leaf samples were macerated by hand or with a pestle, stomached for 1 min, and homogenized samples were serially diluted (10-fold) with 0.1% peptone water. Aliquots (100 µl) were spread-plated in duplicate onto TSA with the corresponding antibiotics, and the plates were incubated at 37°C for 18 h. The microbiological count was calculated and expressed as a mean log CFU per gram of fresh plant tissue (log CFU/g leaf tissue).

The independent experiments were repeated three times and each experiment included three replicate plants per treatment.

3.3.5. Congo red staining of CPS

To detect the presence of capsular polysaccharide in *E. coli* O157:H7 cells, a negative staining based on Maneval's capsule staining method (Corstvet et al., 1982) was performed with some modifications. Bacterial overnight culture of *E. coli* O157:H7 (20 µL) was mixed with one drop of 1% (w/v) Congo red solution on a microscope slide. The mixture was gently spread across the original slide using another clean slide and allowed air-dry for 5 min. The smear was then flooded with Maneval's modified stain (Carolina Biological Supply Company, Burlington, NC) for 1 min and carefully washed with sterile distilled water. After drying completely, the prepared slides were examined at a total magnification of 1,000 × under oil immersion using a light microscope (Olympus BH2, Olympus Co., Tokyo, Japan).

3.3.6. Quantitative analysis of CPS

Cell-associated CPS was extracted and purified by a phenol-water extraction method (Campos et al., 2004). Each *E. coli* O157:H7 strain was grown in 4 mL of TSB at 37°C for 20 h on an orbit shaker (200 rpm) and harvested by centrifugation (5,000 × g, 10 min, 4°C). After the supernatant was removed, the pellets were washed with 1 mL of sterile distilled water (SDW) and resuspended in 500 µL of SDW. The suspensions were serially diluted (1:10) and 100 µL of the dilutions was in duplicate plated onto TSA with

appropriate antibiotics. Following incubation of the plates at 37°C for 20 h, colonies were counted to determine the number of viable cells. The suspensions were preheated at 68°C for 2 min in a water bath (Thermo Scientific, Waltham, MA) and 500 µL of phenol (purity > 89%; Amresco, Solon, OH) was added, then the mixture was continuously incubated at 68°C for 30 min. After cooling the solutions by placing in an ice bath, 500 µL of chloroform (99.8%; Fisher Chemical, Fair Lawn, NJ) was added, vortexed, and then centrifuged at 5,400 × g for 10 min to recover the aqueous phase. CPS in the aqueous phase was precipitated by adding three volumes of absolute ethyl alcohol (EMD Millipore, Billerica, MA) and stored at –20°C for 18 h. Precipitated CPS was harvested by high-speed centrifugation (14,000 × g, 20 min, 4°C) and then resuspended in 500 µL of SDW. CPS was quantified by determining total carbohydrate using a phenol-sulfuric acid method (Saha and Brewer, 1994). In brief, 5% phenol was added to sugar solutions (1:1 v/v) and mixed, followed by rapid mixing with a 5-fold volume of sulfuric acid (98%; Fisher Chemical). The mixtures were placed at room temperature for 30 min and absorbance was measured at 490 nm in a microplate reader (Molecular Devices, Sunnyvale, CA). Using a standard curve of glucose solution, the amount of CPS was calculated as µg CPS per 10^9 CFU of bacterial cells. All samples were measured in triplicate with three independent batches of bacterial culture.

3.3.7. Analysis of pathogenesis-related gene expression by reverse transcription quantitative PCR

Total RNA was extracted from leaves of *Arabidopsis* plants at 8 and 24 h post-inoculation using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the

manufacturer's protocol. Concentrations of RNA samples were measured using a NanoDrop spectrophotometer (2000c, Thermo Scientific). cDNA was synthesized from 1 μg of total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a 20 μL of reaction volume. The thermal cycling conditions of reverse transcription (RT) reaction were at 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and 4°C for infinite hold. After the preparation of cDNA, quantitative PCR (qPCR) was carried out using StepOnePlus™ Real-Time PCR System (Applied Biosystems) with Power SYBR Green PCR Mater Mix (Applied Biosystems), using gene-specific forward and reverse primers (Table 1) (Roy et al., 2013). Final concentrations of cDNA and primers in 25 μL of total reaction volume were 40 ng/μL and 200 nM, respectively. The amplification efficiency of PCR reaction was confirmed with each primer pair of pathogenesis-related 1 (PR1) and Actin8 (ACT8) following the instructions of manufacturer. Cycling parameters were as follows: 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative levels of gene expression were calculated by the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001) using the housekeeping gene of *ACT8* as an internal control. The levels of target gene (*PR1*) were expressed as fold change compared to a mock treatment with sterile distilled water (SDW). The independent experiments were conducted in duplicate and each experiment included three replicate plants per treatment. All samples including NTC (non-template control) were measured in triplicate.

3.3.8. Statistical analysis

All data were evaluated by one-way analysis of variance (ANOVA) using SAS software (SAS University Edition; SAS Institute, Inc., Cary, NC). Statistical significance among the treatment groups of *E. coli* O157:H7 strains were determined as follows. Duncan's multiple range test was used in the analyses of bacterial populations and CPS production. Tukey's honest significance test was used in the analysis of *PR1* gene expression. A significant difference was considered at a P value of < 0.05.

3.4. Results

3.4.1. Influence of surface polysaccharides on the survival of *E. coli* O157:H7 on *Arabidopsis thaliana* and romaine lettuce

To investigate the role of surface polysaccharides in colonization of the enteric pathogen on plants, *Arabidopsis* plants and lettuce plants were inoculated with a wild-type strain of *E. coli* O157:H7 and its corresponding mutants of surface polysaccharides (Δ*yhjN*; cellulose-deficient, Δ*wcaD*; colanic acid-deficient, Δ*waaI*; LPS-truncated). The bacterial populations on the plants were monitored over a 5-day period. Fig. 3.1A shows the populations of *E. coli* O157:H7 strains on *Arabidopsis* plants on day 0, 1, and 5 post-inoculation. The initial mean population of *E. coli* O157:H7 strains on *Arabidopsis* at day 0 was approximately 6.6 log CFU/g leaf tissue ($P > 0.05$). The numbers of each *E. coli* O157:H7 on *Arabidopsis* plants were significantly ($P < 0.05$) decreased over 5 days. By day one post-inoculation, the bacterial populations declined dramatically; ranging from 1.4 log CFU/g (WT) to 2.6 log CFU/g (waaI), which was a much more rapid decline compared with that between day 1 and day 5.

As shown in Fig. 3.1A, the wild-type strain significantly ($P < 0.05$) survived better than colanic acid-deficient and LPS-truncated mutants at both day 1 and day 5 post-inoculation. The effect of surface polysaccharides in the pathogen survival on *Arabidopsis* became more noticeable on day 5; the populations of colanic acid-deficient mutant (2.8 ± 0.2 log CFU/g) and LPS-truncated mutant (2.6 ± 0.5 log CFU/g) were significantly lower ($P < 0.05$) than that of the wild-type strain (3.8 ± 0.4 log CFU/g). This finding demonstrated that colanic acid and LPS may influence the ability of *E. coli* O157:H7 survival or persistence on plants. On the other hand, the cellulose-deficient mutant showed significantly higher populations ($P < 0.05$) than the colanic acid-deficient and LPS-truncated mutants on day 1 and day 5. This indicates that lack of cellulose may not affect the bacterial fitness on *Arabidopsis* plant as much as the lack of colanic acid or a truncated LPS. In addition, it is possible that colanic acid-deficient and LPS-truncated mutants have a reduced fitness on the leaf surface due to environmental factors, which is unrelated to plant defenses.

The colonization study on romaine lettuce (Fig. 3.1B) was subsequently conducted to compare the behavior of *E. coli* O157:H7 strains between the model plant (*Arabidopsis*) and edible crop plant (lettuce). Interestingly, survival trends of *E. coli* O157:H7 strains on lettuce were shown to be similar to those on the *Arabidopsis* plant during the same time period. On day 0, there was no significant difference ($P > 0.05$) in populations of the four *E. coli* O157:H7 strains with a mean population of approximately 6.3 log CFU/g. From the initial populations, the population of all four strains on lettuce consistently declined over the 5-day period, which is a similar manner of decline on *Arabidopsis* plants. With regard to roles of the different polysaccharides of *E. coli*

O157:H7, significantly lower survival ($P < 0.05$) on lettuce was observed in colanic acid-deficient and LPS-truncated mutants compared with the wild-type strain. The populations on lettuce at day 1 were as follows: wild-type (5.2 ± 0.1 log CFU/g), cellulose-deficient mutant (5.0 ± 0.7 log CFU/g), colanic acid-deficient mutant (4.2 ± 0.2 log CFU/g), and LPS-truncated mutant (3.9 ± 0.5 log CFU/g). Along with further reduction of the populations, colanic acid-deficient (2.5 ± 0.5 log CFU/g) and LPS-truncated (2.6 ± 0.2 log CFU/g) mutants showed the lowest populations on lettuce at day 5 post-challenge. This resulted in a total reduction of 3.6 log CFU/g in colanic acid-deficient mutant and LPS-truncated mutant during 5 days. By contrast, the cellulose-deficient mutant survived in the same manner as the wild-type strain. There was no significant difference ($P > 0.05$) in populations at day 1 and 5 post-challenge between the wild-type strain and cellulose-deficient mutant.

3.4.2. Production of capsular polysaccharide (CPS) by *E. coli* O157:H7

To examine whether CPS production contributes to survival of the enteric pathogen on plants, secretion of capsular material by *E. coli* O157:H7 strains was determined by Congo red negative staining (Fig. 3.2) and then quantitatively analyzed (Fig. 3.3). As shown in Fig. 3.2, CPS appeared as clear zones surrounding the *E. coli* O157:H7 cells when cultured in TSB for 20 h. More distinct and larger CPS zones were observed in the wild-type strain and cellulose-deficient mutant compared with the colanic acid-deficient and LPS-truncated mutants. In the analysis of CPS quantification (Fig. 3.3), CPS productions by the wild-type strain and cellulose-deficient mutant (12.1 ± 0.9 and 9.5 ± 0.4 μg CPS/10^9 CFU, respectively) were significantly ($P < 0.05$) greater than those

by the colanic acid-deficient and LPS-truncated mutants (7.1 ± 0.6 and 6.2 ± 1.5 μg CPS/10^9 CFU, respectively). The lower amounts of CPS secreted by colanic acid-deficient and LPS-truncated mutants corresponded with the narrow clear halos shown in Fig. 3.2.

3.4.3. Induction of plant defense response by different components of surface polysaccharides in *E. coli* O157:H7

After confirming the influences of surface polysaccharides of *E. coli* O157:H7 in the differential survival on plants, we further examined the expression level of pathogenesis-related gene (*PR1*) that is a marker gene of plant defense response. To analyze the *PR1* gene expression, reverse transcription quantitative PCR (RT-qPCR) was performed with RNA extracted from leaves of *Arabidopsis* plants inoculated with the *E. coli* O157:H7 strains. The treatment with water was used as a control to assess the relative levels of gene expression. As shown in Fig. 3.4, *E. coli* O157:H7 strains induced significantly ($P < 0.05$) higher defense-related *PR1* gene on *Arabidopsis* compared with the control at 8 h post-inoculation. Plants inoculated with *E. coli* O157:H7 wild-type and cellulose-deficient mutant exhibited a 2.2-fold and a 2.0-fold increase in expression of *PR1* gene at 8 h, respectively, compared with the control. The greater inductions of *PR1* gene expression were observed in plants challenged with colanic acid-deficient mutant (4.1-fold) and LPS-truncated mutant (4.0-fold) at 8 h compared with the control, which are significantly higher levels than those of the wild-type strain and cellulose-deficient mutant ($P < 0.05$). These results indicate that colanic acid-deficient and LPS-truncated mutants induced greater expression of the plant defense gene (*PR1*) that encodes

antimicrobial PR protein, which may contribute to their lower populations on *Arabidopsis* and lettuce compared with wild-type and cellulose-deficient strains (Fig. 3.1). At 24 h post-inoculation, however, levels of *PR1* gene expression were decreased to basal level and there was no significant difference ($P > 0.05$) among the all treatment groups of *E. coli* O157:H7 strains; Control (1.0-fold), wild-type (1.4-fold), colanic acid-deficient (1.0-fold), and LPS-truncated (1.5-fold). The overall findings from the RT-qPCR analysis indicate that different production of surface polysaccharides by *E. coli* O157:H7 can display changes in the induction of plant defense responses.

3.5. Discussion

Foodborne illness and outbreaks associated with the consumption of fresh produce contaminated with human pathogens are common globally. Human enteric pathogens have not yet proven to be phytopathogens. However, recent studies suggest that plants can be good alternate hosts for human pathogens, such as *E. coli* O157:H7 and *Salmonella* that are major concerns for produce safety and public health (Melotto et al., 2014). It becomes more important to elucidate the molecular responses of plants to human enteric pathogens since plant immunity regulates survival and persistence of microorganism on a plant. Researchers found that human pathogenic bacteria can mount plant defense responses even though they do not cause visual symptoms of disease on plant leaves (Meng et al., 2013; Seo and Matthews, 2012).

Bacterial polysaccharides are known to play important roles in interactions between enteric pathogens and biotic or abiotic surfaces (Barak et al., 2007; Matthysse et al., 2008). However, information on precise functions of bacterial polysaccharides in

evading plant defense responses or triggering the plant immunity is very limited. In the present study, we determined the roles of surface polysaccharides of *E. coli* O157:H7 on the bacterial survival/persistence on plants with the differential induction of plant defense response. Overall, the results of the study revealed that *E. coli* O157:H7 populations were gradually decreased on both *Arabidopsis* and lettuce plants over 5 days (Fig. 3.1). Results showed that *E. coli* O157:H7 strains do not multiply aggressively on *Arabidopsis* plants, which may be associated with innate defense responses of the plants. There was no significant difference ($P > 0.05$) in the weights of plant samples between day 0 and day 5, indicating that weight changes did not affect the decline of *E. coli* O157:H7 populations over the time period (data not shown). In contrast to the behavior of *E. coli* O157:H7, phytopathogens exhibit a different survival pattern. For instance, numbers of *Pseudomonas syringae* pv. tomato DC3000 on *Arabidopsis* plants remained constant during a 5-day period with visible infection symptoms such as chlorosis and necrosis (Seo and Matthews, 2012). Moreover, *S.* Typhimurium was shown to persist better on plants with lower induction of *PR1* gene expression and stomatal immunity compared with *E. coli* O157:H7, which suggests that plants may recognize and respond differently depending on genus/species of human enteric pathogens (Roy et al., 2013).

In Fig. 3.1A and B, the dramatic reductions of *E. coli* O157:H7 populations between day 0 and day 1 may suggest that PAMP-triggered immunity restricted bacterial colonization. A genome-wide analysis of cellular transcriptional response of plant showed that *E. coli* O157:H7 elicits expression of PAMP-responsive genes, indicating an induction of PTI in *Arabidopsis* (Thilmony et al., 2006). It was demonstrated that cell surface structures of *E. coli* O157:H7, including flagella and curli fimbriae, can promote

plant defense responses, resulting in reduction of bacterial colonization on *Arabidopsis* plants (Seo and Matthews, 2012). Flagellin subunit of *S.* Typhimurium was shown to activate PTI in tobacco plants (*Nicotiana benthamiana*) (Meng et al., 2013). These findings indicate that structural components (i.e. PAMPs) of human pathogens are recognized by plant defense system, thereby limiting the survival or growth of pathogens on plants.

Regarding the influence of different surface polysaccharides on *E. coli* O157:H7 survival on plants, deficiencies in colanic acid and of LPS structure resulted in reduced survival with a greater induction of *PR1* gene expression (Fig. 3.1 and 3.4). This result suggests that colanic acid and LPS may impact bacterial survival and persistence on plants. Similarly, it was shown that O-antigen capsule of *S.* Enteritidis contributes to colonization in alfalfa sprouts (Barak et al., 2007) and colanic acid of *E. coli* O157:H7 aids the retention or growth on lettuce cut ends, cucumber and tomato epidermis (Mathews et al., 2014). In this study, cellulose (*yhjN* gene) did not influence the bacterial survival on both *Arabidopsis* and lettuce. However, a gene regulating cellulose synthesis (*bcsA*) of *S.* Enteritidis was associated with biofilm formation and facilitated the attachment/colonization on alfalfa sprouts (Barak et al., 2007). The discrepancies in the roles of bacterial polysaccharide components in binding or colonization are possibly owing to differences in bacterial species or serotypes, varieties of plants, plant cultivation method, and experimental conditions such as inoculation level and exposure time.

Interestingly, we observed the differential CPS production and *PR1* gene expression by the different strains of *E. coli* O157:H7. The *PR1* gene is regulated by salicylic acid-mediated pathway of plant basal immunity and is known as a molecular

marker of systemic acquired resistance (Durrant and Dong, 2004). Results in this study indicate that better survival of wild-type strain and cellulose-deficient mutant of *E. coli* O157:H7 on plants may be due to greater amounts of CPS production and lower induction of plant defense response than those of colanic acid-deficient and LPS-truncated mutants (Fig. 3.2 and 3.3). Larger clear zones indicating CPS around cells were more evident for the wild-type strain and cellulose-deficient mutant compared with the other two mutants (Fig. 3.2). A possible explanation for the results is that the CPS structure may mask the outer cell moieties of bacteria, thereby leading to decreased recognition of PAMPs that can trigger the plant defense responses and consequently resulting in a greater survival on plants. When *E. coli* O157:H7 was cultured in or exposed to soil or manure, the cells produced greater amounts of CPS compared to those grown solely in LB medium, which exhibited lower GUS activity (an indication of plant defense response) and better colonization on *Arabidopsis* plants (Seo and Matthews, 2014). Furthermore, the improved production of CPS or extracellular polysaccharides (EPS) may enhance the bacterial survival on plants by providing a physical barrier against the plant host defense metabolites including antimicrobial compounds (reactive oxygen species and phytoalexins) or defense-related proteins (chitinase and β-1,3-glucanase) (D'Haeze and Holsters, 2004; Tarchevsky, 2001). For example, EPS of *Azorhizobium caulinodans* restricted the entry into the organism of hydrogen peroxide (H_2O_2) produced by the plant host (D'Haeze et al., 2004). CPS of *Klebsiella pneumoniae* was shown to be involved in the protection of the bacteria against antimicrobial peptides and proteins although CPS is thought to be a virulence factor in the *K. pneumoniae* infection (Campos et al., 2004). The polyanionic nature of bacterial EPS can affect the

suppression of PAMP-triggered plant immunity by means of chelating intracellular calcium ions, resulting in reduction of plant defense signaling (Aslam et al., 2008).

Another intriguing finding in this study was that behavior of *E. coli* O157:H7 strains on romaine lettuce was similar to that on *Arabidopsis thaliana*. Indeed, the survival trend of *E. coli* O157:H7 on lettuce was consistent with that on *Arabidopsis* plants (Fig. 3.1A and B). This finding suggests that *Arabidopsis* may serve as a suitable model plant for studying the interactions between *E. coli* O157:H7 and leafy vegetables. Genome sequences of *Arabidopsis thaliana* have been completely characterized (Arabidopsis Genome Initiative, 2000), so that the genomic approach using *Arabidopsis* plants can provide extensive information on the molecular mechanisms of plant interactions to human enteric pathogens.

With regard to inoculation method, dip-inoculation was used in the present study to closely mimic the passive infection process of human enteric pathogens without use of surfactants. Methods commonly used for inoculating *Arabidopsis* plants include syringe pressure infiltration, spray-inoculation, and dip-inoculation (Katagiri et al., 2002). Bacterial pathogens are generally able to enter the plant host tissues through natural stomatal openings or wounds. However, the syringe infiltration method may cause injury of foliar tissue and bypass the natural entry or infection process because it forces bacteria to penetrate into the apoplast (Underwood et al., 2007). The spray-inoculation method may not be sufficient to yield effective endophytic colonization of bacteria because hairy leaf surface that repels water may lead to entry failure of inoculum droplets into plant tissues (Kangatharalingam et al., 2003).

In conclusion, this study provides evidences that surface polysaccharides of *E. coli* O157:H7 have an important role in survival of the enteric human pathogen on plants through mitigation of plant defense responses. Different production of surface polysaccharides in *E. coli* O157:H7 cells may influence the survival ability and induction of plant defense responses. In particular, CPS structure of *E. coli* O157:H7 cells may be involved in protection of organism and evasion of plant host defenses possibly by masking PAMPs, thereby improving the survival. Better understanding of the interaction between human pathogens and plants, especially how the plant defense responses regulate the colonization or survival of foodborne pathogens, will be helpful to devise new intervention strategies in the pre-harvest environment for ensuring the safety of fresh produce.

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Table 3.1. Bacterial strains or primers used in this study

Strain or primers	Genotype/phenotype characteristic	Reference
E. coli O157:H7		
86-24 WT	wild-type, Sm^r Nal^r	Tarr et al., 1989
86-24 yhjN	Δ*yhjN*, Sm^r Nal^r (cellulose-deficient)	Matthysse et al., 2008
86-24 wcaD	Δ*wcaD*, Sm^r Nal^r (colanic acid-deficient)	Matthysse et al., 2008
86-24 waaI	Δ*waaI*, Sm^r Km^r (LPS-truncated)	Torres and Kaper, 2003
Primers	Sequence (5'-3')	
ACT8 (Forward)	TTCCGGTTACAGCGTTTGGAGAGA	Roy et al., 2013
ACT8 (Reverse)	AACGCGGATTAGTGCCTCAGGTAA	Roy et al., 2013
PR1 (Forward)	CTTGTTCTTCCCTCGAAAGCTCAAGATAGC	Roy et al., 2013
PR1 (Reverse)	GAGCATAGGCTGCAACCCTCTC	Roy et al., 2013

Sm^r, streptomycin resistant; Nal^r, nalidixic acid resistant; Km^r, kanamycin resistant.

All *E. coli* O157:H7 strains were curli-negative based on CRI agar method.

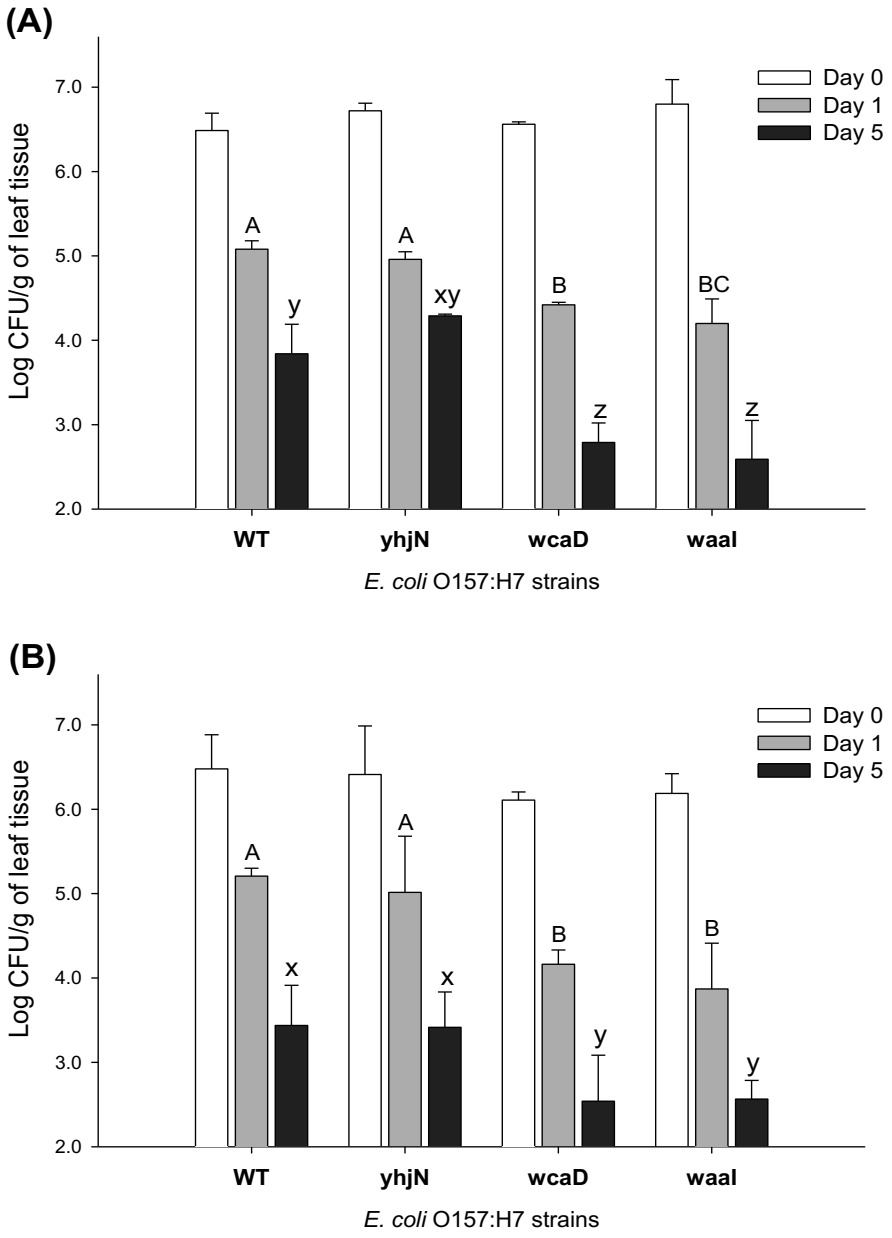

Figure 3.1. Populations of *E. coli* O157:H7 wild-type and its corresponding mutants on *Arabidopsis* plants (A) and romaine lettuce (B) on day 0, 1, and 5 post-inoculation. *E. coli* O157:H7 strains include wild-type (WT), cellulose-deficient (yhjN), colanic acid-deficient (wcaD), and LPS-truncated (waaI).

Figure 3.1 (Continued). Data represents the mean of populations from three independent experiments with three replicate plants (n = 9). Error bars indicate the standard deviations from the three separate experiments. Different letters indicate significant differences at $P < 0.05$. Uppercase letters (A, B, C) show the significant differences on day 1 between strains and lowercase letters (x, y, z) show the significant differences on day 5 between strains. Significant differences by strain between day 1 and day 5 are indicated by different letters. There was no significant difference in populations on day 0 among the four strains of *E. coli* O157:H7 ($P > 0.05$).

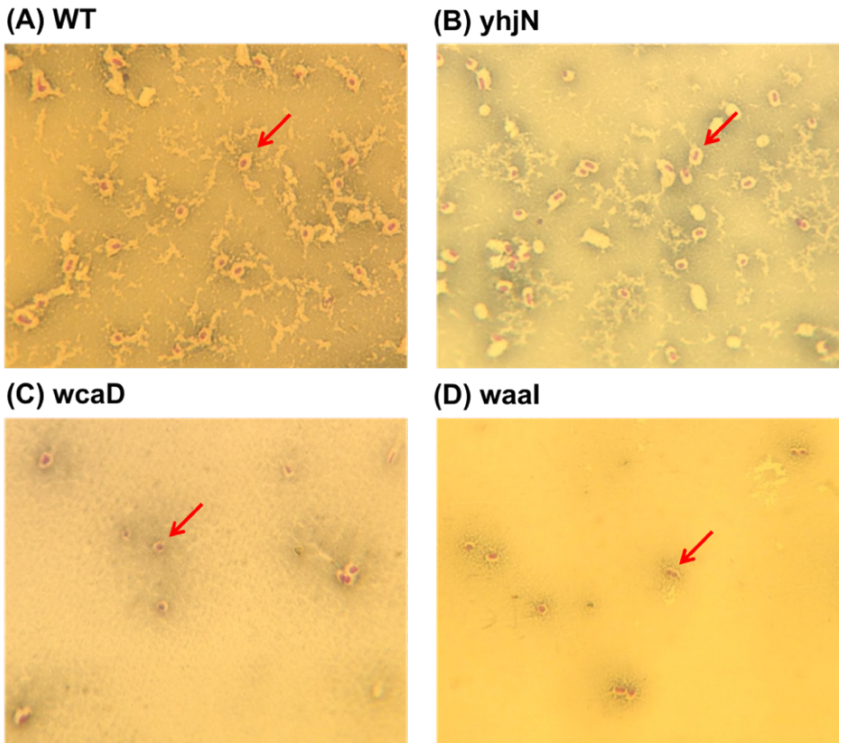

Figure 3.2. Congo red negative staining of capsular polysaccharide (CPS) of *E. coli* O157:H7 strains; wild-type (A), cellulose-deficient (B), colanic acid-deficient (C), and LPS-truncated (D). Clear halos surrounding cells suggest the presence of CPS as denoted by arrows.

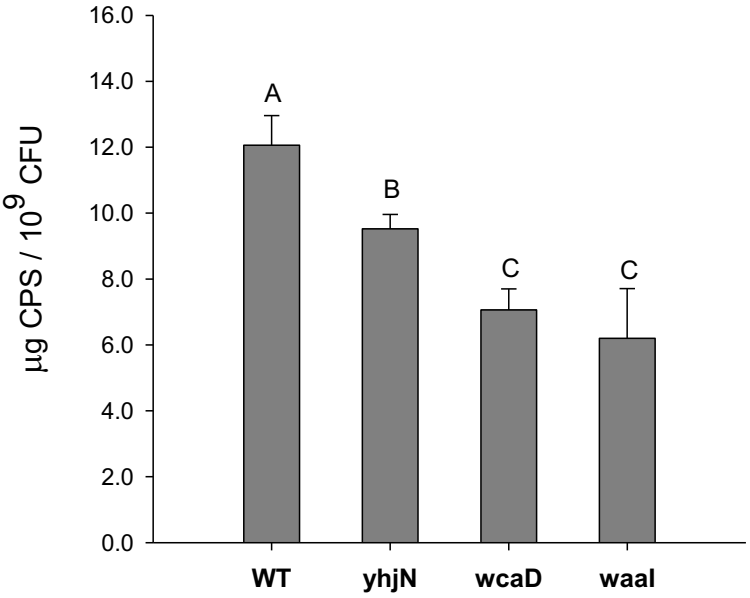

Figure 3.3. Quantitative analysis of capsular polysaccharide (CPS) production by *E. coli* O157:H7 strains: wild-type (WT), cellulose-deficient (yhjN), colanic acid-deficient (wcaD), and LPS-truncated (waaI). Data represents the mean of CPS production from three independent experiments with triplicate measurements. Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$.

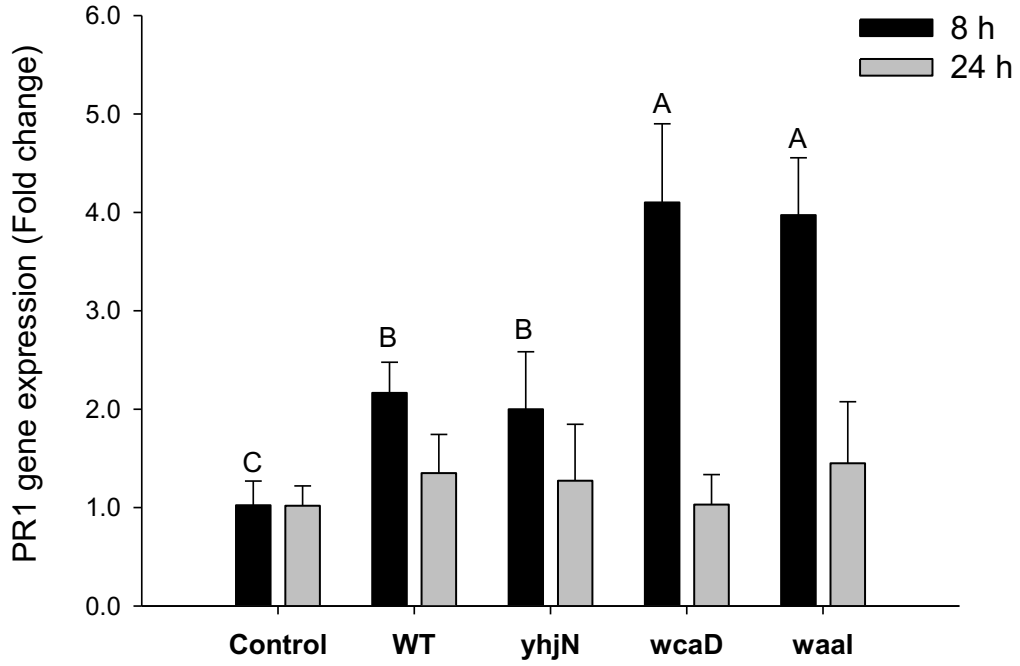

Figure 3.4. RT-qPCR analysis on expression levels of pathogenesis-related gene (*PR1*) by *Arabidopsis* plants at 8 (black) and 24 h (gray) post-inoculation in response to different strains of *E. coli* O157:H7; wild-type (WT), cellulose-deficient (yhjN), colanic acid-deficient (wcaD), and LPS-truncated (waaI). Data represents the mean fold change over control (mock treatment with SDW) from six replicate plants (n = 6). Error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$. There was no significant difference in the levels of *PR1* gene expression at 24 h among the four strains of *E. coli* O157:H7 ($P > 0.05$).

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CHAPTER 4.

Survival and interaction of *Escherichia coli* O104:H4 on *Arabidopsis thaliana* and lettuce: Influence of plant defense response and bacterial capsular polysaccharides

Hyein Jang and Karl R. Matthews

4.1. Abstract

Shiga toxin-producing *Escherichia coli* (STEC) has been associated with illnesses and outbreaks by fresh vegetables, prompting a growing public health concern. Most studies regarding interactions of STEC on fresh produce focused on *E. coli* O157:H7 strains. Limited information is available about survival or fitness of *E. coli* O104:H4 strain, an emerging pathogen that was linked to one of the largest outbreaks of hemolytic uremic syndrome in 2011. This study aimed to evaluate survival ability of *E. coli* O104:H4 strains on *Arabidopsis thaliana* plant and lettuce, and investigate potential influence of plant defense response and capsular polysaccharide (CPS) on the bacterial fitness on plant. Populations of *E. coli* O104:H4 strain (RG1, C3493, and LpfA) on *Arabidopsis* and lettuce were significantly ($P < 0.05$) greater than those of *E. coli* O157:H7 strains (7386 and sakai) at day 5 post-inoculation, indicating *E. coli* O104:H4 strains may have better survival ability on the plants. In addition, the *E. coli* O104:H4 strains produced significantly ($P < 0.05$) higher amounts of CPS compared with the *E. coli* O157:H7 strains. RG1 strain (1.5-fold) exhibited significantly ($P < 0.05$) lower expression of pathogenesis-related gene (*PR1*) indicating induction of plant defense response compared with *E. coli* O157:H7 strains 7386 (2.9-fold) and sakai (2.7-fold). Collectively, the results in this study suggests that different level of CPS production and plant defense response initiated by each STEC strain might influence the bacterial survival or persistence on plants. The present study provides better understanding of survival behavior of STEC, particularly *E. coli* O104:H4 strains, using a model plant and vegetable under pre-harvest environmental conditions with plant defense response.

4.2. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) strains can cause severe enteric diseases in human and foodborne outbreaks (Kaper et al., 2004; Smith et al., 2014). The clinical symptoms caused by STEC infection are typically diarrhea or can progress to hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS), which is related to high rate of hospitalization (Gyles, 2007; Nataro and Kaper, 1998). *E. coli* O157:H7 has traditionally been the most common serotype of STEC linked to foodborne disease outbreaks. However, infections with non-O157 STEC are noticeably recognized with greater frequency; incidence rate of non-O157 STEC infections were increased while the rate of O157 STEC infections decreased between 2000 and 2010 in the United States (Gould et al., 2013). Epidemiological studies showed that non-O157 serotypes may be responsible for 20–50% of STEC infections in the United States (Johnson et al., 2006) and proportion of non-O157 infections among confirmed cases of human STEC infections increased from 45.1% (2012) to 51.1% (2013) in Europe (EFSA, 2015). Therefore, the public health concern regarding the potential risk of non-O157 STEC strains has been worldwide raised to food industry and regulatory officials (van Elsas et al., 2011).

As an emerging strain of non-O157 STEC, *E. coli* O104:H4 was responsible for very large outbreaks of HUS and bloody diarrhea in 2011, predominantly in Germany and 14 other European countries, the United States, and Canada (WHO, 2011). The outbreaks resulted in 4,075 infections including 908 HUS cases and 50 deaths (CDC, 2013; WHO, 2011). In the United States, six cases of O104:H4 infections including one death with HUS have been reported and five cases were linked to travel to Germany

(CDC, 2013). Raw fenugreek sprouts were identified as the vehicle of transmission (EFSA, 2011). This newly observed *E. coli* O104:H4 was found to present the unique combination of genomic features of two virulence pathotypes, enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAEC). It contained Shiga toxin gene 2 (stx_2) and several marker genes of EAEC such as *aggR* but was negative for other EHEC typical markers such as stx_1 and *eae* (intimin) (Bielaszewska et al., 2011). The novel combination of virulence profiles and relevant phenotypes might lead to large outbreaks with augmented pathogenic potential (Bielaszewska et al., 2011).

Recent researches indicate that plant can be a potential habitat refuge for human enteric pathogens (Brandl et al., 2013), and more importantly fresh produce can serve as an important vehicle for transmission of STEC (Berger et al., 2010; Tyler and Triplett, 2008). Plants and vegetable crops can be contaminated with human pathogens such as STEC in pre- and post-harvest environments, which may lead to subsequent illnesses and outbreaks (Barak and Schroeder, 2012; Jung et al., 2014; Lynch et al., 2009). Since most fresh produce is consumed raw or minimally processed, contaminated produce can bring a potential health risks (Beuchat, 2006; Olaimat and Holly, 2012). It is quite evident that consumption of fresh produce contaminated with both O157 and non-O157 STEC can cause serious diseases in human (Cooley et al., 2007; van Elsas et al., 2011). Therefore, better understanding on interactions between STEC and plants or vegetables has become increasingly important. In particular, factors influencing fitness of human pathogens including survival/colonization in plants and molecular mechanisms of plant defense responses need to be elucidated (García and Hirt, 2014).

In recent years, the areas of microbial food safety and plant pathology have been integrated to gain deep knowledge about interactive mechanisms between human enteric pathogens and plant defense responses (Fletcher et al., 2013). *Arabidopsis thaliana* challenged with *E. coli* O157:H7 and *Salmonella* Typhimurium was shown to induce salicylic acid (SA)-dependent plant defense responses (Iniguez et al., 2005; Melotto et al., 2006; Roy et al., 2013). Bacterial cell surface structures of *E. coli* O157:H7 and *S.* Typhimurium, including flagella and extracellular polysaccharides, have been shown to have a role in activation of plant defense responses, thereby restricting bacterial colonization on plants (García et al., 2014; Seo and Matthews, 2012; Seo and Matthews, 2014, Meng et al., 2013). However, influence of bacterial surface structures on plant defense responses remains largely unknown.

Several studies have reported physiology, virulence factors, or pathogenicity of *E. coli* O104:H4 (Islam et al., 2016; Piérard et al., 2012), and the growth or fate in meat foods (Böhnlein et al., 2016; Luchansky et al., 2013; Porto-Fett et al., 2013) and radish sprouts (Xiao et al., 2014). Understanding the survival mechanism of *E. coli* O104:H4 on plant tissue is important in designing control strategies for crop safety. However, very little is known about behavior of survival and persistence of the emerging pathogen *E. coli* O104:H4 on plant systems, particularly with respect to plant defense response. In this study, we investigated the survival of *E. coli* O104:H4 strains compared with *E. coli* O157:H7 strains on *Arabidopsis thaliana* (a plant model) as well as on romaine lettuce (an edible crop plant) with plant defense response. Moreover, a possible role of bacterial capsular polysaccharide (CPS) in the plant-pathogen interaction was investigated.

4.3. Materials and methods

4.3.1. Bacterial strains and media

Three strains of *E. coli* O104:H4 (RG1, C3493, and LpfA) and two strains of *E. coli* O157:H7 (7386 and sakai) were used in this study (Table 4.1). The *E. coli* strains were kindly provided as follows: RG1 and C3493, U.S. FDA (Jackson et al., 2012); LpfA, University of Texas Medical Branch (Ross et al., 2015); 7386 and sakai (Yun et al., 2013). Each strain was grown in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD) overnight, and streaked onto Sorbitol MacConkey agar (Difco, MD) and incubated at 37°C for 20 h. A confirmed colony of each strain was transferred onto tryptic soy agar (TSA; Difco) and cultured in TSB (Difco) at 37°C for 20 h to prepare frozen stocks (20% glycerol, –70°C storage). The bacteria were cultured on TSA or in TSB at 37°C for 20 h with the following concentrations of antibiotics; ampicillin (100 μg/mL) for *E. coli* O104:H4 and nalidixic acid (100 μg/mL) for *E. coli* O157:H7.

4.3.2. Curli expression

Curli production of *E. coli* O104:H4 and *E. coli* O157:H7 strains was investigated using Congo red indicator (CRI) agar method (Hammar et al., 1996). CRI agar contained Bacto agar (Difco; 15 g/L), yeast extract (Bacto, Becton Dickinson, Sparks, MD; 1 g/L), casamino acids (Bacto; 10 g/L) with Congo red (MP Biomedicals, Solon, OH; 20 mg/L) and Coomassie brilliant blue (Bio-Rad Laboratories, Hercules, CA; 10 mg/L). Overnight cultures were streaked on the CRI agar. Following incubation at 25°C for 48 h, curli

expression was determined based on color of colonies; white or colorless indicates curli-negative and red represents curli-positive.

4.3.3. Plants and growth conditions

Arabidopsis thaliana and romaine lettuce (*Lactuca sativa* L. var. *longifolia*) were used to assess the colonization and survival of various *E. coli* strains on plants. *Arabidopsis thaliana* Columbia ecotype (Col-0, CS 70000) seeds were obtained from Arabidopsis Biological Resource Center (ABRC; Columbus, OH), while the romaine lettuce seeds (Parris Island Cos) was purchased from W. Atlee Burpee & Co. (Warminster, PA). Prior to planting, *Arabidopsis* seeds were submerged in sterile distilled water (4°C, 3 days) to promote germination and synchrony. Planting of the seeds and cultivation of the *Arabidopsis* was undertaken according to the standard guideline published by ABRC (ABRC, 2013). Sunshine Redi-earth professional growing mix soil (Sun Gro, Agawam, MA) and Fafard Canadian growing mix 2 (Sun Gro) were used for cultivation of *Arabidopsis* and lettuce plants, respectively. Growth conditions in a climate-controlled research greenhouse (Rutgers University, New Brunswick, NJ) were under 16 h-photoperiod (21 ± 2°C/day, 19 ± 2°C/night, with a relative humidity 65 ± 5%).

4.3.4. Inoculum preparation and bacterial challenge of plants

The *E. coli* O104:H4 and *E. coli* O157:H7 strains were cultured in two 30 mL volumes of TSB in the presence of relevant antibiotics at 37 °C for 20 h with agitation at 200 rpm. Bacterial cultures were centrifuged at 4,200 × g for 8 min (Allegra^{TM} 21R,

Beckman Coulter, Palo Alto, CA) and the supernatant was removed. The pellet was washed in 0.1% sterile peptone water (30 mL) twice, and then resuspended in sterile distilled water (540 mL) to achieve a final concentration of approximately 1.5×10^8 CFU/mL. For the bacterial challenge of the plants, 4-week old whole *Arabidopsis* and lettuce plants were dip-inoculated in the inoculum for 30 s and 60 s, respectively. *E. coli* O104:H4 and *E. coli* O157:H7 populations were determined on day 0, 1, and 5 following inoculation (Seo and Matthews, 2012). For each time point, whole *Arabidopsis* leaves (9 to 11) were aseptically collected, weighed, placed into a sterile sample bag (Whirl-Pak, Nasco, Fort Atkinson, WI), and added 5 mL of 0.1% peptone water added. The lettuce samples (5 to 6 leaves) were weighed, 10-fold diluted with 0.1% peptone water (w/v), and then homogenized (maceration by hand or with a pestle and stomaching for 1 min). After serial dilution (1:10), 100 µl aliquots were plated onto TSA with antibiotics in duplicate and incubated at 37°C for 18 h. Each experiment was performed independently two times and included three replicate plants per treatment.

4.3.5. Congo red staining of CPS

To examine capsular polysaccharide (CPS) of *E. coli* O104:H4 and *E. coli* O157:H7 cells, a negative staining method based on Maneval's capsule staining was used (Corstvet et al., 1982). Briefly, one drop of 1% Congo red solution and overnight *E. coli* cultures (20 µL) were mixed on a clean microscope slide and carefully spread across the slide. After 5 min drying, the smear was stained with Maneval's modified dye (Carolina Biological Supply Company, Burlington, NC) for 1 min and gently washed by sterile

distilled water. The dried slides were examined at 1,000 × magnification under oil immersion with a light microscope (Olympus BH2, Olympus Co., Tokyo, Japan).

4.3.6. Quantitative analysis of CPS

Extraction and purification of cell-associated CPS were performed with phenol-water extraction (Campos et al., 2004). The *E. coli* strains (O104:H4 and O157:H7) were grown in TSB (4 mL) at 37°C for 20 h with 200 rpm of agitation prior to harvest by centrifugation (5,000 × g, 10 min, 4°C). After removal of the supernatant, the pellets were washed in sterile distilled water (SDW; 1 mL). Resuspended in SDW (500 µL), serially diluted (10-fold), and 100 µL plated onto TSA in duplicate, and the plates were incubated at 37°C for 20 h to detect the number of bacteria (CFU/mL). The suspensions were placed in a water bathe (Thermo Scientific, Waltham, MA) at 68 °C for 2 min for preheating. The mixture with 500 µL of phenol (Amresco, Solon, OH) was further incubated at 68°C for 30 min and cooled down in an ice bath. For recovery of the aqueous phase, 500 µL of chloroform (99.8%; Fisher Chemical, Fair Lawn, NJ) was added, vortexed, and centrifuged (5,400 × g for 10 min). The CPS within the aqueous phase was precipitated by addition of three volumes of absolute ethanol (EMD Millipore, Billerica, MA) before storage at –20°C for 18 h. The precipitated CPS was collected through centrifugation at 14,000 × g for 20 min (4°C) with resuspension of SDW (500 µL). Quantification of CPS was performed using a phenol-sulfuric acid method to determine total carbohydrate (Saha and Brewer, 1994). The quantity of CPS was calculated as µg CPS per 10^9 CFU of bacterial cells using glucose solution standard curve.

All samples were analyzed in triplicate with three independent batches for each bacterial culture.

4.3.7. Determination of pathogenesis-related gene expression with RT-qPCR

Total RNA was extracted from *Arabidopsis* leaves at 8 h after inoculation with an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA concentrations were determined with a NanoDrop spectrophotometer (2000c, Thermo Scientific). One microgram of total RNA was used to synthesize cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with a 20 μL reaction volume. For reverse transcription (RT) reaction, thermal cycling conditions were as follows: 25°C (10 min), 37°C (120 min), 85°C (5 min), and 4°C hold. After synthesis of the cDNA, quantitative PCR (qPCR) was performed by a StepOnePlus™ Real-Time PCR System (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems). Forward and reverse primers used in this study were shown in Table 4.2. In a total reaction volume (25 μL), the final concentrations of cDNA and primers were 40 ng/μL and 200 nM, respectively. Cycling conditions are single cycle of 95°C (10 min), 40 cycles of 95°C (15 s) and 60°C (1 min). Relative gene expression levels were calculated using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001) with *ACT8* housekeeping gene serving as an internal control. Pathogenesis-related 1 (*PR1*; a target gene) expression was relatively expressed as a fold-change compared with mock treatment with water. The independent experiments were conducted in duplicate and each experiment included three replicate plants per treatment. Three replicates of plant

samples were analyzed and all samples including NTC (non-template control) were measured in triplicate.

4.3.8. Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SAS software (SAS University Edition; SAS Institute, Inc., Cary, NC). Duncan's multiple range test was used to evaluate statistical difference, among the treatment groups of *E. coli* strains, in the analyses of bacterial population on plants, CPS production, and *PR1* gene expression. Statistical significance was considered at a *P* value below 0.05.

4.4. Results

4.4.1. Different survival between *E. coli* O104:H4 and *E. coli* O157:H7 on plants

To compare the survival ability of *E. coli* O104:H4 (RG1, C3493, and LpfA) and *E. coli* O157:H7 strains (7386 and sakai) on plants, the bacterial populations in plant leaf tissues were determined over 5 days. Figure 4.1A shows the colonization of *E. coli* strains on *Arabidopsis* plant on day 0, 1, and 5 post-inoculation. The initial populations of *E. coli* O104:H4 and *E. coli* O157:H7 at day 0 were approximately 6.6 and 6.5 log CFU/g leaf tissue, respectively, and there was no significant difference among the *E. coli* strains on day 0 ($P > 0.05$). The populations of each *E. coli* strain on *Arabidopsis* were gradually decreased over a 5-day period, showing significant ($P < 0.05$) reduction between day 1 and 5.

In Fig. 4.1A, *E. coli* O104:H4 strains (RG1, C3493, and LpfA) showed better survival ability on *Arabidopsis* compared with *E. coli* O157:H7 strains (7386 and sakai) at day 1 and 5 post-inoculation. On day 5, the bacterial numbers of RG1 (4.1 ± 0.5 log CFU/g), C3493 (3.6 ± 0.4 log CFU/g), and LpfA (3.4 ± 0.1 log CFU/g) were significantly ($P < 0.05$) higher than those of 7386 (2.3 ± 0.3 log CFU/g) and sakai (2.3 ± 0.5 log CFU/g). With respect to influence of long polar fimbriae (Lpf) of *E. coli* O104:H4, there was no significant difference in the populations between C3493 (wild-type) and LpfA (isogenic mutant of C3493; lacking Lpf) on both day 1 and 5 ($P > 0.05$), which demonstrates that long polar fimbriae of *E. coli* O104:H4 did not have an important role on the survival on plant.

Fig. 4.1B shows the colonization study on romaine lettuce demonstrating the fitness or survival behavior of *E. coli* O104:H4 and O157:H7 strains on vegetable. The survival trends of *E. coli* O104:H4 and *E. coli* O157:H7 on lettuce were analogous to those on *Arabidopsis* (a model plant). From the mean initial population of *E. coli* strains (approximately 6.7 log CFU/g) at day 0, the bacterial numbers were consistently declined over 5 days with a significant ($P < 0.05$) reduction between day 1 and 5 within each strain. At day 1 post-inoculation, RG1 strain showed significantly ($P < 0.05$) higher populations compared with two *E. coli* O157:H7 strains (7386 and sakai). Along with the continuous decline, different survival ability between *E. coli* O104:H4 and *E. coli* O157:H7 strains on lettuce was evident on day 5; RG1 (3.8 ± 0.1 log CFU/g), C3493 (4.3 ± 0.3 log CFU/g), and LpfA (4.1 ± 0.4 log CFU/g) strains significantly ($P < 0.05$) survived better compared with 7386 (2.7 ± 0.1 log CFU/g), and sakai (2.8 ± 0.3 log CFU/g). This resulted in a total reduction of 4.1 log CFU/g in 7387 strain and 3.7 log CFU/g in sakai

over 5 days while RG1, C3493, and LpfA had a total reduction range of 2.5 to 3.0 log CFU/g.

4.4.2. Analysis of capsular polysaccharide (CPS) produced by *E. coli* O104:H4 and *E. coli* O157:H7

CPS production by *E. coli* O104:H4 and *E. coli* O157:H7 cells were examined by Congo red negative staining (Fig. 4.2) and quantitatively evaluated (Fig. 4.3). Clear halos surrounding the cells demonstrate the presence of CPS when each strain was cultured in TSB for 20 h. In Fig. 4.2, *E. coli* O104:H4 cells (RG1, C3493, and LpfA) represented larger size of CPS (clear zone) compared with *E. coli* O157:H7 cells (7386 and sakai). In the analysis of CPS quantification (Fig.4.3), three *E. coli* O104:H4 strains were shown to produce significantly ($P < 0.05$) greater amount of CPS compared with *E. coli* O157:H7 strains as follows: RG1 (12.3 ± 1.0 μg CPS/10^9 CFU), C3493 (13.3 ± 1.1 μg CPS/10^9 CFU), LpfA (11.9 ± 1.0 μg CPS/10^9 CFU), 7386 (6.2 ± 0.7 μg CPS/10^9 CFU), and sakai (4.5 ± 0.5 μg CPS/10^9 CFU). The microscopic observation of CPS staining revealed the presence or secretion of capsular material, which accords with the quantified amounts of CPS produced by each *E. coli* strain (Fig. 4.2 and 4.3).

4.4.3. Induction of plant defense response by *E. coli* O104:H4 and *E. coli* O157:H7

To investigate induction level of plant defense response, expression of pathogenesis-related gene (*PR1*, a marker gene of *Arabidopsis* plant immunity) was analyzed by a reverse transcription quantitative PCR (RT-qPCR). Fig. 4.4 shows the

relative expression level of *PR1* gene in *Arabidopsis* plant leaves at 8 h after inoculation with each *E. coli* strain, compared to water treatment (control). Plants inoculated with *E. coli* O157:H7 strains, 7386 (2.9-fold) and sakai (2.7-fold), exhibited a significantly ($P < 0.05$) greater expression of *PR1* gene than RG1 of *E. coli* O104:H4 (1.5-fold). Other *E. coli* O104:H4 strains C3493 and LpfA resulted in a 1.7-fold and a 1.9-fold significantly ($P < 0.05$) higher level of *PR1* gene expression compared to control, respectively. However, there was no significant difference ($P > 0.05$) between the *E. coli* O104:H4 strains and control.

4.5. Discussion

Fresh produce can be transmission vectors of STEC strains, which is associated with a growing burden of foodborne outbreaks (Berger et al., 2010; Lynch et al., 2009). Studies regarding attachment or fitness of *E. coli* O157:H7 on fresh produce are relatively well-documented, but there is a continuing need to explore survival mechanisms of non-O157 STEC such as an emerging pathogen *E. coli* O104:H4.

In this study, we compared the survival behavior of *E. coli* O104:H4 and *E. coli* O157:H7 strains on *Arabidopsis* and romaine lettuce, and further determined induction of plant defense response that regulates microbial survival/persistence to understand molecular responses of plants to STEC strains. The populations of *E. coli* O104:H4 and *E. coli* O157:H7 strains on both *Arabidopsis* and lettuce were shown to gradually reduced over 5 days (Fig. 4.1A and B), which indicates that the STEC strains might have less fitness to plant compared with phytopathogens. For instance, previous research demonstrated that *Pseudomonas syringae* pv. tomato DC3000, a phytopathogen, persists

on *Arabidopsis* plant with no reduction of population during 5 days (Seo and Matthews, 2012). In terms of comparison in survival ability between *E. coli* O104:H4 and *E. coli* O157:H7, *E. coli* O104:H4 strains survived better compared with *E. coli* O157:H7 strains on both *Arabidopsis* and lettuce at day 5 post-inoculation ($P < 0.05$). Similarly, Markland et al. (2012) showed that *E. coli* O104:H4 was detectable at 10 day on basil plant whereas *E. coli* O157:H7 did not recovered, indicating *E. coli* O104:H4 isolates may have enhanced fitness to plant. In addition, *E. coli* O104:H4 strain was shown to persist longer than *E. coli* O157:H7 on fermented sausages up to 60 days (Böhnlein et al., 2016). *E. coli* O104:H4 strains have been reported to produce stable biofilm on abiotic surfaces (Nagy et al., 2016) and *in vivo* with higher density of colonization compared to *E. coli* O157:H7 (Safadi et al., 2012). Biofilm formation of *E. coli* O104:H4 might be associated with potential mechanisms of interactions with plant or other food-related environments in helping the strains to persist and survive.

It is important to understand roles of bacterial surface polysaccharide in plant-pathogen interactions since the outermost structure may initially encounter plant host defense and environments (D'Haeze and Holsters, 2004). In this study, capsular polysaccharide (CPS; tightly associated with cell surface) produced by *E. coli* O104:H4 and O157:H7 was examined using congo red staining and quantitatively measured. It revealed that *E. coli* O104:H4 strains (RG1, C3493, and LpfA) showing better survival produced significantly ($P < 0.05$) higher amounts of CPS with larger size of CPS surrounding cells, compared with *E. coli* O157:H7 strains 7386 and sakai (Fig. 4.2 and 4.3). Bacterial CPS can provide physical layer to adverse environmental conditions such as oxidative stress, desiccation, and antimicrobial peptides or host immune responses

(Campos et al., 2004; Whitfield, 2006). In other words, higher level of CPS production might contribute to survival ability on plants because CPS or extracellular polysaccharides can act as protective barriers to plant defense-related metabolites such as reactive oxygen species, phytoalexins, and hydrolytic enzymes (chitinase and β-1,3-glucanase) (D'Haeze and Holsters, 2004; Tarchevsky, 2001). Another possible explanation regarding role of CPS in the bacterial survival is that CPS structure may negatively affect induction of plant defense responses by masking conserved bacterial cell surface structures (known as pathogen-associated molecular patterns; PAMPs) triggering plant immunity, although the details have not been fully elucidated. It is shown that polyanionic properties of bacterial extracellular polysaccharides could suppress PAMP-triggered immunity in plant through chelation of intracellular calcium ions, thereby decreasing plant defense signaling (Aslam et al., 2008).

In the interactions between plant host and pathogen, plant defense responses can be key factors influencing the survival/persistence of pathogens. Upon recognition of structural components of microorganisms (called as PAMPs or MAMPs; microbe-associated molecular patterns) through pattern recognition receptors (PRRs), plant immune responses can be activated (Pel and Pieters, 2013). For instance, research demonstrated that flagella of human enteric pathogens such as *E. coli* O157:H7 and *Salmonella* can induce plant defense responses, consequently limiting the bacterial colonization on plants (García et al., 2014; Meng et al., 2013; Seo and Matthews, 2012). In this study, we observed different expression level of *PR1* gene on *Arabidopsis* leaves between *E. coli* O104:H4 and *E. coli* O157:H7 challenges (Fig. 4.4). The plant defense gene (*PR1*) encodes antimicrobial PR protein and is a molecular marker of systemic

acquired resistance (SAR; a wide spectrum of plant immunity) (Durrant and Dong, 2004). *E. coli* O104:H4 RG1 strain induced lower expression of *PR1* gene compared with *E. coli* O157:H7 strains (7386 and sakai), suggesting the lower induction level of plant defense response may contribute to greater colonization and survival on plant.

In order to extensively understand the survival mechanisms of *E. coli* O104:H4, various environmental factors should be considered. It has been shown that non-O157 strains, including *E. coli* O104:H4, exposed to sub-lethal osmotic, acid, or chlorine stresses brought improved bacterial growth on produce (Yoo et al., 2015) and higher cytotoxicity with expression of virulence genes of *stx1*, *stx2*, and *eae* (Yoo et al., 2017). Treatment with hydrogen peroxide to STEC strains on lettuce showed downregulated expression of genes associated with oxidative stress and virulence (Mei et al., 2017). Moreover, *E. coli* O104:H4 was shown to have higher cell viability than *E. coli* O157:H7 under conditions of sodium nitrite (Böhnlein et al., 2016). These studies provide information about resistance or virulence abilities of *E. coli* O104:H4 that may be related to different behavior of survival and persistence. However, most of the studies have been conducted *in vitro* or with post-harvest produce. Therefore, additional information on adaptation mechanisms to environmental conditions that a pathogen may encounter during grwoth is required, which can serve to account for differences in survival or fitness as well as developing pre-harvest intervention strategies.

Table 4.1. Bacterial strains used in this study

Strain	Description
E. coli O104:H4	Amp^r
RG1	wild-type, clinical isolate, bloody diarrhea, Republic of Georgia, 2009
C3493	wild-type, clinical isolate during German sprout outbreak, HUS, US, 2011
LpfA	Δ*LpfA1*, isogenic mutant of C3493, lacking long polar fimbriae
E. coli O157:H7	Nal^r
7386	wild-type, romaine lettuce outbreak, US, 2008
sakai	wild-type, sprout outbreak, Japan, 1996

Amp^r, ampicillin resistant (100 μg/mL); Nal^r, nalidixic acid resistant (100 μg/mL).
C3493, LpfA, 7386, and sakai strains were curli-negative but RG1 strain was curli-positive based on CRI agar method.

Table 4.2. PCR primer sequences used in this study

Primers	Sequences (5'-3')	Reference
ACT8 (Forward)	TTCCGGTTACAGCGTTTGGAGAGA	Roy et al., 2013
ACT8 (Reverse)	AACGCGGATTAGTGCCTCAGGTAA	
PR1 (Forward)	CTTGTTCTTCCCTCGAAAGCTCAAGATAGC	
PR1 (Reverse)	GAGCATAGGCTGCAACCCTCTC	

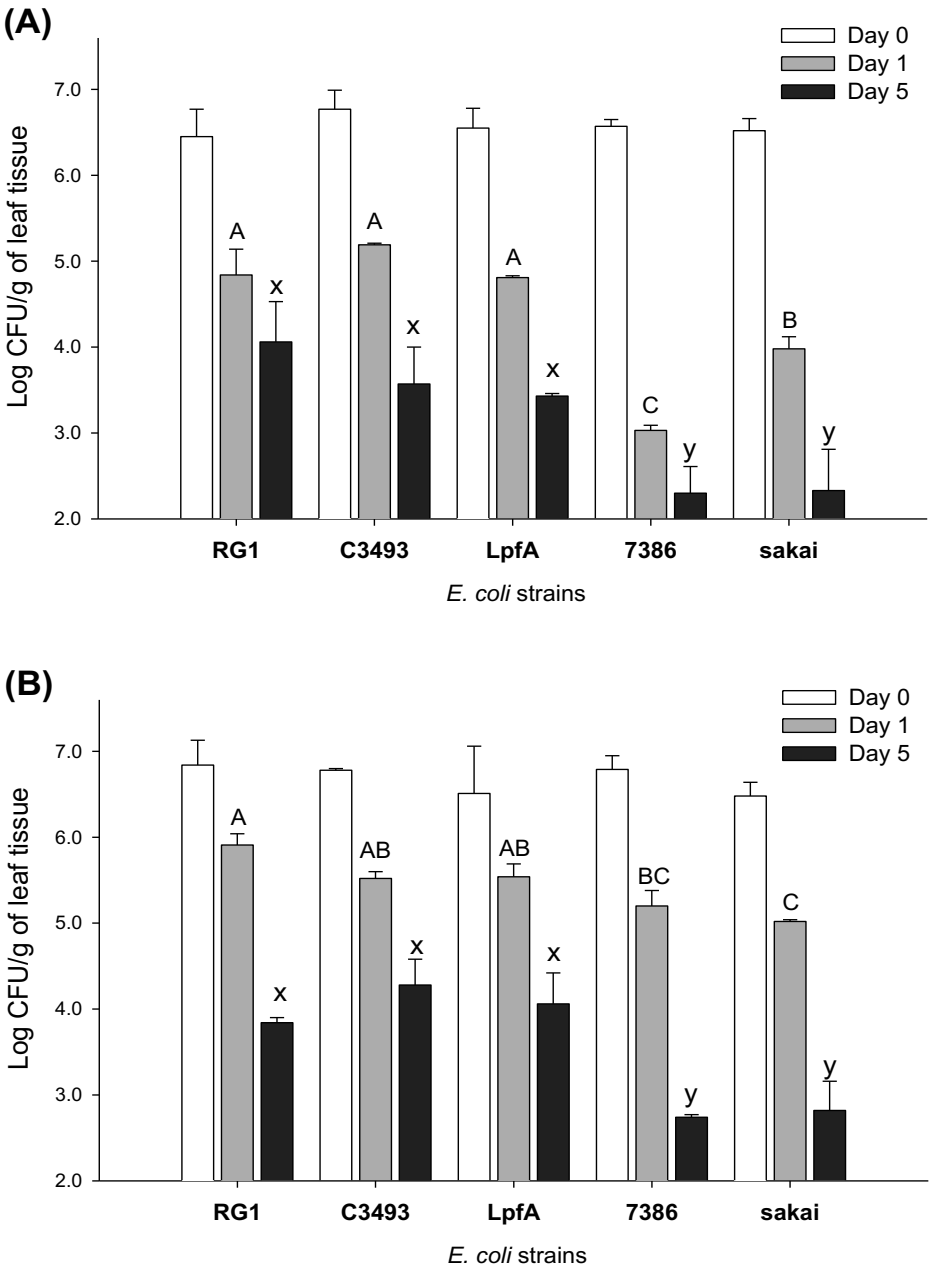

Figure 4.1. Populations of *E. coli* O104:H4 and *E. coli* O157:H7 strains on *Arabidopsis thaliana* plants (A) and romaine lettuce (B) at day 0, 1, and 5 post-inoculation. *E. coli* O104:H4 strains include RG1, C3493, and LpfA. *E. coli* O157:H7 strains include 7386 and sakai.

Figure 4.1 (Continued). Data shows the mean of bacterial populations from two independent experiments with three replicate plants and error bars indicate the standard deviations. Different letters indicate significant differences at $P < 0.05$. Uppercase letters (A, B, C) show the significant differences on day 1 between strains and lowercase letters (x, y, z) show the significant differences on day 5 between strains. Significant differences by strain between day 1 and day 5 are indicated by different letters. There was no significant difference in populations among the five *E. coli* strains of on day 0 ($P > 0.05$).

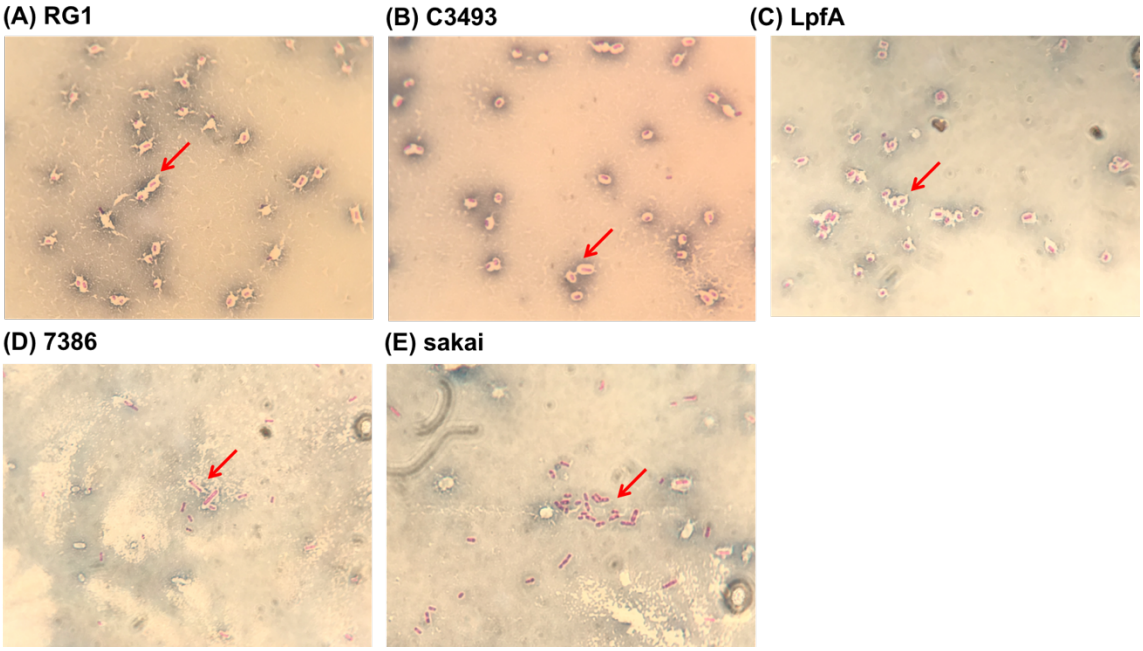

Figure 4.2. Congo red negative staining of capsular polysaccharide (CPS) produced by *E. coli* O104:H4 and *E. coli* O157:H7 strains; RG1 (A), C3493 (B), LpfA (C), 7386 (D), and sakai (E). Clear zones surrounding cells demonstrate the presence of CPS as denoted by arrows.

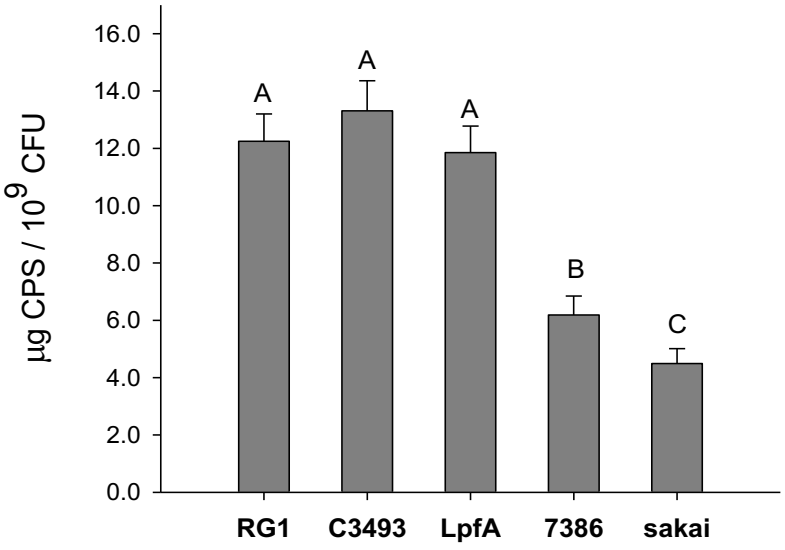

Figure 4.3. Quantification of capsular polysaccharide (CPS) produced by *E. coli* O104:H4 (RG1, C3493, and LpfA) and *E. coli* O157:H7 strains (7386 and sakai). Data represents the mean of CPS production and standard deviation (error bars) from three independent experiments. Different letters indicate significant differences at $P < 0.05$.

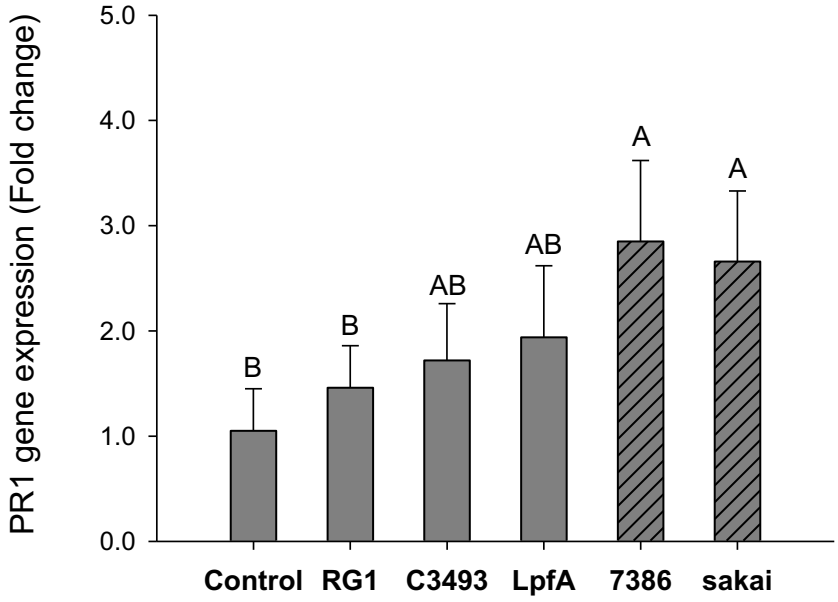

Figure 4.4. Relative expression levels of pathogenesis-related gene (*PR1*) in *Arabidopsis thaliana* plants at 8 h after challenge with water (control), *E. coli* O104:H4 strains (RG1, C3493, and LpfA), and *E. coli* O157:H7 strains (7386 and sakai). Data represents the mean of fold change and standard deviation (error bars) from three independent experiments. Different letters indicate significant differences at $P < 0.05$.

4.6. Reference

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