

The role of the bacterial effector protein DspE in initiation of soft-rot caused by
Pectobacterium carotovorum on leaves of solanaceous plants

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THESIS ABSTRACT

Pectobacterium species are enterobacterial plant pathogens that cause soft rot disease in diverse plant species. The type III secretion system (T3SS) of *Pectobacterium carotovorum* subsp. *carotovorum* (*P. carotovorum*) appears to secrete only one effector protein, DspE. HrpL, the alternative sigma factor responsible for regulating the T3SS operon, and DspE are required for *P. carotovorum* pathogenesis on host leaf tissue. In this work, we present data that support the hypothesis that the role of DspE is to initiate pathogenesis by causing host cell death via autophagy. To initiate autophagy, DspE requires its full length and WxxxE-like motifs, which are important for effector proteins in diverse bacterial pathogens of plants and animals. Further, analysis of *P. carotovorum* gene expression *in planta* indicated that HrpL and DspE expression activates expression of a large number of genes outside of the T3SS operon, suggesting that host cell death caused by DspE subsequently activates expression of a large number of genes involved in soft-rot pathogenesis. Deletion of *dspE* eliminates the ability *P. carotovorum* to cause symptoms on leaves, however, when leaf tissue was constantly misted with water after inoculation, the *dspE* mutant strain was able to initiate disease, suggesting that acquisition of *dspE* and the T3SS enables *P. carotovorum* to initiate disease at lower ambient humidity.

DEDICATION

To my wife, Kate...

To my parents...

To my family...

Thank you!

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LIST OF ABBREVIATIONS

- AHL**- Acyl homoserine lactone
- Acyl-HSL QS**- Acyl-homoserine lactone quorum sensing
- D**- Aspartic acid
- DFI**- Differential fluorescence induction
- ERMRS**- Endoplasmic reticulum membrane retention signal
- ET**- Ethylene
- ETI**-Effector triggered immunity
- FACS**- Fluorescence activated cell sorter
- GM**- Genetically modified
- E**- Glutamic acid
- GEF**- Guanine nucleotide exchange factor
- hrp**- Hypersensitive response and pathogenicity
- HR**- Hypersensitive response
- JA**- Jasmonic acid
- LRR**- Leucine-rich repeat
- PCWDE**- Plant cell wall degrading enzymes
- PTI**- Pathogen associated molecular pattern triggered immunity
- RT-qPCR**- reverse transcriptase quantitative PCR
- RLK**- Receptor-like threonine kinase
- RER**- Relative expression ratio
- SA**- salicylic acid
- SRE**- Soft-rot *Enterobacteriaceae*

W- tryptophan

T3SS- Type three secretion system

Y- Tyrosine

Chapter 1- Introduction to Soft Rot Pathology

The soft rot enterobacteriaceae (SRE) are broad host range pathogens of many plant species and can be found infecting crops in a wide range of climates (Toth, Bell, Holeva, and Birch 2003). The SRE include two genera, *Pectobacterium* and *Dickeya*, and *P. carotovorum* is one of the most common and widespread species within this group (Toth, Bell, Holeva, and Birch 2003). These pathogens regularly incur large economic losses in fruit, vegetable, and ornamental crop agriculture. Soft rot has been responsible for severe losses to the Wisconsin potato industry in particular, to the extent that the Wisconsin Potato and Vegetable Growers Association (WPVGA) has financed the creation of a research facility devoted to developing crop storage methods that prevent or reduce losses due to soft rot and similar diseases. There are currently no effective control methods for these pathogens, but insights into the molecular mechanisms by which plant pathogens cause disease can lead to the development of novel biotechnological approaches to crop protection (Dodds and Rathjen 2010). The type III secretion system is critical for pathogenesis of many pathogens, but little is known concerning its role in soft rot pathogenesis. Therefore the goal of this work was to define the role of the type III secretion system, and its secreted effector protein DspE, in *Pectobacterium carotovorum* pathogenesis.

Pectobacterium* and the soft rot *Enterobacteriaceae

Soft rot enterobacteria are responsible for economically significant losses in many crops both during the growing season, causing a stem disease known as black leg in

potato, and causing soft rot in stored vegetable crops (Perombelon and Kelman 1980; Czajkowski, Pérombelon, van Veen, and van der Wolf 2011). These bacteria are characterized by their coordinate production of a large array of plant cell wall degrading enzymes (PCWDEs) secreted primarily through the type II secretion system; though other virulence factors are secreted as well (Charkowski et al. 2012). The SRE are separated into two recently defined genera, *Pectobacterium* (formerly *Erwinia carotovora*) and *Dickeya* (formerly *Erwinia chrysanthemi*) (Samson et al. 2005). SRE are considered necrotrophic plant pathogens as they obtain nutrients from the dead host cells. In contrast, pathogens classified as biotrophs thrive on nutrients obtained from live host cells. A third group, hemibiotrophs, act as biotrophs early in infection and later switch to a necrotrophic strategy.

SRE are considered archetypical broad host range necrotrophs (Mengiste 2012). They can be found on plant surfaces and in soil, from which they enter the host through wound sites or natural opening in the plant surface. The pathogen can also be introduced via contaminated seed tubers used in potato production (Toth, Bell, Holeva, and Birch 2003). Following entry, they reside inside the plant tissue until environmental conditions, such as free water and oxygen availability, become suitable for pathogenesis. When conditions become suitable for disease, they coordinately activate expression of a large number of virulence factors (Table 1), causing host cell death and tissue maceration characteristic of soft rot (Fig. 2). SRE utilize multiple plant cell wall degrading enzymes (PCWDE), such as pectinases, cellulases, and proteases, which break down plant cell walls and release nutrients for bacterial growth (Toth and Birch 2005; Perombelon and Kelman 1980). The ability of these pathogens to communally regulate production of

large amounts of these PCWDE at key points during pathogenesis is essential for successful infection. This activity is coordinated in large part by the cell density dependent regulatory mechanism known as quorum sensing (QS) (Liu et al. 2008). The constitutively expressed gene *expI*, homologous to *luxI* in *Vibrio fischeri*, encodes an enzyme that produces an acyl-homoserine lactone (acyl-HSL) at low basal levels, which acts as a small diffusible signal. Concentration of the acyl-HSL increases with bacterial density, and when the concentration of the molecule reaches a threshold level (indicating the population density is sufficient to initiate pathogenesis), expression of PCWDE and other pathogenesis associated genes are activated. Further, the acyl-HSL product of *expI* positively regulates its own expression, thus creating a positive feedback loop, and resulting in the dramatically increased production of the signaling molecule, and consequently activating production of large quantities PCWDE (Liu et al. 2008).

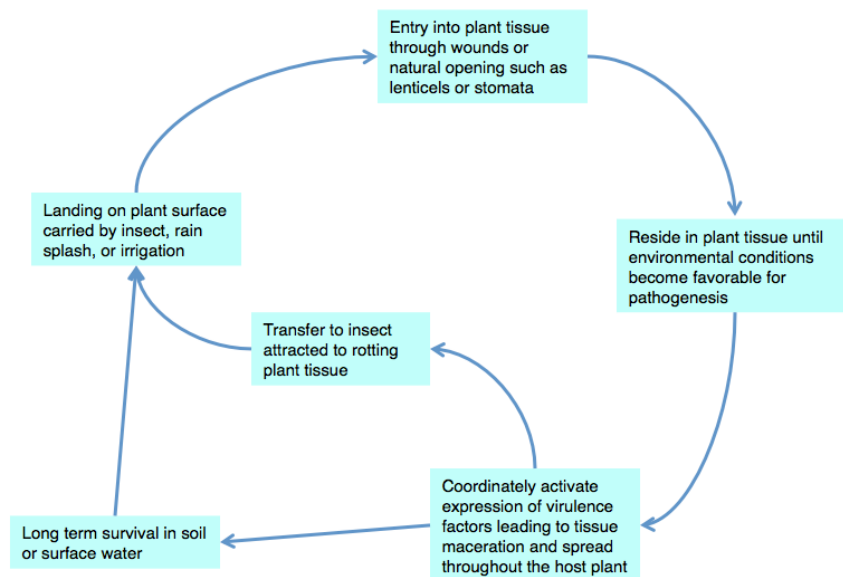


Figure 11 Soft rot enterobacteriaceae life cycle

This study focuses on *Pectobacterium carotovorum* as a model system for necrotrophic plant-microbe interactions as it is considered one of the most important SRE

due to its broad host range and global distribution (Toth, Bell, Holeva, and Birch 2003). In addition to its agricultural significance, this gram-negative bacterium is an excellent candidate for study as it grows quickly, and provides consistent results in virulence assays. As members of the *Enterobacteriaceae* family, which also includes human pathogens, they are amenable to genetic manipulation using techniques established for *Escherichia coli* (Glasner et al. 2008; Kim, Thammarat, Lommel, Hogan, et al. 2011). The genome of *P. carotovorum* has been sequenced and is publically available, further supporting the utility of this pathogen as a model organism for bacterial soft rot.

Plant Immunity

Plants in the field are attacked by a wide variety of bacterial and fungal pathogens. To defend themselves, they have developed diverse mechanisms, some of which are activated only in response to certain pathogens. Regulation of these defenses is important for plant fitness, as some of these defense responses have deleterious effects on the host, and activation of inappropriate defenses may enhance, rather than limit, disease.

The plant cell wall is a dynamic set of polymers that encases the host cell and serves as the first line of defense against pathogenic attack. Plants can alter the composition and molecular structure of cell walls to enhance resistance to pathogen invaders. For example, altering pectin methylation in cell walls by overexpressing pectin methylesterase inhibitors in *Arabidopsis* enhances resistance to the fungal necrotrophic pathogen *Botrytis cinerea*, as does decreasing acetylation in cell wall polymers (Lionetti et al. 2007; Manabe et al. 2011). Also, cell wall components released by PCWDE can

trigger defense responses in neighboring cells, bolstering the immune response and increasing the likelihood of containing the infection (Aziz et al. 2007).

Active plant defense responses begin with recognition of the pathogen by the host, often through microbe (or pathogen) associated molecular pattern recognition. Recognition can occur through binding of a pathogen epitope, such as flagellin, to a host cell surface receptor protein, which then activates the immune response in a process known as pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). Many gram-negative bacterial plant pathogens encode effector proteins, and these effectors are secreted into host cells where they may modify or target for degradation plant proteins required for PTI.

Host cells also encode resistance genes (*R* gene) that perceive the activity of pathogen effector proteins and, if perceived, trigger a stronger immune response known as effector triggered immunity (ETI). A number of plant defense responses that limit pathogen growth and development of disease symptoms are activated following ETI. Early investigations proposed a receptor-ligand model to explain the mechanism by which resistance proteins functioned, and observations of some resistance proteins support this model, such as the interaction between *Arabidopsis* RRS1-R and bacterial pathogen effector Pop2 from *Ralstonia solanacearum*, which results in ETI (van der Hoorn and Kamoun 2008; Deslandes et al. 2003). However, direct binding to pathogen effectors has not been observed for the majority of *R* gene products, leading to the conclusion that the perception of pathogen effectors by *R* proteins can occur through indirect interaction. Consequently, the guard model was proposed in which *R*-proteins detect the biological activities of bacterial effectors on host targets, or perturbations to

host cell homeostasis, rather than directly binding to pathogen effectors themselves (van der Hoorn and Kamoun 2008). This model is supported by studies showing that the proteins encoded by R-genes RPM1 and RPS2 detect effector-induced cleavage of the host RIN4 protein in *Arabidopsis* (Kim et al. 2005).

Later findings showing that bacterial effectors can have multiple host targets, led to development of the decoy model, which proposes that plants evolved proteins that have no role in pathogen virulence, but mimic the properties of host targets targeted by effectors and are monitored by proteins encoded by R-genes. Following recognition of pathogen attack, the host activates a complex set of overlapping immune responses and regulatory networks, as well as synthesizes and releases a multitude of virulence determinants with a range of host targets. Recent work has revealed critical regulators and cellular processes that are involved in plant immunity (Mengiste 2012). These regulators are responsible for specifically activating only those plant defense responses that are effective against the invading pathogen, which can include reinforcement of cell walls through deposition of callose and lignin, as well as production of antimicrobial compounds, in addition to HR.

Through little is known about the mechanisms underlying disease-associated cell death, recent studies indicate that autophagy plays a role in stress responses, including pathogen-induced stress. Autophagy (self-eating) is the process by which cells degrade and recycle proteins and other cellular components. This process is carried out by double membrane acidic vesicles termed autophagosomes that engulf their targets and delivering them to vacuoles for degradation (Levine and Yuan 2005). By generating reserves of nutritional building blocks and maintaining energy homeostasis under stressful

conditions, autophagy increases plant tolerance to a range of environmental conditions, and physiological and pathological stresses (Mizushima 2007).

ETI and PTI often result in the release of reactive oxygen species (ROS) known as the oxidative burst. This burst is a component of the hypersensitive response (HR), which is an immune response that utilizes programmed cell death to combat infection (Glazebrook 2005). The activation of HR causes membrane perturbation leading to calcium influx and pH changes, as well as biosynthesis of antimicrobial compounds and eventually cell death (Cuesta Arenas et al. 2010). *R* gene-mediated resistance is effective against biotrophic and hemibiotrophic pathogens and is associated with activation of a salicylic acid (SA)- dependent signalling pathway. Other plant defense responses are controlled by ethylene (ET) and/or jasmonic acid (JA), and have considerable overlap with plant responses to wounding and herbivory. The relationship between SA, ET and JA signalling pathways is complex, and no single pathway is solely responsible for governing defense against any particular pathogen.

The plant response to necrotrophs

Plants employ both constitutive and induced responses to defend against necrotrophic attack. The cell wall provides basal resistance against necrotrophic infection, and is a primary target of necrotrophic pathogens, which secrete PCWDE that are required for successful infection. Plant defenses include inhibitors that interfere with secreted pathogen enzymes, such as polygalacturanase inhibiting proteins (PGIPs) that counteract pathogen derived polygalacturonases (Ferrari 2002). The cell wall is also

involved in inducing plant defenses, and cell wall fragments released due to damage can contribute to activation of defense against necrotrophs. Pre-treatment of *Arabidopsis* plants with cell wall fragments prior to infection with *B. cinerea* has been shown to increase resistance to infection, though the mechanisms by which these fragments elicit defenses remain unknown (Ferrari et al. 2007).

Successful defense against pathogen attack requires the plant host response to be tailored to the invading pathogen, as

responses that are effective at controlling certain pathogen, may in fact promote disease caused by a different pathogen.

Though the HR response is effective at combating invasion by many biotrophic

pathogens through limiting the availability of living cells from which they can obtain nutrients, this programmed cell death

response can facilitate attack by necrotrophs, by providing them with dead tissue from which to feed (Govrin and Levine 2000) (Fig. 2). Similarly, the kinetics of the oxidative burst play an important role in defining the outcome of pathogen attack, as evidence indicates that ROS produced early in infection promotes resistance to both bio- and necrotrophs, whereas ROS produced later in infection promotes necrotrophic growth and enhances PCWDE activity, but enhances resistance to biotrophs (Lamb and Dixon 1997).

The response elicited by *P. carotovorum* has many of the same characteristics of the HR, such as induction by a type III secreted effector and requirement of a functional

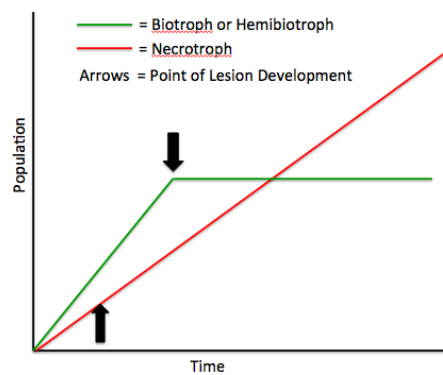


Figure 2 Idealized growth curve showing how lesion development resulting from HR inhibits growth for biotrophic pathogens but stimulates growth for necrotrophs

SGT1 gene in the host plant (Oh, Martin, and Beer 2007). Plant mutants with enhanced cell death show increased resistance to biotrophic pathogens, but increased susceptibility to necrotrophs; though interestingly, HR is associated with resistance to hemibiotrophs *Phytophthora infestans* and *Magnaprothe oryzae* (Veronese et al. 2006; Jia, McAdams, Bryan, Hershey, et al. 2000; Vleeshouwers, van Doijeweert, Govers, Kamoun, et al. 2000). Since this response normally results in the elimination of the pathogen from host tissue, it is possible that the response elicited by *P. carotovorum* differs from the HR in subtle yet important ways. Additionally, the pathogen may encode genes whose products enable *P. carotovorum* to survive the host response. Consequently, inhibition of plant cell death is a possible avenue for developing soft rot resistance, although a greater understanding of the role of DspE is needed to explore this possibility. DspE could cause host cell death through several pathways: DspE could be interacting with a single dominant plant resistance protein or its guarded target, thereby activating programmed host cell death. Alternatively, DspE could be directly toxic to plant cells, causing cell death by disrupting a process essential to maintaining cell viability, such as membrane integrity. To cause cell death, DspE requires the host protein SGT1, a central component of plant defense signaling pathway, suggesting that it is more likely that DspE acts by activating programmed cell death, rather than interfering with cell viability (Oh, Martin, and Beer 2007).

To date only one R-gene has been associated with resistance to a necrotrophic pathogen, the *Arabidopsis* RLM3 gene which confers resistance to *B. cinerea* (Staal, Kaliff, Dewaele, Persson, et al. 2008). This suggests that ETI is not an effective immune response to necrotrophs, and may in fact enhance susceptibility by promoting cell death.

Importantly, genes conferring resistance to biotrophic pathogens have been shown to enhance susceptibility to necrotrophic pathogens (Veronese et al. 2006). The necrotrophic fungi *Periconia circinata*, *Cochliobolus vicotriiae*, *Pyrenophora tritici-repentis*, and *Stagonospora nodorum* take advantage of ETI by producing toxins that bind to host R-like NB-LRR protein to promote pathogenesis (Nagy et al. 2007; Lorang, Sweat, and Wolpert 2007; Faris et al. 2010). Taken together these data indicate that necrotrophs exploit host immune responses to promote virulence. Genes that confer resistance to necrotrophs could work by encoding proteins that deactivate toxins, encode toxin-insensitive alleles or encode proteins that enhance cellular process that restrict cell death (Wolpert, Dunkle, and Ciuffetti 2002; Johal and Briggs 1992; Brandwagt et al. 2000).

Programmed cell death plays an important role in plant defense, and deregulating cell death enhances susceptibility to necrotrophs. Pathways involved in regulating autophagy, a form of programmed cell death, also regulate HR, ROS production and basal immunity (Liu et al. 2005; Kabbage, Williams, and Dickman 2013). *B. cinerea* induces expression of autophagy genes and the formation of autophagosomes in *Arabidopsis* (Lai, Wang, Zheng, Fan, et al. 2011). Mutations in plant autophagy genes enhance *B. cinerea* and *Alternaria brassicicola*-induced necrosis, though interestingly these plant mutants display dramatically increased resistance to powdery mildew (Wang, Wu, and Tang 2011; Lenz et al. 2011). *Sclerotinia sclerotiorum*, which induces formation of autophagosomes, uses oxalic acid to suppress this defense response further highlighting the importance of autophagy in defense against necrotrophic pathogens (Kabbage, Williams, and Dickman 2013).

The role of plant hormones is essential for effective plant immune responses, though their function in modulating resistance to necrotrophs is complex and poorly understood. Many necrotrophic pathogens synthesize plant hormone mimics to modulate host plant defenses (Grant and Jones 2009; Sharon, Elad, Barakat, and Tudzynski 2007). Elevated levels of salicylic acid (SA) have been associated with increased susceptibility to necrotrophic pathogens, though it enhances resistance to biotrophs and hemibiotrophs (Veronese et al. 2006). *B. cinerea* exopolysaccharide promotes pathogenesis by activating the SA pathway and subsequently reduces JA signaling (El Oirdi et al. 2011). The importance of JA in resistance to necrotrophs has been well established, though it is associated with responses to wounding and herbivory. Following attack, JA synthesis increases and results in expression of defense associated genes such as protease inhibitors (El Oirdi et al. 2011; Glazebrook 2005). ET signaling has also been linked to resistance to necrotrophs, and is involved in perception of necrotrophic pathogens and subsequent activation of PTI and cell wall reinforcement (Lloyd et al. 2011; Zhao and Guo 2011). The transcription factor ERF1 integrates signals from JA and ET, and its over expression results in activation of many defense related genes (Berrocal-Lobo, Molina, and Solano 2002). Other hormones are involved in determining the outcome of necrotrophic attack on plant hosts. Absciscic acid deficient mutant plants are resistant to *B. cinerea* infection but are more susceptible to infection by the biotrophic bacterium *Ralstonia solanaceum* (Asselbergh et al. 2007). To fully understand the complex roles of hormones in plant disease, and to distinguish those roles from the vital functions hormones serve to overall plant vitality, quantitative and systems-level temporal and spatial analyses are required (Mengiste 2012).

Plants employ chemical defenses to provide additional protection through synthesis of chemicals known as phytoalexins. Camalexin, an indole derivative of tryptophan, is the best described secondary metabolite involved in *Arabidopsis* defense. Only effective against certain pathogens (including some necrotrophs), it is believed to act by disrupting pathogen membranes (Rauhut and Glawischnig n.d.). *B. cinerea* was recently confirmed to carry an ABC transporter that exports camalexin from the fungal cell, demonstrating the importance of this molecule in plant-pathogen interactions (Stefanato et al. 2009). Glucosinolates are also phytoalexin indole compounds that have been associated with immune responses to necrotrophic pathogens (Bednarek et al. 2009). These are constitutively maintained in plant tissue and are released upon tissue damage. When released, they become hydrolyzed which creates antimicrobial derivatives and further induces plant defenses (Bednarek et al. 2009).

Progress has been made in the development of transgenic soft-rot resistant plants. Effective resistance has been conferred by host expression of phage-T4 lysozyme and chicken lysozyme, pectate lyases, 5-O-glucosyltransferase and AHL lactonases (During 1996; Serrano et al. 2000; Wegener 2001; Lorenc-Kukuła, Jafra, Oszmiański, and Szopa 2005; Dong et al. 2001). However, there is significant public opposition to the development of crops that express genes from a sexually incompatible species (Gaskell 1999). This objection to genetically modified (GM) plants could be addressed by producing cisgenic, rather than transgenic plants, in which resistance genes are sourced from closely related species so new cultivars could have hypothetically arisen through traditional breeding, thus eliminating concern over “unnatural” biochemistry (Schouten, Krens, and Jacobsen 2006).

The Type III secretion system and pathogen effectors

The type III secretion system (T3SS) in SRE plays a crucial role in pathogenesis under certain conditions. The T3SS, often referred to as the *hrp* (hypersensitive response and pathogenicity) system in phytopathogens, is a mechanism utilized by many pathogens to deliver “effector” proteins directly into the host cytosol that assist in promoting bacterial virulence (Francis, Wolf-Watz, and Forsberg 2002). The T3SS has been implicated in pathogenesis and in triggering non-host resistance by multiple strains of SRE (Ham, Bauer, Fouts, and Collmer 1998; Rantakari et al. 2001; Yang et al. 2002). The structure of the T3SS consists of a set of proteins that have evolved from the flagellar secretion system and span the inner and outer membranes of the pathogen and the cell membrane of the host, facilitating direct transfer of proteins from within the pathogen to the host cytosol (Abby and Rocha 2012). These T3-secreted proteins often encode an N-terminal targeting signal, adjacent to a chaperone binding site, that facilitates transport to the T3SS within the bacterial cell (Lloyd, Norman, Rosqvist, and Wolf-Watz 2001; Guo et al. 2005).

Pectobacterium requires the T3SS for leaf infection of solanaceous plants, though it is used for secretion of only a small number of proteins (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011). There are naturally occurring strains of *Pectobacterium* that lack a T3SS, however, these have reduced ability to infect leaves, suggesting that acquisition of a T3SS expanded the range of tissues that could be attacked by *Pectobacterium* (Kim, Ma, Perna, and Charkowski 2009). Extensive genomic and

experimental approaches have failed to reveal any additional T3SS1 effector proteins other than DspE in the *Pectobacterium* genome. This contrasts with the genome of *Pseudomonas syringae*, which encodes up to 30 effectors and which is used for most research focused on understanding the role of the T3SS in plant pathogenesis (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011; Cunnac, Lindeberg, and Collmer 2009). Thus, interfering with the action of DspE is an attractive strategy for control of soft-rot pathogens that utilize this mechanism.

Though a large number of bacterial effectors have been identified, the mechanisms by which they promote pathogenesis are known for only a small number of these proteins. Effectors secreted by biotrophs such as *Pseudomonas syringae* have been studied extensively. Many of these biotroph secreted effectors interfere with host recognition or activation of defense responses (Xiao et al. 2007; He et al. 2006). However, there are many components of the host immune system that are targeted by bacterial effectors other than pathogen perception proteins and the subsequent signaling cascades. The host protein MIN7 is an adenosine diphosphate ribosylation factor (ARF) family guanine exchange factor (GEF) protein involved in vesicle formation, which occurs during the immune response to facilitate release of antimicrobial compounds and cell wall reinforcing molecules. HopM1 from *Pseudomonas syringae* suppresses the immune response by targeting MIN7 for degradation, thereby inhibiting the delivery of antimicrobial compounds and other metabolites associated with the immune response (Nomura et al. 2006).

DspE is conserved among many gram-negative plant pathogens and is required by *P. carotovorum* to cause pathogenesis on leaves (Kim, Thammarat, Lommel, Hogan, and

Charkowski 2011). Homologs of this protein are denoted as AvrE in *Pseudomonas syringae* and WtsE in *Pantoea agglomerans*. In other pathogens, the DspE/AvrE family of proteins suppresses plant defenses, including callose deposition (DebRoy, Thilmony, Kwack, Nomura, et al. 2004). Contrastingly, DspE from *P. carotovorum* induces programmed cell death in leaves of *Nicotiana benthamiana* and many potato species that is indistinguishable from HR. Also, DspE from *P. carotovorum* does not seem to be able to suppress the host immune response, unlike other effectors from this family that can suppress callose deposition (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011). Translocation of DspE into the host cytosol has been demonstrated indirectly through observations that cAMP levels were increased in only host tissue infected with the bacterial pathogen expressing DspE fused to CyaA and not uninfected tissue, or tissue infected with strains deficient in T3SS (Bocsanczy and Nissinen 2004). The first 150 amino acids of DspE are necessary for optimal secretion and DspE requires its chaperone DspF for secretion, a characteristic shared by all members of the AvrE-family effectors (DebRoy, Thilmony, Kwack, Nomura, and He 2004; Ham, Majerczak, Arroyo-Rodriguez, Mackey, et al. 2006; Triplett, Melotto, and Sundin 2009; Gaudriault, Malandrin, Paulin, and Barny 1997). Chaperones of T3SS effectors stabilize and facilitate transport to T3SS machinery by binding to the N-terminus of nascent polypeptides (Ghosh 2004). Additionally, they ensure their bound effectors are not secreted before assembly of the T3SS is completed, and protect nascent effector proteins from proteasomal degradation (Losada and Hutcheson 2005; Tomalka, Stopford, Lee, and Rietsch 2012).

Data supporting the hypothesis that DspE-mediated cell death promotes pathogenesis suggest that inhibition of the plant cell death caused by DspE would increase the resistance of plants to soft rot pathogens (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011). To examine this hypothesis, we first need to further define the role of DspE in soft rot pathogenesis. Previous studies in the Charkowski lab have shown that mutation of the T3SS and deletion of *dspE* inhibit disease development in potato and *N. benthamiana* leaves, suggesting that *P. carotovorum* promotes disease by induction of cell death via this type III secreted effector (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011). These data complement previous evidence supporting the hypothesis that necrotrophic pathogens induce the HR (Glazebrook 2005).

WxxxE family effectors

Results presented in this dissertation indicate that DspE is a member of a recently characterized family of pathogen effector proteins known as the WxxxE-family. Analysis of the sequences from several animal pathogen effectors that mimic host guanine nucleotide exchange factors (GEFs) revealed a common amino acid sequence motif, WxxxE, in which xxx represents any three residues (Alto et al. 2006). For several pathogens, this effector is involved in reorganization of the host cytoskeleton, and initial studies indicated that these proteins function by mimicking activated Rho GTPases (Alto et al. 2006). Though substitutions of biochemically similar Y for W, or D for E, had no effect, substitution of other amino acids inhibited effector function (Alto et al. 2006). The *Salmonella typhimurium* effector SopE2 acts as a GEF that induces rearrangement of the

cytoskeleton through promotion of GDP/GTP exchange with the Rho-family GTPases Rac1 and CDC42 (Hardt, Chen, Schuebel, Bustelo, et al. 1998). Structural analysis revealed that this effector mimics the interaction of the host GEF protein Tiam1 with Rac1 (Williams, Galyov, and Bagby 2004). Though SopE2 is not a WxxxE-family effector, the *S. typhimurium* effector SifA does contain WxxxE motifs, and requires these to modulate the cytoskeleton through interaction with GDP-bound RhoA (Ohlson et al. 2008). SopE2 and SifA lack sequence similarity, however they both share a C-terminal fold required for GEF activity (Ohlson et al. 2008). Little is known of the role of the WxxxE motif, but in SifA the motif appears to stabilize this fold. Additionally, the *E. coli* WxxxE-family effector Map takes a similar conformation when modulating GDP/GTP exchange on the host protein CDC42, and this activity is affected by mutating the WxxxE motif (Huang et al. 2009). Though more work needs to be done investigating the crystal structure of these effectors, it appears that the WxxxE motif is involved in maintaining a protein conformation involved in GEF activity.

Bacterial gene expression during pathogenesis.

Important insights into the mechanisms of pathogenesis can be revealed through analysis of pathogen gene expression during pathogenesis. Through the recent publication of the genomes of many phytopathogens, significant advances have been made in identifying genes important for adapting to changing conditions and understanding their regulation using tiling microarrays and sequencing and quantification of RNA (RNA-Seq). These data provide a picture of an organism's response to different environmental conditions, and help identify important genes governing these responses

(Sorek and Cossart 2010). For example, array-based evaluation of the *Pectobacterium atrosepticum* gene expression in response to an AHL revealed expression change for 26% of genes (Liu et al. 2008). Additionally, changes in gene expression in response to changing environmental conditions, such as those that occur in the host during pathogenesis, can now be examined using real time quantitative reverse transcriptase PCR (qRT-PCR), which requires previous knowledge of the sequence of the genes of interest in order to design probes specific for those genes. Analysis of gene expression *in planta* provides a new perspective through which we can advance our understanding of plant-pathogen interactions. However, there are many technical challenges that must be overcome. These include developing a reliable system for obtaining high quality RNA and fine-tuning experimental conditions so that proper controls can be used and significant data can be obtained. Often each set of experimental conditions is presented with a unique set of solutions to these challenges. This study effectively utilized reverse transcription real-time quantitative PCR to identify genes induced during pathogenesis *in planta*, and identified new highly stable reference gene targets that may be used in future studies of gene expression.

Significance of this study

This study examined the role of DspE in pathogenesis of *P. carotovorum*, which helps advance understanding of the molecular mechanisms of disease and builds towards more effective management of *Pectobacterium*, as well as providing a better overall understanding of necrotrophic pathogenesis. Additionally, this work has significance in

the study of pathogen evolution as it examines how a necrotrophic pathogen uses a homolog of a protein found in hemi-biotrophic pathogens that suppresses the host immune system to instead sabotage plant defenses.

The work presented in this dissertation is divided into two chapters. The Chapter 2 presents the results that advance understanding of the AvrE-family effectors and the role of the T3SS in pathogenesis by necrotrophic bacteria. In this chapter I show the identification of specific conserved amino acid residues and motifs that are required for virulence function of DspE. Chapter 3 presents the results that suggest a mechanism for DspE-mediated cell death, and is one of the first studies to effectively demonstrate the use of quantitative PCR to measure *in planta* gene expression of pathogenesis associated genes in a bacterial pathogen. This research identified bacterial genes involved in pathogenesis by examining bacterial gene expression in leaves during pathogenesis, and provided new insights into the role of water and programmed cell death in necrotrophic plant disease. Each chapter is followed by a discussion of the results obtained, and of implications for future research on this particular *P. carotovorum* effector.

Table 1. Secreted virulence factors of soft rot enterobacteriaceae (Charkowski 2006)

Type I secretion system		
	Metalloproteases	Cell wall degradation and degradation of host proteins that interfere with cell wall degradation
	Adhesins	Bacterial attachment to host
Type II Secretion System		
	Pectinases	Plant cell wall degradation
	Proteases	Plant cell wall degradation
	Cellulases	Plant cell wall degradation
	Harpins	Elicitation of HR
Type III Secretions system		
	Effectors	Modulate host immunity within the host cell
	Harpins	Elicitation of HR
Siderophores		
	Chrysobactin	Iron uptake
	Achromobactin	Iron uptake

REFERENCES

- Abby, S. S., and Rocha, E. P. C. 2012. The non-flagellar type III secretion system evolved from the bacterial flagellum and diversified into host-cell adapted systems. *PLOS Genetics*. 8:e1002983
- Alto, N. M., Shao, F., Lazar, C. S., Brost, R. L., Chua, G., Mattoo, S., et al. 2006. Identification of a bacterial type III effector family with G protein mimicry functions. *Cell*. 124:133–45
- Asselbergh, B., Curvers, K., Franca, S. C., Audenaert, K., Vuylsteke, M., Van Breusegem, F., et al. 2007. Resistance to *Botrytis cinerea* in sitiens, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. *Plant Physiology*. 144:1863–77
- Aziz, A., Gauthier, A., Bézier, A., Poinssot, B., Joubert, J.-M., Pugin, A., et al. 2007. Elicitor and resistance-inducing activities of beta-1,4 cellodextrins in grapevine, comparison with beta-1,3 glucans and alpha-1,4 oligogalacturonides. *Journal of Experimental Botany*. 58:1463–72
- Bednarek, P., Pislewska-Bednarek, M., Svatos, A., Schneider, B., Doubsky, J., Mansurova, M., et al. 2009. A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science*. 323:101–6
- Berrocal-Lobo, M., Molina, A., and Solano, R. 2002. Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. *The Plant Journal*. 29:23–32
- Bocsanczy, A., and Nissinen, R. 2004. DspE, an effector of *Erwinia amylovora* is translocated into plant cells. *Acta Horticulturae*. 704:467–471
- Brandwagt, B. F., Mesbah, L. A., Takken, F. L. W., Laurent, P. L., Kneppers, T. J. A., Hille, J., et al. 2000. A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp. *lycopersici* toxins and fumonisin B1. *Proceedings of the National Academy of Sciences USA*. 97:4961–4966
- Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., et al. 2012. The role of secretion systems and small molecules in soft-rot enterobacteriaceae pathogenicity. *Annual Review of Phytopathology*. 50:425–49
- Charkowski, A. O. 2006. The Soft Rot *Erwinia*. In *Plant-Associated Bacteria*, ed. Gnanamanickam. Dordrecht, The Netherlands: Springer, p. 423–505.
- Cuesta Arenas, Y., Kalkman, E. R. I. C., Schouten, A., Dieho, M., Vredenburg, P., Uwumukiza, B., et al. 2010. Functional analysis and mode of action of phytotoxic Nep1-

like proteins of *Botrytis cinerea*. *Physiological and Molecular Plant Pathology*. 74:376–386

Cunnac, S., Lindeberg, M., and Collmer, A. 2009. *Pseudomonas syringae* type III secretion system effectors: repertoires in search of functions. *Current Opinion in Microbiology*. 12:53–60

Czajkowski, R., Pérombelon, M. C. M., van Veen, J. a., and van der Wolf, J. M. 2011. Control of blackleg and tuber soft rot of potato caused by *Pectobacterium* and *Dickeya* species: a review. *Plant Pathology*. 60:999–1013

DebRoy, S., Thilmony, R., Kwack, Y.-B., Nomura, K., and He, S. Y. 2004. A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proceedings of the National Academy of Sciences of the United States of America*. 101:9927–32

Deslandes, L., Olivier, J., Peeters, N., Feng, D. X., Khounlotham, M., Boucher, C., et al. 2003. Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proceedings of the National Academy of Sciences of the United States of America*. 100:8024–9

Dodds, P. N., and Rathjen, J. P. 2010. Plant immunity: towards an integrated view of plant-pathogen interactions. *Nature Reviews Genetics*. 11:539–48

Dong, Y. H., Wang, L. H., Xu, J. L., Zhang, H. B., Zhang, X. F., and Zhang, L. H. 2001. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature*. 411:813–7

During, K. 1996. Genetic engineering for resistance to bacteria in transgenic plants by introduction of foreign genes. *Molecular Breeding*. 2:297–305

Faris, J. D., Zhang, Z., Lu, H., Lu, S., Reddy, L., Cloutier, S., et al. 2010. A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. *Proceedings of the National Academy of Sciences of the United States of America*. 107:13544–9

Ferrari, S. 2002. Tandemly duplicated *Arabidopsis* genes that encode polygalacturonase-Inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. *The Plant Cell*. 15:93–106

Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F. M., and Dewdney, J. 2007. Resistance to *Botrytis cinerea* induced in *Arabidopsis* by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. *Plant Physiology*. 144:367–79

Francis, M. S., Wolf-Watz, H., and Forsberg, A. 2002. Regulation of type III secretion systems. *Current Opinion in Microbiology*. 5:166–72

Gaskell, G. 1999. Worlds apart? The reception of genetically modified foods in Europe and the U.S. *Science*. 285:384–387

Gaudriault, S., Malandrin, L., Paulin, J.-P., and Barny, M.-A. 1997. DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of *Pseudomonas syringae*, is secreted via the Hrp secretion pathway in a DspB-dependent way. *Molecular Microbiology*. 26:1057–1069

Ghosh, P. 2004. Process of protein transport by the type III secretion system. *Microbiology and Molecular Biology Reviews*. 68:771–95

Glasner, J. D., Marquez-Villavicencio, M., Kim, H.-S., Jahn, C. E., Ma, B., Biehl, B. S., et al. 2008. Niche-specificity and the variable fraction of the *Pectobacterium* pan-genome. *Molecular Plant-Microbe Interactions*. 21:1549–60

Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*. 43:205–27

Govrin, E. M., and Levine, A. 2000. The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Current Biology*. 10:751–757

Grant, M. R., and Jones, J. D. G. 2009. Hormone (dis)harmony moulds plant health and disease. *Science*. 324:750–2

Guo, M., Chancey, S. T., Tian, F., Ge, Z., Jamir, Y., and Alfano, J. R. 2005. *Pseudomonas syringae* type III chaperones ShcO1, ShcS1, and ShcS2 facilitate translocation of their cognate effectors and can substitute for each other in the secretion of HopO1-1. *Journal of Bacteriology*. 187:4257–69

Ham, J. H., Bauer, D. W., Fouts, D. E., and Collmer, A. 1998. A cloned *Erwinia chrysanthemi* Hrp (type III protein secretion) system functions in *Escherichia coli* to deliver *Pseudomonas syringae* Avr signals to plant cells and to secrete Avr proteins in culture. *Proceedings of the National Academy of Sciences USA*. 95:10206–10211

Ham, J. H., Majerczak, D. R., Arroyo-Rodriguez, A. S., Mackey, D. M., and Coplin, D. L. 2006. WtsE, an AvrE-family effector protein from *Pantoea stewartii* subsp. *stewartii*, causes disease-associated cell death in corn and requires a chaperone protein for stability. *Molecular Plant-Microbe Interactions*. 19:1092–102

Hardt, W.-D., Chen, L.-M., Schuebel, K. E., Bustelo, X. R., and Galán, J. E. 1998. *S. typhimurium* encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell*. 93:815–826

- He, P., Shan, L., Lin, N.-C., Martin, G. B., Kemmerling, B., Nürnberger, T., et al. 2006. Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in *Arabidopsis* innate immunity. *Cell*. 125:563–75
- Van der Hoorn, R. a L., and Kamoun, S. 2008. From Guard to Decoy: a new model for perception of plant pathogen effectors. *The Plant Cell*. 20:2009–17
- Huang, Z., Sutton, S. E., Wallenfang, A. J., Orchard, R. C., Wu, X., Feng, Y., et al. 2009. Structural insights into host GTPase isoform selection by a family of bacterial GEF mimics. *Nature Structural and Molecular Biology*. 16:853–60
- Jia, Y., McAdams, S. A., Bryan, G. T., Hershey, H. P., and Valent, B. 2000. Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. *The EMBO Journal*. 19:4004–14
- Johal, G., and Briggs, S. 1992. Reductase activity encoded by the HM1 disease resistance gene in maize. *Science*. 258:985–987
- Kabbage, M., Williams, B., and Dickman, M. B. 2013. Cell death control: the interplay of apoptosis and autophagy in the pathogenicity of *Sclerotinia sclerotiorum*. *PLOS Pathogens*. 9:e1003287
- Kim, H. S., Ma, B., Perna, N. T., and Charkowski, A. O. 2009. Phylogeny and virulence of naturally occurring type III secretion system-deficient *Pectobacterium* strains. *Applied and Environmental Microbiology*. 75:4539–49
- Kim, H. S., Thammarat, P., Lommel, S. A., Hogan, C. S., and Charkowski, A. O. 2011. *Pectobacterium carotovorum* elicits plant cell death with DspE/F but the *P. carotovorum* DspE does not suppress callose or induce expression of plant genes early in plant-microbe interactions. *Molecular Plant-Microbe Interactions*. 24:773–86
- Kim, M. G., da Cunha, L., McFall, A. J., Belkadir, Y., DebRoy, S., Dangl, J. L., et al. 2005. Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defense in *Arabidopsis*. *Cell*. 121:749–59
- Lai, Z., Wang, F., Zheng, Z., Fan, B., and Chen, Z. 2011. A critical role of autophagy in plant resistance to necrotrophic fungal pathogens. *The Plant Journal*. 66:953–68
- Lamb, C., and Dixon, R. A. 1997. The oxidative burst in plant disease resistance. *Annual Review of Plant Physiology and Plant Molecular Biology*. 48:251–275
- Lenz, H. D., Haller, E., Melzer, E., Kober, K., Wurster, K., Stahl, M., et al. 2011. Autophagy differentially controls plant basal immunity to biotrophic and necrotrophic pathogens. *The Plant Journal*. 66:818–30

Levine, B., and Yuan, J. 2005. Autophagy in cell death: an innocent convict? *The Journal of Clinical Investigation*. 115:2679–88

Lionetti, V., Raiola, A., Camardella, L., Giovane, A., Obel, N., Pauly, M., et al. 2007. Overexpression of pectin methylesterase inhibitors in *Arabidopsis* restricts fungal infection by *Botrytis cinerea*. *Plant Physiology*. 143:1871–80

Liu, H., Coulthurst, S. J., Pritchard, L., Hedley, P. E., Ravensdale, M., Humphris, S., et al. 2008. Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. *PLOS Pathogens*. 4:e1000093

Liu, Y., Schiff, M., Czymmek, K., Tallóczy, Z., Levine, B., and Dinesh-Kumar, S. P. 2005. Autophagy regulates programmed cell death during the plant innate immune response. *Cell*. 121:567–77

Lloyd, A., Allwood, W. J., Winder, C. L., Dunn, W. B., Heald, J. K., Cristescu, S. M., et al. 2011. Metabolomic approaches reveal that cell wall modifications play a major role in ethylene-mediated resistance against *Botrytis cinerea*. *The Plant Journal*. 67:852–68

Lloyd, S. A., Norman, M., Rosqvist, R., and Wolf-Watz, H. 2001. *Yersinia* YopE is targeted for type III secretion by N-terminal, not mRNA, signals. *Molecular Microbiology*. 39:520–532

Lorang, J. M., Sweat, T. A., and Wolpert, T. J. 2007. Plant disease susceptibility conferred by a “resistance” gene. *Proceedings of the National Academy of Sciences of the United States of America*. 104:14861–6

Lorenc-Kukuła, K., Jafra, S., Oszmiański, J., and Szopa, J. 2005. Ectopic expression of anthocyanin 5-o-glucosyltransferase in potato tuber causes increased resistance to bacteria. *Journal of Agricultural and Food Chemistry*. 53:272–81

Losada, L. C., and Hutcheson, S. W. 2005. Type III secretion chaperones of *Pseudomonas syringae* protect effectors from Lon-associated degradation. *Molecular Microbiology*. 55:941–53

Manabe, Y., Nafisi, M., Verhertbruggen, Y., Orfila, C., Gille, S., Rautengarten, C., et al. 2011. Loss-of-function mutation of REDUCED WALL ACETYLTATION2 in *Arabidopsis* leads to reduced cell wall acetylation and increased resistance to *Botrytis cinerea*. *Plant Physiology*. 155:1068–78

Mengiste, T. 2012. Plant immunity to necrotrophs. *Annual Review of Phytopathology*. 50:267–94

Mizushima, N. 2007. Autophagy: process and function. *Genes and Development*. 21:2861–73

- Nagy, E. D., Lee, T.-C., Ramakrishna, W., Xu, Z., Klein, P. E., SanMiguel, P., et al. 2007. Fine mapping of the Pc locus of *Sorghum bicolor*, a gene controlling the reaction to a fungal pathogen and its host-selective toxin. *Theoretical and Applied Genetics*. 114:961–70
- Nomura, K., Debroy, S., Lee, Y. H., Pumplin, N., Jones, J., and He, S. Y. 2006. A bacterial virulence protein suppresses host innate immunity to cause plant disease. *Science*. 313:220–3
- Oh, C.-S., Martin, G. B., and Beer, S. V. 2007. DspA/E, a type III effector of *Erwinia amylovora*, is required for early rapid growth in *Nicotiana benthamiana* and causes NbSGT1-dependent cell death. *Molecular Plant Pathology*. 8:255–65
- Ohlson, M. B., Huang, Z., Alto, N. M., Blanc, M.-P., Dixon, J. E., Chai, J., et al. 2008. Structure and function of *Salmonella* SifA indicate that its interactions with SKIP, SseJ, and RhoA family GTPases induce endosomal tubulation. *Cell Host and Microbe*. 4:434–46
- El Oirdi, M., El Rahman, T. A., Rigano, L., El Hadrami, A., Rodriguez, M. C., Daayf, F., et al. 2011. *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *The Plant Cell*. 23:2405–21
- Perombelon, M. C. M., and Kelman, A. 1980. Ecology of the Soft Rot *Erwinias*. *Annual Review of Phytopathology*. 18:361–387
- Rantakari, A., Virtaharju, O., Vähämäki, S., Taira, S., Palva, E. T., Saarilahti, H. T., et al. 2001. Type III secretion contributes to the pathogenesis of the soft-rot pathogen *Erwinia carotovora*: partial characterization of the hrp gene cluster. *Molecular Plant-Microbe Interactions*. 14:962–8
- Rauhut, T., and Glawischnig, E. Evolution of camalexin and structurally related indolic compounds. *Phytochemistry*. 70:1638–44
- Samson, R., Legendre, J. B., Christen, R., Fischer-Le Saux, M., Achouak, W., and Gardan, L. 2005. Transfer of *Pectobacterium chrysanthemi* (Burkholder et al. 1953) Brenner et al. 1973 and *Brenneria paradisiaca* to the genus *Dickeya* gen. nov. as *Dickeya chrysanthemi* comb. nov. and *Dickeya paradisiaca* comb. nov. and . *International Journal of Systematic and Evolutionary Microbiology*. 55:1415–27
- Schouten, H. J., Krens, F. A., and Jacobsen, E. 2006. Cisgenic plants are similar to traditionally bred plants: international regulations for genetically modified organisms should be altered to exempt cisgenesis. *EMBO Reports*. 7:750–3
- Serrano, C., Arce-Johnson, P., Torres, H., Gebauer, M., Gutierrez, M., Moreno, M., et al. 2000. Expression of the chicken lysozyme gene in potato enhances resistance to infection

by *Erwinia carotovora* subsp. *atroseptica*. American Journal of Potato Research. 77:191–199

Sharon, A., Elad, Y., Barakat, R., and Tudzynski, P. 2007. *Botrytis*: Biology, Pathology and Control. In *Botrytis: Biology, Pathology and Control*, eds. Yigal Elad, Brian Williamson, Paul Tudzynski, and Nafiz Delen. Dordrecht: Springer Netherlands, p. 163–179.

Sorek, R., and Cossart, P. 2010. Prokaryotic transcriptomics: a new view on regulation, physiology and pathogenicity. Nature Reviews Genetics. 11:9–16

Staal, J., Kaliff, M., Dewaele, E., Persson, M., and Dixelius, C. 2008. RLM3, a TIR domain encoding gene involved in broad-range immunity of *Arabidopsis* to necrotrophic fungal pathogens. The Plant Journal. 55:188–200

Stefanato, F. L., Abou-Mansour, E., Buchala, A., Kretschmer, M., Mosbach, A., Hahn, M., et al. 2009. The ABC transporter BcatrB from *Botrytis cinerea* exports camalexin and is a virulence factor on *Arabidopsis thaliana*. The Plant Journal. 58:499–510

Tomalka, A. G., Stopford, C. M., Lee, P.-C., and Rietsch, A. 2012. A translocator-specific export signal establishes the translocator-effector secretion hierarchy that is important for type III secretion system function. Molecular Microbiology. 86:1464–81

Toth, I. K., Bell, K. S., Holeva, M. C., and Birch, P. R. J. 2003. Soft Rot *Erwiniae*: from genes to genomes. Molecular Plant Pathology. 4:17–30

Toth, I. K., and Birch, P. R. J. 2005. Rotting softly and stealthily. Current Opinion in Plant Biology. 8:424–9

Triplett, L. R., Melotto, M., and Sundin, G. W. 2009. Functional analysis of the N terminus of the *Erwinia amylovora* secreted effector DspA/E reveals features required for secretion, translocation, and binding to the chaperone DspB/F. Molecular Plant-Microbe Interactions. 22:1282–92

Veronese, P., Nakagami, H., Bluhm, B., Abuqamar, S., Chen, X., Salmeron, J., et al. 2006. The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens. The Plant Cell. 18:257–73

Vleeshouwers, V. G. A. A., van Dooijeweert, W., Govers, F., Kamoun, S., and Colon, L. T. 2000. The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans*. Planta. 210:853–864

Wang, Y., Wu, Y., and Tang, D. 2011. The autophagy gene, ATG18a, plays a negative role in powdery mildew resistance and mildew-induced cell death in *Arabidopsis*. Plant Signaling and Behavior. 6:1408–1410

Wegener, C. B. 2001. Transgenic potatoes expressing an *Erwinia* pectate lyase gene — results of a 4-year field experiment. *Potato Research*. 44:401–410

Williams, C., Galyov, E. E., and Bagby, S. 2004. Solution structure, backbone dynamics, and interaction with Cdc42 of *Salmonella* guanine nucleotide exchange factor SopE2. *Biochemistry*. 43:11998–2008

Wolpert, T. J., Dunkle, L. D., and Ciuffetti, L. M. 2002. Host-selective toxins and avirulence determinants: what's in a name? *Annual Review of Phytopathology*. 40:251–85

Xiao, F., He, P., Abramovitch, R. B., Dawson, J. E., Nicholson, L. K., Sheen, J., et al. 2007. The N-terminal region of *Pseudomonas* type III effector AvrPtoB elicits Pto-dependent immunity and has two distinct virulence determinants. *The Plant Journal*. 52:595–614

Yang, C. H., Gavilanes-Ruiz, M., Okinaka, Y., Vedel, R., Berthuy, I., Boccara, M., et al. 2002. *hrp* genes of *Erwinia chrysanthemi* 3937 are important virulence factors. *Molecular Plant-Microbe Interactions*. 15:472–80

Zhao, Q., and Guo, H.-W. 2011. Paradigms and paradox in the ethylene signaling pathway and interaction network. *Molecular Plant*. 4:626–34

Chapter 2 - The type III secreted effector DspE is required early in *Solanum tuberosum* leaf infection by *Pectobacterium carotovorum* to cause cell death, and requires Wx(3-6)D/E motifs

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ABSTRACT

Pectobacterium species are enterobacterial plant-pathogens that cause soft rot disease in diverse plant species. Unlike hemi-biotrophic plant pathogenic bacteria, the type III secretion system (T3SS) of *Pectobacterium carotovorum* subsp. *carotovorum* (*P. carotovorum*) appears to secrete only one effector protein, DspE. We have previously shown that HrpL, the T3SS regulator, and DspE are required for pathogenesis on leaves. In this work, we identified genes up-regulated by HrpL, visualized expression of *hrpL* in *planta*, and established that DspE causes host cell death. DspE required its full length, and WxxxE-like motifs, which are characteristic of the AvrE-family effectors, for host cell death. We also examined expression in plant leaves and showed that *hrpL* is required for the expression of *dspE* and *hrpN*, as well as the genes *ffh*, *gyrA*, and *pelB*. These data support a model where *P. carotovorum* uses the T3SS early in leaf infection to initiate pathogenesis through elicitation of DspE-mediated host cell death.

INTRODUCTION

Pectobacterium carotovorum is a necrotrophic Gram-negative bacterial pathogen that causes soft rot disease on plant species from over 24 orders of angiosperms, including important crops in the Solanaceae and Brassicaceae. It is a ubiquitous pathogen that survives in soil, surface and ground water, and it has been associated with a variety of invertebrates (Harrison, Quinn, Sells, and Graham 1977; McCarter-Zorner et al. 1984, 1985; Molina, Harrison, and Brewer 1974). *P. carotovorum* attacks plant tissue by secreting an array of plant cell wall degrading enzymes through the type II secretion system (Allen, Reverchon, and Robert-Baudouy 1989; Lagaert, Beliën, and Volckaert 2009; Matsumoto, Jitareerat, Baba, and Tsuyumu 2003). It also has a type III secretion system (T3SS) that is required for pathogenesis in leaves (Kim, Thammarat, Lommel, Hogan, et al. 2011).

The genes encoding the T3SS typically lie in clusters of structural and regulatory genes called *hrp* (hypersensitive response (HR) and Pathogenicity) that tend to be flanked by genes encoding secreted effectors and their chaperones (Chatterjee, Cui, and Chatterjee 2002). In *P. carotovorum* and *Pseudomonas syringae*, an alternative sigma factor, HrpL, is the main regulator of genes in the *hrp* cluster. In *Pseudomonas syringae*, HrpL is known to regulate all type III effector-encoding genes, in addition to the T3SS structural genes (Xiao and Hutcheson 1994).

Among phytopathogenic bacteria, numerous categories of effectors have been described. The AvrE-family of effectors is unusual in that it is widespread among *Pseudomonas*, *Pantoea*, *Dickeya*, *Pectobacterium*, and *Erwinia* spp. AvrE from

Pseudomonas syringae blocks pathogen induced callose deposition along the plant cell wall to enhance virulence on host plants and AvrE causes cell death in leaf tissue (DebRoy, Thilmony, Kwack, Nomura, et al. 2004). DspE and WtsE, AvrE-family effectors from *Erwinia amylovora* and *Pantoea stewartii* respectively, suppress salicylic acid (SA)-mediated host defenses and also cause cell death in leaves (Boureau et al. 2006; DebRoy, Thilmony, Kwack, Nomura, and He 2004; Ham et al. 2008), and these effectors are essential for both *E. amylovora* and *P. stewartii* pathogenesis (Bogdanove, Bauer, and Beer 1998; Ham et al. 2008). There is no evidence that *P. carotovorum* can suppress SA-mediate host defenses or callose deposition (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011). Mutagenesis experiments suggest that DspE is the only effector encoded by *P. carotovorum* and that DspE is required by the necrotrophic *P. carotovorum* to induce necrosis on plant leaves (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011).

In this work, we searched for additional HrpL regulated genes with a promoter-trap screen, but found none outside of the T3SS cluster, further supporting the hypothesis that *P. carotovorum* encodes a single effector, DspE. We found that the *hrpL* gene is required *in planta* for expression of *dspE*, *hrpN* and several additional genes including the virulence factor gene *pelB*. To further define the role of the effector protein DspE in *P. carotovorum* pathogenesis, we used site-directed mutagenesis to identify residues critical for DspE function. Additionally, we found that DspE is sufficient to cause cell death and demonstrated that WxxxD/E motifs are important for this function; supporting the hypothesis that *P. carotovorum* pathogenesis in leaf tissue is initiated by host cell death mediated by DspE.

RESULTS

Confocal microscopy confirms HrpL activity in vivo.

We used confocal microscopy to visualize the activity of a HrpL-responsive reporter during leaf infection. Wild-type *P. carotovorum* WPP14 carrying a Differential Fluorescence Induction (DFI) vector (Chang et al. 2005) with a *dspE* promoter-*gfp* fusion construct was infiltrated into *Arabidopsis thaliana* Col-0. *A. thaliana* leaves were used due to their thinness relative to potato leaves. Leaves were visualized at 5 mins, 30 mins, 1 hours, 3 hours and 7 hours post infiltration, with peak fluorescence observed at 7 hours post infiltration. Later time-points were not examined with confocal microscopy due to leaf tissue maceration and for fear that the long half-life of GFP would skew interpretations of the timing of expression. Fluorescence, visualized 7 hours after infiltration, indicated native HrpL induction and activity, and T3SS expression *in vivo* (Fig. 1.) Importantly, when our *dspE_{pro}-gfp* fusion was carried in a *hrpL* mutant, we did not see fluorescence at any of the sampled time points, indicating that the fluorescence was due to HrpL activity.

Promoter-trap screen identifies HrpL regulated genes.

We used a fluorescence activated cell sorter (FACS)-based promoter trap screen developed by (Chang et al. 2005) to identify T3SS effectors. Previous studies of the T3SS

in *P. carotovorum* had identified the alternative sigma factor, HrpL, as the master regulator of the T3SS and the effector, DspE (Chatterjee, Cui, and Chatterjee 2002). We exploited HrpL as a likely regulator of unidentified effectors and associated virulence factors and performed the FACS-based promoter trap screen as described by (Chang et al. 2005). The *dspE* gene was cloned into the DFI vector (Chatterjee, Cui, and Chatterjee 2002) to serve as a positive control for induction, and peak fluorescence after *hrpL* induction. *dspE* and genome library fragments were inserted into the DFI vector and mobilized by tri-parental mating into *P. carotovorum* WPP14, which already carried *hrpL* on the arabinose-inducible pBAD plasmid and in the chromosome. Cultures were sorted by FACS with 4 tandem sorts to enrich for *hrpL*-dependent fluorescing cells. Approximately 2600 clones were captured and their library fragments were sequenced and aligned to the genome. Sequences aligned to 37 distinct regions (< 4kb in length) of the genome. 17% of the sequences aligned to an arabinose operon responsive to the induction conditions and were therefore disregarded. Randomly selected clones representing each region were independently grown and re-screened by FACS to verify HrpL-dependent fluorescence. The verified clones represented 17 HrpL-inducible regions and a full length ORF was identified in each region. To confirm that these regions were regulated by HrpL, we used qRT-PCR to analyze expression of ORF's contained in these 17 regions in a WPP14 Δ *hrpL* mutant strain carrying *hrpL* fused to an arabinose inducible promoter under inducing conditions. Only 6 genome regions (representing 58% of the sequenced library clones) were confirmed with qRT-PCR, and these all aligned to known HrpL-dependent genes and operons within the T3SS gene cluster (Table 1, Fig. 2).

The region that was most frequently identified, represented by 784 clones, was a 12 member operon within the T3SS cluster. Known *hrp* cluster members and predicted HrpL binding sites were based on a published description of the T3SS cluster. *dspE*, the only previously identified effector protein, was represented in the screen 329 times. *hrpA*, encoding a T3SS-associated pilin, and *hrpF*, a putative T3SS translocon, were represented the least, with only 6 and 4 representative clones, respectively. Only one known HrpL-regulated gene was not identified in the screen, *hrpN*. We cloned *hrpN* into the DFI vector to assess its fluorescent profile. We found that *hrpN* expression was inducible via HrpL; however its basal expression level was higher than 30% of the fluorescence of the library, suggesting the reason it was not captured during the screen (data not shown).

Expression of *dspE* and *hrpN* requires *hrpL* in *planta*.

Our previous mutational analysis demonstrated that the *hrpL* or the *dspE* genes are required for disease and necrosis on plant leaves (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011). In the same study, we found that the T3SS genes *hrpN* and *hrpW* were not required for disease on leaves. To verify the *in planta* dependence of T3SS gene expression on HrpL shown by our *dspE_{pro}-gfp* fusion analysis, we used real-time reverse transcriptase quantitative PCR (real-time RT-qPCR) to compare expression of *dspE* and *hrpN* in wild-type WPP14 versus a Δ *hrpL* strain. Leaves of *N. benthamiana* were inoculated with either the wild-type or mutant strain with a needleless syringe. Total RNA was extracted from infected leaves five hours post-inoculation because leaves

infiltrated with the wild-type strain consistently began to wilt at this time point, indicating activation of pathogenesis. Expression of *hrpN* and *dspE* relative to the validated reference genes *recA* and *proC* were quantified for each strain. Both genes were expressed in leaves and expression of both *dspE* and *hrpN* was reduced more than 59-fold in the $\Delta hrpL$ mutant at 5 hours post inoculation (Fig. 3). In both cases, the target transcripts were produced in the *hrpL* mutant near the limit of detection for real-time RT-qPCR.

DspE alone is able to elicit cell death in leaf tissue.

To study the activity of DspE in the absence of any other effector proteins produced by *P. carotovorum* WPP14, we transiently expressed *dspE* in *N. benthamiana* leaves by agroinfiltration (Goodin, Zaitlin, Naidu, and Lommel 2008; Nakagawa et al. 2007) and verified these results in *Solanum tuberosum* leaves (Bhaskar, Venkateshwaran, Wu, Ané, et al. 2009). Infiltration of *Agrobacterium tumefaciens* carrying the wild-type *dspE* clone (pCH0002) resulted in necrotic lesions that resembled the hypersensitive response (HR) in both *N. benthamiana* and *S. tuberosum* 48 hours post inoculation (Fig. 4). These data establish that DspE is sufficient for HR elicitation. Previous work showed that deletion of *dspE* eliminates the ability of *P. carotovorum* WPP14 to kill leaf tissue (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011), thus it appears that DspE is necessary and sufficient for *P. carotovorum* to cause host cell death.

Deletion analysis and site-directed mutagenesis revealed that the entire length of DspE, and W_{x(3-6)}D/E motifs are required for cell death.

We precisely deleted the N-terminal 330 amino acids and the C-terminal 98 amino acids from DspE. When expressed in *N. benthamiana* leaves, these mutant proteins did not cause necrosis (Fig. 4), suggesting that these deletions destabilize the protein or contain motifs important for function.

Our searches of the entire predicted *P. carotovorum* WPP14 DspE sequence revealed only one WxxxD motif at position 464. However, there are highly conserved tryptophans within 6 residues of an aspartic acid (D) or glutamic acid (E) that aligned to WxxxE motifs found in WtsE at DspE positions W514 and W660 (Fig. 5). Changing either the W or E residue to A in both motifs carried by AvrE and WtsE (DspE homologs from *Pseudomonas syringae* and *Pantoea stewartii*, respectively) resulted in loss of cell killing activity, as long as both WxxxE motifs contained at least one A substitution (Ham et al. 2009). Therefore, we created a series of DspE mutants with various combinations of W to A substitutions at the sites mentioned above. These mutations were constructed by site-directed mutagenesis in plasmid pCH0001; the single mutants were designated DspE-w1 (W464A), DspE-w2 (W514A) and DspE-w3 (W660A). The double mutants were designated DspE-w12, DspE-w13 and DspE-w23; the triple mutant was designated DspE-w123. We found that, like AvrE and WtsE, all of the DspE mutants containing a single substitution retained cell-killing activity (Fig. 4). However, the w12, w13, w23 and w123 mutants failed to induce necrosis (Fig. 4), though in one of five assays the w23 mutants would show attenuated leaf cell death (data not shown). We increased the expression level of these mutants by co-expressing the *Tobacco etch virus* silencing

suppressor P1/Hc-Pro (Johansen and Carrington 2001). Co-expression of wild-type DspE with Hc-Pro caused necrosis one-day post inoculation, compared to 2 days without Hc-Pro, suggesting that an increase in the expression level of the protein resulted in a swifter reaction. It is also possible that the presence of the silencing suppressor affected the leaf sensitivity to DspE and derivatives. Co-expression of w23 with Hc-Pro caused necrosis to consistently appear two days post-inoculation, indicating that the combination of w2 and w3 mutations partially attenuated cell-killing function of the protein, rather than completely eliminating this function.

As with a previous report on WtsE (Ham et al. 2009), attempts to monitor *in planta* production and stability of DspE following *Agrobacterium*-mediated transient gene expression failed to detect DspE or its derivatives (data not shown). However, mutations of two or three amino acids in the w12, w13, w23 and w123 mutant alleles are unlikely to affect expression or stability of DspE, since each mutation alone had no effect on elicitation of plant necrosis. Additionally, the ability of the w23 DspE mutant allele to cause cell death when expression is increased by the presence of Hc-Pro indicates that the mutant protein accumulates and retains attenuated function in leaf cells. Furthermore, similar mutations in DspE homologs did not affect production or secretion (Ham et al. 2009). Together, these data indicate that W_{X(3-6)}D/E motifs are functionally redundant, but required for DspE mediated host cell death.

HrpL is required for the expression of genes outside of T3SS cluster *in planta*.

In addition to the T3SS genes, we examined the effect of the $\Delta hrpL$ mutation on the expression of several other *P. carotovorum* genes *in planta*. Surprisingly, several of these genes were also attenuated in the mutant. The *ffh* and *gyrA* genes are conserved among Gram-negative bacterial species and are often used as reference transcripts for real-time RT-qPCR in *Pectobacterium* species (Takle, Toth, and Brurberg 2007; Glasner et al. 2008). In the course of validating these genes as reference transcripts, we found that expression of *ffh* was reduced 4-fold and *gyrA* was reduced 5.5-fold in the $\Delta hrpL$ mutant (Fig. 6). Since *P. carotovorum* T3SS mutants do not macerate leaves, we also examined the expression of *pelB*, which encodes pectate lyase, and found it to be reduced 67-fold in the $\Delta hrpL$ mutant. Expression of a flagellin gene, *fliC*, was not affected (Fig. 6).

DISCUSSION

We found that *P. carotovorum* subsp. *carotovorum* strain WPP14 (*P. carotovorum* WPP14) activates the T3SS during the early stages of infection of leaf tissue and appears to translocate a single effector, DspE. Nearly all of the genes regulated by HrpL are T3SS genes or homologous to known secreted proteins, including *hrpN*, *hrpK*, *hrpW*, and *dspE*. The *hrpK* gene is separated from the rest of the T3SS gene cluster by insertion of an operon homologous to *hecAB*, which encode bacterial adhesins (Fig. 2).

We also found that *P. carotovorum* DspE is sufficient for killing both tobacco and potato leaf cells when expressed transiently in plant cells. Multiple WxxxD/E motifs are required by *P. carotovorum* DspE for cell-killing activity (Fig. 4), as has been found with

homologs from other bacterial species (Ham et al. 2009). These data support findings from previous studies in which DspE was the only T3SS-effector homolog identified in *P. carotovorum* WPP14 (Kim, Ma, Perna, and Charkowski 2009; Glasner et al. 2008; Kim, Thammarat, Lommel, Hogan, and Charkowski 2011).

We confirmed that HrpL, an alternative sigma factor required for expression of the T3SS and other pathogenesis-associated-genes (Chatterjee, Cui, and Chatterjee 2002), is required for expression of the T3SS genes *dspE* and *hrpN* in plant leaves. Interestingly, the expression of several genes outside of the T3SS cluster is reduced in leaves in our *hrpL* mutant, including *pelB* and the central metabolism genes *ffh* and *gyrA* (Fig. 6). Since no HrpL binding site is evident upstream of these genes, this suggests that plant responses to a functional T3SS affect expression of a wide range of genes not directly regulated by HrpL. Down-regulation of *pelB* is consistent with the no-maceration phenotype we see with T3SS mutants when they are infiltrated into plant leaves (Kim, Ma, Perna, and Charkowski 2009; Kim, Thammarat, Lommel, Hogan, and Charkowski 2011). The down-regulation of central metabolism genes *ffh* and *gyrA* highlights the importance of testing reference genes under the conditions being studied, rather than relying on previously reported reference genes in new experimental conditions (Takle, Toth, and Brurberg 2007).

Cell death is required for *P. carotovorum* WPP14 pathogenesis in leaves and depends upon the HrpL-regulated T3SS and *dspE/F* (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011). We showed, through heterologous gene expression, that DspE is sufficient to cause plant cell death (Fig. 4). Whether cell death is due to direct toxicity of the DspE protein, and/or because DspE function triggers plant programmed cell death,

remains unknown. The hypothesis that DspE is recognized by a host R protein that subsequently activates the hypersensitive response (HR) is supported by the requirement for SGT1 for *P. carotovorum*-mediated leaf cell death (Wang, Uppalapati, Zhu, Dineshkumar, et al. 2010). Further supporting this hypothesis is the observation that DspA/E from *Erwinia amylovora* interacts with several leucine-rich repeat (LRR) receptor-like serine threonine kinases (RLK) (Meng, Bonasera, Kim, Nissinen, et al. 2006) and that silencing of these proteins increases the resistance of apple to *E. amylovora* infection (Borejsza-Wysocka et al. 2006). Additionally, *P. carotovorum* is unable to infect *Arabidopsis* “defense, no death” mutants (Ahn 2007), suggesting that elicitation of leaf cell death is required for *P. carotovorum* pathogenesis. Taken together, this evidence supports a model in which DspE aids the necrotrophic *P. carotovorum* in attacking leaf tissue by eliciting R-protein mediated cell death.

Alternatively, DspE may activate programmed cell death by using WxxxE/D motifs to mimic a plant guanine nucleotide exchange factor (GEF), a class of proteins that regulate many processes in the cell including the HR (Agrawal, Iwahashi, and Rakwal 2003). Other AvrE-family proteins from hemibiotrophic pathogens possess one or two W/YxxxE/D motifs (Ham et al. 2009) and deletion of these motifs eliminates the plant cell death activity of AvrE from *Pseudomonas syringae* and WtsE from *P. stewartii*. This motif is also commonly found in animal pathogen T3SS effectors thought to act as regulators of the Rho family of GTPases by functioning as GEFs (Huang et al. 2009). Rho GTPases are important regulators of many cellular processes including vesicle trafficking and apoptosis; processes that are important for pathogen resistance (Etienne-Manneville and Hall 2002). Thus, disruption of Rho GTPase signaling using

type III effectors is an effective strategy for pathogens to modulate the host response. These two hypotheses, activation of R-protein mediated plant cell death and mimicking a GEF protein, are not mutually exclusive. The relative importance of each pathway to virulence may depend upon the physiological state of the host or the plant species infected by this broad host range pathogen.

We found that DspE relies upon three functionally redundant motifs consisting of a tryptophan (W), and either an aspartic (D) or glutamic acid (E) separated by a spacer region of three to six amino acids (Fig. 5). When the W from at least two of these motifs is substituted with an alanine residue, the cell killing ability of DspE is abolished, though single amino acid substitutions have no effect. The double substitutions are unlikely to affect stability of DspE since each mutation alone had little or no effect on cell killing activity. Furthermore, similar mutations in DspE homologs did not affect production or secretion (Ham et al. 2008). These motifs closely resemble WxxxE motifs important for function of DspE homologs AvrE from *Pseudomonas syringae* DC3000 and WtsE from *Pantoea stewartii* (Fig. 5), which, like DspE, elicit plant cell death (Ham et al. 2009). Further supporting this hypothesis is the observation that AvrE and WtsE suppress callose deposition and have an endoplasmic reticulum membrane retention signal (ERMRS) (Ham et al. 2009). These features indicate that AvrE and WtsE interfere with vesicle trafficking, a process known to be regulated by GEFs.

Notably, unlike AvrE and WtsE, *P. carotovorum* DspE lacks both callose deposition suppressing activity and an ERMRS (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011), indicating that it may lack GEF activity. It is possible that the cell death activity shared between AvrE, WtsE and DspE is caused by a mechanism

independent of callose deposition suppressing activity and does not require an ERMRS. Crystal structure analyses of the animal pathogen WxxxE effectors suggest a structural, instead of a catalytic, role for the WxxxE motif (Ohlson et al. 2008; Huang et al. 2009). Thus, while mutation of the WxxxE motif may result in a loss of GEF activity due to destabilization of a catalytic site, the loss of cell death activity may be attributed to a conformational change that disrupts recognition of the effector or its activities by an R protein. Further studies are needed to determine if DspE possesses GEF activity, whether AvrE or DspE affects vesicular trafficking, and which host proteins interact with DspE.

It appears that acquisition of a T3SS and an AvrE-family effector allowed *P. carotovorum* to expand the range of tissues that it can infect to include plant leaves. The biochemical function of DspE remains unknown, but the lack of an ERMRS and callose suppressing activity suggest that *P. carotovorum* requires only the ability to induce host cell death in order to infect leaf tissue and that this is achieved by maintaining a single effector protein that is active in a wide range of plant species. Additional biochemical characterization of DspE, as well as analysis of *P. carotovorum* ecology is required to further support this model. s

MATERIALS AND METHODS

Bacterial strains and their growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *Pectobacterium carotovorum* strains were grown from single colonies in Luria-Bertani (LB) broth or 2xYT broth in a 250 rpm shaking incubator at 37°C or maintained

on LB plates solidified with 1.5% BD Bacto-agar, containing appropriate antibiotics. The compositions of LB medium, M9 minimal medium, 2xYT media are published in Sambrook and Russell (Sambrook and Russell 2001). The minimal medium for *hrpL* repression was described in (Chang et al. 2005). *Agrobacterium tumefaciens* strains were also grown from single colonies in LB broth in a shaking incubator at 28°C or maintained on LB plates.

Confocal Microscopy.

Cultures were grown over night in LB, washed twice in 10 mM MgCl₂ and re-suspended at an OD₆₀₀ of 1.0 and infiltrated into leaves of 4-5 week old *A. thaliana* Col-0 using a needle-less syringe. Potato leaves were not used because they were too thick to properly mount on microscope slides and visualize with confocal microscope. A Zeiss confocal microscope was used to visualize fluorescent bacteria *in planta* and images were captured and viewed with LSM image browser software (Zeiss).

DNA manipulation, transformation, and sequencing.

A FastDNA® SPIN for Soil Kit (MP Biomedicals, LLC, Solon, Ohio, U.S.A.) with a homogenization time of 5 seconds in a Mini Beadbeater™ (Biospec Products, Inc., Bartlesville, OK, U.S.A.) was used to isolate DNA from *P. carotovorum* grown overnight in LB. Appropriate Qiagen (Valencia, CA, U.S.A.) kits were used for isolation of DNA fragments from agarose gels or polymerase chain reaction (PCR) solutions. Platinum Taq DNA polymerase High Fidelity and Gateway vectors (Invitrogen, Carlsbad, CA, U.S.A.) were used to amplify and clone DNA, following the manufacturer's protocols.

All clones obtained were confirmed by DNA sequencing with an ABI 3730xl capillary electrophoresis sequencer (Applied Biosystems, Foster City, CA, U.S.A.) at the University of Wisconsin Biotechnology Center DNA Sequencing Facility or the University of North Carolina-Charlotte Genome Analysis Facility. Oligonucleotides were purchased from The University of Wisconsin Biotechnology Center DNA Synthesis Laboratory. Transformation of *A. tumefaciens* and *E. coli* DH5 α -ETM was conducted by electroporation in 1mm cuvettes using a GenePulser XcellTM (Bio-Rad Laboratories, Hercules, CA, U.S.A.) apparatus set to 200 Ω and 1.8 kV. Transformation of *E. coli* DH5 α TM-T1^R was conducted by a heat shock protocol following the manufacturer's protocols.

Plasmid construction.

pBAD::*hrpL*WPP14 construction: *hrpL* was PCR amplified using Pfu (Stratagene) then cloned into pCFS40 as described in (Chang et al. 2005). pBAD::*hrpL* exhibited low expression in the absence of arabinose and high expression in the presence of arabinose in minimal medium developed for repression of the native *hrpL* promoter (data not shown). 200 mM of arabinose was optimal for *hrpL* induction. Differential Fluorescence Induction (DFI) Vectors: Construction of DFI vectors was described in (Chang et al. 2005). Briefly, pBBR1-MCS2 was modified to carry RNA terminators, and mutant GFP3, which has an excitation peak corresponding to the argon laser (488 nm) of the FACS. Three stop codons in each frame were annealed and cloned upstream from the Shine-Delgarno sequence as an EcoRI-XbaI fragment, yielding vector 125.1. PCR

products were directly cloned into EcoRI-BamHI digested 125.1 vector following a sticky-end PCR protocol, resulting in DFI vectors.

Library Construction for Promoter-Trap Screen.

DNA was extracted from *P. carotovorum* WPP14, purified by using Epicentre MasterPure DNA extraction kit, and partially digested with either Tsp509I, or *AluI*, *BstUI*, *HaeIII*, and *RsaI*. Fragments from 1–1.6 kb, 1.6–3 kb, and 3–4 kb were extracted and cloned into either EcoRI- or *SmaI*-digested and shrimp alkaline phosphatase (SAP)-treated DFI vectors. *E. coli* colonies carrying approximately 66,000 library fragments were pooled and mated en masse by modified triparental mating with WPP14 + pBAD::*hrpL* and pRK2013.

Promoter-Trap Screen.

The screen was done as described in (Chang et al. 2005). Briefly, we analyzed the DFI library under the inducing conditions of 200 mM arabinose for 22 h before FACS screening. FACS was performed on a MoFlo (Cytomation) and analysis was performed on a FACScan from Becton Dickinson. One ml of culture grown over night in LB was pelleted and then diluted into 400µl of 1× PBS. HrpL-inducible gene fragments were identified with 4 subsequent sorts. The first, in the absence of induction, the least fluorescent cells (30%) were collected. These cells were grown in inducing conditions and a small population (less than 2% of total population) of highly-fluorescent cells was collected; these two sorts were repeated, except final fluorescent cells were collected in individual wells of a 364 well plate. Individual library clones were grown in these plates,

their library fragments were amplified from the DFI vector, and amplicons were sequenced.

Sequencing library clones.

Approximately 2600 clones were sequenced. Candidate HrpL-induced gene fragments were amplified from cells harboring the DFI plasmids using *Taq* polymerase and primers, HYZ163 and HYZ166 (Chang et al. 2005). PCR products were treated with 5 units of exonuclease I and 0.5 unit of SAP at 37°C for 40 min and heat terminated at 80°C for 30 min. Sequences were aligned to the WPP14 genome sequence.

DspE constructs. The GENEART[®] Site-Directed Mutagenesis system (Invitrogen) was used to create W464A, W514A and W660A mutations in *dspE* using pCH0001 for the template plasmid and sequence-specific primers (Tables 2) for each mutation. Double mutants W464A/W514A and W464A/W660A were created using pCH0007 for the template plasmid. The W514A/W660A double mutant was created using pCH0008 for the template plasmid and the triple mutant was created using pCH0016 for the template plasmid. All mutations were confirmed by DNA sequencing.

Plant Growth Conditions.

A. thaliana Col-0 were grown in 9 h day-length lighting conditions in a Conviron growth chamber at 23°C. *Nicotiana benthamiana* was grown in growth chambers at 26°C with a 12 h photoperiod under 40-W cool white fluorescent bulbs with photons at

240 μ mol/m²/s. *S. tuberosum* US-W4 and cv. “Dark Red Norland” plants used for agroinfiltration were grown from certified seed tubers in a greenhouse.

Agroinfiltration assays.

Agrobacterium strains were grown for 48h in a shaking incubator (200 rpm) at 28°C in 5 ml of LB medium supplemented with appropriate antibiotics. One ml of this culture was then added to 50 ml of fresh LB with appropriate antibiotics and then grown overnight in a shaking incubator (200 rpm) at 28°C. The culture was then centrifuged (10 min, 7200 *g*) and resuspended in 5 ml of infiltration buffer (pH 5.7) (10 mM MgCl₂, 10mM MES pH5.6, 150 μ M acetosyringone). The bacterial suspension was diluted with the same buffer to adjust the inoculum concentration to an OD₆₀₀= 0.7. The *A. tumefaciens* inoculum was delivered to *N. benthamiana* and *S. tuberosum* leaves in the same manner described for *P. carotovorum* inoculum. The infected area of the leaf was delimited and labeled with an indelible pen. The treated plants were placed in a growth chamber under ambient humidity and reactions were recorded at 24 and 48 hpi. Each assay was performed in triplicate and all plant assays were repeated at least three times.

Bacterial sample collection from plants and RNA isolation.

Leaves of *N. benthamiana* were infiltrated with 1 ml of a cell suspension of *P. carotovorum* at OD₆₀₀=1.0 made from an overnight culture grown on LBA and suspended in sterile water. For each strain three leaves were infiltrated, and RNA was isolated from each individually. Whole leaves were placed in cooled sterile RNase-free mortars pre-filled with 17.5 ml of DEPC water and 1.25 ml of ice cold EtOH/Phenol stop solution

(5% water-saturated phenol, 95% EtOHl pH<7.0) to stabilize cellular RNA and stop degradation. Plant samples were gently ground with a sterile pestle. Supernatants were collected by pouring ground samples through a tea strainer and transferring the supernatant to two sterile 15 ml falcon tubes. Bacterial RNA was collected from supernatants according to the method described in Jahn et al. 2008 (Jahn, Charkowski, and Willis 2008).

Real-time RT-qPCR

Primers were designed based on the draft genome sequence of WPP14 using the Beacon Designer software (Premier Biosoft International, Palo Alto, CA, U.S.A). Primers were designed in regions of little secondary template structure. Sequence primers are shown in Table 2. Primer efficiency was determined using dilution series of target DNA (Pfaffl, Tichopad, Prgomet, and Neuvians 2004). RNA samples were tested for residual DNA contamination using qPCR and primers targeting the *proC* gene in WPP14. RNA samples that showed quantification cycle (Cq) values >30 cycles were considered to be sufficiently free of chromosomal DNA contamination.

cDNA synthesis was performed using iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Briefly, cDNA synthesis was performed with 1 µg of total RNA in 16 µl of DEPC-water and 4 µl of 5X iScript reaction mix containing a blend of oligo(dT), random hexamer primers and reverse transcriptase. The reaction conditions were performed at 25 °C for 5 m, 42 °C for 30 m, and 85 °C for 5 m. Three independent biological replicates samples per experimental condition were used for the cDNA synthesis. The cDNA reactions were

diluted 10-fold and 24 μ l was used for each reaction that was divided into three wells at 20 μ l per well (technical replicates). The cDNA was quantified in the CFX-96 detection system (Bio-Rad Laboratories) using SsoFast EvaGreen Supermix (Bio-Rad Laboratories). PCR conditions were 98°C for 2 m; 40 cycles of 98°C for 2 s and 55°C for 5 s; followed by a dissociation curve with 80 cycles starting at 70°C for 10 s with a 0.2°C increase per cycle. Primer-dimers and presence of a single product per reaction were evaluated using the dissociation (Melt) curve analysis within the CFX detection system software (Bio-Rad Laboratories).

For relative expression analysis, target gene abundance was internally normalized to two reference gene transcripts (*recA* and *proC*) using the formula: $2^{\Delta Cq(\text{target-reference})}$. The reference transcripts were found to be stable under all experimental conditions and were validated using the *BestKeeper* program (Pfaffl, Tichopad, Prgomet, and Neuvians 2004). The transcripts of five genes (*ffh*, *recA*, *proC*, *fliC*, and *gyrA*) were evaluated using the *BestKeeper* program. Of these, three transcripts (*recA*, *proC*, and *fliC*) were validated as stable reference transcripts under our experimental conditions. Target transcript amounts were reported as relative expression ratio (RER) of target transcript in the $\Delta hrpL$ strain relative to wild-type. To determine the RER of target transcripts, the normalized target RNA value was divided by the average of the normalized values of wild-type samples. This average was designated as the “calibrator” since the variation of all samples, including the individual wild-type samples, was determined relative to this value. This method of calculating the RER was derived from the previously published $2^{-\Delta\Delta Cq}$ formula (Pfaffl, Tichopad, Prgomet, and Neuvians 2004; Rotenberg, Thompson, German, and

Willis 2006). Statistical analysis of RER values was conducted by the unpaired two-tailed t-test with 95% confidence interval using Prism 5.0a software (GraphPad Software, Inc.).

Table 1. HrpL-regulated genes found in the DFI screen. T3SS accessory genes are involved in apparatus assembly. When a hrp cluster operon was identified, the first gene of the operon is listed.

Gene	ASAP ID#	Function	Number of Clones	Minimum number of independent inserts
<i>hrpQ/J</i>	ADT-0002852	Type III secretion system component	784	74
<i>hrpA</i>	ADT-0001735	Type III secretion system component	5	2
<i>hrpF</i>	ADT-0001736	Type III secreted protein	6	2
<i>hrpW</i>	ADT-0002859	Type III secreted protein	149	16
<i>hrpK</i>	ADT-0002735	Type III secretion system component	196	17
<i>dspE</i>	ADT-0003863	Type III secreted effector	329	19

Table 2. Plasmids and bacterial strains used in this study

Plasmids and Strains	Description ^a	Source or reference
<i>Agrobacterium tumefaciens</i>		
GV3101	Vir-, Gm ^r , Rif ^r	Van Larebeke et al. 1974
C58C1 ^D	Vir-, Gm ^r , Rif ^r	Johansen and Carrington, 2001
<i>Escherichia coli</i>		
DH5α-E TM	F- φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rk-, mk+) <i>gal-phoA supE44 λ- thi-1 gyrA96 relA1</i>	Invitrogen Corp
DH5α TM -T1 ^R	F- φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (rk-, mk+) <i>phoA supE44 thi-1 gyrA96 relA1 tonA</i> (confers resistance to phage T1)	Invitrogen Corp
<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>		
WPP14	Wild-type strain isolated from an infected potato stem from a Wisconsin field in 2001	Ma et al. 2007; Yap et al. 2004
WPP477	Sp ^r , Δ <i>dspE/F</i> ::Sp derivative of WPP14	Kim et al. 2011
WPP500	Cm ^r , Δ <i>hrpL</i> ::Cm derivative of WPP14	This work
PLASMIDS		
pCFS40	Tet ^r , contains MCS downstream of arabinose inducible promoter	Chang et al. 2005
pBAD::hrpLWPP14	Tet ^r , contains <i>hrpL</i> under control of an arabinose inducible promoter	This work
pBBR1-MCS2	Km ^r , broad host range cloning vector	Kovach et al. 1995
pRK2013	Km ^r , broad host range triparental mating helper plasmid	Figurski and Helinski, 1979
125.1	Km ^r , DFI vector, derivative of pBBR1-MCS2	This work
pCR [®] 2.1-TOPO [®]	Amp ^r , Km ^r , subcloning vector	Invitrogen Crop
pCR2.1SOE <i>hrpL</i>	Amp ^r , Km ^r , contains 1kb regions flanking WPP14 <i>hrpL</i>	This work
pCR2.1Δ <i>hrpL</i>	Amp ^r , Cm ^r , Km ^r , contains Cm ^r cassette inserted between WPP14 <i>hrpL</i> flanking regions	This work
pDONR221	Km ^r A donor vector for the Gateway [®] cloning system	Invitrogen Corp
pGWB2	Km ^r , Hy ^r , Binary vector for agroinfiltration and <i>in planta</i> expression	Nakagawa et al., 2007

	under control of the <i>Cauliflower mosaic virus</i> 35s promoter	
pCH0001	Km ^r pDONR221:: <i>dspE</i> from <i>P. carotovorum</i> subsp. <i>carotovorum</i> strain WPP14	This work
pCH0002	Km ^r , Hy ^r , pGWB2:: <i>dspE</i> from <i>P. carotovorum</i> subsp. <i>carotovorum</i> strain WPP14	This work
pCH0003	Km ^r pDONR221:: <i>dspE</i> - Δ aa1-330	This work
pCH0004	Km ^r pDONR221:: <i>dspE</i> - Δ aa1529-162	This work
pCH0005	Km ^r , Hy ^r , pGWB2:: <i>dspE</i> - Δ aa1-330	This work
pCH0006	Km ^r , Hy ^r , pGWB2:: <i>dspE</i> - Δ aa1529-162	This work
pCH0007	Km ^r , Derivative of pCH0001 with DspE-W464A	This work
pCH0008	Km ^r , Derivative of pCH0001 with DspE-W514A	This work
pCH0009	Km ^r , Derivative of pCH0001 with DspE-W660A	This work
pCH0010	Km ^r , Derivative of pCH0001 with DspE-W464A/W514A	This work
pCH0011	Km ^r , Derivative of pCH0001 with DspE-W464A/W660A	This work
pCH0012	Km ^r , Derivative of pCH0001 with DspE-W464A/W514A/W660A	This work
pCH0013	Km ^r , Hy ^r , pGWB2:: <i>dspE</i> -W464A	This work
pCH0014	Km ^r , Hy ^r , pGWB2:: <i>dspE</i> -W514A	This work
pCH0015	Km ^r , Hy ^r , pGWB2:: <i>dspE</i> -W660A	This work
pCH0016	Km ^r , Hy ^r , pGWB2:: <i>dspE</i> -W464A/W514A	This work
pCH0017	Km ^r , Hy ^r , pGWB2:: <i>dspE</i> -W464A/W660A	This work
pCH0018	Km ^r , Hy ^r , pGWB2:: <i>dspE</i> -W464A/W660A	This work
pCH0019	Km ^r , Hy ^r , pGWB2:: <i>dspE</i> -W464A/W514A/W660A	This work
pSLJ755I5::HC-Pro _{TEV}	Km ^r	Johansen and Carrington, 2001

^aAmp^r, Cm^r, Gm^r, Rif^r, Sp^r, Km^r, Hy^r indicate resistant to ampicillin, chloramphenicol, gentamycin, rifampicin, spectinomycin, kanamycin and hygromycin, respectively. Appropriate antibiotics were added at the following concentrations: ampicillin (100 µg/ml), chloramphenicol (30 µg/ml) gentamycin (50µg/), hygromycin (50 µg/ml) kanamycin (50 µg/ml) rifampicin (15 µg/mL for *A. tumefaciens*, spectinomycin (50 µg/ml), and tetracycline (5 µg/ml).

Table 3. Real-time RT-qPCR primer sequences and efficiency

Gene Name	Primer Sequence	Efficiency (%)^A
<i>ffh</i>	Forward: 5'-TGGAAACATTGGCAGAGC-3' Reverse: 5'-GACTAACAAGACATCGTAGAAC-3'	105
<i>gyrA</i>	Forward: 5'-CGTGAAACCATCATCGTG-3' Reverse: 5'-TTAACCAGCTCGGCAATT-3'	95
<i>proC</i>	Forward: 5'-GTGCGAATTATGCCAAAC-3' Reverse: 5'-TTATCTGCCTGACTGACG-3'	100
<i>recA</i>	Forward: 5'-GTAACCCTGAAACGACTA-3' Reverse: 5'-CAGTACGACGAATATCCA-3'	92
<i>pelB</i>	Forward: 5'-CTCCGTAACAACAACATT-3' Reverse: 5'-GTACTCTTCCAGTCATCT-3'	102
<i>fliC</i>	Forward: 5'-CGAATCTACCATTACTAACCT-3' Reverse: 5'-AGTCAGCGTCTTCAATAC-3'	99
<i>dspE</i>	Forward: 5'-GTCCTATACCAACCTCAG-3' Reverse: 5'-GCAACGAAGAGAACAAAT-3'	99
<i>hrpN</i>	Forward: 5'-CAGGAGTTGAACAACATTAG-3' Reverse: 5'-CCATCTTACGGTCTTCTT-3'	89
<i>hrpL</i>	Forward: 5'-AATGACCTATCTGGAAGT-3' Reverse: 5'-TTGAAATAATTGCGAACC-3'	95

^APrimer efficiencies were calculated from dilution curve using MyiQ Cyclor software

Table 4. Primers for cloning DspE and derivatives

Primer Name	Description	Sequence
DspE-1f	Sequencing forward primer starting at nt 1nt of <i>dspE</i>	ATGCAGAAAATCCAGCAT
DspE-985f	Sequencing forward primer starting at nt 991 of <i>dspE</i>	TTTTCGGCCAATCAGAAC
DspE-4884r-I	Sequencing reverse primer starting at nt 4890 of <i>dspE</i>	TTAACCGTTAAGCTGTAG
DspE-1f-I	First step forward adapter primer for cloning <i>dspE</i> with Gateway® attB1 site	GGCTTCGAAGGAGATAGAACCATGCAGAAA ATCCAGCATGTT
DspE-985f-I	First step forward adapter primer for cloning <i>dspE</i> Δ1-330aa with Gateway® attB1 site	GGCTTCGAAGGAGATAGAACCATG
DspE F-II universal	Second step adapter primer for cloning <i>dspE</i> with Gateway® attB1 site	GGGGACAAGTTTGTACAAAAAAGCAGGCTT CGAAGGAGATAGA
DspE-4589r-I	First step reverse adapter primer for cloning <i>dspE</i> Δ1530-1628aa with Gateway® attB2 site	GTACAAGAAAGCTGGGTCCTATGCCGTGCGT CT
DspE-4589r-II	Second step reverse adapter primer for cloning <i>dspE</i> Δ1530-1628aa with Gateway® attB2 site	GGGGACCACTTTGTACAAGAAAGCTGGGTC CTATGC
DspE-4905r-I	First step reverse adapter primer for cloning <i>dspE</i> with Gateway® attB2 site	GTACAAGAAAGCTGGGTCCTAATCACCGCG TGTTAT
DspE-4905r-II	Second step reverse adapter primer for cloning <i>dspE</i> with Gateway® attB2 site	GGGGACCACTTTGTACAAGAAAGCTGGGTC CTAATC
DspE-W464A-f	Forward primer for tryptophan to alanine substitution at position 464 in DspE protein	TCGCCAACGCCGGGGGCAAACCTGAGCGAC AGT
DspE-W464A-r	Reverse primer for tryptophan to alanine substitution at position 464 in DspE protein	ACTGTCGCTCAGGTTTGGCCCCGGCGTTGGC GA
DspE-W514A-f	Forward primer for tryptophan to alanine substitution at position 514 in DspE protein	AACGGCAATACCAAAAGCGCAGAGGCATCC AGCGTTGAA
DspE-W514A-r	Reverse primer for tryptophan to alanine substitution at position 514 in DspE protein	TTCAACGCTGGATGCCTCTGCGCTTTTGGTA TTGCCTTG
DspE-W660A-f	Forward primer for tryptophan to alanine substitution at	CTGCCGAAAGCCGACGCACAAAATGCGGCG AAT

position 660 in DspE protein

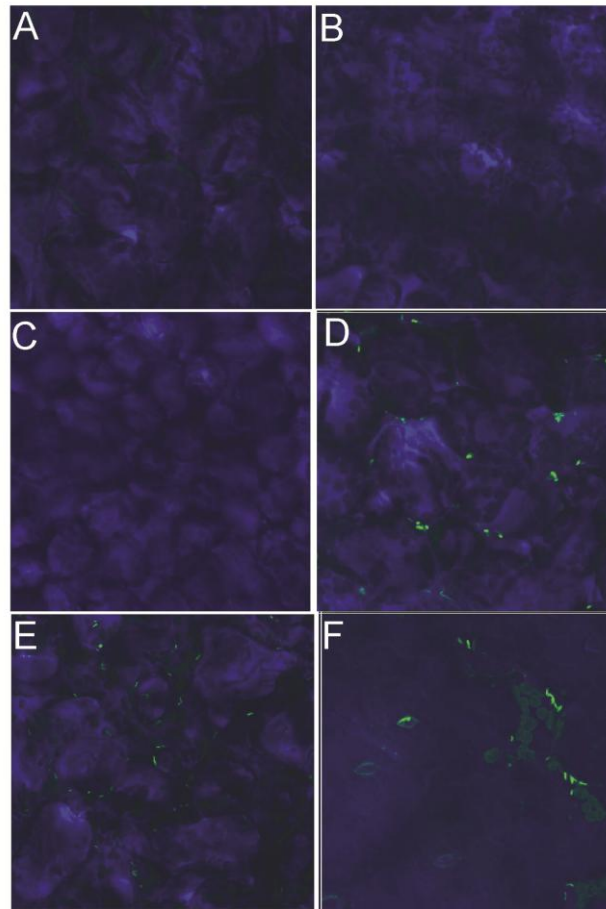


Figure 1. Confocal microscopy images at magnification of 650X of *A. thaliana* Col-0 mesophyll cells 7 hours after infection with *P. carotovorum*. **A.** Mesophyll cells from an uninoculated leaf. **B.** Mesophyll cells from a leaf inoculated with WPP14. **C.** Mesophyll cells infected with WPP14 (pDFI::*dspE_{pro}*GFP). Images are representative of three independent experiments in which at least one leaf was inoculated for each treatment. **D.** Mesophyll cells from a leaf inoculated with 200 mM arabinose and WPP14(pDFI::*dspE_{pro}*GFP) and pBAD::*hrpL* as a positive control. **E and F.** Mesophyll cells infected with WPP14 (pDFI::*dspE_{pro}*GFP). Images are representative of three independent experiments in which at least one leaf was inoculated for each treatment.

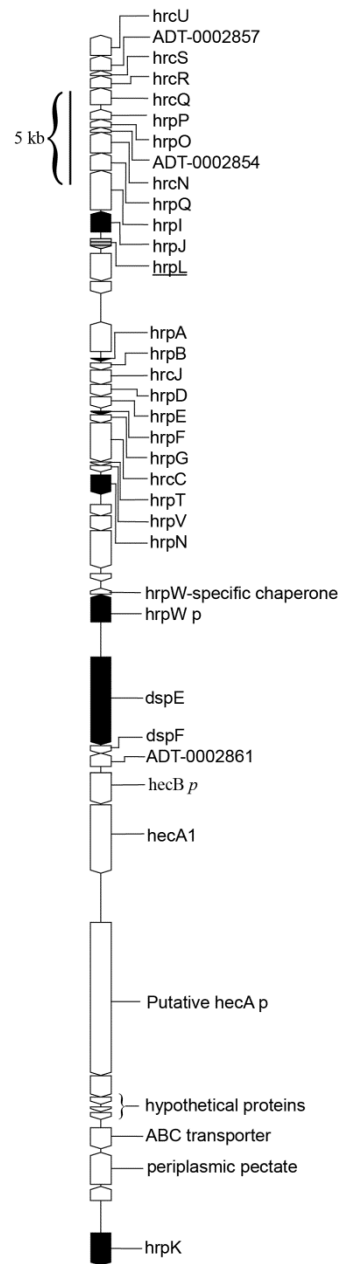


Figure 2. The *hrp* pathogenicity island of *P. carotovorum* strain WPP14 (GenBank accession NZ_ABVT000000000). Black fill denotes detection in the fluorescence based promoter trap screen, and p indicates pseudogenes that have yet to be further examined. This figure was made from a draft genome sequence and it is likely the *hecA1* and putative *hecA* represent a single ORF

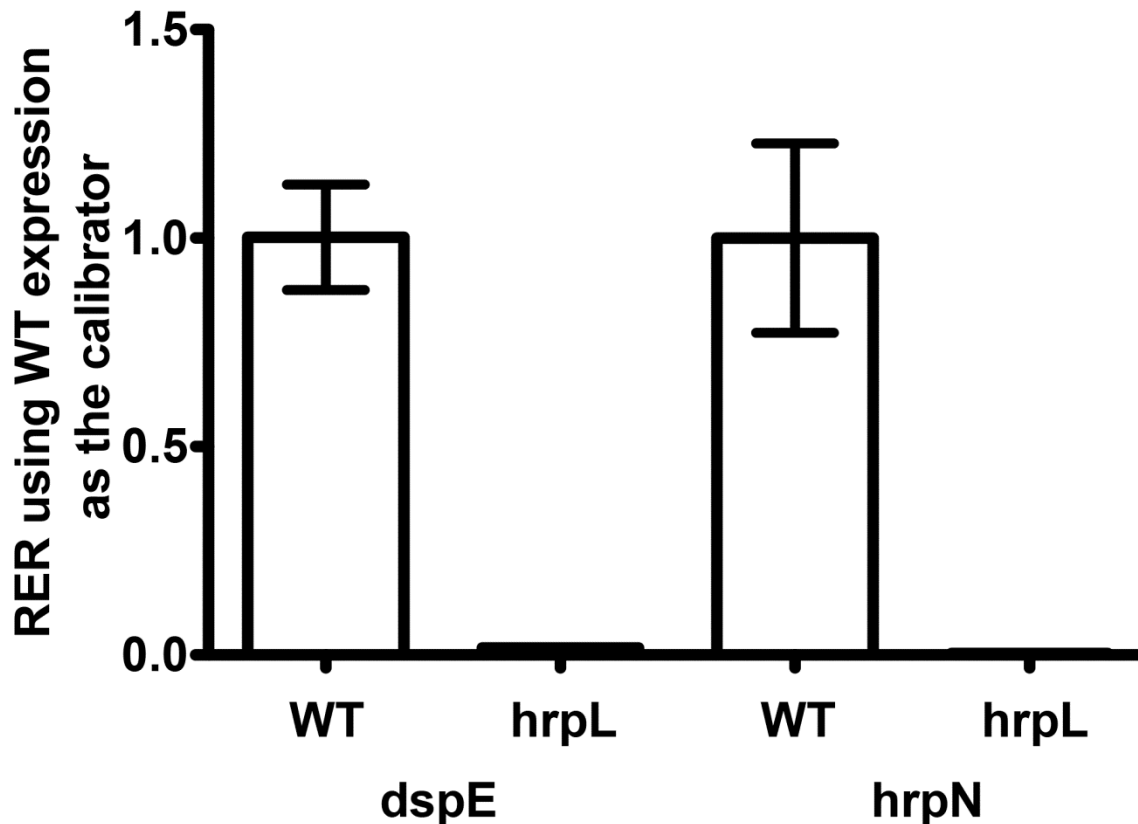


Figure 3. : In planta expression of select *P. carotovorum* genes dspE and hrpL at 5 hpi. Relative expression of the dspE and hrpL transcripts in *N. bethamiana* Col-0 leaves was determined with two validated reference mRNA (recA and proC - Table 2). The mean relative to reference mRNA was used as the calibrator. The difference in the RER means in the hrpL mutant compared to the wild-type for both target transcripts was found to be significant at a 95% confidence interval by ANOVA with a Tukey Post Test using Prism software (Mac version 5.0c, GraphPad Software, inc). Error bars represent standard error of the mean. No differences in expression between the two strains were observed at earlier timepoints.

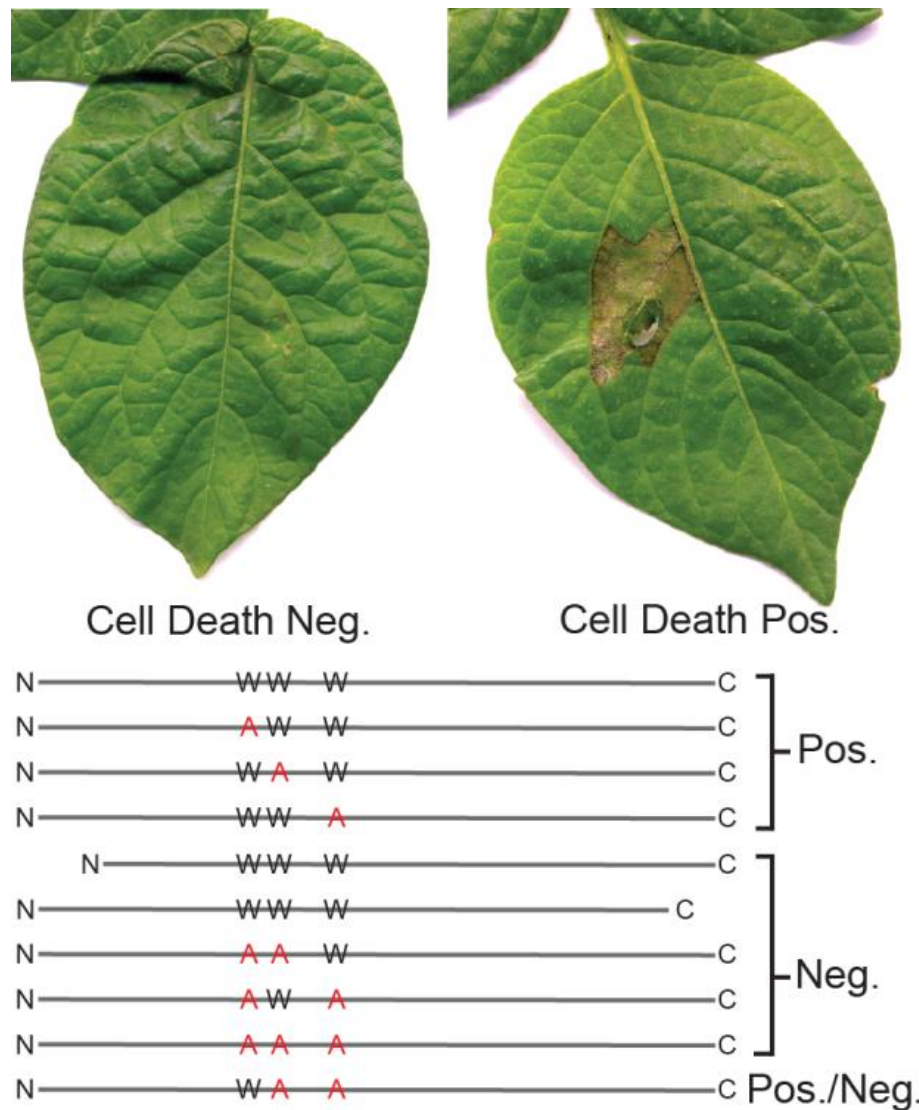


Figure 4. Heterologously expressed DspE causes cell death. Deletion of either the N- or C- terminus, as well as certain tryptophan-to-alanine substitutions, inhibit function. *Agrobacterium* mediated transformation of *S. tuberosum* leaves for expression of DspE and a series of mutant constructs. These leaf images are typical of a positive or negative cell death response. Combination of any two of the W to A substitutions reduced or eliminated function. The derivative containing only the two most downstream substitutions caused an inconsistent cell death response.

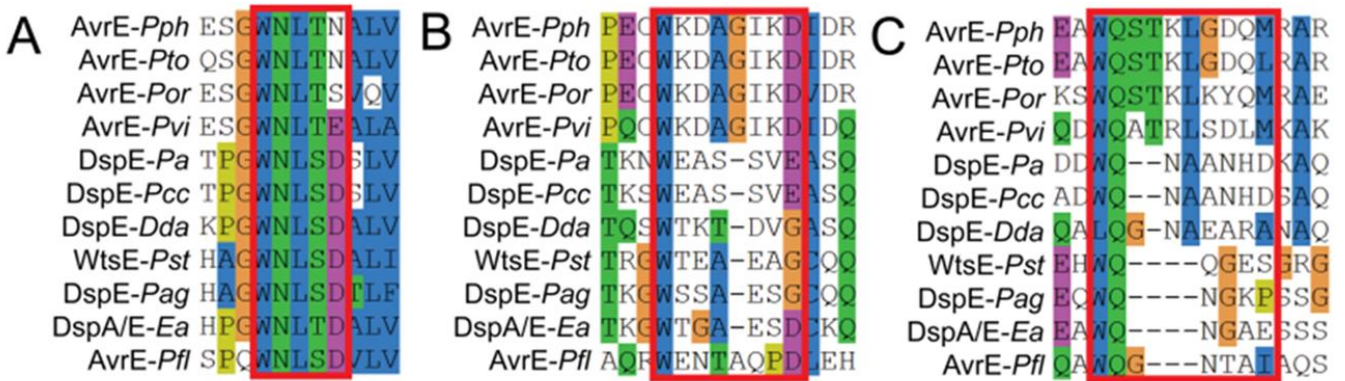


Figure 5. Multiple sequence alignment with hierarchical clustering of DspE homologs in plant pathogenic bacteria. **A.** Conserved tryptophan at position 464, **B.** Conserved tryptophan at position 514, **C.** Conserved tryptophan at position 660 in *P. carotovorum* WPP14 protein sequence. Dashes indicate gaps. Genus and species abbreviations and protein names are as follows *Pph* (*Pseudomonas syringae* pv. *phaseolicola* 1448A , NCBI Ref. Seq. YP_273527.1), *Pto* (*Pseudomonas syringae* pv. *tomato* DC3000, NCBI Ref. Seq. NP_791204.1), *Por* (*Pseudomonas syringae* pv. *oryzae*, NCBI Ref. Seq. ZP_04588443.2), *Pvi* (*Pseudomonas viridiflava*, NCBI Ref. Seq. GenBank accession AAT96164.1), *Pa* (*Pectobacterium atrosepticum* SCRI1043, GenBank accessionYP_050208.1), *Pcc* (*Pectobacterium carotovorum* subsp. *carotovorum* WPP14, NCBI Ref. Seq. ZP_03833468.1), *Dda* (*Dickeya dadantii* 3937, NCBI Ref. Seq. YP_003883118.1), *Pst* (*Pantoea stewartii* subsp. *stewartii*, GenBank accession AAG01467.2), *Pag* (*Pantoea agglomerans* pv. *gypsophilae*, GenBank accession AAF76343.1), *Ea* (*Erwinia amylovora*, GenBank accession AAC04850.1), *Pfl* (*Pseudomonas fluorescens*, GenBank accession AAK74145.1).

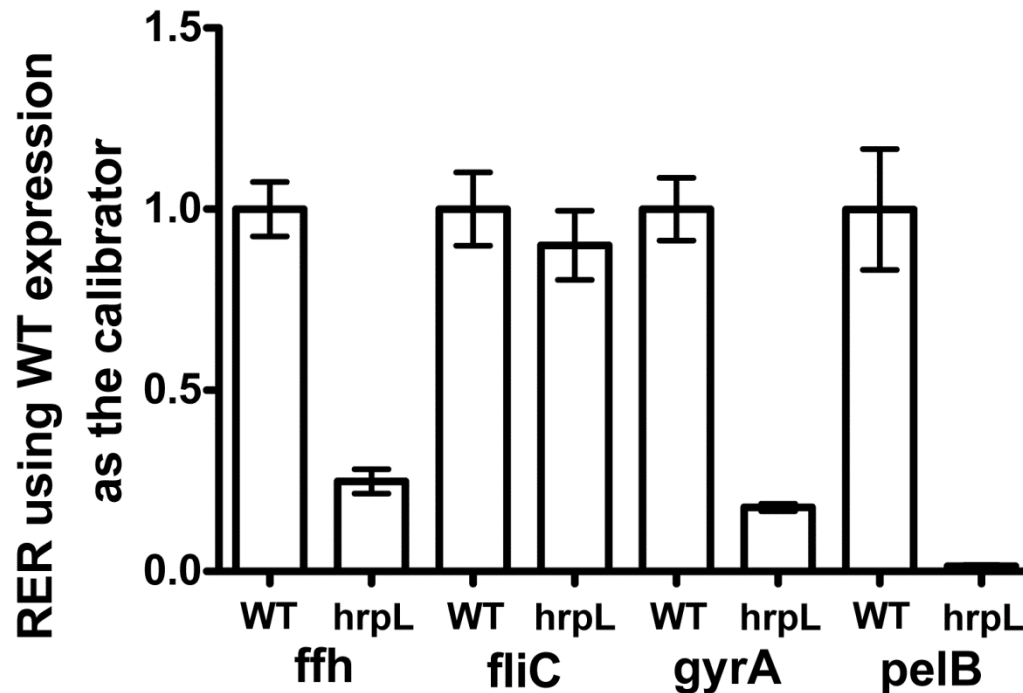


Figure 6 *In planta* expression of select *P. carotovorum* genes *dspE* and *hrpL* at 5 hpi.

Relative expression of the *ffh*, *fliC*, *gyrA* and *pelB* transcripts in *A. thaliana* Col-0 leaves was determined with two validated reference mRNA (*recA* and *proC* – Table 2). The mean relative expression ratio (RER) of the *ffh*, *fliC*, *gyrA* and *pelB* transcripts in the *hrpL* mutant was 0.25, 0.90, 0.18 and 0.015, respectively, compared to expression in the wild-type strain. The mean normalized transcript abundance relative to reference mRNA was used as the calibrator. The difference in the RER means in the *hrpL* mutant compared to wild-type for all target transcripts except *fliC* was found to be significant at a 95% confidence interval by Anova with a Tukey Post Test using Prism software (Mac version 5.0c, GraphPad Software, Inc.). Error bars represent standard error of the mean. No differences in expression between the two strains were observed at earlier timepoints

REFERENCES

- Agrawal, G. K., Iwahashi, H., and Rakwal, R. 2003. Small GTPase “Rop”: molecular switch for plant defense responses. *FEBS Letters*. 546:173–180
- Ahn, I. 2007. Disturbance of the Ca(2+)/calmodulin-dependent signalling pathway is responsible for the resistance of *Arabidopsis dnd1* against *Pectobacterium carotovorum* infection. *Molecular Plant Pathology*. 8:747–59
- Allen, C., Reverchon, S., and Robert-Baudouy, J. 1989. Nucleotide sequence of the *Erwinia chrysanthemi* gene encoding 2-keto-3-deoxygluconate permease. *Gene*. 83:233–241
- Bhaskar, P. B., Venkateshwaran, M., Wu, L., Ané, J.-M., and Jiang, J. 2009. *Agrobacterium*-mediated transient gene expression and silencing: a rapid tool for functional gene assay in potato. *PLOS ONE*. 4:e5812
- Bogdanove, A. J., Bauer, D. W., and Beer, S. V. 1998. *Erwinia amylovora* Secretes DspE, a Pathogenicity Factor and Functional AvrE Homolog, through the Hrp (Type III Secretion) Pathway. *Journal of Bacteriology*. 180:2244–2247
- Borejsza-Wysocka, E. E., Malnoy, M., Aldwinckle, H. S., Meng, X., Bonasera, J. M., and Nissinen, R. M. 2006. The Fire Blight Resistance of Apple Clones in Which DspE-Interacting Proteins Are Silenced. *Acta Horticulturae*. 704:509–513
- Boureau, T., ElMaarouf-Bouteau, H., Garnier, A., Brisset, M.-N., Perino, C., Pucheu, I., et al. 2006. DspA/E, a type III effector essential for *Erwinia amylovora* pathogenicity and growth in planta, induces cell death in host apple and nonhost tobacco plants. *Molecular Plant-Microbe Interactions*. 19:16–24
- Chang, J. H., Urbach, J. M., Law, T. F., Arnold, L. W., Hu, A., Gombor, S., et al. 2005. A high-throughput, near-saturating screen for type III effector genes from *Pseudomonas syringae*. *Proceedings of the National Academy of Sciences of the United States of America*. 102:2549–54
- Chatterjee, A., Cui, Y., and Chatterjee, A. K. 2002. Regulation of *Erwinia carotovora* hrpL(Ecc) (sigma-L(Ecc)), which encodes an extracytoplasmic function subfamily of sigma factor required for expression of the HRP regulon. *Molecular Plant-Microbe Interactions*. 15:971–80
- DebRoy, S., Thilmony, R., Kwack, Y.-B., Nomura, K., and He, S. Y. 2004. A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proceedings of the National Academy of Sciences of the United States of America*. 101:9927–32

- Etienne-Manneville, S., and Hall, A. 2002. Rho GTPases in cell biology. *Nature*. 420:629–635
- Glasner, J. D., Marquez-Villavicencio, M., Kim, H.-S., Jahn, C. E., Ma, B., Biehl, B. S., et al. 2008. Niche-specificity and the variable fraction of the *Pectobacterium* pan-genome. *Molecular Plant-Microbe Interactions*. 21:1549–60
- Goodin, M. M., Zaitlin, D., Naidu, R. a, and Lommel, S. a. 2008. *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions. *Molecular Plant-Microbe Interactions*. 21:1015–26
- Ham, J. H., Majerczak, D. R., Ewert, S., Sreerekha, M.-V., Mackey, D., and Coplin, D. L. 2008. WtsE , an AvrE-family type III effector protein of *Pantoea stewartii* subsp. *stewartii*, causes cell death in non-host plants. *Molecular Plant-Microbe Interactions*. 9:633–643
- Ham, J. H., Majerczak, D. R., Nomura, K., Mecey, C., Uribe, F., He, S.-Y., et al. 2009. Multiple activities of the plant pathogen type III effector proteins WtsE and AvrE require WxxxE motifs. *Molecular Plant-Microbe Interactions*. 22:703–12
- Harrison, M., Quinn, C., Sells, I. A., and Graham, D. 1977. Waste potato dumps as sources of insects contaminated with soft rot coliform bacteria in relation to re-contamination of pathogen-free potato stocks. *Potato Research*. 20:37–52
- Huang, Z., Sutton, S. E., Wallenfang, A. J., Orchard, R. C., Wu, X., Feng, Y., et al. 2009. Structural insights into host GTPase isoform selection by a family of bacterial GEF mimics. *Nature Structural and Molecular Biology*. 16:853–60
- Jahn, C. E., Charkowski, A. O., and Willis, D. K. 2008. Evaluation of isolation methods and RNA integrity for bacterial RNA quantitation. *Journal of Microbiological Methods*. 75:318–24
- Johansen, L. K., and Carrington, J. C. 2001. Silencing on the spot. Induction and suppression of RNA silencing in the *Agrobacterium*-mediated transient expression system. *Plant Physiology*. 126:930–8
- Kim, H. S., Ma, B., Perna, N. T., and Charkowski, A. O. 2009. Phylogeny and virulence of naturally occurring type III secretion system-deficient *Pectobacterium* strains. *Applied and Environmental Microbiology*. 75:4539–49
- Kim, H. S., Thammarat, P., Lommel, S. A., Hogan, C. S., and Charkowski, A. O. 2011. *Pectobacterium carotovorum* elicits plant cell death with DspE/F but the *P. carotovorum* DspE does not suppress callose or induce expression of plant genes early in plant-microbe interactions. *Molecular Plant-Microbe Interactions*. 24:773–86

- Lagaert, S., Beliën, T., and Volckaert, G. 2009. Plant cell walls: Protecting the barrier from degradation by microbial enzymes. *Seminars in Cell and Developmental Biology*. 20:1064–73
- Matsumoto, H., Jitareerat, P., Baba, Y., and Tsuyumu, S. 2003. Comparative study of regulatory mechanisms for pectinase production by *Erwinia carotovora* subsp. *carotovora* and *Erwinia chrysanthemi*. *Molecular Plant-Microbe Interactions*. 16:226–37
- McCarter-Zorner, N. J., Franc, G. D., Harrison, M. D., Michaud, J. E., Quinn, C. E., Sells, I. A., et al. 1984. Soft rot *Erwinia* bacteria in surface and underground waters in southern Scotland and in Colorado, United States. *Journal of Applied Bacteriology*. 57:95–105
- McCarter-Zorner, N. J., Harrison, M. D., Franc, G. D., Quinn, C. E., Sells, I. A., and Graham, D. C. 1985. Soft rot *Erwinia* bacteria in the rhizosphere of weeds and crop plants in Colorado, United States and Scotland. *Journal of Applied Bacteriology*. 59:357–368
- Meng, X., Bonasera, J. M., Kim, J. F., Nissinen, R. M., and Beer, S. V. 2006. Apple proteins that interact with DspA/E, a pathogenicity effector of *Erwinia amylovora*, the fire blight pathogen. *Molecular Plant-Microbe Interactions*. 19:53–61
- Molina, J. M., Harrison, D., and Brewer, J. W. 1974. Transmission of *Erwinia carotovora* var. *atroseptica* by *Drosophila melanogaster* Meig. I. Acquisition and transmission of the bacterium. *American Potato Journal*. 19:52–61
- Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., et al. 2007. Development of series of gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. *Journal of Bioscience and Bioengineering*. 104:34–41
- Ohlson, M. B., Huang, Z., Alto, N. M., Blanc, M.-P., Dixon, J. E., Chai, J., et al. 2008. Structure and function of *Salmonella* SifA indicate that its interactions with SKIP, SseJ, and RhoA family GTPases induce endosomal tubulation. *Cell Host and Microbe*. 4:434–46
- Pfaffl, M. W., Tichopad, A., Prgomet, C., and Neuvians, T. P. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnology Letters*. 26:509–15
- Rotenberg, D., Thompson, T. S., German, T. L., and Willis, D. K. 2006. Methods for effective real-time RT-PCR analysis of virus-induced gene silencing. *Journal of Virological Methods*. 138:49–59
- Sambrook, J., and Russell, D. W. 2001. *Molecular cloning: a laboratory manual*.

Takle, G. W., Toth, I. K., and Brurberg, M. B. 2007. Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen *Pectobacterium atrosepticum*. BMC Plant Biology. 7:50

Wang, K., Uppalapati, S. R. A. O., Zhu, X., Dinesh-kumar, S. P., and Mysore, K. S. 2010. SGT1 positively regulates the process of plant cell death during both compatible and incompatible plant – pathogen interactions. Molecular Plant Pathology. 11:597–611

Xiao, Y., and Hutcheson, S. W. 1994. A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. Journal of Bacteriology. 176:3089–91

Chapter 3 - *Pectobacterium carotovorum* requires autophagy like host cell death elicited by the pathogen effector DspE- to activate expression of a large number of pathogenesis associated genes under water limiting conditions

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ABSTRACT

Pectobacterium carotovorum is a gram-negative phytopathogenic bacterium that causes soft rot on diverse plant species. The alternative sigma factor HrpL is required during pathogenesis for up-regulation of *hrp* type III secretion system (T3SS) gene expression, leading to plant cell death mediated by the type III effector DspE. Here we show plant cell death mediated by DspE results in altered expression of numerous *P. carotovorum* virulence and central metabolism genes. Although expression of *ohr*, which detoxifies H₂O₂, is altered, addition of H₂O₂ to leaves infiltrated with a *P. carotovorum dspE* mutant did not restore wild-type gene expression. DspE causes water-soaking symptoms prior to plant cell death, and water availability played a role in *P. carotovorum* gene expression. Increasing free water availability restored wild-type *P. carotovorum* gene expression and negated the requirement for the T3SS to establish disease in leaves. Dyes specific for autophagy-associated cell structures indicate that cell death caused by DspE resembles autophagy. Additionally, we found that maceration promotes pathogen dissemination through physical contact with surfaces, though *P. carotovorum* is not able to persist in

lesions. Our results suggest that DspE and the T3SS function to effectively lower the environmental humidity at which *P. carotovorum* can initiate pathogenesis in leaves.

INTRODUCTION

Pectobacterium species cause soft rot disease on plant species from over 24 orders of angiosperms, with potato being the most economically important host (Toth, Bell, Holeva, and Birch 2003). This gram-negative pathogen is widespread in agricultural systems and can be isolated from soil, surface and ground water, and a variety of insects and snails (Harrison, Quinn, Sells, and Graham 1977; McCarter-Zorner et al. 1984, 1985; Molina, Harrison, and Brewer 1974). Acyl-homoserine lactone (AHL)-mediated quorum sensing plays a key role in *Pectobacterium* virulence and affects regulation of approximately one quarter of *Pectobacterium* genes, including expression of the plant cell wall degrading enzymes (PCWDE) responsible for the characteristic maceration symptoms caused by this genus (Liu et al. 2008). Although required for maceration symptoms, PCWDE production is not sufficient for disease. Several other virulence factors also contribute, including proteins required for iron acquisition (Smits and Duffy 2011), defense against plant antimicrobial compounds (Bell et al. 2004), and the type III secretion system (T3SS) (Kim, Ma, Perna, and Charkowski 2009). Quorum sensing, in concert with other environmental perception mechanisms, works through central regulators to coordinate production of this diverse array of virulence factors.

Pectobacterium carotovorum is considered the most important *Pectobacterium* species due to its host range and global distribution (Toth, Bell, Holeva, and Birch 2003). The T3SS plays an important role in *P. carotovorum* pathogenesis on leaves of solanaceous plants (Kim, Ma, Perna, and Charkowski 2009; Kim, Thammarat, Lommel, Hogan, et al. 2011; Hogan, Mole, Grant, Willis, et al. 2013). This secretion system is a complex protein translocation mechanism used by many animal- and plant-interacting

bacteria to deliver type 3 effector (T3E) proteins into host cells. These effectors cause a cascade of events necessary to establish bacterial infection (Francis, Wolf-Watz, and Forsberg 2002). The genes encoding the T3SS in plant pathogens are denoted *hrp* (for Hypersensitive Response (HR) and Pathogenicity), and they are encoded in a cluster of structural and regulatory genes flanked by genes encoding secreted proteins and their chaperones (Chatterjee, Cui, and Chatterjee 2002). The *hrp* and effector genes are coordinately regulated by the alternative sigma factor HrpL in *P. carotovorum*, so that deletion of *hrpL* from the genome prevents the pathogen from producing a functional type III system.

Pectobacterium strains lacking a T3SS have a slight reduction in virulence in potato stems (Holeva et al. 2004), but the greatest effect of the T3SS on virulence is in plant leaves; T3SS mutations almost entirely eliminate pathogenicity in potato and tobacco leaves. Despite extensive genomic and experimental approaches, only a single T3SS effector, DspE, has been identified in the *Pectobacterium* genome, and deletion of this single effector gene severely compromises the ability of *P. carotovorum* to cause symptoms on leaves (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011). Additionally, transient expression of DspE causes host cell death in leaves, though the mechanism remains unclear (Hogan, Mole, Grant, Willis, and Charkowski 2013).

The mechanism of T3E-mediated plant cell death is yet to be determined, though recent studies indicate that autophagy, which is a form of programmed cell death involved in stress response, plays a role in plant immunity (Lai, Wang, Zheng, Fan, et al. 2011). Cells use autophagy to degrade and recycle cellular components, and it is involved in the HR and production of reactive oxygen species (Kabbage, Williams, and Dickman

2013; Liu et al. 2005; Williams and Dickman 2008). Mutations in autophagy genes increase susceptibility to necrotrophic fungi (Lenz et al. 2011), and these pathogens actively suppresses the formation of autophagosomes. However, no reported studies have examined autophagy in response to attack by bacterial necrotrophs (Kabbage, Williams, and Dickman 2013).

Unlike most families of type 3 effectors encoded by phytopathogenic bacteria, the DspE/AvrE/ WtsE-family is widespread across species including *Pseudomonas*, *Pantoea*, *Dickeya*, *Pectobacterium*, and *Erwinia* spp. Little is known about the function of this class of effectors other than their ability to contribute to virulence of diverse bacterial plant pathogens, including *Erwinia amylovora*, *Pantoea stewartii*, *Pseudomonas syringae*, and *P. carotovorum* (Boureau et al. 2006; DebRoy, Thilmony, Kwack, Nomura, et al. 2004; Ham et al. 2008; Bogdanove, Bauer, and Beer 1998). DspE homologs from hemi-biotrophic bacterial pathogens interfere with plant defenses. For example, the *Pseudomonas syringae* AvrE blocks pathogen-induced callose deposition along the plant cell wall (DebRoy, Thilmony, Kwack, Nomura, and He 2004). The *E. amylovora* and *Pantoea stewartii* DspE homologs suppress salicylic acid (SA)-mediated host defenses (Boureau et al. 2006; DebRoy, Thilmony, Kwack, Nomura, and He 2004; Ham et al. 2008). Unlike the DspE homologs from hemibiotrophs, there is no evidence that *P. carotovorum* DspE suppresses SA-mediated host defenses or callose deposition (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011).

DspE homologs from *Pseudomonas syringae*, *E. amylovora*, *Pantoea stewartii*, and *P. carotovorum* cause plant cell death when expressed transiently in leaves (Ham et al. 2009; Oh, Martin, and Beer 2007; Hogan, Mole, Grant, Willis, and Charkowski 2013)

and our previous results suggest that *P. carotovorum* takes advantage of DspE-mediated leaf cell death to initiate pathogenesis. Therefore, we chose to further explore the effects of DspE on plant-bacterial interactions to discover advantages provided by DspE-mediated plant cell death. The role that host cell death plays in pathogenesis remains to be defined, but it may contribute to transmission and be required for spread throughout the host.

We found that deletion of DspE resulted in loss of expression of numerous virulence and central metabolism genes when *P. carotovorum* was infiltrated into plant leaves. One of these genes, *ohr*, detoxifies H₂O₂, suggesting that release of this molecule by plant cells may affect bacterial gene regulation. However, co-infiltration of H₂O₂ with a *P. carotovorum* DspE mutant did not restore wild-type bacterial gene expression. We also examined expression of genes that encode proteins which act as central regulators of pathogenesis associated genes. KdgR, a transcriptional repressor, is involved in activating expression of pathogenesis associated genes in response to pectin, but expression of this gene is not altered during the switch to an active pathogenic state. We noted that expression of *expI*, which encodes AHL synthase, was much lower in the DspE mutant than the wild-type strain. The expression of the *expI* operon is up regulated by AHL by an ExpR-mediated feedback loop, suggesting that DspE-mediated plant cell death promotes this feedback loop. We found that increasing free water availability by misting water onto inoculated leaves restored the DspE mutant to wild-type gene expression and symptom development phenotypes, suggesting that increasing water availability in leaf tissue, and not cell death itself, explains how DspE promotes *P. carotovorum* pathogenesis in leaves. DspE-mediated cell death causes the formation of

autophagy associated structures, indicating that DspE may function by activating this programmed cell death pathway. DspE mediated cell death doesn't seem to promote growth of the pathogen, and *P. carotovorum* is not able to persist in macerated leaf tissue; however, tissue maceration following cell death promotes dissemination of the pathogen through physical contact, indicating a role for cell death in the pathogen life cycle.

RESULTS

Identification of stable reference gene targets under relevant experimental conditions.

To establish appropriate genes to normalize expression in planta, we identified and tested several gene candidates in the *P. carotovorum* WPP14 genome (Glasner 2003) based on previous work (Hogan, Mole, Grant, Willis, and Charkowski 2013; Takle, Toth, and Brurberg 2007). Previously, we found that expression of *proC* was significantly more stable than the other reference gene targets tested (*recA*, *fliC*). We initially used *recA* as a second reference gene because it was valid according to the *BestKeeper* program.

However, the standard deviation of *recA* was notably higher than *proC* (Table 1), so we trialed additional genes to find one with stability equivalent to *proC*. Genes identified as promising candidates were similar to *proC* in that they all appear to be encoded in single gene operons. Of the genes tested, *argA* proved to be the most stable. Surprisingly, expression of many of the candidate genes was down regulated in the $\Delta hrpL$ and $\Delta dspE$ mutant strains relative to the wild-type WPP14 strain (Fig. 1), further highlighting the importance of testing reference genes under the conditions being studied rather than relying solely upon previously reported reference genes.

We examined eight genes involved in amino acid biosynthesis as candidate reference genes and found that four of them showed significantly higher levels of expression *in planta* in the wild-type WPP14 strain compared to the *hrpL/dspE* mutant strains (Fig. 1). Even though *proC*, *argA*, *argG* and *lysA* expression remained stable, a large number of genes involved in amino acid biosynthesis did exhibit *dspE*-dependent up-regulation, including *asnA*, *argI*, *glyA*, and *ilvC*. The previously established *in planta* reference gene *gyrA* (Takle, Toth, and Brurberg 2007) encoding DNA gyrase also exhibited *dspE* dependent up-regulation.

Comparison of virulence gene expression between wild-type and mutant *P. carotovorum* strains.

DspE is required for expression of many *P. carotovorum* virulence and central metabolism genes. Deletion of DspE does not cause a growth defect in media or *in planta* at the site of inoculation (Fig.4 and S1) (Kim, Thammarat, Lommel, Hogan, and Charkowski 2011) indicating that observed differences in gene expression between the wild-type and mutant strains is not due to an overall growth defect, but a defect specifically in the regulation of pathogenesis-associated genes. It is possible that the *dspE* mutant strains are defective in their ability to spread throughout the host. However we only monitored *in planta* growth within the inoculated area of leaf tissue. We examined the effect of *dspE* and *hrpL* deletions on expression of twenty *P. carotovorum* genes in bacteria inoculated into *Nicotiana benthamiana* leaves. These genes, with the exception of the candidate reference genes involved in amino acid biosynthesis, were chosen because they potentially made contributions to pathogenesis and included genes

encoding PCWDE, antimicrobial resistance enzymes, and regulators of these genes (Fig. S2) (Table 4).

Initially we took samples at 3 hours post inoculation, preceding tissue maceration, which occurs at 5 to 8 hours post inoculation, and found no differences in expression of target genes between wild-type and mutant strains. We found that in samples taken when tissue maceration began to occur, the reference genes were stably expressed for both the wild-type and mutant strains, and some of the target genes showed significant differences in expression between the two strains (data not shown). Later time points were not used because it was impossible to sample a consistent quantity of tissue, which following maceration becomes very fragile.

Unlike wild-type *P. carotovorum*, the DspE mutant strain does not macerate leaf tissue so we chose to examine genes from different PCWDE families. The pectate lyases *pelB* and *pelI*, the protease *prtW*, and the cellulase *celV* were expressed at a lower level in the DspE mutant than in the wild-type strain *in planta* (Fig. 1). Not all PCWDE were up regulated in the wild-type strain. Neither the cellulase *celB* nor the polygalacturonases *pehA* or *pehN* showed significant *dspE*-dependent regulation.

It appears that *P. carotovorum* is able compensate for the release of reactive oxygen species characterizing the plant immune response so we examined expression of genes encoding two different classes of antimicrobial resistance proteins. *In planta* up-regulation of the organic hydrogen peroxide reductase *ohr* depended upon *dspE*, but expression of the superoxide dismutase *sodA* was not affected by the *dspE* deletion.

DspE is unlikely to directly regulate these diverse virulence and central metabolism genes, so we also assessed the expression of two key virulence gene

regulators. There was not a significant difference in *kdgR* expression, but accumulation of *expI* mRNA was significantly decreased in the $\Delta dspE$ and $\Delta hrpL$ mutants (Fig. 1).

Oxidative stress does not stimulate virulence gene expression.

DspE-dependent up-regulation of pathogenesis associated genes is not due to hydrogen peroxide released during the plant defense response. We hypothesized that HrpL and DspE do not directly regulate this diverse group of genes as previous studies have shown that HrpL regulates only genes associated with the T3SS. This hypothesis is further supported by the lack of HrpL binding sites associated with central metabolism genes and plant cell wall degrading genes (Hogan, Mole, Grant, Willis, and Charkowski 2013; Glasner et al. 2008), and the lack of any previous evidence from any plant pathogenic bacterium that DspE is a regulatory protein. Rather, we hypothesized that the central metabolism genes and PCWDE genes were responding to changes in plant physiology induced by DspE. One possible candidate for affecting bacterial gene expression is the burst of reactive oxygen that occurs as part of the plant defense response. Because the H_2O_2 detoxifying *Ohr* was differentially regulated, we examined the effect of co-infiltrating *N. benthamiana* leaves with hydrogen peroxide and wild-type *P. carotovorum* or a $\Delta dspE$ mutant strain to see if hydrogen peroxide up-regulated expression of pathogenesis-associated genes. We first determined that the maximum concentration of hydrogen peroxide that *P. carotovorum* can tolerate in culture is 0.5 mM. This concentration was below the maximum concentration that *N. benthamiana* can tolerate when infiltrated into leaves, which we determined was higher than 13.5 mM. We observed that the gene expression patterns were the same with or without co-infiltration

of 0.5mM hydrogen peroxide (Fig. 2). Thus, hydrogen peroxide released during the hypersensitive response is unlikely to compensate for the loss of DspE in activation of PCWDE expression.

Free water stimulates expression of virulence genes and promotes pathogenesis

Symptom and gene expression differences caused by deletion of *dspE* were complemented by addition of free water (Fig. 3). Water-soaked lesions are a common symptom of infection by many bacterial plant pathogens and are characterized as dark, irregular, translucent areas of tissue that are saturated with water. We observed water-soaked lesions caused by transient expression of DspE in host cells and infection with wild-type *P. carotovorum* (Hogan, Mole, Grant, Willis, and Charkowski 2013). We hypothesized that water released from dying host cells due to DspE-mediated host cell death during the initial stages of pathogenesis activates expression of *P. carotovorum* virulence genes, perhaps by increasing efficiency of AHL signaling. Following inoculation with *P. carotovorum* wild-type and the $\Delta dspE$ mutant strains, leaves were misted with water every 20 minutes to maintain a constant film of free water on leaf surfaces. The addition of free water restored up-regulation of pathogenesis-associated genes in the $\Delta dspE$ mutant strain (Fig. 3) and restored the ability of the mutant to macerate tissue (data not shown).

Water soaked lesions facilitate bacterial movement through plant tissue and enhance transmission to new hosts. We compared physical transmission of *P. carotovorum* from macerated and un-macerated leaf tissue. We used toothpicks to simulate insect legs

by lightly touching the surface of leaf tissue, and dilution plated a water wash from the toothpicks onto LBA to quantify the amount of bacteria picked up from the rotting tissue. We found that 50 fold more *P. carotovorum* cells are transmitted from water soaked lesions on infiltrated leaf tissue as compared to non-water soaked infiltrated tissue (Fig. 4). We also examined the growth and survival of *P. carotovorum* in lesions and were unable to recover any bacteria once the lesion became desiccated, approximately 48 hours after the initiation of pathogenesis (data not shown).

DspE elicits production of autophagosomes.

Autophagy has been linked to plant defense against a number of pathogens (Liu et al. 2005) and the effector A2 from the fungal pathogen *Sclerotinia sclerotiorum* elicits production of autophagosomes (Kabbage, Williams, and Dickman 2013).

Autophagosomes can be visualized using the fluorescent dyes LysoTracker, which stains acidic compartments, and monodansylcadaverine (MDC), which stains double membrane autophagosomes (Contento, Xiong, and Bassham 2005) . We vacuum infiltrated these dyes at concentrations of 1 μ M and 100 μ M respectively into leaf tissue 24 hours post inoculation with *Agrobacterium* carrying either wild-type or non-functional mutant *dspE*. We observed punctate fluorescent structures indicating the presence of autophagosomes in stained samples from leaves expressing wild-type *dspE* but not from leaves expressing the non-functional *dspE* (Fig. 5). These data supports the hypothesis that DspE induces cell death by activating autophagy in host cells.

DISCUSSION

P. carotovorum requires the T3SS, and its sole known effector DspE, to cause maceration symptoms in plant leaves, suggesting that this broad host range necrotrophic pathogen benefits from DspE-mediated plant cell death. We found that a *P. carotovorum* DspE mutant was unable to up-regulate numerous virulence and central metabolism genes when inoculated into leaves. The loss of DspE can be compensated for by the addition of free water to the leaf surface, suggesting that water-soaking symptoms observed may be caused by DspE to promote expression of virulence and central metabolism genes. One of the genes differentially expressed by wild-type and *dspE* mutants is *expI*, which is required for production of the acyl-HSL quorum sensing signal and which is involved in regulating approximately 25% of *Pectobacterium* genes (Liu et al. 2008). Our data suggest that DspE-induced water soaking increases efficiency of AHL signaling and result in up-regulation of virulence and central metabolism genes in leaves, thereby promoting pathogenesis. The mechanism by which DspE causes water-soaking and plant cell death remains unknown, but we provide evidence that DspE-mediated cell death elicits activation of autophagic programmed cell death. We also show that *Pectobacterium* does not survive in macerated leaf lesions, but that it is easily spread from macerated leaves through physical contact.

Assessing *P. carotovorum* gene regulation in leaves by RT-qPCR required identification of suitable reference genes. Although several studies have proposed identification of suitable reference genes for a wide variety of conditions (Hommais et al. 2011; Takle, Toth, and Brurberg 2007), no gene has a constant expression level; thus it is important to identify valid reference genes for every experimental condition. We found

that the previously established reference genes *gyrA* and *ffh* were not stable under our experimental conditions (data not shown). We used *proC* and *recA* initially in our studies, but decided to search for an additional reference gene as *recA* was very close to the threshold limit for standard deviation according to the *BestKeeper* program.

Alternatively, we found *proC* expression is very stable, perhaps because it is in a single gene operon and because of its role in central metabolism encoding a component of amino acid biosynthesis. We surveyed the WPP14 genome (Glasner et al. 2008) for genes with these attributes and tested the stability of each of these candidate reference genes. We found that *argA* is the most stable of the genes tested under our experimental conditions (Table 1). Several of the candidate reference genes, including four involved in amino acid biosynthesis, and one coding for DNA gyrase, are expressed at significantly higher levels in the wild-type strain than the two mutant strains, suggesting that increased expression of genes involved in central metabolism contributes to pathogenesis.

Following establishment of reliable reference genes, we examined expression of 11 virulence genes of interest with RT-qPCR to compare expression of these genes during pathogenesis in wild-type, and *dspE* mutant strains. We found that increased expression of genes encoding PCWDE is not uniform. Genes coding for the protease PrtW and the pectate lyases PelI and PelB are significantly up regulated in the wild-type strain relative to the mutants, while genes coding for the polygalacturonases PehA and PehN are not. These differences are not restricted to pectinases, as *celV* expression is significantly up regulated in the wild-type strain while relative to the mutants while *celB* expression is the same across strains. Differences in gene expression are likely a

reflection of the specificity of different pathogenesis genes for different tissues, stages of infection, or hosts, and this may account for the broad host range of *P. carotovorum*.

DspE is unlikely to directly regulate these genes, so we examined DspE-dependent expression of two regulators. The gene encoding KdgR, a virulence gene repressor, is expressed equivalently across the three strains. In contrast, *expI*, which encodes AHL synthase, showed significantly higher expression in the wild-type strains, which could explain the significant gene expression changes caused by deletion of *hrpL* and *dspE* since the *Pectobacterium* quorum sensing system affects regulation of approximately one quarter of the genome. Previous studies have shown that *pelB*, *prtW*, *celB*, *celV*, *pehA*, *pehN* and *kdgR* are all regulated by quorum sensing in culture medium (Liu et al. 2008), though no previous studies have examined expression of genes involved in central metabolism. Our results do not show significantly elevated expression of *celB*, *pehA* and *pehN* *in planta* in the wild-type *P. carotovorum* relative to the *dspE* mutant strains, suggesting a time or a tissue specific role for these macerating enzymes.

We hypothesize that the DspE-dependent expression of bacterial genes *in planta* is the result of the effects of DspE on plant cells and not due to direct regulation by DspE. However, the nature of the signals generated by the dying plant cell that activate bacterial gene expression remains unclear. Alternatively, it is possible that DspE could be acting within the bacterial cell to directly regulate gene expression. However, there has been no data presented over the past twenty years of research on DspE family proteins, acyl-HSL QS, and PCWDE gene regulation to support this hypothesis. For example, an extensive survey for genes regulated by HrpL in *P. carotovorum* did not identify any central

metabolism genes, any PCWDE genes, or *expI* (Hogan, Mole, Grant, Willis, and Charkowski 2013).

Since expression of *ohr* (organic hydroperoxide reductase) was significantly elevated in wild-type but not the mutant strain, we hypothesized that H₂O₂ concentration might affect PCWDE and central metabolism gene expression. However, co-infiltration of H₂O₂ with *hrpL* or *dspE* mutants did not restore expression of PCWDE or central metabolism gene expression to wild-type levels, providing no evidence that H₂O₂ concentration is the DspE-mediated signal for the phenomena that we observed.

We observed that the amount of time at which the leaves infiltrated with wild-type began to wilt varied inversely with the relative humidity (data not shown). Further, we observed that when the relative humidity was above 70%, leaves infiltrated with the $\Delta dspE$ strain would occasionally become macerated. Based on these observations, in combination with recent developments in the understanding of the role of water in phyllosphere pathogenesis (Beattie 2011; Toth and Birch 2005) we hypothesize that free water in leaves is required for activation of pathogenesis-associated genes and the release of free water into the apoplastic space resulting from DspE-mediated cell death allows *P. carotovorum* to activate pathogenesis under environmental conditions that would otherwise be too dry for pathogenesis to occur. DspE family proteins have been implicated in water-soaking symptoms common to many bacterial leaf pathogens and available water increases efficacy of soluble extracellular signals, such as the QS compound, acyl-HSL (Beattie 2011). Indeed, we were able to restore wild-type expression levels of PCWDE and central metabolism genes by simply misting water onto leaves infiltrated with *hrpL* and *dspE* mutants (Fig. 3).

Though free water appears essential for initiation of pathogenesis, it is likely additional signals are involved, including release of peptides and other cell components, and environmental signals such as changes in pH and oxygen. If cell death was all that was needed for *P. carotovorum* to initiate pathogenesis, a toxin should be sufficient rather than production of a complex and metabolically costly secretion system. It is likely that additional signals from the DspE-mediated HR are advantageous to *P. carotovorum*, such as induction of SA by the HR, which suppresses the jasmonic acid/ethylene (JA/ET) pathways that are known to inhibit *P. carotovorum* pathogenesis (Glazebrook 2005) .

We found the initiation of maceration also promotes spread of the pathogen by physical contact (Fig. 4). These data support the hypothesis that the T3SS increases fitness of *P. carotovorum* by allowing it to be released from leaves in the absence of excess free water. In combination with previous results showing that *P. carotovorum* produces a known insect-attracting aromatic compound (Marquez-Villavicencio, Weber, Witherell, Willis, et al. 2011) and can be transmitted by insects from infected tubers to wounded potato stems (Kloepper, Brewer, and Harrison 1981), this finding supports the hypothesis that mechanical transmission of the pathogen by physical contact, including potential insect transmission, is an important mechanism for colonizing new hosts. The recent widespread use of insect resistant transgenic corn has eliminated stalk rot caused by soft rot bacteria in maize (Dalmacio, Lugod, Serrano, and Munkvold 2007), suggesting that this strategy could be used against soft rot in potato and other host crops.

Recent studies have investigated the role of autophagy in necrotrophic fungal interactions with plants (Lai, Wang, Zheng, Fan, and Chen 2011; Lenz et al. 2011; Kabbage, Williams, and Dickman 2013). Here we show that necrotrophic bacterial

pathogens activate this process. Necrotrophic plant pathogens benefit from host cell death, therefore autophagy could play a role in promoting pathogenesis (Hofius, Munch, Bressendorff, Mundy, et al. 2011). Though *P. carotovorum* cannot hydrolyze starch as a source of energy (Billing and Baker 1963), autophagy could play a role in converting starch to sugar, which would benefit *P. carotovorum* by increasing osmotic pressure causing cell lysis, in addition to making the energy stored in starch available to the pathogen (Nakayama et al. 2012). The role of autophagy in response to necrotrophic pathogenesis is unclear since *Arabidopsis* autophagy mutants have elevated susceptibility to necrotrophic fungi (Lenz et al. 2011). The animal pathogens *Coxiella burnetii* and *Legionella pneumophila* take advantage of autophagy by using autophagosomes to provide a niche for bacterial replication (Mizushima, Levine, Cuervo, and Klionsky 2008). Our data showing formation of autophagosomes in response to DspE suggests that *P. carotovorum* shares the strategy of utilizing autophagic programmed cell death to promote disease development.

Taken together, these results support a model in which, under conditions of low environmental humidity, transfer of DspE to the host cytosol by the T3SS activates autophagic programmed cell death resulting in water-soaking that enhances quorum sensing and enables tissue maceration. This water soaking activates expression of genes associated with infection, including a specific set of PCWDE and a subset of central metabolism genes. Plant cell death triggers production of SA, and therefore inhibits JA/ET signaling pathways, which have been shown to be involved in defense against necrotrophs (Glazebrook 2005). Increased expression of bacterial virulence genes in response to increased availability of free water, activation by the QS inducer ExpI, and

down-regulation of JA/ET plant defenses via SA, leads to leaf tissue maceration. The leaf maceration then allows *P. carotovorum* pathogenesis to progress through the host plant tissue and facilitates physical transmission to new hosts by releasing *P. carotovorum* from the leaf for transmission by water, insects, machinery, or plant-to-plant contact.

The specific target of DspE in plant cells that leads to plant cell death remains unknown, though our results support that its target is part of the host autophagy pathway. Identification of a binding partner for DspE, and localization of DspE within the host cell are important for dissecting the events underlying DspE-mediated host cell death, and will possibly aid in defining some of the mechanisms underlying resistance to *P. carotovorum* in wild *Solanum* species. Attacking virulence rather than viability of pathogen cells is an attractive strategy for disease management, and the methodology used in this study provides a system in which inhibitors of activation of pathogenesis could be assayed. Knock-out plants deficient in autophagy genes should be tested to determine whether specific components of the autophagy pathway are required for DspE-mediated cell death, and may lead to the development of soft rot resistant cultivars by identifying knock-out lines with enhanced disease resistance. Additionally, integrated pest management and methods that interfere with insect attraction to diseased plants or bacterial uptake could prove effective at reducing crop losses due to soft rot (Charkowski et al. 2012).

MATERIALS AND METHODS

Bacterial strains and their growth conditions.

Bacterial strains and plasmids used in this study are listed in Table 3. *Escherichia coli* and *Pectobacterium carotovorum* strains were grown from single colonies in Luria-Bertani (LB) broth in a 250 rpm shaking incubator at 37°C or maintained on LB plates solidified with 1.5% BD Bacto-agar, containing appropriate antibiotics. The composition of LB medium is published in Sambrook and Russell (Sambrook and Russell 2001). The minimal medium for *hrpL* repression was described in (Chang et al. 2005).

Agrobacterium tumefaciens strains were also grown from single colonies in LB broth in a shaking incubator at 28°C or maintained on LB plates.

Plant Growth Conditions.

Nicotiana benthamiana was grown in growth chambers at 26°C with a 12 h photoperiod under 40-W cool white fluorescent bulbs with photons at 240 μ mol/m²/s.

Agroinfiltration assays.

Agrobacterium strains were grown for 48h in a shaking incubator (200 rpm) at 28°C in 5 ml of LB medium supplemented with appropriate antibiotics. One ml of this culture was then added to 50 ml of fresh LB with appropriate antibiotics and then grown overnight in a shaking incubator (200 rpm) at 28°C. The culture was then centrifuged (10 min, 7200 *g*) and resuspended in 5 ml of infiltration buffer (pH 5.7) (10 mM MgCl₂, 10 mM MES pH5.6, 150 μ M acetosyringone) and incubated for 3 to 5 hours in a shaking incubator (200rpm) at 28 °C. The bacterial suspension was diluted with the same buffer to adjust the inoculum concentration to an OD₆₀₀= 0.7. The *A. tumefaciens* inoculum was delivered to *N. benthamiana* and *S. tuberosum* leaves in the same manner described for *P.*

carotovorum inoculum. The infected area of the leaf was delimited and labeled with an indelible pen. The treated plants were placed in a growth chamber under ambient humidity and reactions were recorded at 24 and 48 hpi. Each assay was performed in triplicate and all plant assays were repeated at least three times.

Bacterial sample collection from plants and RNA isolation.

Leaves of *N. benthamiana* were infiltrated with 1 ml of a cell suspension of *P. carotovorum* at OD₆₀₀=1.0 made from an overnight culture grown on LB agar and suspended in sterile water. For each strain, at least three leaves were infiltrated and RNA was isolated from each individually. Whole leaves were placed in cooled sterile RNase-free mortars pre-filled with 17.5 ml of DEPC water and 1.25 ml of ice cold EtOH/phenol stop solution (5% water-saturated phenol, 95% EtOH pH<7.0) to stabilize cellular RNA. Plant samples were gently ground with a sterile pestle. Supernatants were collected by pouring ground samples through a tea strainer and transferring the supernatant to two sterile 15 ml falcon tubes. Bacterial RNA was collected from supernatants according to the method described in Jahn et al. 2008 (Jahn, Charkowski, and Willis 2008).

Real-time RT-qPCR.

Primers were designed based on the draft genome sequence of WPP14 using the Beacon Designer software (Premier Biosoft International, Palo Alto, CA, U.S.A). Primers were designed in regions of little secondary template structure. Sequence primers are shown in Table 2. Primer efficiency was determined using dilution series of target DNA (Pfaffl, Tichopad, Prgomet, and Neuvians 2004). RNA samples were tested for residual DNA

contamination using qPCR and primers targeting the *proC* gene in WPP14. RNA samples that showed quantification cycle (Cq) values >30 cycles were considered to be sufficiently free of chromosomal DNA contamination.

cDNA synthesis was performed using iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Briefly, cDNA synthesis was performed with 1 µg of total RNA in 16 µl of DEPC-water and 4 µl of 5X iScript reaction mix containing a blend of oligo(dT), random hexamer primers and reverse transcriptase. The reaction conditions were performed at 25 °C for 5 m, 42 °C for 30 m, and 85 °C for 5 m. Three independent biological replicates samples per experimental condition were used for the cDNA synthesis. The cDNA reactions were diluted 10-fold and 24 µl was used for each reaction that was divided into three wells at 20 µl per well (technical replicates). The cDNA was quantified in the CFX-96 detection system (Bio-Rad Laboratories) using SsoFast EvaGreen Supermix (Bio-Rad Laboratories). PCR conditions were 98°C for 2 m; 40 cycles of 98°C for 2 s and 55°C for 5 s; followed by a dissociation curve with 80 cycles starting at 70°C for 10 s with a 0.2°C increase per cycle. Primer-dimers and presence of a single product per reaction were evaluated using the dissociation (Melt) curve analysis within the CFX detection system software (Bio-Rad Laboratories).

For relative expression analysis, target gene abundance was internally normalized to two reference gene transcripts (*recA* or *argA* and *proC*) using the formula: $2^{\Delta Cq(\text{target-reference})}$. The reference transcripts were found to be stable under all experimental conditions and were validated using the *BestKeeper* program (Pfaffl, Tichopad, Prgomet, and Neuvians 2004). The transcripts of ten genes (*argA*, *asnA*, *argG*, *argI*, *glyA*, *ilvC*,

lysA, *gyrA*, *recA* and *proC*) were evaluated using the *BestKeeper* program. Of these, three transcripts (*recA*, *proC*, and *argA*) were validated as stable reference transcripts under our experimental conditions. Target transcript amounts were reported as relative expression ratio (RER) of target transcript in the $\Delta hrpL$ and $\Delta dspE$ strains relative to wild-type. To determine the RER of target transcripts, the normalized target RNA value was divided by the average of the normalized values of wild-type samples. This average was designated as the “calibrator” since the variation of all samples, including the individual wild-type samples, was determined relative to this value. This method of calculating the RER was derived from the previously published $2^{-\Delta\Delta Cq}$ formula (Pfaffl, Tichopad, Prgomet, and Neuvians 2004; Rotenberg, Thompson, German, and Willis 2006). Statistical analysis of RER values was conducted by the unpaired two-tailed t-test with 95% confidence interval using Prism 5.0a software (GraphPad Software, Inc.).

Mechanical transmission from macerated tissue.

To determine if mutations affected bacterial transmission from macerated *Nicotiana benthamiana* leaves, bacterial populations obtained through mechanical transmission were quantified. Leaves of *N. benthamiana* were infiltrated with 1 ml of a cell suspension of wild-type *P. carotovorum* WPP14, *dspE* mutant, or *hrpL* mutant strains at $OD_{600} = 0.3$ made from an overnight culture grown on LBA and suspended in sterile water. Infiltrated plants were incubated in the growth chamber until macerated tissue formed on the wild-type infiltrated plants. A sterile toothpick was stabbed into the inoculation site on the leaf and then agitated in 500 μ L of sterile water in a 1.5 mL sterile microcentrifuge tube. Bacterial populations obtained were quantified by dilution plating on LB agar medium,

incubating the plates at 37°C for 24 hours, and counting the resulting colonies. Three replicates were used to calculate means and standard errors. Statistical analysis of values was conducted by the unpaired two-tailed t-test with 95% confidence interval using Prism 5.0a software (GraphPad Software, Inc.)

Table 1. Real-time RT-qPCR primer sequences, efficiency, and stability of candidate reference genes.

Gene Name	Primer Sequence	Efficiency (%)^A	BestKeeper SD^B
<i>argA</i>	Forward: 5'-CAACTTCTCTAGCATCGTAA-3' Reverse: 5'-TAGTGAGGCTCGTAGTGA-3'	97	0.57
<i>asnA</i>	Forward: 5'-AAACAACAGCAAATCAGC-3' Reverse: 5'-GCCTGGACTTCAATCAAT-3'	106	1.39
<i>argG</i>	Forward: 5'-AGATGTCCGAGTTTATGT-3' Reverse: 5'-ATATTGGAATCGGTGGAA-3'	100	1.34
<i>argI</i>	Forward: 5'-CCGTTCTATAAGCGTCATT-3' Reverse: 5'-TATCGGCTTTCAGTTTCG-3'	100	1.00
<i>glyA</i>	Forward: 5'-TTAACCTGTCGGGCAAAC-3' Reverse: 5'-AGCCATTTTCGTCGTAGTC-3'	114	1.42
<i>gyrA</i>	Forward: 5'-CGTGAAACCATCATCGTG-3' Reverse: 5'-TTAACCAGCTCGGCAATT-3'	95	1.38
<i>ilvC</i>	Forward: 5'-GAATGATGACGTGAAACT-3' Reverse: 5'-CCGTTATCAAAGTATTCCT-3'	108	1.06
<i>lysA</i>	Forward: 5'-GAATCCAGGATTTGGTCAT-3' Reverse: 5'-TACCAGATACCGTGCTTG-3'	97	1.14
<i>proC</i>	Forward: 5'-GTGCGAATTATGCCAAAC-3' Reverse: 5'-TTATCTGCCTGACTGACG-3'	100	0.65
<i>recA</i>	Forward: 5'-GTAACCCTGAAACGACTA-3' Reverse: 5'-CAGTACGACGAATATCCA-3'	92	0.94

^APrimer efficiencies were calculated from dilution curve using MyiQ Cyclor software.^BBestKeeper standard deviation must be below 1.0 to be consider stable.

Table 2. Real-time RT-qPCR primer sequences and efficiency

Gene Name	Primer Sequence	Efficiency (%)^A
<i>pelB</i>	Forward: 5'-CTCCGTAACAACAACATT-3' Reverse: 5'-GTACTCTTCCAGTCATCT-3'	102
<i>pelI</i>	Forward: 5'-CATGCTGATGCTGCTGAA-3' Reverse: 5'-TACACATCCTGGCGAACA-3'	108
<i>prtW</i>	Forward: 5'-TCAGATGTCGCTAATATCA-3' Reverse: 5'-GAGTATCGTAGTCCATCC-3'	114
<i>celB</i>	Forward: 5'-CACCTCCAATACCAACAAC-3' Reverse: 5'-ACTCAACGCTGCTGATAT-3'	120
<i>celV</i>	Forward: 5'-TATATTTCCAATCCCTCC-3' Reverse: 5'-GTTATCCGACAAGATATG-3'	124
<i>pehA</i>	Forward: 5'-CAATCAGGCAAGCGAGTT-3' Reverse: 5'-CGGGAATCAGAAGCAAATG-3'	116
<i>pehN</i>	Forward: 5'-AGTAACATAAGGCTGGAT-3' Reverse: 5'-CATCGGCAATACTGATAA-3'	102
<i>kdgR</i>	Forward: 5'- CAGATTTAGATAAACAACCCGATT-3' Reverse: 5'-CTCTTCACCTAATGCCTGTAA-3'	100
<i>ohr</i>	Forward: 5'-AGTATTATACGTTGCTCAT-3' Reverse: 5'-GGGTCGTTAATTTAATATCA-3'	110
<i>sodA</i>	Forward: 5'-GCTGCCTTATGCTTATGA-3' Reverse: 5'-CCTGATGGTGTTTGGAAAT-3'	101
<i>expI</i>	Forward: 5'-GAAGGTAATTATCTGGAATC-3' Reverse: 5'-TAATATCGTCAGCATAGG-3'	99

^APrimer efficiencies were calculated from dilution curve using MyiQ Cyclor software.

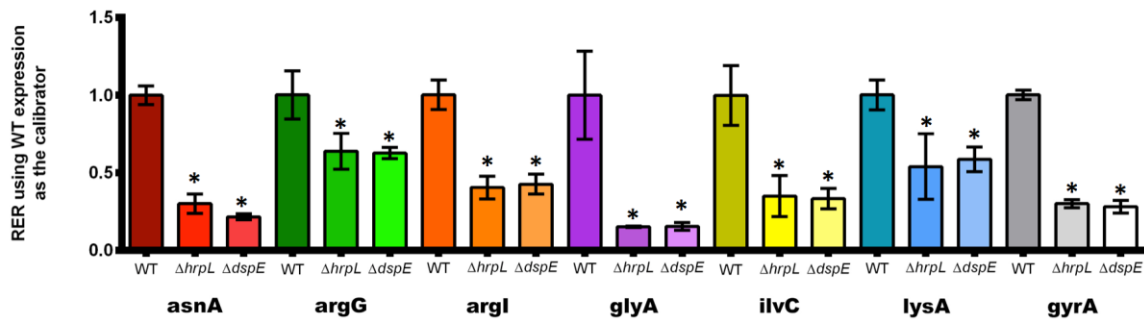
Table 3 Bacterial strains used in Chapter 3

Plasmids and Strains	Description^a	Source or reference
<i>Agrobacterium tumefaciens</i>		
GV3101	Vir-, Gm ^r , Rif ^r	Van Larebeke et al. 1974
C58C1 ^D	Vir-, Gm ^r , Rif ^r	Johansen and Carrington, 2001
<i>Escherichia coli</i>		
DH5α-E TM	F- φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rk-, mk+) <i>gal-phoA supE44 λ- thi-1 gyrA96 relA1</i>	Invitrogen Corp
DH5α TM -T1 ^R	F- φ80 <i>lacZ</i> ΔM15 Δ(<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (rk-, mk+) <i>phoA supE44 thi-1 gyrA96 relA1 tonA</i> (confers resistance to phage T1)	Invitrogen Corp
<i>Pectobacterium carotovorum</i> subsp. <i>carotovorum</i>		
WPP14	Wild-type strain isolated from an infected potato stem from a Wisconsin field in 2001	Ma et al. 2007; Yap et al. 2004
WPP477	Sp ^r , Δ <i>dspE/F</i> ::Sp derivative of WPP14	Kim et al. 2011
WPP500	Cm ^r , Δ <i>hrpL</i> ::Cm derivative of WPP14	Kim et al. 2011

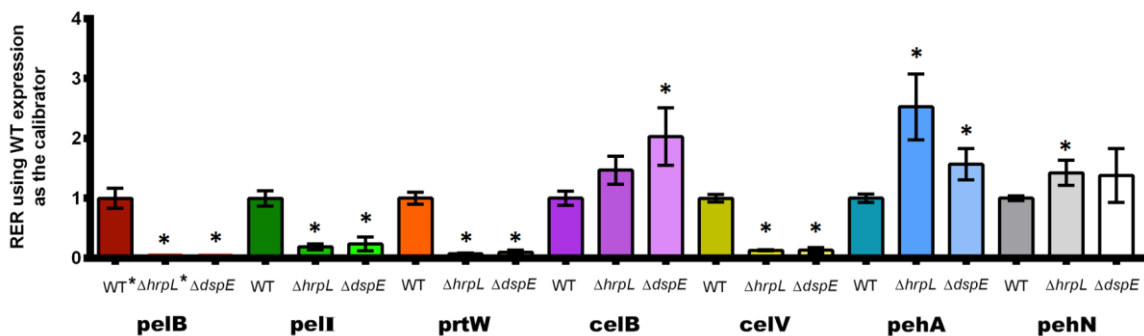
Table 4 Genes that were examined for *in planta* expression.

Gene name	Product	Function	Reference
asnA	Asparagine synthetase	Asparagine biosynthesis	(Humbert and Simoni 1980)
argA	N-acetylglutamate synthase	arginine biosynthesis	(Leisinger and Haas 1975)
argI	Ornithine carbamoyltransferase	arginine biosynthesis	(Legrain, Stalon, and Glansdorff 1976)
glyA	Serine hydroxymethyltransferase	biosynthesis of purines, thymidine, methionine, choline and lipids	(Stover and Schirch 1990)
ilvC	Acetohydroxy acid isomeroreductase	biosynthesis of isoleucine and valine	(Hill and Duggleby 1999)
lysA	Diaminopimelate decarboxylase	lysine biosynthesis	(Stragier, Richaud, Borne, and Patte 1983)
gyrA	DNA gyrase subunit A	DNA unwinding	(Neumann and Quiñones 1997)
pelB	Pectate lyase B	Plant cell wall degradation	(Lei et al. 1987)
pelI	Pectate lyase I	Plant cell wall degradation	(Shevchik, Robert-Baudouy, and Hugouvieux-Cotte-Pattat 1997)
prtW	Extracellular protease	Maceration of plant tissue	(Marits, Kõiv, Laasik, and Mäe 1999)
celB	Cellulase	Plant cell wall degradation	(Saul et al. 1990)
celV	Cellulase	Plant cell wall degradation	(Cooper and Salmond 1993)
pehA	Polygalacturonase	Plant cell wall degradation	(Saarilahti et al. 1990)
pehN	Polygalacturonase	Plant cell wall degradation	(Hugouvieux-Cotte-Pattat, Shevchik, and Nasser 2002)
kdgR	Transcriptional repressor	Regulates genes involved in pectin catabolism	(Reverchon, Nasser, and Robert-Baudouy 1991)
ohr	Organic hydrogen peroxide reductase	Resistance to oxidative damage	(Mongkolsuk, Praituan, Loprasert, Fuangthong, et al. 1998)
sodA	Superoxide dismutase	Resistance to oxidative damage	(Takeda and Avila 1986)
expI	Autoinducer synthase	Regulation of cell density dependent processes	(Pirhonen, Flego, Heikinheimo, and Palva 1993)

Central Metabolism



Plant Cell Wall Degrading Enzymes



Other Virulence-Associated Genes

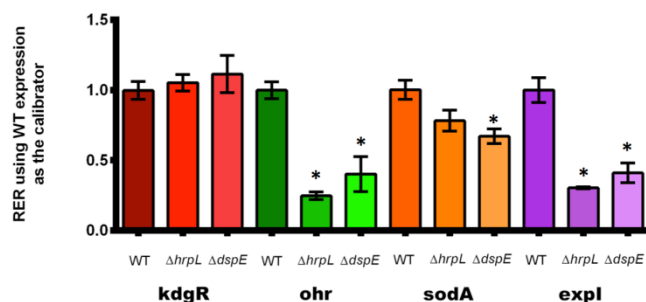


Figure 1. *In planta* expression of select *P. carotovorum* genes in wild-type (WT) and *hrpL* and *dspE* deletion strains during pathogenesis. Relative expression of the *asnA*, *argG*, *argI*, *glyA*, *ilvC*, *lysA*, *gyrA*, *pelB*, *pelI*, *prtW*, *celB*, *celV*, *pehA*, *pehN*, *kdgR*, *ohr*, *sodA*, and *expl* transcripts in *N. benthamiana* leaves was determined with two validated reference mRNA (*argA* and *proC* – Table 1). The mean relative expression ratio (RER) of the gene transcripts in the *hrpL* and *dspE* mutants is relative to the mean normalized transcript abundance of the reference mRNA for each gene. Error bars depict standard error from the mean; asterisks denote values whose means were significantly different than the WT at the 95% confidence interval by ANOVA with a Tukey Post Test using Prism software (Mac version 5.0c, GraphPad Software, inc).

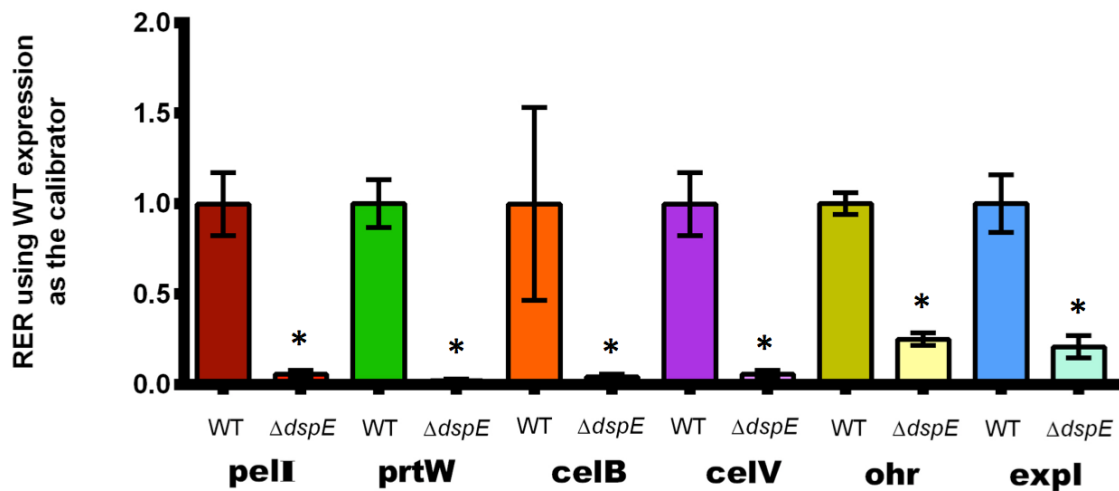


Figure 2.- In planta expression of select *P. carotovorum* genes in wild-type (WT) and *dspE* deletion strains during pathogenesis when coinfiltrated with 0.5mM H₂O₂.

Relative expression of the *pelI*, *prtW*, *celV*, *pehA*, *pehN*, *kdgR*, *ohr*, *sodA*, and *expI* transcripts in *N. benthamiana* leaves was determined with two validated reference mRNA (*argA* and *proC* – Table 1). The mean relative expression ratio (RER) of the gene transcripts in the *hrpL* and *dspE* mutants is relative to the mean normalized transcript abundance of the reference mRNA for each gene. Error bars depict standard error from the mean; asterisks denote values whose means were significantly different than the WT at the 95% confidence interval by ANOVA with a Tukey Post Test using Prism software (Mac version 5.0c, GraphPad Software, inc).

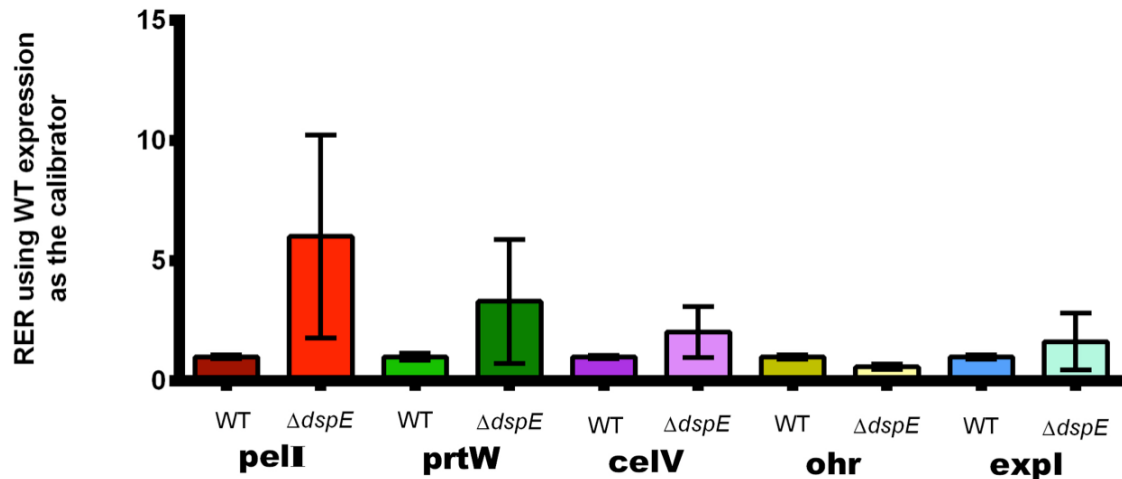


Figure 3. - *In planta* expression of select *P. carotovorum* genes in wild-type (WT) and *dspE* deletion strains during pathogenesis under wet conditions. Relative expression of the *pell*, *prtW*, *celV*, *ohr*, and *expl* transcripts in *N. benthamiana* leaves was determined with two validated reference mRNA (*argA* and *proC* – Table 1). The mean relative expression ratio (RER) of the gene transcripts in the *hrpL* and *dspE* mutants is relative to the mean normalized transcript abundance of the reference mRNA for each gene. Error bars depict standard error from the mean; no genes were significantly differently expressed between the WT and mutant strains at the 95% confidence interval by ANOVA with a Tukey Post Test using Prism software (Mac version 5.0c, GraphPad Software, inc).

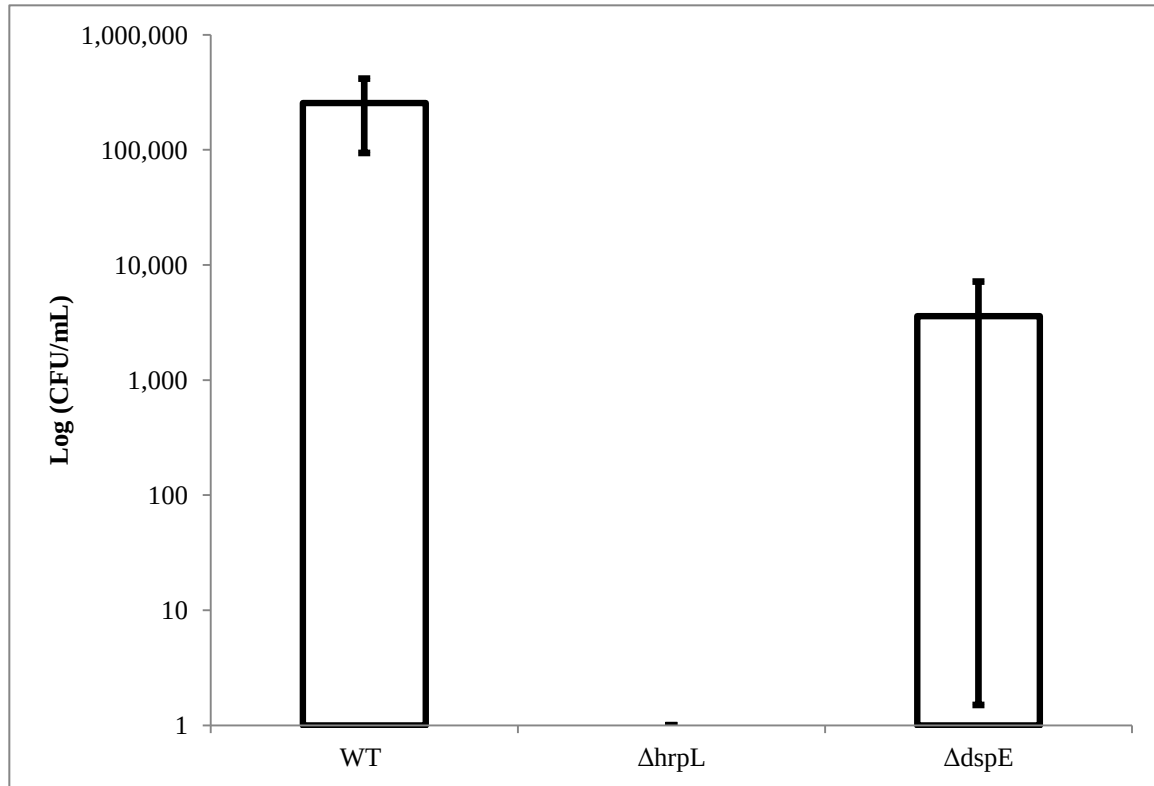


Figure 4. Mechanical transmission is enhanced by macerating tissue. Counts of bacteria obtained by lightly touching a toothpick on infiltrated tissue and agitating in 50 μ L sterile H₂O. 50X more wild-type *P. carotovorum* were obtained than *hrpL* and *dspE* mutant strains. Only the wild-type infiltrated tissue became macerated. Error bars depict standard deviation from the mean. Wild-type was significantly different from the two mutant strains using Student's unpaired t test ($p < 0.05$), though the mutant strains were not significantly different.

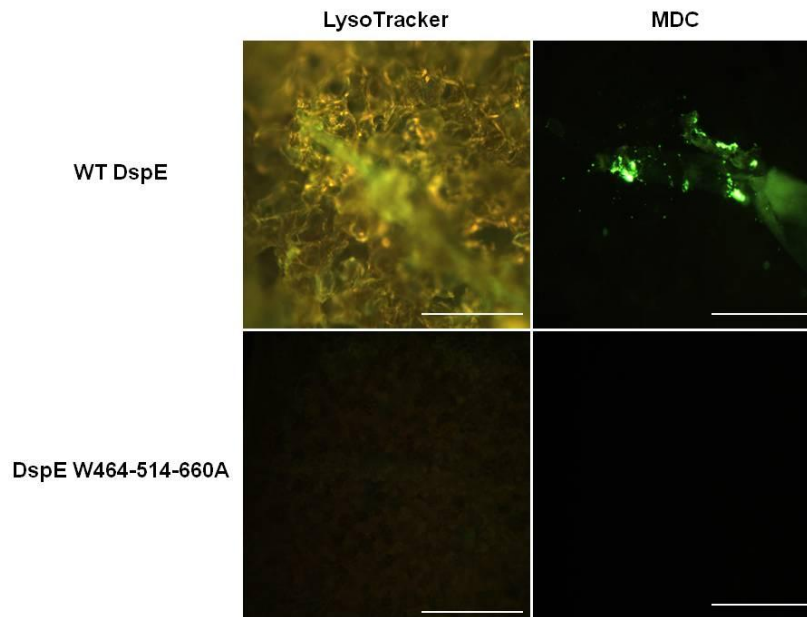


Figure 5 Transiently expressed *P. carotovorum* DspE induces autophagic structures in leaves. *P. carotovorum* DspE and its non-functional mutant derivative containing three tryptophan-to-alanine substitutions (W464-514-660A) were transiently expressed in *N. benthamiana* leaf tissue using *Agrobacterium*-mediated expression. 24 hours post inoculation; leaves were stained with 1 μ M final concentration LysoTracker Blue DND-22(Invitrogen) in $\frac{1}{2}$ MS for 1 hour or 100 μ M final concentration MDC (Sigma) in PBS for 30 min. Fluorescence was visualized using an Olympus IX81 inverted fluorescence confocal microscope (Olympus systems, Germany), with an excitation wavelength of 373nm and an emission wavelength of 422 nm for LysoTracker blue and an excitation wavelength of 335 nm and an emission wavelength of 508 nm for MDC. Images were collected using an Olympus DP 70 camera and processed with Olympus DP Controller software, version 2.2.1.227. Scale bar = 10 μ m.

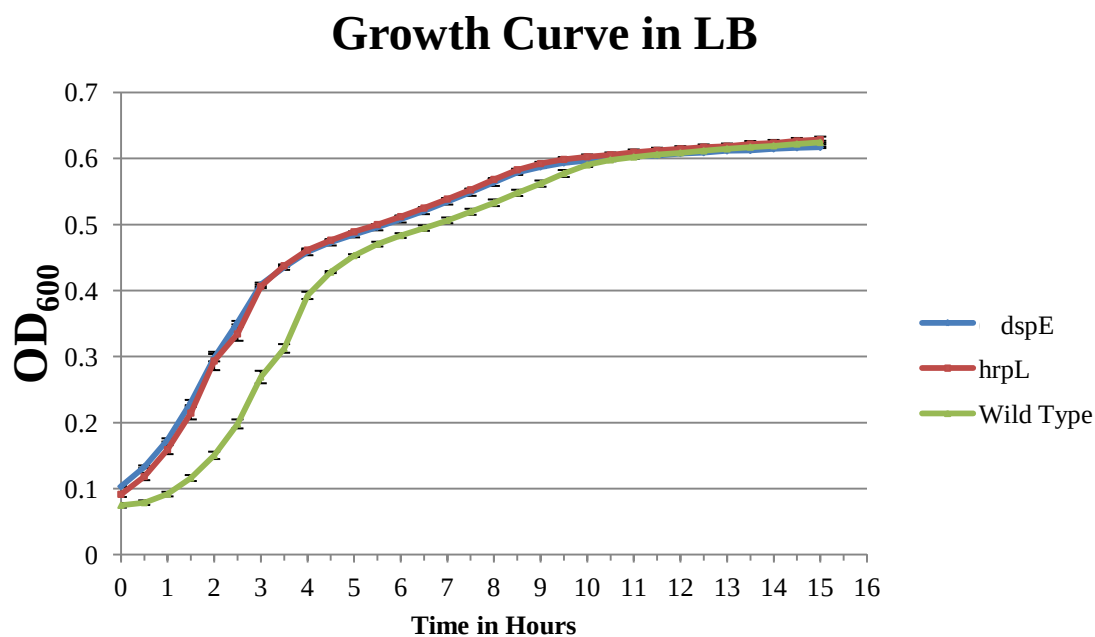


Figure S1 Growth of *P. carotovorum* and mutant derivatives in rich and minimal medium. *P. carotovorum* and mutant derivatives lacking *dspE* or *hrpL* were grown in rich LB or M9 minimal medium and growth was continuously monitored in a Biotek Synergy 2 Multi-mode Microplate Reader with 37°C incubation and shaking at 200 rpm.

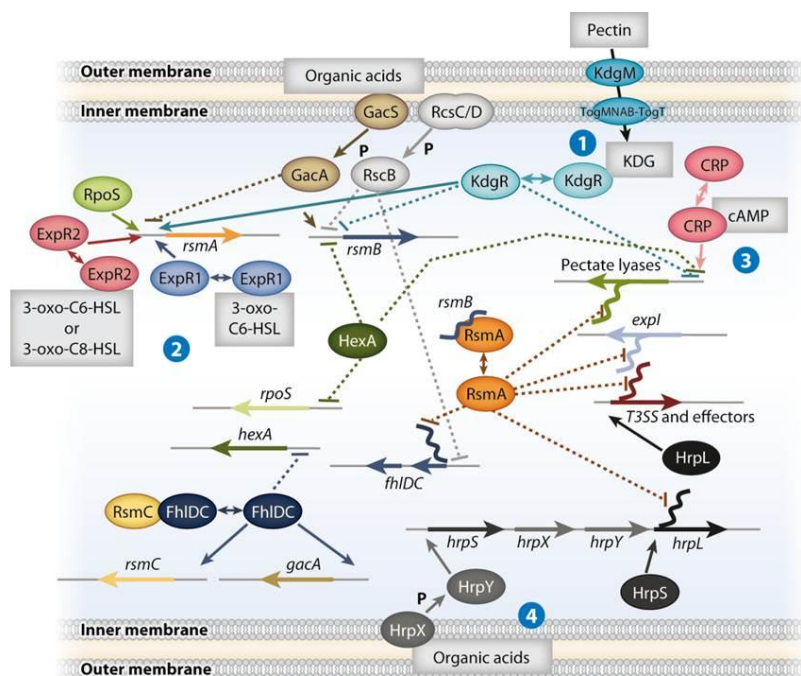


Figure S2 *Pectobacterium*. Integration of small-molecule signals into regulation of exoprotein production. Regulatory proteins are shown as ovals. Upregulation is indicated by an arrow and downregulation by a dashed line. mRNA is shown as a curved line. Small molecules are indicated by labeled gray boxes. 1) Pectin is catabolized and 2-keto-3-deoxygluconate (KDG) is produced: KdgR repression of pectate lyases and *rsmB* is released, whereas *rsmA* is no longer activated, resulting in exoprotein expression. 2) AHL is detected: ExpR binding of AHL (3-oxo-C6-HSL or 3-oxo-C8-HSL) ends ExpR-mediated induction of *rsmA*, resulting in exoprotein production. 3) Glucose levels are low: The cAMP-CRP complex activates pectate lyase production. 4) Organic acids are detected: GacAS represses *rsmA* and induces *rsmB*. The HrpXY two-component system may also detect organic acids, activating the HrpXY-HrpS-HrpL cascade and inducing T3SS genes. Expression tied to the motility regulator FhlDC: *gacA* and *rsmC* are induced, whereas *hexA* is repressed. This results in repression of *hexA* and *rsmA* and induction of *rsmB*, which is followed by expression of exoproteins.

This suggests that exoproteins are secreted by motile cells. Whether motility and the T3SS are co-regulated remains unknown. Control by RscBCD: This system represses *rsmB*, thereby reducing exoprotein production. Abbreviation: P, phosphorylation. This figure was taken from Charkowski et al 2012.

REFERENCES

- Beattie, G. a. 2011. Water relations in the interaction of foliar bacterial pathogens with plants. *Annual Review of Phytopathology*. 49:533–55
- Bell, K. S., Sebaihia, M., Pritchard, L., Holden, M. T. G., Hyman, L. J., Holeva, M. C., et al. 2004. Genome sequence of the enterobacterial phytopathogen *Erwinia carotovora* subsp. *atroseptica* and characterization of virulence factors. *Proceedings of the National Academy of Sciences of the United States of America*. 101:11105–10
- Billing, E., and Baker, L. A. E. 1963. Characteristics of *Erwinia* -like Organisms found in Plant Material. *Journal of Applied Bacteriology*. 26:58–65
- Bogdanove, A. J., Bauer, D. W., and Beer, S. V. 1998. *Erwinia amylovora* Secretes DspE, a Pathogenicity Factor and Functional AvrE Homolog , through the Hrp (Type III Secretion) Pathway. *Journal of Bacteriology*. 180:2244–2247
- Boureau, T., ElMaarouf-Bouteau, H., Garnier, A., Brisset, M.-N., Perino, C., Pucheu, I., et al. 2006. DspA/E, a type III effector essential for *Erwinia amylovora* pathogenicity and growth in planta, induces cell death in host apple and nonhost tobacco plants. *Molecular Plant-Microbe Interactions*. 19:16–24
- Chang, J. H., Urbach, J. M., Law, T. F., Arnold, L. W., Hu, A., Gombor, S., et al. 2005. A high-throughput, near-saturating screen for type III effector genes from *Pseudomonas syringae*. *Proceedings of the National Academy of Sciences of the United States of America*. 102:2549–54
- Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., et al. 2012. The role of secretion systems and small molecules in soft-rot enterobacteriaceae pathogenicity. *Annual Review of Phytopathology*. 50:425–49
- Chatterjee, A., Cui, Y., and Chatterjee, A. K. 2002. Regulation of *Erwinia carotovora* hrpL(Ecc) (sigma-L(Ecc)), which encodes an extracytoplasmic function subfamily of sigma factor required for expression of the HRP regulon. *Molecular Plant-Microbe Interactions*. 15:971–80
- Contento, A. L., Xiong, Y., and Bassham, D. C. 2005. Visualization of autophagy in *Arabidopsis* using the fluorescent dye monodansylcadaverine and a GFP-AtATG8e fusion protein. *The Plant Journal*. 42:598–608
- Cooper, V. J., and Salmond, G. P. 1993. Molecular analysis of the major cellulase (CelV) of *Erwinia carotovora*: evidence for an evolutionary “mix-and-match” of enzyme domains. *Molecular and General Genetics*. 241:341–50

- Dalmacio, S. C., Lugod, T. R., Serrano, E. M., and Munkvold, G. P. 2007. Reduced Incidence of Bacterial Rot on Transgenic Insect-Resistant Maize in the Philippines. *Plant Disease*. 91:346–351
- DebRoy, S., Thilmony, R., Kwack, Y.-B., Nomura, K., and He, S. Y. 2004. A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. *Proceedings of the National Academy of Sciences of the United States of America*. 101:9927–32
- Francis, M. S., Wolf-Watz, H., and Forsberg, A. 2002. Regulation of type III secretion systems. *Current Opinion in Microbiology*. 5:166–72
- Glasner, J. D. 2003. ASAP, a systematic annotation package for community analysis of genomes. *Nucleic Acids Research*. 31:147–151
- Glasner, J. D., Marquez-Villavicencio, M., Kim, H.-S., Jahn, C. E., Ma, B., Biehl, B. S., et al. 2008. Niche-specificity and the variable fraction of the *Pectobacterium* pan-genome. *Molecular Plant-Microbe Interactions*. 21:1549–60
- Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology*. 43:205–27
- Ham, J. H., Majerczak, D. R., Ewert, S., Sreerekha, M.-V., Mackey, D., and Coplin, D. L. 2008. WtsE, an AvrE-family type III effector protein of *Pantoea stewartii* subsp. *stewartii*, causes cell death in non-host plants. *Molecular Plant-Microbe Interactions*. 9:633–643
- Ham, J. H., Majerczak, D. R., Nomura, K., Mecey, C., Uribe, F., He, S.-Y., et al. 2009. Multiple activities of the plant pathogen type III effector proteins WtsE and AvrE require WxxxE motifs. *Molecular Plant-Microbe Interactions*. 22:703–12
- Harrison, M., Quinn, C., Sells, I. A., and Graham, D. 1977. Waste potato dumps as sources of insects contaminated with soft rot coliform bacteria in relation to re-contamination of pathogen-free potato stocks. *Potato Research*. 20:37–52
- Hill, C. M., and Duggleby, R. G. 1999. Purified recombinant *Escherichia coli* ketol-acid reductoisomerase is unsuitable for use in a coupled assay of acetohydroxyacid synthase activity due to an unexpected side reaction. *Protein expression and purification*. 15:57–61
- Hofius, D., Munch, D., Bressendorff, S., Mundy, J., and Petersen, M. 2011. Role of autophagy in disease resistance and hypersensitive response-associated cell death. *Cell Death and Differentiation*. 18:1257–62
- Hogan, C. S., Mole, B. M., Grant, S. R., Willis, D. K., and Charkowski, A. O. 2013. The type III secreted effector DspE is required early in solanum tuberosum leaf infection by

Pectobacterium carotovorum to cause cell death, and requires Wx(3-6)D/E motifs. ed. Stefan Bereswill. PLOS ONE. 8:e65534

Holeva, M. C., Bell, K. S., Hyman, L. J., Avrova, A. O., Whisson, S. C., Birch, P. R. J., et al. 2004. Use of a pooled transposon mutation grid to demonstrate roles in disease development for *Erwinia carotovora* subsp. *atroseptica* putative type III secreted effector (DspE/A) and helper (HrpN) proteins. *Molecular Plant-Microbe Interactions*. 17:943–50

Hommais, F., Zghidi-Abouzid, O., Oger-Desfeux, C., Pineau-Chapelle, E., Van Gijsegem, F., Nasser, W., et al. 2011. lpxC and yafS are the most suitable internal controls to normalize real time RT-qPCR expression in the phytopathogenic bacteria *Dickeya dadantii*. PLOS ONE. 6:e20269

Hugouvieux-Cotte-Pattat, N., Shevchik, V. E., and Nasser, W. 2002. PehN, a polygalacturonase homologue with a low hydrolase activity, is coregulated with the other *Erwinia chrysanthemi* polygalacturonases. *Journal of Bacteriology*. 184:2664–73

Humbert, R., and Simoni, R. D. 1980. Genetic and biomedical studies demonstrating a second gene coding for asparagine synthetase in *Escherichia coli*. *Journal of Bacteriology*. 142:212–20

Jahn, C. E., Charkowski, A. O., and Willis, D. K. 2008. Evaluation of isolation methods and RNA integrity for bacterial RNA quantitation. *Journal of Microbiological Methods*. 75:318–24

Kabbage, M., Williams, B., and Dickman, M. B. 2013. Cell death control: the interplay of apoptosis and autophagy in the pathogenicity of *Sclerotinia sclerotiorum*. PLOS Pathogens. 9:e1003287

Kim, H. S., Ma, B., Perna, N. T., and Charkowski, A. O. 2009. Phylogeny and virulence of naturally occurring type III secretion system-deficient *Pectobacterium* strains. *Applied and Environmental Microbiology*. 75:4539–49

Kim, H. S., Thammarat, P., Lommel, S. A., Hogan, C. S., and Charkowski, A. O. 2011. *Pectobacterium carotovorum* elicits plant cell death with DspE/F but the *P. carotovorum* DspE does not suppress callose or induce expression of plant genes early in plant-microbe interactions. *Molecular Plant-Microbe Interactions*. 24:773–86

Kloepper, J. W., Brewer, J. W., and Harrison, M. D. 1981. Insect transmission of *Erwinia carotovora* var. *carotovora* and *Erwinia carotovora* var. *atroseptica* to potato plants in the field. *American Potato Journal*. 58:165–175

Lai, Z., Wang, F., Zheng, Z., Fan, B., and Chen, Z. 2011. A critical role of autophagy in plant resistance to necrotrophic fungal pathogens. *The Plant Journal*. 66:953–68

- Legrain, C., Stalon, V., and Glansdorff, N. 1976. *Escherichia coli* ornithine carbamoyltransferase isoenzymes: evolutionary significance and the isolation of lambdaargF and lambdaargI transducing bacteriophages. *Journal of Bacteriology*. 128:35–8
- Lei, S. P., Lin, H. C., Wang, S. S., Callaway, J., Wilcox, G., Lei, S., et al. 1987. Characterization of the *Erwinia carotovora* pelB gene and its product pectate lyase. *Journal of Bacteriology*. 169
- Leisinger, T., and Haas, D. 1975. N-Acetylglutamate synthase of *Escherichia coli* regulation of synthesis and activity by arginine. *The Journal of Biological Chemistry*. 250:1690–3
- Lenz, H. D., Haller, E., Melzer, E., Kober, K., Wurster, K., Stahl, M., et al. 2011. Autophagy differentially controls plant basal immunity to biotrophic and necrotrophic pathogens. *The Plant Journal*. 66:818–30
- Liu, H., Coulthurst, S. J., Pritchard, L., Hedley, P. E., Ravensdale, M., Humphris, S., et al. 2008. Quorum sensing coordinates brute force and stealth modes of infection in the plant pathogen *Pectobacterium atrosepticum*. *PLOS Pathogens*. 4:e1000093
- Liu, Y., Schiff, M., Czymmek, K., Tallóczy, Z., Levine, B., and Dinesh-Kumar, S. P. 2005. Autophagy regulates programmed cell death during the plant innate immune response. *Cell*. 121:567–77
- Marits, R., Kõiv, V., Laasik, E., and Mäe, A. 1999. Isolation of an extracellular protease gene of *Erwinia carotovora* subsp. *carotovora* strain SCC3193 by transposon mutagenesis and the role of protease in phytopathogenicity. *Microbiology*. 145 (Pt 8:1959–66
- Marquez-Villavicencio, M. D. P., Weber, B., Witherell, R. A., Willis, D. K., and Charkowski, A. O. 2011. The 3-hydroxy-2-butanone pathway is required for *Pectobacterium carotovorum* pathogenesis. *PLOS ONE*. 6:e22974
- McCarter-Zorner, N. J., Franc, G. D., Harrison, M. D., Michaud, J. E., Quinn, C. E., Sells, I. A., et al. 1984. Soft rot *Erwinia* bacteria in surface and underground waters in southern Scotland and in Colorado, United States. *Journal of Applied Bacteriology*. 57:95–105
- McCarter-Zorner, N. J., Harrison, M. D., Franc, G. D., Quinn, C. E., Sells, I. A., and Graham, D. C. 1985. Soft rot *Erwinia* bacteria in the rhizosphere of weeds and crop plants in Colorado, United States and Scotland. *Journal of Applied Bacteriology*. 59:357–368
- Mizushima, N., Levine, B., Cuervo, A. M., and Klionsky, D. J. 2008. Autophagy fights disease through cellular self-digestion. *Nature*. 451:1069–75

- Molina, J. M., Harrison, D., and Brewer, J. W. 1974. Transmission of *Erwinia carotovora* var. *atroseptica* by *Drosophila melanogaster* Meig. I. Acquisition and transmission of the bacterium. *American Potato Journal*. 19:52–61
- Mongkolsuk, S., Praituan, W., Loprasert, S., Fuangthong, M., and Chamnongpol, S. 1998. Identification and characterization of a new organic hydroperoxide resistance (ohr) gene with a novel pattern of oxidative stress regulation from *Xanthomonas campestris* pv. *phaseoli*. *Journal of Bacteriology*. 180:2636–43
- Nakayama, M., Kaneko, Y., Miyazawa, Y., Fujii, N., Higashitani, N., Wada, S., et al. 2012. A possible involvement of autophagy in amyloplast degradation in columella cells during hydrotropic response of *Arabidopsis* roots. *Planta*. 236:999–1012
- Neumann, S., and Quiñones, A. 1997. Discoordinate gene expression of *gyrA* and *gyrB* in response to DNA gyrase inhibition in *Escherichia coli*. *Journal of Basic Microbiology*. 37:53–69
- Oh, C.-S., Martin, G. B., and Beer, S. V. 2007. DspA/E, a type III effector of *Erwinia amylovora*, is required for early rapid growth in *Nicotiana benthamiana* and causes NbSGT1-dependent cell death. *Molecular Plant Pathology*. 8:255–65
- Pfaffl, M. W., Tichopad, A., Prgomet, C., and Neuvians, T. P. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnology Letters*. 26:509–15
- Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E. T. 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *The EMBO Journal*. 12:2467–76
- Reverchon, S., Nasser, W., and Robert-Baudouy, J. 1991. Characterization of *kdgR*, a gene of *Erwinia chrysanthemi* that regulates pectin degradation. *Molecular Microbiology*. 5:2203–2216
- Rotenberg, D., Thompson, T. S., German, T. L., and Willis, D. K. 2006. Methods for effective real-time RT-PCR analysis of virus-induced gene silencing. *Journal of Virological Methods*. 138:49–59
- Saarilahti, H. T., Heino, P., Pakkanen, R., Kalkkinen, N., Palva, I., and Palva, E. T. 1990. Structural analysis of the *pehA* gene and characterization of its protein product, endopolygalacturonase, of *Erwinia carotovora* subspecies *carotovora*. *Molecular Microbiology*. 4:1037–44
- Sambrook, J., and Russell, D. W. 2001. *Molecular cloning: a laboratory manual*.

- Saul, D. J., Williams, L. C., Grayling, R. A., Chamley, L. W., Love, D. R., and Bergquist, P. L. 1990. *celB*, a gene coding for a bifunctional cellulase from the extreme thermophile “*Caldocellum saccharolyticum*”. *Applied and Environmental Microbiology*. 56:3117–3124
- Shevchik, V. E., Robert-Baudouy, J., and Hugouvieux-Cotte-Pattat, N. 1997. Pectate lyase PelI of *Erwinia chrysanthemi* 3937 belongs to a new family. *Journal of Bacteriology*. 179:7321–30
- Smits, T. H. M., and Duffy, B. 2011. Genomics of iron acquisition in the plant pathogen *Erwinia amylovora*: insights in the biosynthetic pathway of the siderophore desferrioxamine E. *Archives of Microbiology*. 193:693–9
- Stover, P., and Schirch, V. 1990. Serine hydroxymethyltransferase catalyzes the hydrolysis of 5,10-methenyltetrahydrofolate to 5-formyltetrahydrofolate. *The Journal of Biological Chemistry*. 265:14227–33
- Stragier, P., Richaud, F., Borne, F., and Patte, J. C. 1983. Regulation of diaminopimelate decarboxylase synthesis in *Escherichia coli*. I. Identification of a *lysR* gene encoding an activator of the *lysA* gene. *Journal of Molecular Biology*. 168:307–20
- Takeda, Y., and Avila, H. 1986. Structure and gene expression of the *E. coli* Mn-superoxide dismutase gene. *Nucleic Acids Research*. 14:4577–89
- Takle, G. W., Toth, I. K., and Brurberg, M. B. 2007. Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen *Pectobacterium atrosepticum*. *BMC Plant Biology*. 7:50
- Toth, I. K., Bell, K. S., Holeva, M. C., and Birch, P. R. J. 2003. Soft Rot *Erwiniae*: from genes to genomes. *Molecular Plant Pathology*. 4:17–30
- Toth, I. K., and Birch, P. R. J. 2005. Rotting softly and stealthily. *Current Opinion in Plant Biology*. 8:424–9
- Williams, B., and Dickman, M. 2008. Plant programmed cell death: can’t live with it; can’t live without it. *Molecular Plant Pathology*. 9:531–44

Chapter 4 – Future Directions

AvrE-family effectors are among the most important virulence factors in plant pathogenic bacteria. In the previous chapters, I demonstrate that WxxxD/E-like motifs, common to most AvrE-family effectors, are critical for the AvrE family effector DspE to elicit host cell death during *P. carotovorum* infection on leaves. I also show that *P. carotovorum* increases expression of a specific yet diverse set of pathogenesis associated genes to cause tissue maceration characteristic of soft rot pathogens. I believe that these results provide a valuable foundation for future study of the virulence function of DspE and other AvrE-family effectors, and the molecular events occurring during necrotrophic pathogenesis.

The research presented in this dissertation provides insights into the molecular events governing infection by *P. carotovorum*, which can be applied to other necrotrophic pathogens. Here, I discuss future work that is needed to further advance our understanding of DspE and necrotrophic plant pathogens.

In my research, I attempted to identify genes that confer resistance to *Pcc* by breeding a wild resistant potato species to a susceptible domestic variety to create a population in which susceptibility and resistance could be linked with specific genomic loci (Appendix A). Unfortunately, I was unable to produce suitable progeny from these crosses (Appendix A). Experiments are currently being conducted in the Charkowski lab to analyze a parthenogenic haploid population derived from tetraploid potato cultivars *Silverton* and *White Lady* to determine if resistance and susceptibility can be correlated with segregation of genomic loci. Identification of genes that govern the plant response to *Pcc* will further understanding of *Pcc* pathogenesis and possibly DspE function, since

genes conferring resistance might do so by interfering with DspE action, or other critical components of the *Pcc* infection strategy.

My research shows that DspE relies upon Wxx₍₃₋₆₎D/E motifs (Chapter 2). I focused on motifs that aligned with those previously analyzed in AvrE-family homologs. However, there are three conserved WxxxD/E-like motifs that I did not analyze. Two of these have spacer regions of seven amino acid residues, and I did not determine if mutating these motifs would affect DspE function. At these positions in DspE homologs of *Pantoea agglomerans*, *Pantoea stewartii* and *Ewinia amylovora* the spacer region is only six amino acid residues, so it is possible that insertions occurred at this location in DspE following acquisition of the effector gene, due to the different host range and infection strategies these pathogens. Further, one of the motifs I did analyze, WEASSVE, is upstream of two glutamic acid residues; I did not distinguish which of these residues was important for function. Further investigations into the nature of these additional WxxxD/E-like motifs would be informative, and it would be interesting to determine whether the flexible nature of the spacer region in the plant pathogen effectors is shared by homologous effectors found in animal pathogens.

Computer predictions of the tertiary structure of DspE with three distinct protein threading programs (FUGUE, HHpred and Phyre2) predicted a highly conserved double β -propeller domain in the N-terminal portion of the protein. Mutational analysis in DspA/E from *E. amylovora* indicated that this domain was important for function of the effector (Sabrina, Stéphane, Tristan, Marie-Noëlle, et al. 2013). The N-terminal domain of DspA/E in *E. amylovora* is known to interact with four similar putative leucine-rich repeat receptor-like serine/threonine kinases from apple (Meng, Bonasera, Kim, Nissinen,

et al. 2006), which could be mediated by the β -propeller structure. Further, these predicted structures indicated the presence of a peroxisome targeting signal unique to DspE in *P. carotovorum*. AvrE-family effectors suppress callose deposition (Boureau et al. 2006) and WxxxD/E-like effectors interact with membranes (Alto et al. 2006). Thus the predicted peroxisome targeting signal may be important for DspE to suppress peroxide mediated or other defenses through disruption of vesicle traffic. Mutational analysis of these regions could provide valuable insight into the mechanisms underlying DspE-mediated host cell death.

Many aspects of DspE activity remain unknown. All structural information derives from computer modeling algorithms, and no crystal structure has been created for DspE or any AvrE family effector. Further, the localization and host target(s) of DspE and many other AvrE-family effectors remain unknown due to their toxicity, which results in cell death and collapse of cellular structure. Host targets of DspE could be identified by expressing DspE in susceptible hosts containing single gene deletions. Deletion of specific genes that result in suppression of the toxicity of DspE would indicate that they are important for DspE function, and identify them as potential host target proteins. My results indicating that autophagy is activated by DspE, highlight genes involved in this pathway as prime targets for future knock-out studies. Many such single gene knockout lines have been developed in *Solanum* species and *Arabidopsis*, and these genes must be tested to fully elucidate the network of host proteins involved in DspE-mediated host cell death. Yeast could be a valuable host to utilize, as deletion lines have been developed for almost every gene in the yeast genome. However, it would be essential for DspE to be toxic in yeast, and this has not yet been determined.

My work analyzing the physical transmission of *P. carotovorum* from rotted leaves suggests that water soaked lesions provide a very efficient mechanism for the pathogen to spread to new hosts through physical contact. It is very likely that water splash caused by rain or by overhead irrigation could facilitate transmission, especially since I have shown in chapter 3 that development of macerated tissue is favored by wet conditions. Also, my results, in combination with previous work indicating that *P. carotovorum* produces butane-diol (Marquez-Villavicencio, Weber, Witherell, Willis, et al. 2011), a known insect attractant, support a model in which insects vector the pathogen through attraction to land on rotted tissue, and subsequently carry the pathogen on their body to new hosts. A study specifically analyzing insect transmission of *P. carotovorum* is needed to confirm the validity of this hypothesis.

Inhibiting expression of virulence genes is an attractive strategy for disease management as this places weak selective pressure upon pathogen populations in comparison to lethal pesticides (Lee, Almqvist, and Hultgren 2003). Inhibition of the T3SS is an ideal target for this strategy as it is not required for growth outside of plants (Nordfelth, Kauppi, Norberg, Wolf-Watz, et al. 2005). Some progress has been made using inhibitors derived from plant phenolic compounds (Yamazaki et al. 2012), and QS based SRE-resistance mechanisms have been successfully implemented in plants (Dong et al. 2001), but these have not been accepted for commercial use (Charkowski et al. 2012). There are many challenges in developing virulence inhibitors, but the work presented in chapter 3 provides a powerful tool for quantification of responses in expression of many virulence genes to various stimuli, which will allow future research

to dissect the mechanisms by which small molecules act, and the degree to which they are effective in blocking virulence activation (Charkowski et al. 2012).

A complete understanding of the host targets and functional mechanisms of DspE could have significant practical implications. For example, soft-rot resistant plants could be developed that express mutated versions of targeted proteins that are no longer sensitive to DspE action. Characterizing the biochemical function of DspE could lead to the identification of chemical compounds that prevent pathogenesis by disrupting DspE-mediated cell death. There is much research that remains to be performed to accomplish the goal of fully understanding soft-rot pathogenesis, and developing soft-rot resistant crops. However, significant progress has been made, and new research is continuing to bring the goal of enhanced resistance to soft-rot pathogens closer.

REFERENCES

- Alto, N. M., Shao, F., Lazar, C. S., Brost, R. L., Chua, G., Mattoo, S., et al. 2006. Identification of a bacterial type III effector family with G protein mimicry functions. *Cell*. 124:133–45
- Boureau, T., ElMaarouf-Bouteau, H., Garnier, A., Brisset, M.-N., Perino, C., Pucheu, I., et al. 2006. DspA/E, a type III effector essential for *Erwinia amylovora* pathogenicity and growth in planta, induces cell death in host apple and nonhost tobacco plants. *Molecular Plant-Microbe Interactions*. 19:16–24
- Charkowski, A., Blanco, C., Condemine, G., Expert, D., Franza, T., Hayes, C., et al. 2012. The role of secretion systems and small molecules in soft-rot enterobacteriaceae pathogenicity. *Annual Review of Phytopathology*. 50:425–49
- Dong, Y. H., Wang, L. H., Xu, J. L., Zhang, H. B., Zhang, X. F., and Zhang, L. H. 2001. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature*. 411:813–7
- Lee, Y. M., Almqvist, F., and Hultgren, S. J. 2003. Targeting virulence for antimicrobial chemotherapy. *Current Opinion in Pharmacology*. 3:513–519
- Marquez-Villavicencio, M. D. P., Weber, B., Witherell, R. A., Willis, D. K., and Charkowski, A. O. 2011. The 3-hydroxy-2-butanone pathway is required for *Pectobacterium carotovorum* pathogenesis. *PLOS ONE*. 6:e22974
- Meng, X., Bonasera, J. M., Kim, J. F., Nissinen, R. M., and Beer, S. V. 2006. Apple proteins that interact with DspA/E, a pathogenicity effector of *Erwinia amylovora*, the fire blight pathogen. *Molecular Plant-Microbe Interactions*. 19:53–61
- Nordfelth, R., Kauppi, A. M., Norberg, H. A., Wolf-Watz, H., and Elofsson, M. 2005. Small-molecule inhibitors specifically targeting type III secretion. *Infection and Immunity*. 73:3104–14
- Sabrina, S., Stéphane, G., Tristan, B., Marie-Noëlle, B., and Marie-Anne, B. 2013. Mutational analysis of a predicted double β -propeller domain of the DspA/E effector of *Erwinia amylovora*. *FEMS Microbiology Letters*. 342:54–61
- Yamazaki, A., Li, J., Zeng, Q., Khokhani, D., Hutchins, W. C., Yost, A. C., et al. 2012. Derivatives of plant phenolic compound affect the type III secretion system of *Pseudomonas aeruginosa* via a GacS-GacA two-component signal transduction system. *Antimicrobial Agents and Chemotherapy*. 56:36–43

Appendix I- Breeding for Potato Resistance Genes

I attempted to identify genetic loci in wild *Solanum* species that could be associated with resistance or susceptibility to *P. carotovorum* by breeding wild *P. carotovorum* resistant diploid *Solanum* species with the susceptible diploid *S. tuberosum* US-W4. Our hypothesis was that loci that segregate with susceptible or resistant disease phenotypes in progeny plants would be associated with genes that influence this plant-pathogen interaction. After obtaining phenotypes of progeny plants, their genomes would be assayed using the SolCap Potato Infinium SNP chip (Hamilton et al. 2011), which contains probes for over 9,000 single nucleotide polymorphisms for *S. tuberosum*, to identify loci that segregate with resistance or susceptible phenotypes. A previous student in the Charkowski lab, Hye-Sook Kim, had previously assayed a large number of wild *Solanum* species obtained from the United States Potato Genebank for resistance to *P. carotovorum*. Of the resistant species, I identified those that were diploid and had an endosperm balance number of 2, which is reproductively compatible with the susceptible diploid *S. tuberosum* US-W4. The following is the list of wild *Solanum* species I obtained and their reference (PI) numbers in the ARS-GRIN database: *S. albornozii* 561636, *S. andreaeanum* 320345, *S. berthaultii* 558129, *S. bolivense* 597736, *S. candolleianum* 365353, *S. chacoense* 414143, *S. chromatophilum* 310943, *S. microdontum* 545901, *S. microdontum* 500036, *S. violaceimarmoratum* 258856. I retested these species for resistance to stem infection by placing cuttings in water inoculated with *P. carotovorum*, and tested for resistance to leaf infection by injecting leaves with OD₆₀₀=0.3 leaves. Only the *S. microdontum* 500036 species was resistant to stem infection, and the *S. violaceimarmoratum* 258856 was the only leaf resistant species.

I crossed these species to US-W4 by collecting pollen from the wild species and placing on the stigma of US-W4 flowers. From these crosses I obtained viable progeny. I grew 100 progeny plants, which I tested for stem and leaf resistance. For all progeny tested, the phenotype was inconsistent (i.e. none of the progeny plants had a consistent resistant or susceptible phenotype over multiple rounds of pathogen testing). I hypothesized that this was due to a complex mixture of genes from diverse background and resolved to backcross these progeny plants to US-W4. I chose not to attempt backcrosses to the wild male parent as these produced small flowers that were much more fragile and difficult to pollenate compared to the flowers on US-W4. I was unable to obtain progeny from these backcrosses. I hypothesize that the progeny plants produced by my initial crosses was sterile.

REFERENCES

Hamilton, John P., et al. "Single nucleotide polymorphism discovery in elite north american potato germplasm." *BMC genomics* 12.1 (2011): 302

Appendix II- CED-9 does not affect DspE-mediated host cell death

I hypothesized that DspE might activate apoptosis to cause cell death. To test this hypothesis I used transiently expressed DspE and a non-functional mutant derivative in the leaves of wild-type tobacco plants, and plants expressing the anti-apoptotic gene *ced-9* from *Caenorhabditis elegans*. Both wild-type and *ced-9* expressing tobacco were identically susceptible to DspE but not to the non-functional mutant derivative. This result does not conclusively demonstrate that DspE does not activate apoptosis to cause cell death.