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IDENTIFICATION AND ANALYSIS OF INDUCED GENES FROM *ERWINIA AMYLOVORA* AND *MALUS* X *DOMESTICA* DURING FIRE BLIGHT INFECTION

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By

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ABSTRACT

IDENTIFICATION AND ANALYSIS OF INDUCED GENES FROM *ERWINIA AMYLOVORA* AND *MALUS* X *DOMESTICA* DURING FIRE BLIGHT INFECTION

By

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Fire blight, caused by the bacterial pathogen *Erwinia amylovora*, is a difficult disease to manage due to the virulent nature of the pathogen, disease susceptibility of most popular apple varieties, and the lack of known resistance genes in *Malus* spp. The goals of my research are to identify and characterize genes from *E. amylovora* that are induced during infection and to identify genes in *Malus* spp. that are associated with disease resistance. I utilized an *in vivo* expression technology screen to identify *in planta* upregulated *E. amylovora* genes, two of which had homology to pseudopilins from a type II secretion operon and an endo-polygalacturonase (*peh*). Deletion mutants of both the *peh* gene and $outD_{EA}$ were constructed to further analyze the contribution of type II secretion to the *E. amylovora-Malus* spp. interaction. This work resulted in the first report of a polygalacturonase enzyme influencing virulence of *E. amylovora*. Some apple cultivars, such as Red Delicious, exhibit tolerance to fire blight. Because resistance to fire blight appears to be a quantitative trait in apple, I chose to identify genes associated with resistance using suppressive subtractive hybridization. I generated 183 unique expressed sequence tags (ESTs) from cultivar Red Delicious during infection that are absent or repressed in the fire blight sensitive cultivar Gala. Temporal expression analysis identified 21 of the ESTs which are induced in Red Delicious of which eight show differential expression in other apple cultivars of varying resistance to fire blight. Further

functional characterization of these genes will shed new light on signaling cascades that lead to a successful resistance response; in addition these genes can be converted into molecular markers for mapping onto linkage maps of apple for use in marker assisted selection of *E. amylovora* resistance in apple breeding projects.

To my husband Eric, who never stopped smiling.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LITERATURE REVIEW

Fire blight is a necrogenic disease of rosaceous plants caused by the gram negative bacterium, *Erwinia amylovora*. In the United States, fire blight can be a devastating disease on non-native hosts such as domestic apple, pear and quince due to the lack of resistance in popular cultivars and limited control options. Due to the limited amount of control options available, there is interest in the molecular mechanisms of fire blight in order to generate novel control measures. Initial genetic research of the pathogen *E. amylovora* has identified major pathogenicity factors such as the secreted effector protein DspE, a type III secretion system and production of the exopolysaccharide amylovoran (18, 21, 26, 82, 127). The discovery of these pathogenicity factors relied mainly on the creation and mapping of non-pathogenic mutants using phage insertional mutagenesis (239). Research investigating the host response to fire blight infection has demonstrated the induction of known pathogenesis related (PR), antioxidant and phenylpropanoid pathway genes (31, 35, 153, 248, 249). Currently, no resistance genes for fire blight have been identified; however absolute resistance does exist in *Malus* species related to domestic apple and certain cultivars of domestic apple show elevated levels of resistance to fire blight. Due to the increased ease and availability of genomic and genetic techniques, I chose to look at the fire blight pathosystem on a larger genomic scale in order to identify additional virulence factors from *E. amylovora* and resistance response genes from *Malus* × *domestica* cultivar Red Delicious.

Epidemiology and control of fire blight

Fire blight was first described on rosaceous fruit trees in New York around 1780, and is thought to be indigenous to North America. The disease moved westward along with the movement of European settlers bringing nursery stock from the eastern United States, until fire blight was present throughout the entire United States and part of Canada. As apple and pear nursery stock were exported from the United States, fire blight spread worldwide to Europe, the Middle East, and New Zealand (32).

Fire blight is disseminated in orchards by either insects or wind and by the use of contaminated tools and epidemics can develop when both humidity and temperature are high during bloom. In late spring to early summer, over-wintering cankers will start to exude bacterial ooze produced by *E. amylovora* that can be spread to flowering trees by pollinating insects such as bees or by wind and or rain. Once *E. amylovora* is transferred to a blossom, the bacteria can survive briefly as epiphytes, multiplying on the surface of stigmas (241). After this brief epiphytic phase, *E. amylovora* enters the plant through nectarthodes located in the floral cup. The blossom typically becomes necrotic at this point, and this mode of infection is referred to as blossom blight. Bacteria that enter through the nectarthodes may also infect immature fruit that develop from an infected blossom, creating secondary sources of inoculum as bacterial ooze exudes from the infected fruit (119). Again, wind and rain can carry droplets of ooze that contain embedded *E. amylovora* to wounds on stems and leaves created by wind or mechanical damage. Infection through the stems and leaves results in the movement of *E. amylovora* to the vascular tissue, where surrounding parenchyma tissue collapses and disrupts water

and solute transport. The disruption of the vascular system contributes to the formation of shoot blight which is typified by a shepard's crook that quickly becomes necrotic and also may exude bacterial ooze (241). Movement of *E. amylovora* through the vascular system can continue down into the rootstock, killing the vascular system in a susceptible rootstock which will eventually kill the entire tree, this is commonly referred to as rootstock blight (168). Shoot blight will typically form cankers in woody stem tissue that are the primary source of over-wintering inoculum mentioned above. There are also cases of *E. amylovora* surviving in budwood that could also act as primary sources of inoculum (241).

Control of fire blight is often through the use of cultural practices, copper sprays and antibiotics. Cultural practices are comprised of the selection of resistant rootstocks and scions, reduction of inoculum by pruning of diseased tissue and cankers from the orchard and sanitation of tools used for pruning. In respect to chemical control, the application of copper sprays can only be used before leaf set, due to phytotoxicity, which limits copper sprays to only controlling primary *E. amylovora* populations in the early summer (182). Streptomycin is the most commonly used antibiotic for fire blight control, which is used to control blossom blight and to reduce inoculum levels after violent rain storms that cause tissue damage (157). Development of streptomycin resistance has reduced the effectiveness of streptomycin applications for controlling fire blight.

Streptomycin resistance in *E. amylovora* populations has developed through two independent means. The first report of streptomycin resistant *E. amylovora* in California was due to a single point mutation in the streptomycin binding site of ribosomal protein S12 (48). In addition to the isolation of ribosomal point mutations, some streptomycin

resistant *E. amylovora* populations in California carry plasmid pEa8.7 that contains the streptomycin resistance genes *strA-strB*. Streptomycin resistance in Michigan however has arisen due to the horizontal transfer of plasmid pEA34 which harbors the transposon Tn*5393* that expresses the streptomycin resistance genes *strA-strB* under the control of a promoter located in the insertion element IS*1133* (49). The transposon Tn*5393* has also jumped to the ubiquitous plasmid pEa29 and to the chromosome of some field isolates and as a result, fewer streptomycin resistant *E. amylovora* are found that harbor the plasmid pEa34 (155, 158).

Molecular mechanisms of E. amylovora pathogenicity

As mentioned before, three major pathogenicity factors have been identified for *E. amylovora*. First is the capability to secrete effector proteins using the type III secretion system. Type III secretion utilizes a needle-like structure to translocate proteins across the bacterial inner and outer membranes into the host cell. This secretion system is highly conserved among both animal and plant pathogenic bacteria and is independent of Sec-mediated protein secretion (39). In *E. amylovora* the type III secretion system is organized into *hrp* (hypersensitive response and pathogencity) operons (27, 127, 253).

Regulation of the *hrp* operons ensures that the type III secretion machinery is only expressed during conditions that mimic the apoplast of the host such as low nutrient content and low pH. The two-component sensor *hrpXY* locus is responsible for the first known signaling step for *hrp* expression. The sensor protein, HrpX is theorized to be anchored to the inner membrane and responds to favorable *hrp*-inducing stimuli by phosphorylating HrpY. Activated response regulator protein HrpY contains a helix-turn-

helix motif that is then able to bind upstream of the *hrpL* gene to induce transcription along with sigma factor 54 and another enhancer protein HrpS (252). HrpL is the main activator of transcription of the structural *hrp* operons and effector protein genes that contain *hrp* boxes upstream of the gene (125). Interestingly enough, recent evidence suggests that HrpL downregulates flagellar biosynthesis due to the observed increased motility and flagella formation in *hrpL* mutants (44)

All bacteria that posses functional type III secretion systems share a core set of nine conserved *hrp* genes that were renamed as *hrc* (hypersensitive response and conserved) genes. This includes *hrcC* which encodes the outer membrane pore forming protein, *hrcJ* which encodes a lipoprotein, five *hrc* genes encoding inner membrane proteins, an ATPase homologue *hrcN* and the gene *hrcQ* that encodes a protein that may be secreted (125). Another *hrp* gene that are known to form the type III secretion apparatus in *E. amylovora* is *hrpA* which was demonstrated to encode the pilin that forms the type III secretion pilus in *E. amylovora*, mutants in *hrpA* were non-pathogenic and known type III secreted proteins remained in the cytoplasm (118).

Besides the type III secretion system, a secreted effector protein, DspE is the second pathogenicity factor of *E. amylovora*. Originally, *dspE* mutants were observed as disease specific mutations, with no effect on the HR when the mutants were inoculated on tobacco (193). It was later demonstrated that *dspE* mutants could elicit an HR on tobacco especially in highly virulent strains of *E. amylovora* and also on soybean leaves. In addition, the transfer of the *dspEF* locus to *Pseudomonas syringae* pv. *glycinea* rendered it avirulent on soybean (26). DspE was demonstrated to rely on its chaperone DspF for secretion and that secretion was of DspE used type III secretion (25, 82). Later

studies determined that the chaperone DspF interacts with and protects DspE from degradation (83). Genetic analysis determined that the upstream sequence of *dspE* contains a *hrp* box, and expression of *dspE* is HrpL-dependant and coordinated with expression of the neighboring *hrp* operons (82).

Amylovoran, the main exopolysaccharide produced by *E. amylovora* is the third major pathogenicity factor. Biosynthetic genes for amylovoran production are organized into the *ams* operon that consists of 12 genes. Regulation of the ams operon is by a two-component regulatory system RcsB/C (2, 19, 123), and the global regulator protein H-NS which inhibits the production of both amylovoran and levan (103). Amylovoran is comprised mainly of a repeating galactose backbone with galactose and glucuronic acid side chains (84). The composition of sugar monomers in amylovoran can change amongst *E. amylovora* isolates from differing host tissues, such as *E. amylovora* isolates from *Rubus* spp. that are unable to infect apple or pear (148). Mutants in key genes of the amylovoran biosynthetic operon were non-pathogenic and unable to move or increase cell numbers *in planta* (18, 28). This exopolysaccharide is thought to obstruct the host vascular tissue, have a protective role for *E. amylovora* from oxidative burst and desiccation and also a possible role for masking outer membrane proteins from the host (84, 128, 148).

Virulence factors of E. amylovora

Virulence factors also contribute to infection by *E. amylovora* including additional effector proteins HrpN and $AvrRpt2_{EA}$ (253, 268), the iron-binding siderophore desferrioxamine (60), and genes required for metabolism of sorbitol (3),

sucrose (29) and galactose (161). The effector protein HrpN was the first type III secreted protein from E. amylovora to be described and insertional mutants were non-pathogenic on immature pear fruit (253). Immunogold labelling of HrpN protein demonstrated that HrpN is secreted into the host apoplast, but not delivered into the host cells (193). Recently, $HrpN_{3937}$ from *Erwinia chrysanthemi* was determined to act as an aggregation factor and is required for the successful formation of the pellicle (262). It remains to be determined if HrpN from *E. amylovora* is involved in the formation of a biofilm *in planta*.

Recently $AvrRpt2_{EA}$, an additional effector protein contributing to virulence was described by Zhao *et al.* (267). Deletion mutagenesis of $avrRpt2_{EA}$ affected virulence and bacterial cell count in immature pear fruits. Additionally, the transfer of $avrRpt2_{EA}$ to *Pseudomonas syringae* pv. *tomato* DC3000 increased its ability to infect *Arabidopsis rps2* mutants, indicating that $AvrRpt2_{EA}$ is recognized by RPS2 (267).

The ability to scavenge for iron in nutrient-limiting environments is also required for *E. amylovora* virulence under certain conditions. The siderophore desferrioxamine is secreted into the extracellular milieu to complex iron for uptake by the ferrioxamine receptor FoxR. Siderophores are also important for protection of bacterial cells against the host oxidative burst (73). Insertional mutants of desferrioxamine *dfoA* and ferrioxamine receptor *foxR* were hindered in growth under iron limiting conditions. In addition, both mutants were reduced in their ability to colonize and infect apple blossoms (60).

Erwinia amylovora also secretes other virulence factors that affect its interaction with host plants. A metalloprotease (PrtA) which is accompanied by its own type I

secretion machinery was identified in the supernatant fraction from a highly proteolytic strain of *E. amylovora* (E8). Although this metalloprotease did not contribute greatly to virulence of *E. amylovora*, the ability of *prtA* mutants to escape out of xylem vessels and into xylem parenchyma and movement through parenchyma was reduced (265). Another component of *E. amylovora* exopolysaccharide is levan, a β-2, 6-fructan produced by the secreted enzyme levansucrase. Upon secretion to the extracellular milieu, levansucrase produces levan from extracellular sucrose and releases glucose as a by-product. Mutants that do not produce levan are not significantly altered in virulence, although one mutant exhibited reduced virulence under high sucrose conditions (20, 85). Levansucrase is not modified in any manner for its secretion and a putative porin downstream of the *lsc* gene is thought to have a role in its secretion (69).

Type II secretion in phytopathogenic bacteria

E. amylovora also possesses a full *sec* transport system which supports other secretion systems such as type II, type IV and type V (auto-transporter) secretion (188, 238, 266). Type II secretion (T2S) is an important virulence factor in Gram-negative animal pathogens and phytopathogens such as *Pectobacterium carotovorum*, *E. chrysanthemi*, *Ralstonia solanacearum*, and *Xanthomonas campestris*. The T2S operon was identified first from *Klebsiella oxytoca* by transferring a plasmid encoding the T2S operon from *K. oxytoca* to *Escherichia coli* to demonstrate that T2S proteins were required for the secretion of the enzyme pullulanase (215). Transfer of a cosmid containing the T2S from *E. chrysanthemi* to *E. coli* was also used to demonstrate the ability of the T2S to secrete various extracellular enzymes (138). Genome sequencing

has made it possible to identify the presence of T2S genes in many gram negative bacteria other than pathogenic bacteria (50). However, the comparison of known functional T2S systems highlights 12 to 15 proteins expressed from the operon are required for secretion across the outer membrane (215, 219, 220). Although the gene naming convention has differed slightly amongst species possessing a T2S system, the majority of the gene products follow the *Klebsiella* gene designation as A through O plus S (50, 219, 220), with proteins CDEFGHIJKLMNO acting as the core of 12 essential T2S components (50). Here the general nomenclature of the T2S operon, "general secretion pathway" (Gsp) will be followed.

Overall, the core T2S proteins are used to facilitate the transport of folded proteins from the periplasm across the outer membrane. The T2S protein GspD is a member of the secretin protein family, which includes proteins from T3SS and type IV pilus generation. This group of related proteins function to form pores in the outer membrane for protein secretion (238). In some cases where a GspS homologue is present in the T2S operon, an extension of the GspD C-terminus is required for GspS interaction, stabilizing GspD multimers in the outer membrane (54, 183, 228). Electron microscopy analysis of *K. oxytoca* GspD (PulD) multimers with and without GspS (PulS) show a stacked complex with an apparent occlusion in the center of the complex, which is hypothesized to be the N-terminal of GspD (183, 184). This evidence, combined with the observed channel activity of multimeric GspD/ GspS complexes after the application of an electrical current now indicates that GspD may act as a voltage-induced gated channel rather than a passive pore for protein transport (183). There are examples, however, of species that do not posses a GspS homologue in their T2S operon, such as *Pseudomonas*

aeruginosa and *X. campestris*, where their GspD homologue is still able to localize to the outer membrane (228), however the absence of a GspS homologue does not mean that a pilot protein coordinating the insertion of GspD multimers into the outer membrane does not exist (219).

A likely source for energy in the secreton is GspE, a T2S protein theorized to be localized in the cytoplasm and demonstrated to act as an ATPase in *Vibrio cholerae* (43). GspE has been demonstrated to interact with the inner membrane bound GspL in *V. cholerae* (EpsL) and *E. chrysanthemi* ($OutL_{Ech}$) (221), and that conformational changes occur with both $OutE_{Ech}$ and $OutL_{Ech}$ in *E. chrysanthemi* (198). GspL has also been shown to interact with another inner membrane bound protein, GspM, through immunoprecipitation experiments and GspE-GspL-GspM tripartite complexes are thought to involved in the assembly of the entire secreton or just the transport and assembly of a T2S pilus that would bring T2S secreted proteins to the GspD/GspS complex for secretion (43, 198).

Other critical components of T2S are inner membrane bound with some degree of periplasmic domains, such as the major pseudopilin, GspG and minor pseudopilins GspH-J, named for their homology to type IV pilins. Pseudopilins are processed by GspO and are capable of forming protein-protein interactions amongst each other in vitro (67, 145). GspG homologues are able to form pilus-like structures when overexpressed (70, 106, 223); this has led to speculations of pseudopilins forming a pilus that polymerizes to push secreted enzymes through the GspD pore (220).

Exoproteins secreted by type II secretion

Specificity of exoproteins secreted through the T2S has been observed in *K. oxytoca*, *P. carotovorum* and *E. chrysanthemi*. In all three systems, mutations of GspC or GspD could not be complemented by homologous proteins from other systems (173, 197, 228). This led to the hypothesis that they determine the specificity of secreted proteins. The N-terminus of $OutD_{Ech}$ from *E. chrysanthemi* was determined through a protein deletion series to interact with secreted endogenous proteins, and was not able to interact with T2S secreted proteins from *P. carotovorum* (228). In the system of *K. oxytoca*, however, the N-terminal of $OutD_{Ech}$ from *E. chrysanthemi* fused to a truncated PulD protein was still able to secrete pullulanase, indicating that the requirements for secretion specificity may differ from species to species (95).

Therefore, the contribution of GspC to secretion specificity must not be overlooked. GspC and its homologues are inner membrane bound proteins with large periplasm-associated domains (34). Based on amino acid sequence analysis of GspC homologues, a PDZ motif is present near the C-terminal of GspC homologues from *K. oxytoca* (PulC), *P. carotovorum* ($OutC_{Eca}$) and *E. chrysanthemi* ($OutC_{Ech}$). Certain mutations of the PDZ motif of PulC with the addition or deletion of amino acids closest to the C-teminus rendered the T2S secreton non-functional (196). When domains from $OutC_{Eca}$ and $OutC_{Ech}$ were swapped to form fusion proteins, the PDZ motif was shown to be involved in the secretion of species specific exoproteins in *E. chrysanthemi* (34).

The search for a type II secretion signal in exoproteins has had some success, with regions responsible for secretion across the outer membrane determined for pullulanase

of *K. oxytoca*, PelC of *E. chrysanthemi* and PehA of *P. carotovorum* (76, 137, 190). Recent work with PehA from *P. carotovorum* indicates that the secretion signal needed for species dependant secretion is a three-dimensional motif (189). No easily identifiable T2S signal has been determined by amino acid sequence comparisons as of yet.

Exoproteins secreted by phytopathogenic bacteria include proteinases, amylase, pectinases, cellulases and polygalacturonases (50). Polygalacturonases present in phytopathogenic bacteria fall into two categories, endo- and exo-polygalacturonases. The former catalyze the random hydrolysis of pectic acid forming oligogalacturonates while the latter catalyze the terminal hydrolysis of pectic acid forming galacturonic acid monomers (115). While endo-polygalacturonase activity has not been detected in *E. chrysanthemi* (109), endo-polygalacturonase activity is a known virulence factor in *P. carotovorum* (216), *R. solanacearum* (61, 107) and *Agrobacterium vitis* (210).

Analysis of the secretome of *E. chrysanthemi* identified 14 T2S secreted proteins, including 11 pectinases, cellulase, rhamnogalacturonan lyase, a novel esterase and a novel Avr-like protein (122). Sequence analysis of *Ralstonia solanacearum* has identified 6 exoproteins including three polygalacturonases, a pectin methylesterase, and two cellulases (139). Previously, *E. amylovora* was described not to possess any cell wall degradation abilities (226); however, recent studies have described the presence of a type II secretion gene, *outF*, based on Southern hybridization to an $outF_{Eca}$ homologue and weak CelA activity (207).

Host response to fire blight infection

Interest in the genetic mechanisms of the host response to fire blight infection has increased due to the limited control options available to growers. Venisse *et al.* demonstrated that wild type *E. amylovora* and an *ams* mutant were capable of inducing the oxidative burst in pear and a susceptible apple cultivar's leaves, whereas the type III secretion mutant was unable to induce an oxidative burst (248, 249). Furthermore, in another study, Venisse *et al.* were able to link the secretion of two effector proteins, HrpN and DspE to the elicitation of the oxidative burst of pear (247). When the levels of expression of phenylpropanoid genes were observed in a resistant and susceptible cultivar of apple, it appeared that effector proteins were also down-regulating some of the flavanol biosynthesis genes in the susceptible cultivar (249). Based on these studies, the elicitation of an oxidative burst by the host appears to aid infection by *E. amylovora*, and the lack of down-regulation of flavanol biosynthesis genes appears to be related to resistance (248, 249).

Other studies concentrated on *pathogenesis-related* (*PR*) genes and their expression after chemical elicitation or infection in apple. Use of a salicylic acid analogue, acibenzolar-S-methyl (ASM) was demonstrated to induce *PR-1*, *PR-2*, *PR-8* in cultivar Jonathan, *PR-2* and *PR-10* isoforms in cultivar Golden Delicious (35, 153, 269). When apple cultivar Gala was treated with ASM, there was no noticeable induction of *PR-1a*, *PR-2*, *PR-5* or *PR-8*, the authors explain that this may be because in the previous studies, apple seedlings were used compared to the use of shoots on woody tissue for the Gala experiment. The induction of *PR-2*, *PR-5* and *PR-8* were the strongest however after inoculation with E. amylovora 48 and 96 hours post inoculation (31).

Plant proteins interacting with the effector protein DspE have been identified from *Malus* × *domestica* using a yeast two-hybrid system. Four of the proteins, DIPM 1 to 4 are serine threonine kinases with a leucine rich repeat (LRR) motif (159). This is similar to other receptor-like kinase (RLK)-LRR resistance (R) genes such as *Xa21* from rice, *rpg1* from barley and *FLS2* from *Arabidopsis* (37, 55, 134). The site of interaction between DIPMs and DspE is the intercellular RLK domain, in contrast to other RLK-LRR family members where the interacting site is the extracellular LRR domain. The RLK-LRR DIPMs were present in all hosts of fire blight tested, both resistant and susceptible, alluding that the DIPM proteins are not R genes that lead to a successful resistance response (159).

Cloning and mapping of RGAs from apple species is another way to identify resistance-related genes. Classic resistance genes encode proteins with a nucleotide binding site (NBS) and a LRR domain that participates in protein-protein interactions (13). These resistance genes are now hypothesized to be guard proteins that monitor changes to other proteins that are the true targets of pathogen effectors (181). Resistance gene analogues from apple in two studies were cloned from sequences amplified based on degenerate primers in the nucleotide binding site (NBS) (14, 135). In one study, after the cloning of 27 sequences from RGAs, 18 of those sequences were successfully converted into molecular markers and mapped the locations of the RGAs on linkage maps (14). The technique NBS-profiling was proven useful by identifying and mapping of 23 RGAs at the same time in F1 progeny, adding to the number of RGA markers available for mapping (42). In a recent study, expressed sequence tags (ESTs)

homologous to RGAs were converted into expressed sequence tagged site (E-STS) markers, facilitating the mapping of ESTs onto linkage maps (175).

Groups have also sought to map quantitative trait loci (QTLs) in apple and pear for fire blight resistance, to aid in breeding efforts. The first QTLs identified for fire blight resistance were located in a resistant cultivar of European pear. Four QTLs were identified, two on linkage group two, one on linkage group four and another on linkage group nine. Two molecular makers, one co-localizing with the QTL on linkage group two-b and the other co-localizing with linkage group four are resistance gene analogue markers, indicating that nucleotide binding site (NBS) type resistance genes may be involved in fire blight resistance (65). Calenge *et al.* were able to identify a major QTL in apple that accounts for 34.3 to 46.6 % of the resistance phenotype. This QTL was located on linkage group seven, with no RGA markers co-localizing with the QTL (41). This indicates a high probability that the main genetic component of fire blight resistance is not R gene mediated. Khan *et al.* also identified a major QTL for fire blight resistance on linkage group seven that accounted for 37.5% to 38.6% of the resistance phenotype. Both QTLs identified on linkage group seven were in roughly the same area and were observed using two different strains of E. amylovora for infected, therefore this QTL is likely to be a major fire blight resistance locus (41, 124). Furthermore, areas of the pear QTLs on linkage groups two and nine were determined to be involved in digenic interactions with other linkage groups, thus in apple, the contribution of the pear QTLs are in combination with other loci (41, 65).

Although a major QTL for fire blight resistance has been determined, the exact nature of that resistance is still unknown. Since no currently cloned RGAs are located in

that region of linkage group seven, there are no mapped candidate genes for resistance in apple. The identification of the DspE interacting apple proteins is an insight into the potential innate immunity response of Rosaceous plants, however RLK-LRR proteins are unlikely to be involved in resistance to fire blight, either. In order to identify other genes involved in the resistance response to fire blight, attention should be given to genes other than the traditional R gene types.

Using plant genomics to complement genetic analysis of resistance loci

Major food crops such as rice, wheat, barley, maize, potatoes and soybeans still suffer losses due to plant pathogens on a global scale, despite the best efforts of breeding programs and the availability of pesticides (108, 235). The search for durable resistance is not a novel concept in plant biology and the overall goal of disease resistance breeding programs is to identify chromosomal regions conferring this type of resistance (121, 163). Identification of useful disease resistance QTLs are further complicated by rapid evolution of some pathogens, by overcoming released resistant cultivars in a matter of years (163, 233). In the search for the ultimate resistance gene, a great wealth of genomic material pertaining to plant pathogen interactions has been generated for pathogens and plants in model and crop species.

Currently, there are four complete plant genome sequences available. The first plant genome sequence to be released was the model plant *Arabidopsis thaliana* in 2000 (110), followed by the genome sequence of two rice types (*Oryza sativa* L ssp. *indica* and *O. sativa* L ssp. *japonica*) in 2002 (89, 263) and most recently, the genome of the model tree *Populus trichocarpa* (245). These genome sequences are invaluable for establishing

areas of synteny with other related plants and using the genome sequences for molecular marker development and choosing candidate genes from the collinear area. With increased ability to obtain large scale expression profiling of pathogen-induced genes, more studies are also seeking to identify the global gene expression response of crop species to various pathogens. Expressed sequence tag libraries are available for rice infected with rice blast (*Magnaporthe grisea*), potato leaves infected with late blight (*Phytophthora infestans*), wheat infected with Fusarium head blight (*Fusarium graminearum*), apple infected with apple scab (*Venturia inaequalis*) and nodule tissues of the legume model species *Lotus japonicus* (10, 114, 178, 213). In addition, there are a great deal of subtractive libraries produced from infected tissue, aimed at identifying ESTs in infected tissue that are only involved in the resistance response in: rice (144, 258), wheat (94, 129, 146), potato (242), barley (177), tomato (87), apple (58) and coffee (74).

Resistance (R) genes were first described by Flor in his seminal work that established the gene for gene theory where an incompatible response (disease resistance) occurs when an R gene "recognizes" an avirulence gene from a pathogen (reviewed in 55). More recently, a "guard hypothesis" has been proposed where the R genes are monitoring the status of a particular protein, and upon a change to that protein mediated by a pathogen effector protein, the R gene initiates a signaling cascade (55).

The identification of a large number of R genes involved in gene for gene interactions has allowed for the classification of types of R genes and their conserved motifs. The most common R genes were those with a nucleotide-binding site (NBS) and leucine rich repeats (LRR) that are split into two types, those with coiled coil (CC)

domains at their N-terminal or those with the animal-like Toll and interleukin receptors (TIR). The conservation of motifs in the NBS-LRR regions of these R genes has allowed researchers to clone resistance gene analogues (RGAs) from numerous plant species (156).

In addition to the identification of R genes, characterization of disease response genes in the model system, *A. thaliana*, is useful for a reference point in which to identify similar mechanisms in crop species. The majority of the disease-related (DR) genes from *Arabidopsis* are involved in the signaling pathways that coordinate defense and were identified by mutant analysis (97). Defense signaling pathways are classified into defense against biotrophs or necrotrophs for simplicity, although the true picture of how a plant perceives a pathogen is assuredly more complicated (88). Very briefly, signaling cascades for defense against biotrophic pathogens typically occur through CC-NBS-LRR or TIR-NBS-LRR R genes that initiate an oxidative burst, activate MAP kinases, induce salicylic acid production and the induction of PR gene expression through NPR1 (97). Detection of a necrotroph will initiate jasmonic acid and ethylene responsive pathways that induce gene expression through ethylene and jasmonic acid induced transcription factor ERF1, and jasmonic acid induced transcription factors RAP2.6 and JIN1. In addition, host detection of a necrotroph will also activate a MAP kinase pathway that leads to the downregulation of the salicylic response (88, 97).

As useful as genetic and genomic data are for assessing the molecular pathways and interactions leading up to a resistance response, the question of how that information can be applied is not often answered. Bridging gaps in between basic knowledge of plant pathogen interactions and applied knowledge of resistance phenotypes in available

germplasm is vital to identifying genes that coordinate durable resistance. In this literature review I will examine the coordination of large scale genomics projects and resistance phenotype analysis and the promise of these methods in yielding crops with durable resistance.

Resistance gene analogues

Approximately 149 NBS-LRR gene homologues were identified from *Arabidopsis* after the genome sequence was released. A physical map of the locations of these RGAs shows areas of 43 RGA clusters on the chromosomes, the result of localized gene duplication (162). While evolutionary insights leading to the duplication and distribution of RGAs are critical for the understanding of the development and maintenance of disease resistance in plants, the function of the majority of RGAs is unknown; function has only been determined for twelve NBS-LRR genes (105). As such, once an RGA is found to contribute to disease resistance, it is then known as an R gene. The mapping of RGAs to areas of QTL localization for disease resistance could help to identify the potential function of these genes. Mapping of RGAs to QTL regions has also been conducted in major crops such as rice, soybean, potato, rapeseed, and barley (reviewed in 195). RGAs are also currently being used as candidate genes in disease resistance QTL analysis in rice (202, 255, 256), potato (108, 233), and crucifers (231, 236).

Candidate gene analysis with crop-specific RGAs

Candidate gene analysis involves the identification of previously characterized defense-related genes, such as those identified already in *Arabidopsis* (13, 55), or the identification of RGAs before disease resistance QTL analysis. The candidate genes are then converted into molecular markers for use in the QTL analysis and for placement onto the genetic map. Those candidate genes that co-localize with the disease resistance QTLs can be further analyzed without the need for map based cloning (reviewed in 195).

In addition to the potential for an RGA to be identified as a functional R gene, RGAs may also be used as molecular markers for other resistance loci, since RGA clusters have mapped to resistance loci as is the case with *Arabidopsis* (1, 234). Therefore, caution must be exercised on assumptions from co-localizing candidate genes and the function of the candidate gene and phenotype must be confirmed using other methods such as Northern analysis or the use of transgenic plants for phenotype analysis. In the case of the *Rpg1* resistance gene to stem rust in barley, RGAs mapped near *Rpg1*, but attempts to use synteny with rice failed to identify any candidate genes in that area (12) and a non-traditional R gene, *Rpg1* was later cloned using positional cloning and was confirmed as being absent in the rice genome (37).

RGAs were used in the identification and cloning of a resistance gene *RB* from *Solanum bulbocasta* after other R genes that were bred into domestic potato (*Solanum tuberosum*) failed (233). Cloning of the *RB* resistance gene from *S. bulbocasta* used both traditional map-based cloning techniques, along with long-range PCR targeting the identified candidate gene RGAs from genomic DNA of resistant *S. bulbocasta*. The BAC carrying the *RB* allele was unstable in *E. coli*; therefore the complete *RB* gene was

amplified from genomic DNA and cloned into a binary vector for plant transformation and validation as the gene conferring resistance in previous QTL analysis (174). Insertion of *RB* as a transgene into a late blight-susceptible potato variety conferred resistance to all races of *P. infestans*, including one race that had overcome all previous R genes (233).

In maize, a combination of RGA mapping and transposon mutational analysis was used to clone a resistance gene from the *rp1* locus. RGAs had previously been mapped to the maize physical map and one RGA, *PIC20* had mapped to the *rp1* locus which is responsible for maize resistance to common rust (*Puccinia sorghi*). The detection of an insertion into the R gene *Rp1-D* was detected by the *PIC20* probe, and led to the subsequent cloning and characterization of *Rp1-D* (53).

Candidate gene analysis in rice has been useful for the identification of rice RGAs and defense-related genes that co-localize with QTLs for bacterial blight resistance (*Xanthomonas oryzae* pv. *oryzae*) and rice blast (*Magnaprothe grisea*). One study compiled a group of 118 molecular markers based on RGAs and previously determined defense-related (DR) genes (202). In total, six of the candidate gene markers co-localized with QTLs for bacterial blight resistance: five were RGAs and one was a defense-related gene, oxalate oxidase. A higher number of candidate genes with functional characterization co-localized with previously determined blast resistance QTLs (202). Due to the low number of defense-related markers associating with the bacterial blast resistance QTLs, caution must be exercised with choosing potential candidate genes for screening, the higher number of DR markers that associated with the fungal (blast)

resistance QTLs suggests that the authors selected genes that were more likely to be involved in fungal, not bacterial disease resistance in rice.

A second candidate gene approach to QTL analysis determined which RGAs and defense-related genes co-localized with rice blast resistance QTL in advanced backcross populations of the rice cultivar Vandana (256). Advanced backcross populations were used so that lines with promising resistance already possessed the positive agronomic traits from the susceptible parent, and could be released with minimal further selection and breeding (237). Seven markers were associated with blast resistance when a single blast isolate was used for infection; including two candidate genes and one RGA. A higher number of candidate genes were associated with resistance when multiple blast isolates were used for inoculation; there were four candidate genes and two RGAs. In addition, one of the candidate genes, a NBS-LRR gene, was also determined to have a digenic effect with two other NBS-LRR genes and the RGA homologue (256). The increased number of candidate genes identified as being significant to resistance during multiple isolate pressures is interesting, since this more closely mimics natural field conditions that new cultivars face. Particularly since there were digenic effects detected between NBS-LRR genes, supporting the hypothesis that R gene diversity is driven in part, by pathogen diversity (105). Overall, the localization of the RGAs with rice resistance QTLs is interesting; however it requires further characterization of the RGAs to determine their involvement in the resistance response.

Comparative genomics for R gene discovery

Comparative genomics is also a potentially useful method to identify collinear resistance genes between two related species where the function of the R gene is known in one of the species. The identification of an R gene in tomato; the model Solanaceous plant, against Fusarium wilt was used to identify the potato homologue based on extensive synteny between the genetic maps of tomato and potato. By aligning the genetic maps of the I2 resistance locus from tomato and the R3 resistance locus from potato, the identification of the collinear R gene, *R3a* was determined in potato (108). This was the first known report of an R gene being cloned based on synteny.

Since *Arabidopsis* is also a crucifer, RGAs derived from the *Arabidopsis* genome are of interest to groups working with agriculturally important crucifers. Because no R genes have been cloned from *Brassica napus* or its progenitors, *Brassica rapa* and *Brassica oleracea*, previously identified RGAs from *Arabidopsis* (33) were selected as R-EST clones and mapped onto a *B. napus* genetic linkage map in order to determine areas of collinearity, which were found to exhibit conserved gene content and order (231). Similarly, for *B. rapa*, markers based on known open reading frames (ORFs) from *Arabidopsis* were used to map areas of collinearity between clubroot (*Plasmodiophora brassicae*) resistance QTLs and the corresponding segment of an *A. thaliana* chromosome that is known to have R gene clusters (236). Overall for *B. napus*, the inheritance of R genes on chromosomal segments from the *Brassica* progenitors have contributed to R gene duplication, however these areas are still collinear to *Arabidopsis*. Once areas are identified in *B. napus* as being related to resistance and in the case of *B.*

rapa QTLs, researchers can take advantage of the genomic data from *Arabidopsis* to identify candidate genes (231, 236).

The areas of synteny determined above for *B. napus* were used for comparative mapping with *Arabidopsis* against the LmR1 QTL for blackleg (*Leptosphaeria maculans*) (154). LmR1 had previously been mapped so as to narrow the QTL down to a locus, however the identity of the gene is unknown; using EST markers that co-localize with this locus has helped narrow the candidate genes down to seven possible RGAs. The EST markers that the LmR1 locus co-segregates with are a WD-40 repeat family protein and a nucleoporin family protein (154). Even though the WD-40 repeat family protein wasn't considered a candidate gene, coincidentally one of the QTLs for clubroot resistance in *B. rapa* also encompasses a WD-40 repeat family protein sequence (236). Even though there are no reports of WD-40 proteins being involved in resistance, this would be a potential candidate gene based on position.

Candidate gene analysis using crop-specific EST data

Attempts at using comparative genomic analysis for the identification of resistance genes against Fusarium head blight (FHB) proved difficult for wheat, due in part to a lack of collinearity between the QTL in wheat and the corresponding area in rice. The breakdown of synteny at that region was due to rearrangements of the chromosomes in wheat and rice (140). As such, a previous attempt at using synteny between wheat and rice to identify candidate R genes or DR genes failed, in part because there were no annotated R or DR genes present in that chromosomal area of rice. A new approach to find candidate genes for the FHB resistance QTL utilized the vast amount of

EST sequences available for wheat that are mapped onto chromosomes. A low stringency tBLASTx search returned three ESTs with homology to R genes from other cereal species. One of the ESTs; homologous to a barley R gene functional against rust, was found to be polymorphic between parents used for analysis, and is a good candidate for the R gene responsible for the QTL (227).

Synthesis of genomic and genetic data: broad spectrum disease resistance

Overall synthesis of existing information on disease resistance QTLs, cDNA libraries and the genome sequence can also yield positional candidates for disease resistance. By combining QTL data for resistance to fungal, bacterial and viral pathogens against a genomic map, areas of multiple QTL congregation and RGA clustering are hypothesized to be involved in broad spectrum resistance (255). RGAs were identified by nucleotide homology to other known NBS-LRR sequences and amino acid similarity to a conserved domain. In addition to mapping QTLs, RGAs and known R genes, a digital Northern approach was used to analyze the occurrence of ESTs from inoculated leaf tissue by using full length cDNAs to perform BLASTn searches against the EST libraries. Full length cDNA with significantly different numbers of EST matches were placed on the genomic map in order to determine if they also co-localize with broad spectrum disease resistance regions. Four chromosomal areas were identified by the concurrent mapping of QTLs along with other genetic and genomic data. Positional candidate genes from each of these areas were determined based on homology to annotated *Arabidopsis* proteins with homology to the translated cDNA sequences from

that particular chromosomal segment (255). Further narrowing of the areas on the chromosome that QTLs encompass is still required for better introgression of these broad spectrum disease resistance areas into new cultivars, but this was a first important step in the melding of current breeding and genomic data for rice for disease resistance.

Concluding Remarks

Since the majority of the molecular mechanisms of *E. amylovora* virulence are still elucidated by mutational analysis, I chose to use an *in vivo* expression technology (IVET) approach to identify genes upregulated in *E. amylovora* during colonization and virulence. This work is reported on in chapter one and pertains to the diversity of upregulated genes in *E. amylovora* that are required for cellular processes during virulence beyond just plant-microbe interactions. In the IVET screen, genes from a type II secretion operon and an endo-polygalacturonase were identified. Since type II secretion was previously though not to be important for *E. amylovora* virulence, I chose to characterize both the type II operon and endo-polygalacturonase and to determine their contribution to virulence in chapter two. Finally, with the increased amount of genomic data available for plant systems, scientists are able to apply knowledge about disease response in model plant systems to crop plant systems. The use of RGAs and defense related genes as molecular markers and candidate genes for QTL analysis is helping to identify new introgressible R genes for agricultural use. However, in a slow growing crop species such as apple, the ability to identify resistance loci from QTLs is not easy and is time consuming. In order to identify discrete genes that are involved in the resistance response to *E. amylovora*, I chose to use a suppression subtractive

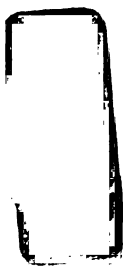
hybridization library combined with expression analysis with cDNA macroarrays. Chapter three concerns these genes that are upregulated in the moderately resistance cultivar Red Delicious, and are part of the resistance response.

CHAPTER 1

Identification of *Erwinia amylovora* Genes Induced During Infection of Immature Pear Tissue

ABSTRACT

I used a modified *in vivo* expression technology (IVET) system to identify *E. amylovora* genes that are activated during infection of immature pear tissue, and identified 394 unique *p*ear *f*ruit-*i*nduced (*pfi*) genes on the basis of sequence similarity to known genes. Known virulence genes, including *hrp/hrc* components of the type III secretion system, the major effector gene *dspE*, type II secretion, levansucrase *lsc*, and regulators of levansucrase and amylovoran biosynthesis were upregulated during pear tissue infection. Known virulence factors previously identified in *E. (Pectobacterium) carotovora* and *Pseudomonas syringae* were identified for the first time in *E. amylovora* and included HecA hemagglutin family adhesion, Peh polygalacturonase, new effector $HopPtoC_{EA}$, and membrane-bound lytic murein transglycosylase $MltE_{EA}$. An insertional mutation within *hopPtoCEA* did not result in reduced virulence; however, an $mltE_{EA}$ knockout mutant was reduced in virulence and growth in immature pears.



INTRODUCTION

Erwinia amylovora is the causative agent of fire blight, a devastating necrotic disease affecting apple, pear and other rosaceous plants. Entry of the bacterium into plants can occur via flower blossoms, actively growing young shoots or through wounds. Upon entry, the fire blight pathogen moves through intercellular spaces towards the xylem, and also the cortical parenchyma (247). Symptoms often appear as water-soaked tissue that rapidly wilts and becomes necrotic, leading to the characteristic "shepherd's crook". As a member of the *Enterobacteriaceae*, *E. amylovora* is related to many important human and animal pathogens such as *Escherichia coli*, *Yersinia pestis*, *Y. enterocolitica, Salmonella enterica* and *Shigella flexneri.*

Like many other Gram-negative plant pathogenic bacteria, *E. amylovora* produces a type III Hrp secretion (TTSS) apparatus that delivers effector proteins into host plants (125). The TTSS in *E. amylovora* controls the ability of *E. amylovora* to cause disease in susceptible host plants and to elicit the hypersensitive response (HR) in resistant and non-host plants. Most *hrp* genes have been found to encode proteins involved in gene regulation or in assembly of the TTSS apparatus (4, 99, 125).

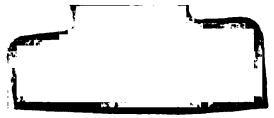
The TTSS of *E. amylovora* secretes several virulence proteins, including HrpA, HrpN, HrpW and disease-specific protein DspA/E (hereafter referred to as DspE) (25, 26, 82, 125, 126, 251, 252). The HrpA protein is the major structural protein of a pilus called the Hrp pilus, which is the extracellular part of the TTSS (117). DspE, HrpN, and HrpW proteins are effector proteins of the TTSS and are believed to be injected directly into host cells (25, 26).

Additional *E. amylovora* virulence factors that contribute to pathogenesis and plant colonization include the exopolysaccharides amylovoran and levan, iron-scavenging siderophore desferrioxamine, metalloprotease PrtA, multidrug efflux pump AcrAB, and carbohydrate metabolism genes specifically involved in the utilization of sorbitol, sucrose and galactose (3, 29, 38, 161, 264). Transcriptional regulators of the amylovoran and levan biosynthetic operons have also been identified (22, 51, 264) and are required for the expression of the biosynthetic machinery for the exopolysaccharides (19, 68, 123, 264). *E. amylovora* pathogenesis is also subject to global regulation by the small regulatory RNA *rsmB* which functions by titrating and countering the activity of the repressor protein RsmA; this system is reported to positively regulate exopolysaccharide production, motility and pathogenicity (147). In addition, *E. amylovora* strains contain a ubiquitous nonconjugative plasmid of 28-30 kb designated pEA29; laboratory-derived plasmid-cured strains exhibit a reduction in virulence (155). pEA29 encodes several potential virulence genes including a thiamine-biosynthetic operon that is proposed to influence amylovoran production (155).

Genetic analysis of virulence genes in *E. amylovora* have been performed mostly through the production and screening of mutants. Additionally, most of the genes discovered so far have been identified from mutant screening under controlled conditions. However, it is not feasible to mimic all of the nutrient and defense conditions *in vitro* to characterize all the genes from *E. amylovora* required for infection and colonization of plants. There is a need then, for a high-throughput method of screening for genes that are involved in virulence and growth *in planta* of *E. amylovora*.

In the last decade, many gene expression technologies including *in vivo* expression technology (IVET) have been developed to identify gene expression profiles of organisms during interactions with various host environments (8, 101, 150). IVET screening theoretically scans the entire genome, and through the use of appropriate environmental conditions and different strategies, can yield large numbers of potentially important genes (201). IVET screens have identified genes upregulated upon infection with enteric human and animal pathogens such as *Salmonella enterica*, *Shigella flexneri*, and *Y. enterocolitica* (15, 150, 171, 261). IVET systems have also been used to identify genes expressed during plant infection by *Xanthomonas campestris*, *E. chrysanthemi*, *Pseudomonas syringae* and *Ralstonia solanacearum* (24, 36, 96, 172, 187, 254), phyllosphere colonization by *P. syringae* (151), and saprophytic colonization by *P. fluorescens* (200, 225).

Like many plant pathogenic bacteria, *E. amylovora* can infect different host tissues at different stages of disease development. *E. amylovora* not only infects blossoms, leaves, and succulent shoots, but also immature fruits of susceptible hosts. The bacterium also grows epiphytically on stigmas and endophytically inside plant tissue. The maintenance of large numbers of apple trees for study of *E. amylovora* pathogenesis is quite difficult due to the extensive greenhouse and growth chamber space required. As an alternative, many researchers have utilized immature pear fruits to study *E. amylovora* infection (19, 26, 85). Immature pear infection is initiated through a wound inoculation; wound colonization is a frequently-utilized mechanism of *E. amylovora* infection in nature (247). Immature pear assays, either using intact pear fruits or pear slices, have



been used successfully to analyze virulence effects of several *E. amylovora* genes (26, 85, 125).

Although key virulence factors contributing to fire blight have been identified, little knowledge is available of the global host-regulated genes of *E. amylovora* during infection. To gain a better understanding of the molecular mechanism governing *E. amylovora*–host plant interactions, we undertook a comprehensive genome-wide examination of gene expression patterns during host infection to uncover pathogenesis strategies of the organism and to lay the groundwork for future studies examining the expression and function of critical virulence genes during infection of different host tissues and survival within the host. Several known virulence and pathogenesis factors were identified using this modified IVET screen, along with new potential virulence genes that were previously only described in other bacterial pathosystems. We also confirmed that infection of immature pear tissue by *E. amylovora* required the major pathogenicity factors of the bacterium.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids utilized in this study are listed in Table 1. *Erwinia amylovora* wild type (WT) and mutant strains and *E. coli* strains were grown in Luria Bertani (LB) medium at 28°C and 37°C, respectively. Antibiotics were added to the culture medium at the following concentrations: rifampicin, 100 μg/ml; kanamycin, 30 μg/ml; gentamicin, 10μg/ml and ampicillin, 100 μg/ml. Oligonucleotide primers used for polymerase chain reaction (PCR) and sequencing in this study are also listed in Table 1.

DNA manipulation and sequence analysis. Plasmid DNA purification, PCR amplification of genes, isolation of fragments from agarose gels, restriction enzyme digestion, T4 DNA ligation, and Southern hybridization were performed using standard molecular procedures (217). Chromosomal DNA was isolated using a genomic DNA purification kit (Qiagen, Valencia, CA). TAIL-PCR was performed using the degenerate primers AD1, AD2 and AD3 as described previously (142) and PCR products from secondary and tertiary nested PCR were used for sequencing. DNA sequencing was performed at the Genomic Technology Support Facility at Michigan State University. The oligonucleotide primer Aj1585 corresponding to the 5' end of *uidA* gene was used for sequencing fragment inserts cloned into the pGCM0 plasmid. Sequence management and contig assembly were conducted using DNAStar software (DNAStar Inc., Madison, WI, USA). Database searches were conducted using the BLAST programs at NCBI (www.ncbi.nlm.nih.gov/BLAST). Percent similarity was also calculated using the

BLAST program (7). Amino acid alignments were done with ClustalW, v. 1.83 (European Bioinformatics Institute, Cambridge, UK).

TABLE 1. Bacterial strains, plasmids and primers used in this study

Strains, plasmids, primers	Relevant characters or sequences (5'—3')[a]	Reference or source
Erwinia amylovora		
Ea110	Wild type, isolated from apple	(155)
Ea110$^-$	Ea110, cured of pEA29	(155)
Ea1189	Wild type, isolated from apple	(38)
CFBP1430	Wild type, isolated from *Crataegus*	(82)
M52 (Ea *dspA*)	CFBP1430, *dspA::uidA*-km, Km^R	(82)
Ea 110 *hrpA*	Ea110 Δ*hrpA* Km^R	(117)
ZYC1-3 (Ea *hopPtoC*$_{EA}$)	*hopPtoC::Km;* partial deletion and Km^R–insertional mutant of *hopPtoC*$_{EA}$ of Ea1189, Km^R	This study
ZYE3-11 (Ea *mltE*$_{EA}$)	*mltE::Km;* partial deletion and Km^R–insertional mutant of *mltE*$_{EA}$ of Ea1189, Km^R	This study
E. coli		
DH10B	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*Δ139 Δ(*ara, leu*)7697 *gal*U *gal*K λ - *rps*L (Str^R) *nup*G	Invitrogen
S17-1	*recA pro hsdR* RP4-2-Tc::Mu-Km::Tn7	(253)
S17-1 λ pir	λ -pir lysogen of S17-1	(253)
Plasmids		
pBluescript II SK(+)	Ap^R, cloning vector	Stratagene
pGem3zf+	Ap^R, cloning vector	Promega
pCAM140	Sm^R, Sp^R, Ap^R, R6k origin, mTn5SS*gusA*40	(253)
pCAM140-MCS	Ap^R, R6K origin, pCAM140 derivative without mini-Tn5, contains the multiple cloning site of pBluescript II SK (+)	(38)
pX1918GT	*xylE*-Gm^R fusion cassettes-containing plasmid flanked by inverted repeats of the pUC19 MCS	(224)
pBSL15	Km cassette flanked by inverted repeats of the pUC18 MCS	(8)
pGCM0	Gm^R cassette with downstream transcriptional terminator and *gusA* with upstream translational stop codons in pGem3zf+	This study

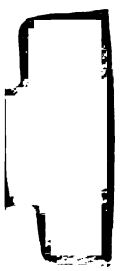
TABLE 1 (con't)

Strains, plasmids, primers	Relevant characters or sequences (5'—3')[a]	Reference or source
pZYF2	570 bp *dspE* promoter in opposite orientation relative to *uidA* in pGCM0	This study
pZYF8	570 bp *dspE* promoter in correct orientation relative to *uidA* in pGCM0	This study
Primers[b]		
Aj1388	CCCAAGCTTGGTGCGCCAGGAGAGTTGTTG (*Hin*d III)	
Aj1389	AAAACTGCAGTGATTGATTGACGGACCAGTATTATTATC (*Pst*I)	
Aj1390	CCGGAATTCCGAATTGACATAAGCCTGTTCGG (*Eco*RI)	
Aj1391	CGGGGTACCTGGACGCGGCCGATCACCTGGCCGTTG (*Kpn*I)	
Aj1585	GATAATAATACTGGTCCGTCAATC	
Aj1565	CGGTTTACAAGCATAAAGCTGGGCAACGGCC	
DspE1	TCCCCCGGGCAGTGAGGGGGGGCAGACTTTTTTTTAACC (*Sma*I)	
DspE2	TCCCCCGGGTATCTTCGCCGCTGCCACCTTTCACCATTG (*Sma*I)	
PtoC1	TCCCCGCGGGCGGGCTGTTGGTCTTGCTCT (*Sac*II)	
PtoC2	TGCTCTAGACTCTGGCAAAATTCAACTGA (*Xba*I)	
PtoC3	CCGGAATTCCATGGCAGGGACCCGCAGTTTG (*EcoR*I)	
PtoC4	CCGCTCGAGGGCTGATGGCGGGTTAGTCTGTCG (*Xho*I)	
MltE1	TCCCCGCGGTGAATAGTGCGTGGCGTGATGTGC (*Sac*II)	
MltE2	TGCTCTAGATTAATCATTGCAATCGCCTCGTC (*Xba*I)	
MltE3	CCGGAATTCTTACCAGCACGTGCAGACAAAACA (*EcoR*I)	
MltE4	CCGCTCGAGCCGGATGGATCTGGTGAGGGGCGC (*Xho*I)	
AD1	NTCGASTWTSGWGTT	
AD2	NGTCGASWGANAWGAA	
AD3	WGTGNAGWANCANAGA	

[a] Km^R, Ap^R, Gm^R, Sp^R, Sm^R = kanamycin, ampicillin, gentamicin, spectinomycin, and streptomycin resistance, respectively.

[b] Underlined nucleotides are restriction sites added and the restriction enzymes are indicated at the end of primers. Mixed nucleotides: S = C+G; W = A+T; N = A+T+C+G.

Immature pear infection assays. Immature pears are routinely used to examine the pathogenicity of naturally-occurring isolates or bacterial mutants of *E. amylovora* (26). In order to confirm that infection of immature pear required major pathogenicity factors as previously reported (26), we inoculated wounded immature pear fruits with *E. amylovora* M52 (CFBP1430 *dspE*) and Ea110 *hrpA* mutants and monitored for symptom development and *in planta* bacterial growth. Bacterial suspensions of all strains were



grown overnight in LB broth , harvested by centrifugation, and resuspended in 0.5X sterile phosphate buffered-saline (PBS) with the cells adjusted to approximately 1 x 10^4 colony-forming units (CFU)/μl (OD_{600} = 0.1 and then diluted 100 times) in PBS. Immature pears (*Pyrus communis* L. cv. 'Bartlett') were surface sterilized with 10% bleach and pricked with a sterile needle as described previously (155). Wounded pears were inoculated with 2 μl of cell suspensions and incubated in a humidified chamber at 28°C. Symptoms were recorded at 2, 4, 6, and 8 days post inoculation. For bacterial population studies, the pear tissue surrounding the inoculation site was excised by using a #4 cork borer as described previously (26) and homogenized in 0.5 ml of 0.5 X PBS. Bacterial growth within the pear tissue was monitored by dilution-plating of the ground material on LB medium amended with the appropriate antibiotics. For each strain tested, fruits were assayed in triplicate, and each experiment was repeated.

Construction of the genomic library of transcriptional fusions to *uidA*. We used *E. amylovora* Ea110$^-$ (cured of the ubiquitous plasmid pEA29) as the source of chromosomal DNA for the IVET experiments. We excluded pEA29 genes from this study because an analysis of the expression of pEA29-encoded genes during infection will be presented in a separate report (McGhee and Sundin; unpublished). To create a library of transcriptional fusions, chromosomal DNA from *E. amylovora* Ea110$^-$ was partially digested with *Hae*III, and fragments between 800 bp and 2 kb in length were separated by electrophoresis and gel-purified. The purified fragments were ligated into pGCM0 prepared by *Sma*I digestion and transformed into WT *E. amylovora* Ea110 (containing pEA29) by electroporation. The use of WT strain Ea110 was necessary

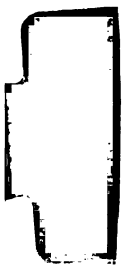
because the ubiquitous pEA29 plasmid contributes to *E. amylovora* virulence (155).

The 6.2-kb pGCM0 reporter vector was constructed by cloning the *aacC1* gene (conferring resistance to gentamicin) into the *Eco*RI and *Kpn*I sites and the promoter-less *uidA* (β-glucuronidase) reporter gene into *Pst*I and *Hin*dIII sites of pGem3zf through multiple cloning steps (Figure 1A). The *aacC1* gene was amplified from plasmid pX1918GT by PCR using the primer pair Aj1390 and Aj1391, whereas the promoter-less *uidA* gene was amplified from plasmid pCAM140 using the primer pair Aj1388 and Aj1389. A transcriptional terminator sequence, also from pX1918G, was located immediately downstream of the *aacC1* gene, and we added translational termination codons in all three reading frames upstream of the *uidA* gene. The pGCM0 vector was first digested with *Sma*I and the ends were dephosphorylated with calf intestinal alkaline phosphatase (CIAP) and checked for self-ligation before ligation with *E. amylovora* chromosomal DNA fragments. After ligation, DNA was introduced into Ea110 by electroporation, and transformants growing on LB medium amended with gentamicin and ampicillin were randomly collected and plasmids were recovered. The randomness of the inserts in the IVET collection was confirmed by checking insert size from 30 random colonies through restriction digestion and PCR (data not shown).

As a control, we cloned a 570-bp fragment containing the *dspE* promoter into pGCM0. The fragment was amplified by PCR from strain Ea110 using the primer pair DspE1 and DspE2. The resulting 570-bp product was cleaved with *Sma*I and ligated into pGCM0 in both orientations. The resulting plasmids were designated as pZYF2 ($dspE^{rev}$::pGCM0, *dspE* promoter in opposite orientation to *uidA*) and pZYF8 ($dspE^{for}$::pGCM0, *dspE* promoter in correct orientation to *uidA*), respectively, and each

plasmid was introduced into strain Ea110 by electroporation.

Screening of the *E. amylovora* IVET library using a GUS-based microtiter plate assay. An *in vivo* microtiter plate assay was developed for screening of the *E. amylovora* IVET library (Figure 1B). Briefly, approximately 19,200 transformants in strain Ea110 were randomly collected and initially screened for GUS activity on LB plates containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (Xgluc). After incubation at 25 °C for 48 h, bacteria were transferred individually using a 48-pin colony transfer apparatus and inoculated onto immature pear discs (3mm) in 96-well microtiter plates. Intact pears were surface sterilized using 10% bleach for 10 min and rinsed three times with sterile water. Discs were cut from pears using a #2 cork borer and immediately immersed into microtiter plate wells containing 25 µl 0.5X PBS buffer to avoid oxidation. The microtiter plates were then covered with AirPore tape (Qiagen, Valencia, CA) after inoculation and incubated in a humidity chamber at 25 °C for 48h. After incubation, a qualitative GUS assay was performed as described below. Transformants showing GUS activity on pear tissue but not on LB plates were selected and re-screened on LB plates containing Xgluc and re-inoculated onto pear discs in 96-well microtiter plates. Confirmed differentially-expressing transformants were again selected and stored at -70 °C in glycerol stocks for further analysis. Plasmids were isolated from the consistent differentially expressed transformants and were end sequenced to identify the genes or promoter regions. Transformants showing GUS activities on both LB plates and on pear tissues were assumed to contain constitutively expressed fusions and were not analyzed further in this study.



Construction of *hopPtoC*$_{EA}$ and *mltE*$_{EA}$ mutants. For the construction of *hopPtoC*$_{EA}$ and *mltE*$_{EA}$ mutants, the sequences of the putative ORFs defined by the corresponding clones were determined and used to design primers to amplify fragments of the genes and its upstream and downstream sequences. Primer pairs PtoC1 and PtoC2, PtoC3 and PtoC4 were used to amplify 590 bp and 670 bp fragments from *E. amylovora* strain Ea1189 corresponding to the upstream and downstream of *hopPtoC*$_{EA}$ gene, respectively. Primer pairs MltE1 and MltE2, MltE3 and MltE4 were used to amplify 700 bp and 560 bp fragments from *E. amylovora* strain Ea1189 corresponding to the upstream and downstream of *mltE*$_{EA}$ gene, respectively. The two fragments for each ORF were cloned into pBluescript-II SK(+) through multiple cloning steps with corresponding restriction enzyme digestion (*Sac*II and *Xba*I; *EcoR*I and *Xho*I, respectively). The whole fragment was excised using *Sac*II and *Xho*I, gel purified and cloned into the suicide vector pCAM-MCS (38) digested with the same enzymes. The resulting plasmids were digested with *Sma*I and ligated with a 1.2 kb fragment of the *aph* gene (conferring kanamycin resistance) released from plasmid pBSL15. The final plasmids were designated as pZYC8 and pZYE8, respectively and introduced into *E. amylovora* strain Ea1189 by electroporation. Transconjugants resistant to kanamycin (Km) were selected. To further exclude mutants resulting from single crossover events, transformants were selected on LB plates supplemented with Km and onto LB with ampicillin (Ap). Km-resistant and Ap-sensitive colonies were selected and their genotypes were confirmed by hybridization or PCR analysis.

GUS Assays. The β-glucuronidase (GUS) reporter gene (*uidA*) on pGCM0 was used to monitor promoter activity of IVET clones both *in vitro* and *in vivo*. Qualitative GUS activity of IVET clones was monitored visually by the development of a blue color within 48 h of cells on LB medium containing 1 mM Xgluc. Qualitative GUS activity of IVET clones grown on pear slices in microtiter plates after 48 h at 25 °C was also monitored visually by adding 10 μl of 20mM Xgluc into the wells followed by incubation for 30 min at 37 °C. The development of a blue color indicated GUS activity.

To monitor the expression of IVET clones in pear tissue, quantitative GUS activity of bacteria in either culture or in pear tissue was determined as described previously (26, 116) using 4-methylumbelliferyl-β-D-glucuronide (MUG) as a substrate and 0.2 M Na_2CO_3 as stop buffer. Briefly, *E. amylovora* strains containing the IVET clones were grown on LB medium, resuspended in 0.5 × PBS, and inoculated in immature pear fruits as described above. At 0, 24, and 48 h post inoculation, the pear tissue surrounding the inoculation site was excised using a #4 cork borer and homogenized in 0.5 ml 0.5 × PBS. Forty microliters of homogenate was mixed with 160 μl of GUS extraction buffer. Reactions were stopped by Na_2CO_3 addition and fluorescence was measured using a SAFIRE fluorometer (TECAN Boston, Medford, MA). Bacterial cell numbers in the sample were estimated by dilution plating, and GUS activity (μmol of 4-methylumbelliferone (MU) produced per min) was normalized per 10^9 CFU (26). Three replicate fruits for each strain were tested and the experiment was repeated.

GenBank Accession numbers. Nucleotide sequence data reported for the *hopPtoC*$_{EA}$

and $mltE_{EA}$ genes were deposited in the GenBank database under the accession no. AY887538 and AY887539.

RESULTS

Development of an IVET system and identification of E. amylovora upregulated genes during immature pear infection

Our immature pear infection results clearly demonstrated that infection of immature pear by *E. amylovora* required major pathogenicity factors (Figure 2). At 48 h after inoculation, *E. amylovora* strains CFBP 1430 and Ea110 produced water-soaking symptoms in pears with visible bacterial ooze (data not shown). Two different strains were used in this initial experiment because of the availability of mutants of these strains. This work also confirmed that growth and symptom development of WT strains CFBP1430 and Ea110 were similar during immature pear inoculation (Figure 2). Four days after inoculation, inoculated immature pears showed necrotic lesions and bacterial ooze formation (Figure 2A). After eight days, the entire pears showed necrosis, turning black with copious ooze production at the inoculation site (Figure 2A). In contrast, disease symptoms were not observed on immature pears inoculated with either the *E. amylovora hrpA* or *dspE* mutants (Figure 2A). Disease symptoms caused by WT strains on immature pear were correlated with high levels of bacterial growth in pear tissue during the four days post inoculation (Figure 2B); however, the CFBP1430 *dspE* mutant M52 grew only slightly in pears, representing an approximately 10^5-fold reduction relative to the WT CFBP1430 strain. Populations of the *hrpA* mutant declined quickly after inoculation, indicating that the *hrpA* mutant was not able to survive in immature pear (Figure 2B).

Verification that infection of immature pear required major pathogenicity factors

of *E. amylovora* facilitated the development of a simple, high-throughput IVET system to identify *E. amylovora* genes induced during colonization and infection. We used cores of immature pear tissue in a microtiter plate format, a system that was conducive to handling large numbers of samples.

To develop an immature pear fruit assay, we used the β-glucuronidase gene *uidA* as a reporter (116) in the vector pGCM0 which was constructed as described in Materials and Methods (Figure 1A). The vector was verified with a control construct containing the *dspE* promoter in both orientations (Table 1). The *dspE* promoter was previously reported to be strongly induced during immature pear infection (26). GUS activity was not observed after two days growth in LB medium for either Ea110(pZYF8) (*dspE* promoter in correct orientation to *uidA*) or Ea110(pZYF2) (*dspE* promoter in opposite orientation to *uidA*). However, GUS activity was observed for strain Ea110(pZYF8) in qualitative assays two days following inoculation onto immature pear discs but not following inoculation of strain Ea110(pZYF2) (data not shown). GUS activity was not observed for the WT Ea110 strain containing the empty pGCM0 vector on either LB medium or in pear discs.

To identify *E. amylovora* genes expressed during colonization and infection of pear discs, we constructed a library of 0.8 to 2-kb fragments of genomic DNA of Ea110$^-$ (cured of PEA29) in pGCM0 and introduced the library into WT Ea110 by electroporation. In order to screen for differentially-expressed promoter fusions, we developed an *in-planta* pear disc microtiter plate assay (Figure 1B). Strain Ea110$^-$ containing library clones were first grown on LB/Xgluc medium for two days, visually

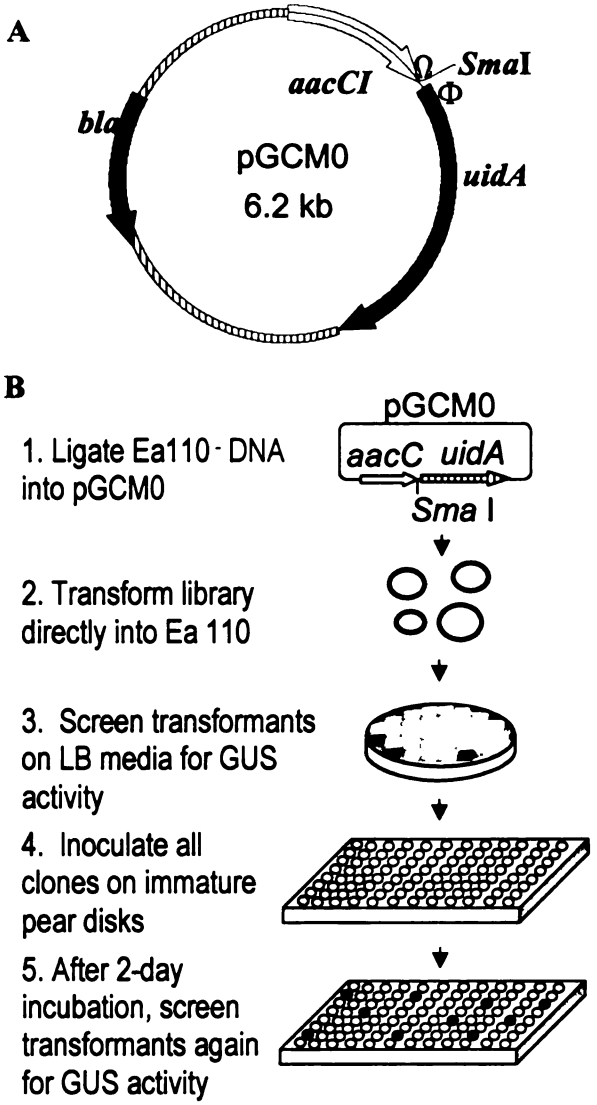


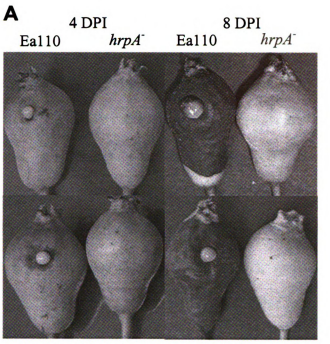
FIGURE 1. Overview of the IVET screen for *E. amylovora* genes induced during infection of immature pear discs. **(A)** Schematic map of the IVET vector pGCM0. The 6.2-kb pGCM0 vector was constructed by cloning the *aacC1* gene (conferring resistance to gentamicin) into the *Eco*RI and *Kpn*I sites and the promoter-less *uidA* (β-glucuronidase) reporter gene into *Pst*I and *Hin*dIII sites of pGem3zf through multiple cloning steps. The Φ symbol represents translational terminator codons in all three reading frames upstream of the *uidA* gene; and the symbol Ω represents a transcriptional terminator sequence immediately downstream of the *aacC1* gene. The *Sma*I site was used for ligation of random chromosomal inserts. **(B)** A library (19,200 clones) of *Sma*I chromosomal DNA fragments (0.8 to 2 kb) from *E. amylovora* was constructed in pGCM0, transformed into *E. amylovora* Ea110 and screened individually for GUS activity on LB medium amended with Xgluc. A 96-well microplate containing slices of pear tissue was inoculated with Ea100 containing random IVET fusion clones and incubated for 48h at 25°C. Clones exhibiting GUS activity on pear discs but not on LB+Xgluc medium were selected and the plasmids were recovered for further analysis.

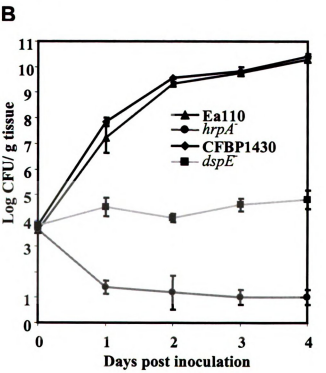
monitored for GUS activity, and then inoculated onto pear discs in microtiter plates (Figure 1B). GUS activity was qualitatively detected after two days of incubation at 25°C. Only clones that showed high GUS activity in pear discs but no GUS activity on LB plates were recognized as pear-upregulated clones. Those differentially-expressed clones were screened again on LB/Xgluc plates and pear discs to confirm the results. A total of 19,200 transcriptional fusion clones were screened on both LB/Xgluc medium and pear discs, and 498 clones (2.5%) were repeatedly found to differentially express GUS activity on pear discs in this qualitative assay.

Sequence analysis of E. amylovora genes upregulated in immature pear tissue.

We determined the sequence of the inserts from the 498 clones and identified the putative genes induced following BLAST searches of the non-redundant GenBank database. Of the 498 inserts sequenced, a total of 55 genes were identified two or more times and 12 clones contained either an intragenic sequence or with the putative gene present in the incorrect orientation. Although it is possible that these 12 clones may contain cryptic promoter sequences as has been shown in a previous study with *P. fluorescens* (66), we separated the clones from the others in the current study and did not subject them to further analysis. Thus, a total of 394 unique putative pear-inducible genes were identified, and these *pear fruit-induced* (*pfi*) genes could be divided into nine putative functional groups, including host-microbe interactions (3.8%), stress response (5.3%), regulatory (11.9%), cell surface (8.9%), transport (13.5%), mobile elements - phage (1.0%), metabolism (20.3%), nutrient acquisition and synthesis (15.5%), and unknown or hypothetical proteins (19.8%).

Figure 2. Symptoms and bacterial growth of *Erwinia amylovora* WT strains and *hrpA* and *dspE* mutants in immature pear. **(A)** Symptoms caused by *Erwinia amylovora* Ea110, CFBP1430 and corresponding *hrpA* and *dspE* mutants in immature pear. DPI: days post inoculation. **(B)** Bacterial growth of *Erwinia amylovora* WT EA110, CFBP1430 and *hrpA* and *dspE* mutants during infection of immature pears. The growth of bacterial strains was monitored at 0, 1, 2, 3, 4 days after inoculation. Data points represent the means of three replicates ± SE. Similar results were obtained in a second independent experiment.


A
4 DPI
8 DPI
Ea110
hrpA-
Ea110
hrpA-
CFBP1430
dspE-
CFBP1430
dspE-


B
Log CFU/ g tissue
Days post inoculation
Ea110
hrpA-
CFBP1430
dspE-
0
1
2
3
4
5
6
7
8
9
10
11

The majority of the putative gene-products identified as inducible during infection of pear tissue shared high amino acid similarity with that of proteins from *Yersinia* spp., *Salmonella* spp., *E. coli*, *Shigella* spp., and *Erwinia* spp. (Table 2). Several known virulence factors previously reported in *E. amylovora* such as the TTSS genes *hrpGF*, *hrpL*, *hrpX* and genes encoding known or new effector proteins DspE and $HopPtoC_{EA}$ were upregulated during pear infection (Table 2). Other known *E. amylovora* virulence genes identified as upregulated in this study were levansucrase (*lsc*), regulator of levansucrase (*rlsA*), amylovoran regulator (*rcsA*), and zinc-binding metalloprotease (*prtA*) (Table 2). In addition, genes encoding polygalacturonase (*peh*), a hemaggulatinin-family adhesion (*hecA*), and membrane-bound lytic murein transglycosylase (*mltE*) were identified for the first time in *E. amylovora* Ea110 (Table 2). Peh and HecA are important virulence factors in *E. chrysanthemi (205)*, and MltE plays a role in the virulence of *P. syringae* (24). A total of 54 upregulated genes identified were homologs of genes identified in IVET studies performed with other bacterial plant or animal pathogens (Table 2).

Type II secretion system (T2SS) genes similar to *Yersinia enterocolitica yts1IJ* (112), a known virulence factor, and one of five major protein secretion systems in many pathogenic bacteria, were identified for the first time in *E. amylovora*. Yts1IJ are known type 4 pilin-like proteins (pseudopilins). Interestingly, the type II secretion system is dependent on the general secretory pathway (GSP), i. e. the Sec secretion pathway (112). In our study, the major preprotein translocase SecA (ATPase), molecular chaperone SecB and membrane proteins SecDF of the GSP were also induced during infection of pears along with the T2SS (Table 2). Furthermore, the *peh* gene, encoding an enzyme that is

known to be secreted by the T2SS, was also up-regulated (Table 2), indicating that a functional T2SS is present in *E. amylovora* and to a greater extent could contribute to the virulence of this pathogen.

Transport genes (*pfi* 16 to 51) including general, ion, sugar, amino acid, peptide, and nucleotide transport proteins were induced in pear tissues (Table 2). Some of the transporters may belong to the type I secretion system that is known to be involved in secreting toxins, proteases and lipases and are potential virulence factors in *E. amylovora*. Cell surface proteins including inner, periplasmic, and outer membrane proteins, lipoproteins, flagella and polysaccharide proteins were also induced during pear tissue infection (*pfi* 52 to 72; Table 2). These membrane proteins may be involved in protein secretion and membrane maintenance. The sensor component (*envZ, pfi* 94) of a two-component regulatory system and cognate outer membrane protein genes that this system regulates *ompA* (*pfi* 65) and *ompC* (*pfi* 64) were also differentially expressed in pears compared to LB medium.

Under unfavorable conditions such as nutritional stress or exposure to a host defense response, bacterial pathogens respond by over-expressing stress response genes. Several stress response genes (*pfi* 75 to 90) were identified in our screen (Table 2). These genes included DNA repair or protection (*mutS*, *recA*, *sulA*), carbon starvation, heat or phage shock and antioxidant genes (such as *grpE* and *ahpC*). These results suggest that pear tissue at least initially is not a favorable habitat for *E. amylovora* growth and /or that DNA damage and the neutralization of plant-derived reactive oxygen species are involved in virulence and *in planta* growth.

The sensor component of a two component regulatory system, *grrS* (*pfi* 93), was

identified as upregulated in this study. GrrS is a homolog of GacS which, along with GacA, globally regulate a network of virulence functions in *E. carotovora*, including the production of quorum-sensing signaling molecules (72). Besides amylovoran and levansucrase regulators (*rcsA* and *rlsA*), and genes encoding sensor component of a two-component regulatory system (*grrS* and *envZ*), our screen identified *fliZ*, a positive regulator of the flagellar biosynthetic operon in enterobacteria, as upregulated. Other regulatory genes (*pfi* 96 to 126) and genes involved in sequence mobilility (*pfi* 73 to 74) and metabolism and nutrient scavenging (*pfi* 127 to 157) were identified in our screen and listed in Table 2. We recovered several metabolic genes that are potential precursors for the siderophore desferrioxamine biosynthesis in this study (*pfi* 153 to 157). It is probable that under unfavorable conditions, the bacterium itself adjusts and overcomes nutrient and iron deficiencies.

The large number of unknown or hypothetical proteins identified in this IVET screen (78 genes, 19.8%) indicates the future possibilities of characterizing novel virulence traits in *E. amylovora* and assigning functions to these proteins. A complete genome sequence of *E. amylovora* is expected soon. When an annotated genome sequence is released, we will make a listing available upon request of the gene numbers of the unknown or hypothetical proteins identified in this study.

TABLE 2. Selected list of *Erwinia amylovora* genes induced during infection of immature pear tissue[a]

pfi no.	*Gene*[b]	*Organism*	Blastx_BestHit[c]	GenBank Acc. no.	Previously reported[d]
Type III secretion (TTSS) and effectors					
1	*dspE**	*Erwinia amylovora*	DspE/pathogenicity factor	AAC04850	Ech, (261)
2	*hrpGF*	*E. amylovora*	Type III secretion HrpG/F	AAB49178	Pst, (24)
3	*hrpL**	*E. amylovora*	Type III sensor kinase HrpL	AAD24682	
4	*hrpX**	*E. amylovora*	Type III secretion HrpX	AAD24681	
5	*hopPtoC*	*Pseudomonas syringae pv. tomato*	Type III effector HopPtoC	NP_790436	Pst, (24)
General secretion pathway (GSP)					
6	*secA**	*Salmonella typhimurium*	Preprotein translocase SecA	NP_459141	
7	*secDF**	*Yersinia pestis*	Protein-export membrane protein SecDF	NP_406663	Apl, (150)
8	*secB**	*Escherichia coli*	Protein-export protein SecB	NP_756294	
Type II secretion (T2SS) and proteins secreted by T2SS					
9	*yts1IJ*	*Yersinia enterocolitica*	Type II secretion Yts1IJ protein	CAC83035	Rso,Ech; (36, 261)
10	*lsc**	*E. amylovora*	Levansucrase	CAA52972	
11	*peh*	*Pectobacterium carotovorum*	Peh (Polygalacturonase)	BAA74431	Pst, (24)
Proteins known to be secreted by other secretion systems					
12	*fhaB**	*Escherichia coli*	Putative adhesin/hemagglutinin/hemolysin	AAQ19127	Rso, (36)
13	*hecA*	*Erwinia chrysanthemi*	Hemolysin/hemagglutinin-like protein HecA	AAN38708	Rso, (36)
14	*mltE*	*Yersinia pestis*	Membrane-bound lytic murein transglycosylase	NP_405972	Pst, (24)
15	*prtA**	*Erwinia amylovora*	Zinc-binding metalloprotease	CAB42873	
General, ion, sugar, amino acid, nucleotide transport					
16	*abc*	*Yersinia pestis*	ABC transporter ATP-binding protein (Inorganic ion transport)	NP_404687	Ech, (261)
17	*pstA*	*Yersinia pestis*	Putative phosphate transport system permease	NP_406342	
18	*ppx*	*Pantoea agglomerans*	Exopolyphosphatase	AAQ14878	Sty, (171)
19	*ftn*	*Yersinia pestis*	Cytoplasmic ferritin (an iron storage protein)	NP_669829	
20	*kefB*	*Salmonella typhimurium*	K^+/H^+ antiporter	NP_462361	Rso, (36)

TABLE 2 (con't)

pfi no.	*Gene*[b]	*Organism*	Blastx_BestHit[c]	GenBank Acc. no.	Previously reported[d]
21	*c3774**	*Escherichia coli*	Ferric enterobactin transport ATP-binding protein	NP_755645	
22	*btuB*	*Serratia marcescens*	Outer membrane receptor for iron transport	AAL50647	
23	*Gmet1925*	*Geobacter metallireducens*	ABC-type nitrate/sulfonate/bicarbonate transport system	ZP_00081179	
24	*exbB*	*Salmonella enterica*	Biopolymer transport ExbB protein	NP_457552	
25	*kgtP**	*Salmonella typhimurium*	Alpha-ketoglutarate permease	NP_461589	Rso, (36)
26	*ECs2608*	*Escherichia coli*	ATP-binding component of high-affinity L-arabinose transport system	NP_310635	
27	*citA*	*Pseudomonas aeruginosa*	Citrate transporter	NP_254163	
28	*rbsD*	*Yersinia pestis*	High-affinity D-ribose permease	NP_403673	
29	*gntU*	*Salmonella typhimurium*	Low-affinity gluconate permease	NP_462442	
30	*mglA**	*Salmonella typhimurium*	methyl-galactoside transport protein	NP_461134	
31	*emrB*	*Escherichia coli*	Multidrug resistance protein B	NP_755121	Rso/Eco; (36, 261)
32	*mdl*	*Klebsiella pneumoniae*	Multidrug resistance-like ATP-binding protein	CAA07091	
33	*putP**	*Salmonella sp.*	Proline permease	AAA99282	
34	*Avin4550**	*Azotobacter vinelandii*	Permeases of the major facilitator superfamily	ZP_00092807	Pfl, (230)
35	*rbsB*	*Streptococcus agalactiae*	Ribose ABC transporter, periplasmic D-ribose-binding protein	NP_687150	
36	*livK*	*Yersinia pestis*	High-affinity leucine-specific-binding periplasmic protein;branched-chain amino acid ABC transport system	NP_667760	
37	*ECs3191*	*Escherichia coli*	Histidine transport system membrane protein M	NP_311218	
38	*lysP*	*Shigella flexneri*	Lysine-specific permease	NP_708053	
39	*YPO3257**	*Yersinia pestis*	Amino acid ABC transporter, periplasmic protein	NP_406727	Rso, (36)
40	*metC**	*E. coli*	Beta-cystathionase	AAA69175	
41	*lysC*	*Yersinia pestis*	Lysine-sensitive aspartokinase III	NP_407170	
42	*avtA**	*Escherichia coli*	Valine--pyruvate aminotransferase	NP_756255	

TABLE 2 (con't)

pfi no.	*Gene*[b]	*Organism*	Blastx_BestHit[c]	GenBank Acc. no.	Previously reported[d]
43	*oppA*	*Yersinia pestis*	Oligopeptide ABC transporter; periplasmic binding protein	NP_669341	
44	*oppC*	*Yersinia pestis*	Oligopeptide transport system permease protein	NP_405727	
45	*tsx*	*Salmonella*	Nucleoside-specific channel-forming protein	NP_455008	
46	*hemH*	*Yersinia enterocolitica*	Ferrochelatase	AAC60760	
47	*uup*	*Salmonella typhimurium*	Putative ABC transporter ATPase component	NP_460036	Rso, (36)
48	*sbmA*	*Salmonella typhimurium*	Putative ABC transporter membrane protein	NP_459371	Rso, (36)
49	*y0619**	*Yersinia pestis*	Putative periplasmic binding transport protein	AAM84207	
50	*hemT*	*Yersinia enterocolitica*	Hemin binding protein (periplasm binding protein -haemin transport)	CAA54866	
51	*yadH**	*Salmonella typhimurium*	Putative transport protein	NP_459178	
Bacterial cell surface and transmembrane					
52	*nlpA**	*Escherichia coli*	Lipoprotein-28	NP_418117	Rso, (36)
53	*dacB*	*Yersinia pestis*	D-alanyl-D-alanine carboxypeptidase/penicillin-binding protein 4	NP_668015	Rso, (36)
54	*kpsC*	*Escherichia coli*	Capsule polysaccharide export protein	P42217	Eco, (232)
55	*dinF*	*Salmonella enterica*	DNA-damage-inducible membrane protein	NP_458536	Rso, (36)
56	*corB**	*Salmonella enterica*	Putative membrane protein	NP_457149	Ech, (261)
57	*ytfK**	*Salmonella typhimurium*	Putative cytoplasmic protein	NP_463267	Ech, (261)
58	*YPO2305*	*Yersinia pestis*	Putative exported protein	NP_405842	
59	*tolA*	*Salmonella typhimurium*	Tol import system inner membrane protein	NP_459732	
60	*tolR*	*Erwinia chrysanthemi*	TolR protein inner membrane protein interacting with TolA and TolQ	CAC82707	
61	*matE**	*Salmonella enterica*	Putative inner membrane protein	NP_456567	Rso, (36)
62	*STM0278**	*Salmonella typhimurium*	Putative periplasmic protein	NP_459276	Rso, (36)
63	*ydiY*	*Salmonella typhimurium*	Putative outer membrane protein (salt inducible)	NP_460293	
64	*ompC*	*Escherichia coli*	Outer membrane protein C (Porin OmpC)	Q54471	
65	*ompA**	*Salmonella enterica*	Putative outer membrane protein (OmpA)	NP_458280	

TABLE 2 (con't)

pfi no.	*Gene*[b]	*Organism*	Blastx_BestHit[c]	GenBank Acc. no.	Previously reported[d]
66	*Z1931**	*Escherichia coli*	Outer membrane protein 3b (a), protease VII	NP_287408	
67	*ptrA**	*Yersinia pestis*	Protease III precursor	NP_404633	
68	*mrdB*	*Escherichia coli*	Rod shape-determining protein RodA	NP_752655	
69	*flgN*	*Shigella flexneri*	Flagella synthesis protein FlgN	NP_836779	
70	*fliG*	*Shigella flexneri*	Flagellar motor switch protein FliG	NP_707824	
71	*fliM*	*Escherichia coli*	Flagellar motor switch protein FliM	NP_754254	
72	*ycgB*	*Escherichia coli*	Putative sporulation protein	NP_287427	
Mobile elements - phage					
73	*STY4666**	*Salmonella enterica*	Probable phage integrase	NP_458745	
74	*intD*	*Escherichia coli*	Prophage DLP12 integrase	NP_415069	
Stress response					
75	*mutS*	*Yersinia pestis*	DNA mismatch repair protein MutS	NP_406817	Yen, (112)
76	*recA*	*Yersinia pestis*	RecA protein	NP_406773	Sau, (150)
77	*sulA*	*Enterobacter aerogenes*	Cell division inhibitor, SOS regulon gene	C29016	
78	*dps*	*Serratia marcescens*	DNA protection during starvation protein (Dsp)	AAO47741	
79	*cstA*	*Salmonella enterica*	Carbon starvation protein	NP_458955	
80	*ycbP**	*Escherichia coli*	Sulfate starvation-induced protein 4 /FMN reductase	NP_753005	
81	*ahpC**	*Photorhabdus luminescens*	Alkyl hydroperoxide reductase, small subunit (antioxidant)	NP_931108	
82	*cyoB**	*Escherichia coli*	Cytochrome o ubiquinol oxidase subunit I	NP_286173	
83	*ydiJ*	*Salmonella typhimurium*	Putative oxidase	NP_460330	
84	*bacA*	*Salmonella typhimurium*	Bacitracin resistance protein	NP_462120	Ech, (261)
85	*pqiB*	*Yersinia pestis*	Paraquat-inducible protein B	NP_670048	
86	*grpE*	*Photorhabdus luminescens*	GrpE protein (HSP-70 cofactor) (heat shock protein B25.3)	NP_930590	Rso, (36)
87	*y1165*	*Yersinia pestis*	Putative cold-shock protein	NP_668491	
88	*pspB**	*Yersinia enterocolitica*	Phage shock protein B	AAG22114	

TABLE 2 (con't)

pfi no.	*Gene*[b]	*Organism*	Blastx_BestHit[c]	GenBank Acc. no.	Previously reported[d]
89	*pspC*	*Yersinia enterocolitica*	Phage shock protein C	AAG22115	
90	*tsr**	*Salmonella typhimurium*	Methyl-accepting chemotaxis protein I	NP_463392	Ech, (261)
Regulation					
91	*rlsA*	*Erwinia amylovora*	RlsA protein/ lysR homolog	CAA10420	
92	*rcsA**	*Erwinia amylovora*	Colanic acid capsular biosynthesis activation protein A	A45828	
93	*grrS**	*Serratia plymuthica*	Putative global response regulation sensor kinase	AAL11449	
94	*envZ**	*Yersinia pestis*	Histidine kinase/EnvZ/osmolarity sensor protein	NP_403793	Sty, (101)
95	*fliZ*	*Yersinia pestis*	Putative alternative sigma factor regulatory protein	NP_405408	
96	*gntR*	*Salmonella enterica*	gluconate utilization operon repressor	NP_458376	
97	*deoR*	*Salmonella enterica*	DeoR /deoxyribose operon repressor	NP_455391	
98	*metJ*	*Photorhabdus luminescens*	Met repressor (Met regulon regulatory protein MetJ)/repressor of the methionine regulon	NP_931917	
99	*hlfK*	*Shigella flexneri*	Protease specific for phage lambda cII repressor	NP_710039	
100	*yqhC**	*Escherichia coli*	Putative AraC-type regulatory protein	NP_289587	
101	*ybiQ*	*Shigella flexneri*	Transcriptional regulator mntR	NP_706694	
102	*budR*	*Raoultella terrigena*	HTH-type transcriptional regulator	P52666	
103	*c5058**	*Escherichia coli*	Putative transcriptional regulator LysR	NP_756910	Ech, (261)
104	*PSPTO3576*	*Pseudomonas syringae pv. tomato*	TetR family transcriptional regulator	NP_793355	
105	*YPO3913**	*Yersinia pestis*	TetR-family transcriptional regulatory protein	NP_407358	
106	*YPO0736*	*Yersinia pestis*	Putative regulatory protein	NP_404367	
107	*YPO0315**	*Yersinia pestis*	Regulatory protein (multiple antibiotics resistance)	NP_403966	
108	*fnr*	*Escherichia coli*	Fumarate and nitrate reduction regulatory protein	NP_753709	
109	*glnK*	*Yersinia pestis*	Nitrogen regulatory protein P-II	NP_406618	

TABLE 2 (con't)

pfi no.	*Gene*[b]	*Organism*	Blastx_BestHit[c]	GenBank Acc. no.	Previously reported[d]
110	*modE*	*Escherichia coli*	Molybdate uptake regulatory protein	NP_286482	
111	*pspF*	*Salmonella enterica*	Psp operon transcriptional activator	NP_455812	
112	*hlyB*	*Yersinia pestis*	Putative hemolysin activator protein	NP_406025	
113	*rbsR**	*Shigella flexneri*	Regulator for rbs operon	NP_839112	
114	*Psyr0512*	*Pseudomonas syringae*	Rhs family protein	ZP_00124230	
115	*STY4601**	*Salmonella enteric*	Putative regulator of late gene expression	NP_458684	
116	*kdpE*	*Salmonella typhimurium*	Response regulator in two-component regulatory system with KdpD	AAL19646	
117	*PA2177*	*Pseudomonas aeruginosa*	Probable sensor/response regulator hybrid	NP_250867	
118	*y3531**	*Yersinia pestis*	Putative kinase	NP_670828	
119	*YPO0014*	*Yersinia pestis*	Putative type II homoserine kinase/YihE	NP_403681	
120	*Avin4532*	*Azotobacter vinelandii*	Serine/threonine protein kinase	ZP_00092789	
121	*Bcep3208*	*Burkholderia fungorum*	Sugar kinases, ribokinase family	ZP_00030390	
122	*NE2503*	*Nitrosomonas europaea*	TonB-dependent receptor protein	NP_842492	
123	*hrpA*	*Escherichia coli*	ATP-dependent helicase hrpA	P43329	Rso, (36)
124	*dbpA**	*Yersinia pestis*	ATP-dependent RNA helicase	NP_405343	
125	*rpoN*	*Klebsiella pneumoniae*	RNA polymerase sigma-54 factor	P06223	Rso, (36)
126	*rpoD*	*Pantoea agglomerans*	RNA polymerase sigma-70 subunit	AAL11450	
Metabolism and nutrient scavenging					
127	*ilvI**	*Yersinia pestis*	Acetolactate synthase III	NP_670937	Pst, (24)
128	*purF*	*Escherichia coli*	Amidophosphoribosyltransferase	NP_288886	Rso, (36)
129	*argG**	*Photorhabdus luminescens*	Argininosuccinate synthase	NP_931904	Rso, (36)
130	*pheA*	*Erwinia herbicola*	Chorismate mutase / prephenate dehydratase	S26053	Ech, (261)
131	*hemB*	*Salmonella enterica*	delta-aminolevulinic acid dehydratase	NP_454967	Rso, (36)
132	*glgX*	*Escherichia coli*	Glucose-1-phosphate adenylyltransferase	NP_756082	Rso, (36)
133	*murB**	*Escherichia coli*	Oxidoreductase /UDP-N-acetylpyruvoylglucosamine reductase	AAA24185	Rso/Pst; (24, 36)

TABLE 2 (con't)

pfi no.	*Gene*[b]	*Organism*	Blastx_BestHit[c]	GenBank Acc. no.	Previously reported[d]
134	*pksC**	*Mycobacterium leprae*	Polyketide synthase PksC	S73013	Rso, (36)
135	*YPO2195*	*Yersinia pestis*	Putative acyl-CoA thioester hydrolase	NP_405738	Pst, (24)
136	*ygfZ*	*Salmonella typhimurium*	Putative aminomethyltransferase	NP_461964	Pst, (24)
137	*YPO2310*	*Yersinia pestis*	Putative carboxypeptidase	NP_405847	Pst, (24)
138	*truD*	*Yersinia pestis*	Putative hydrogenase subunit	AAM84415	Pst, (24)
139	*aceE*	*Salmonella enterica*	Pyruvate dehydrogenase E1 component	NP_454766	Pst, (24)
140	*rhsD*	*Yersinia pestis*	RhsD protein	NP_667608	
141	*hemD*	*Salmonella typhimurium*	Uroporphyrinogen III synthase	NP_462823	
142	*pyrG**	*Salmonella enterica*	CTP synthase (UTP--ammonia ligase)	NP_457342	Rso, (36)
143	*dnaQ**	*Escherichia coli*	DNA polymerase III, epsilon chain	NP_752198	Rso, (36)
144	*argA*	*Yersinia pestis*	Amino-acid acetyltransferase	NP_404636	Vch, (160)
145	*pepN**	*Salmonella typhimurium*	Aminopeptidase N	NP_460031	Rso, (36)
146	*glnA**	*Yersinia pestis*	Glutamine synthetase	NP_671098	Rso/Eco, (36, 261)
147	*cheR*	*Yersinia pestis*	Glutamate methyltransferase	NP_669155	
148	*moaC**	*Salmonella enterica*	Molybdenum cofactor biosynthesis protein C	NP_455345	Pfl, (230)
149	*moeC*	*Yersinia pestis*	Molybdopterin biosynthesis protein	NP_669976	
150	*YPO2420**	*Yersinia pestis*	Probable formyl transferase	NP_405953	
151	*YPO2174**	*Yersinia pestis*	Putative nucleotide sugar dehydrogenase	NP_405718	
152	*YPO1009*	*Yersinia pestis*	Probable peptidase (PepT)	NP_286807	
153	*fbp*	*Escherichia coli*	Fructose-1,6-bisphosphatase	NP_757176	
154	*gnd*	*Yersinia pestis*	Gluconate-6-phosphate dehydrogenase	NP_669932	
155	*zwf*	*Yersinia pestis*	Glucose-6-phosphate dehydrogenase	NP_405618	
156	*pckA*	*Salmonella typhimurium*	Phosphoenolpyruvate carboxykinase	NP_462403	
157	*ECs0188*	*Escherichia coli*	Lysine decarboxylase 2	NP_308215	

[a]Hypothetical and unknown genes were not listed.

[b]Gene name designations were based on originally-reported gene products that shared high similarity to *pfi* clone sequences. Asterisks after the gene name indicated that two or more clones were identified for the same gene in the experiment.

[c]Predicted proteins or functions based on similar proteins identified using BlastX searches.

[d]Genes identified to be induced in other bacteria-plant or animal systems using similar IVET screen or other *in vivo* expression systems. Rso: *Ralstonia solanacearum*; Ech: *Erwinia chrysanthemi*; Pst: *Pseudomonas syringae* pv. *tomato*; Pfl: *Pseudomonas fluorescens*; Sen: *Salmonella enterica*; Sty: *Salmonella typhimurium*; Sau: *Staphylococcus aureus*, Apl: *Actinobacillus pleuropneumoniae*, Vch: *Vibrio cholerae,* Eco: *Escherichia coli*. Number indicated the reference.

Quantitative expression analysis of selected pear up-regulated genes

To verify that the pear-upregulated gene promoters identified using the qualitative IVET assay are induced in pear, quantitative GUS activity for six strains containing *pfi* promoter constructs and for promoter constructs containing the *dspE* promoter (in both directions) was monitored 24 and 48 hours after infection of immature pear. The positive control $dspE^{for}$ promoter in pZYF8 was highly induced in pear after infection at both 24 and 48 hr post inoculation (Table 3), whereas the negative control $dspE^{rev}$ promoter in pZYF2 showed very low GUS expression (Table 3). Most of the *pfi* clones tested showed varying degrees of induction of promoter activity at 24 h and 48 h after infection of immature pear (Table 3). The *pfi* 43 clone (*oppA*) was found to be highly induced at both 24 and 48 h after inoculation; whereas *pfi* 5 ($hopPtoC_{EA}$), *pfi* 9 (*yts1IJ*), *pfi* 91 (*rlsA*), and *pfi* 93 (*grrS*) were only induced at 48 h post inoculation. The clone containing hypothetical protein (*sav2932*), on the other hand, was strongly induced at 24 h post inoculation with expression tailing off at 48 h (Table 3).

TABLE 3. Expression of IVET clones after inoculation of immature pear fruit

pfi clone or strain	Gene homolog	0 hpi[a]	24 hpi[a]		48 hpi[a]	
		GUS activity[b]	GUS activity[b]	Fold induction[c]	GUS activity[b]	Fold induction[c]
pZYF2	*dspE* [rev d]	u.l.[f]	u.l.[f]	0	u.l.[f]	0
pZYF8	*dspE* [for e]	1.0 ± 2.1	282.0 ± 145.5	282.0	118.3 ± 18.4	118.3
pfi5	*hopPtoC*	10.8 ± 6.7	11.8 ± 4.4	1.1	90.1 ± 43.3	8.4
pfi9	*ytslIJ*	37.1 ± 2.0	74.7 ± 34.9	2.0	128.0 ± 48.8	3.5
pfi43	*oppA*	1.0 ± 0.9	91.4 ± 27.4	91.4	138.9 ± 25.2	138.9
pfi91	*rlsA*	1.0 ± 1.3	u.l.[f]	0	19.2 ± 12.6	19.2
pfi93	*grrS*	77.7 ± 2.3	30.2 ± 7.0	0.4	106.3 ± 35.8	2.4
	sav2932[g]	107.4 ± 3.5	852.2 ± 380.3	7.9	86.2 ± 40.4	0.8

[a] Hours post inoculation (hpi).

[b] GUS activity is shown in μmol of 4-methylumbelliferyl produced min^{-1} 10^9 CFU^{-1}. Data represented the mean of three measurements ± standard error. Similar results were obtained in a second independent experiment.

[c] Fold induction is shown as GUS activity at 24 or 48 hpi/GUS activity at 0 hpi.

[d] *dspE* promoter in opposite direction of *uidA* gene.

[e] *dspE* promoter in correct direction of *uidA* gene.

[f] u.l.: Under detection limit of vector control.

[g] *sav2932* encodes a hypothetical protein and is not listed in Table 2.

Construction and analysis of knockout mutants

Although the IVET experiments were conducted in the *E. amylovora* Ea110 background and an Ea110 *hrpA* mutant was available, we were unsuccessful in subsequent attempts to construct chromosomal knockout mutants in this strain. Thus we utilized the strain Ea1189 for mutant construction. Although the similarity of genetic backgrounds of these strains is currently unknown, the virulence of the two strains is similar (data not shown) and the overall genome diversity of *E. amylovora* is relatively low and therefore, we hypothesize that the expression of promoters identified as upregulated in Ea110 would be comparable to that in Ea1189. We chose the $hopPtoC_{EA}$ and $mltE_{EA}$ genes as candidates for insertional mutagenesis using allele marker exchange

to investigate the potential roles of those genes in virulence. The full sequences of these genes and their corresponding upstream and downstream sequences were obtained by various methods including fully sequencing available clone inserts or by recovering additional flanking DNA sequences using thermal asymmetric interlaced (TAIL-) PCR. Sequence analysis showed that the deduced amino acid sequence of the *hopPtoC*$_{EA}$ gene shared 77% similarity with that of *hopPtoC*$_{PST}$ gene from *P. syringae* pv. *tomato* (data not shown). The deduced amino acid sequence of *E. amylovora mltE*$_{EA}$ gene showed 75% similarity with that of *mltE*$_{YP}$ gene from *Yersinia pestis* (data not shown). As described in Materials and Methods, we were able to generate insertional mutants for *hopPtoC*$_{EA}$ and *mltE*$_{EA}$ genes and tested the effect of these mutations on pathogenesis and bacterial growth in immature pear.

The *hopPtoC*$_{EA}$ mutant ZYC1-3 was fully virulent like the WT Ea1189 strain upon infection of immature pear (Figure 3A). The symptoms caused by the *hopPtoC*$_{EA}$ mutant also progressed similarly to the wild type strain and caused typical symptoms, i. e. necrotic lesions and the production of bacterial ooze (Figure 3A). In addition, we were unable to detect significant difference in bacterial growth upon infection of immature pear for the *hopPtoC*$_{EA}$ mutant (Figure 3B). In contrast, the *mltE*$_{EA}$ knockout mutant (ZYE3-11) was slightly reduced in virulence and bacterial growth (Figure 3A and 3B). Although the mutant caused similar symptoms as the wild type strain, symptom progression was slightly reduced (Figure 3A). There was no difference for bacterial growth of the *mltE*$_{EA}$ mutant compared to that of the wild type strain two days after inoculation, however, cell counts of the *mltE*$_{EA}$ mutant were 5 to 10 fold less than that of the WT strain at three and four days after inoculation (Figure 3B).

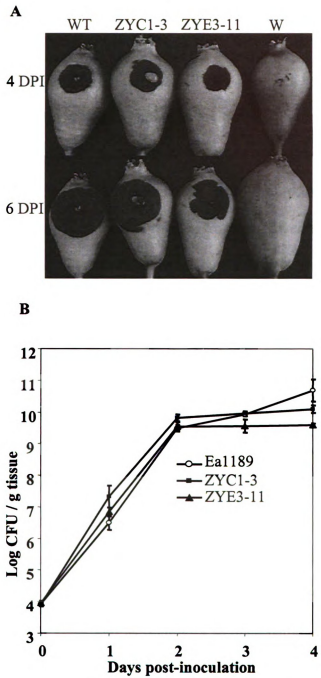


Figure 3. Symptoms and growth of *Erwinia amylovora* WT Ea1189 and corresponding *hopPtoC*$_{EA}$ and *mltE*$_{EA}$ mutants in immature pear. **(A)** Symptoms caused by *Erwinia amylovora* Ea1189 and *hopPtoC*$_{EA}$ (ZYC1-3) and *mltE*$_{EA}$ (ZYE3-11) mutants in immature pear. WT: Wild type strain; W: water control. DPI: days post inoculation. **(B)** Bacterial growth of *Erwinia amylovora* WT Ea1189, and *hopPtoC*$_{EA}$ (ZYC1-3) and *mltE*$_{EA}$ (ZYE3-11) mutants during infection of immature pears. The growth of strains was monitored at 0, 1, 2, 3, 4 days after inoculation. Data points represent the means of three replicates ± SE. Similar results were obtained in a second independent experiment.

DISCUSSION

We utilized a simplified qualitative IVET approach to scan the *E. amylovora* genome and recovered 394 unique chromosomal genes with increased expression during infection of pear fruit tissue. As expected, this study highlighted the importance of type III secretion in *E. amylovora* pathogenesis with the recovery of genes encoding regulatory and structural components of the Hrp type III secretion system and effector proteins. While we did not recover all of the currently known *hrp*-regulated genes in *E. amylovora*, our results are similar to those of other IVET studies with plant pathogenic bacteria. For example, IVET studies of *E. chrysanthemi* and *P. syringae* pv. tomato identified two and eight *hrp*-regulated genes, respectively (24, 261). These findings validated our approach and suggested that a detailed analysis of the genes recovered in this study would further reveal additional determinants involved in the pathogenesis of the fire blight bacterium.

The *dspEF* operon, encoding the major effector and pathogenicity factor DspE and its cognate chaperone DspF, was recovered multiple times in our analysis and shown by quantitative expression analysis to be highly expressed during pear infection (Table 3). The importance of DspE and its homologs to plant pathogenesis is well known in a number of pathosystems (26, 77, 143, 170, 243) although the elucidation of the function(s) of this large protein remains unknown. DspE was recently shown to contribute to the suppression of salicylic-acid-mediated basal immunity (57); effector suppression of the host defense response is rapidly becoming recognized as an important strategy of bacterial plant pathogenesis (5). We identified a new putative effector

HopPtoC$_{EA}$ in this study, an ortholog of HopPtoC from *P. syringae* pv. *tomato* (224). As with many effectors from *P. syringae*, a knockout mutant of HopPtoC$_{EA}$ in *E. amylovora* Ea1189 was not reduced in virulence presumably due to functional redundancy with other effectors in the *E. amylovora* genome. The other known *E. amylovora* effectors HrpN and HrpW were not identified as upregulated in this study; although the roles of *hrpN* and *hrpW* in the pathogenicity of *E. amylovora* were reported to differ during infection of immature pear fruit (126, 252). It is tempting to speculate that additional effector proteins may exist in the genome of *E. amylovora* and contribute to the virulence of the bacterium.

The importance of type II secretion in *E. amylovora* pathogenesis was also highlighted with the identification of the upregulation of genes of the *yts1IJ* operon and components of the general secretion pathway. Type II secretion is a cooperative process initially dependent upon the secretion of enzymes into the periplasm by the general secretion pathway followed by targeted secretion through the type II apparatus (9, 220). In *Y. enterocolitica*, the Yts1 protein secretion apparatus is unique to highly pathogenic species, is important for virulence in a mouse model, and shares homology with type II secretion clusters from *E. chrysanthemi* and *E. carotovora* (17, 112). Peh (polygalacturonase), an enzyme thought to be secreted by the T2SS, was also upregulated and recovered in our IVET screen (122). While the importance of polygalacturonase to virulence in soft-rotting *Erwinia* spp. is well known (122), the role of cell wall degrading enzymes in *E. amylovora* pathogenesis is currently still unknown. In addition, the upregulation of MltE, a specialized cell-wall-degrading enzyme was interesting in that the function of this enzyme is to generate localized openings of the bacterial

peptidoglycan envelope for export of bulky materials including possibly toxins and fimbrial proteins and to allow the efficient assembly and anchoring of supramolecular transport complexes such as T2SS and TTSS in the cell envelope (63, 130). As in *P. syringae* (24), we found that *E. amylovora* MltE made a small contribution to virulence.

We identified three additional upregulated enzymes in our IVET assay that are potentially secreted from the cell including levansucrase Lsc and the adhesin-like protein HecA which belongs to a class of external virulence factors that is widely distributed among plant and animal pathogens. HecA from *E. chrysanthemi* contributes to attachment, aggregation, and epidermal cell killing and is thought to be involved in the earliest stages of *E. chrysanthemi* pathogenesis (212). Levansucrase, an enzyme that directs the synthesis of levan from sucrose, has a known effect on the virulence of *E. amylovora* during pear seedling infection (85). The PrtA metalloprotease contributes to *E. amylovora* virulence in an apple leaf infection assay and is apparently dependent upon the type I Prt machinery for secretion (122, 265). These results demonstrate the importance of TTSS, T2SS and of other external virulence factors in *E. amylovora* infection of fruit tissue.

A total of 5.3% of the IVET genes identified were placed in the functional category of stress response including genes involved in the response to reactive oxygen species, both heat and cold shock, and carbon and sulfate starvation. *E. amylovora* apparently induces an initial host defense response early after infection (247, 248); the bacterium is capable of surviving this plant oxidative burst with the initial plant cell death and nutrient leakage thought to provide the impetus for further spreading of the pathogen within the plant. The role of individual proteins in oxidative stress survival is currently

unknown in *E. amylovora*, however, the alkyl hydroperoxide reductase AhpC is a known virulence factor in several plant pathogenic bacteria contributing to protection from oxidative stress from an active plant defense response (169).

We recovered a multitude of transporters functioning in the uptake of iron, sugars, amino acids, and inorganic ions. The induction of these systems during infection indicates that *E. amylovora* elaborates various factors as needed to colonize host tissues. Iron availability is critical to most bacterial pathogens and the siderophore desferrioxamine is a virulence factor in *E. amylovora* (73). We recovered three upregulated proteins involved in iron transport or storage. It is probable that under unfavorable conditions, the bacterium itself adjusts and overcomes nutrient and iron deficiencies. Several upregulated transport proteins recovered were ABC transporters which is potentially significant because ABC transporters both directly and indirectly affect virulence of bacterial pathogens (80). While most of the transporters were involved in uptake, the multidrug resistance proteins EmrB (*pfi* 31) was also upregulated and presumably functions in the efflux of plant-derived toxins encountered during infection. The role of the AcrAB efflux pump in *E. amylovora* virulence and tolerance of phytoalexins including phloretin, naringenin, and quercetin was recently reported (38). Thus, it is possible that many of these ABC transporters are involved in the virulence of *E. amylovora*. In conjunction with the number of upregulated transporters found, a large proportion of the genes identified in this study were involved in metabolism (20.3%) and nutrient acquisition (16%). These frequencies may be associated with the host tissue (immature pear fruit) chosen for study; however, a number of genes we identified were

also identified in other IVET studies involving *E. coli, P. fluorescens, P. syringae, R. solanacearum*, and *V. cholerae* (Table 2; 24, 36, 150, 261).

About 12% of the genes identified in this study were involved in regulation, which is a similar ratio to that identified in an IVET examination of *E. chrysanthemi* infection (261). Previously-known *E. amylovora* transcription factors that were upregulated included RcsA, an activator (along with RcsB) of amylovoran production (251) and RlsA, an activator of levan production (264) along with the capsular polysaccharide export protein KpsC. This further confirms that the production of both amylovoran and levan in *E. amylovora* is induced during infection. Another important regulator, GrrS (global response regulator sensor in a two-component regulatory system), is a homolog of GacS which, along with GacA, globally regulate a network that controls exoenzyme and secondary metabolite (toxin) production in *Pseudomonas* spp., virulence functions in *E. carotovora*, and also regulates the production of quorum-sensing signalling molecules (45, 72, 203). GacA/GacS-regulated networks also function by positively controlling the transcription of small regulatory RNAs, transcriptional activators, and alternative sigma factors such as HrpL (45, 100). In *E. amylovora*, the small regulatory RNA *rsmB* titrates the repressor RsmA in a system that affects exopolysaccharide production and therefore, pathogenicity (147).

EnvZ is the sensor component of the OmpR/EnvZ two component regulatory system that is very important in regulating various cellular components such as outer memberane proteins OmpC and OmpA, which is also upregulated in this study. In *Salmonella* spp., OmpR-EnvZ regulates another two component system SsrA-SsrB that in turn regulates the type III secretion system produced by *Salmonella* pathogenicity island

2 (Spi-2; 133). EnvZ is a transmembrane sensor that predominantly responds to acidic pH conditions and subsequently phosphorylates OmpR which functions as a transcriptional activator in the expression of the *ssrAB* genes (79). SsrA is second sensor protein that is responsive to acidic pH and also detects low osmolarity conditions and the absence of Ca^{2+} ions, all environmental conditions within macrophages where the Spi-2 type III secretion system is exclusively expressed (79). In *E. amylovora*, the structural components of the TTSS encoded by the Hrp regulon are regulated by the two component system HrpX and HrpY, which direct the expression of the σ^{54}-dependent, enhancer-binding protein HrpS (251). Both HrpY and HrpS function in activating the expression of the alternate sigma factor HrpL, thereby regulating the various genes and operons of the Hrp regulon which contains HrpL-dependent promoter sequences (251). The expression of HrpX and HrpS is regulated by low pH, low nutrients, and low temperature conditions, mimicking the plant apoplast, but also representing conditions that suggest a two component regulatory system such as OmpR-EnvZ could further regulate the *hrpXY* operon despite no direct evidence to support this claim. Interestingly, both *hrpX* and *hrpL*, along with EnvZ were found to be upregulated during infection of immature pear in this study (Table 2).

Among the bacterial cell surface and transmembrane upregulated proteins, three flagellar proteins FliG, FliM, and FlgN were upregulated. The trait of motility is not required for *E. amylovora* pathogenesis, however, motility does increase blossom infectivity, particularly at lower cell concentrations (16). Furthermore, a homolog of *Y. pestis* FliZ, a positive regulator for the flagellar biosynthetic operon and an alternative sigma factor, was also found to be upregulated in our study. In *Salmonella enterica*

serovar Typhimurium, FliZ upregulates HilA, which in turn activates production of several invasion proteins encoded within the *Salmonella* pathogenicity island 1 (113). Finally, the contribution of cell shape to virulence was also highlighted by the recovery of an *E. coli* RodA homolog; *E. amylovora* mutants with TnPhoA insertions within the *rodA-pbpA* operon were previously reported to be avirulent (165).

In summary, our IVET screen successfully identified a variety of genes upregulated during fruit infection by *E. amylovora*. We utilized a modified IVET method in this study which is different from many other IVET studies in that we did not impose a rigorous selection step, i.e. one that necessitates rescue of an essential phenotype, in our gene identification work. An advantage of our approach is that we screened clones individually which would remove potential competition among fusion clones in vivo if inoculated as a pool. The successful identification of a large number of known virulence genes of *E. amylovora* in this study further validated our approach. However, because of the qualitative nature of the gene identification step, through β-glucoronidase staining and visualization of gene expression on pear slices and agar medium, it is possible that this methodology may have resulted in some artifacts. Nevertheless, the main goal of this work, as in other IVET analyses, was to identify potentially important genes in the *E. amylovora* infection process that could be subjected to further detailed studies to clearly delineate the role of these genes in pathogenesis.

We further confirmed that the TTSS and its major effector protein DspE are essential for full virulence in *E. amylovora* during infection of immature pear. We also found a complete and functional T2SS and its potential secreted proteins in *E. amylovora* for the first time. We identified a new putative effector, external virulence factors such as HecA which were previously unknown in *E. amylovora*, and discovered a number of putative regulatory proteins that may influence the regulation of virulence factors on a global level and eventually contribute to the virulence of the bacterium. We can now ask questions concerning the comparative regulation of critical genes identified in this study during infection of other host tissues, particularly blossoms and shoots. It is possible that *E. amylovora* the pathogen may utilize differential virulence strategies depending upon the host tissue encountered. Of interest to us also is the expression profile of these same genes during infection of highly susceptible versus fire blight-tolerant apple varieties.

CHAPTER 2

Taking a page from macerogenic bacteria: Functional characterization of an *Erwinia amylovora* endo-polygalacturonase and its effect on virulence

ABSTRACT

In a previous *in vivo* expression technology (IVET) screen of induced *Erwinia amylovora* genes during infection (Chapter 1), two genes associated with type II secretion (T2SS), the pseudopilins *outIJ* and an endo-polygalacturonase *peh* were identified. Here I describe the contribution of T2SS to virulence, using deletion mutants of $outD_{EA}$ and *peh*. In particular, *E. amylovora peh* was reduced in virulence during apple leaf inoculations when cells were not introduced into the main vascular tissue of leaves. The activity of both the T2SS operon promoter and *peh* promoters were also determined under differing conditions using flourometric methods. Additionally, the *peh* gene was confirmed to encode a protein with polygalacturonase activity. His-tagged polygalactuonase was present in the supernatant of *E. amylovora*, demonstrating secretion to the extracellular milleu. Overall, this is the first report of a polygalacturonase enzyme influencing virulence of *E. amyolvora*.

INTRODUCTION

Erwinia amylovora is a Gram-negative phytopathogenic bacterium that infects members of the Rosaceae family. This necrogenic pathogen causes tissue necrosis and additional symptoms including water soaking, wilt, and cankers. Infection can occur either by entry through nectarthodes in the base of flowers, or through wounds on young, actively growing tissue. Entry into the plant via wounding is followed by multiplication in the cortical parenchyma, and the expression of disease promoting genes (246). Ultrastructural analyses have shown bacteria-dependent plasmolysis by 24 hours post infection, of xylem parenchyma. As the infection progressed, the extensive plasmolysis appeared to lower the turgor pressure around xylem vessels resulting in the bursting of cells (90). Later studies have demonstrated that infection and symptom development depends on the secretion of extracellular proteins from *E. amylovora*.

Major pathogenicity factors of *E. amylovora* include a type III secretion system (T3SS), the type III effector DspA/E, and the exopolysaccharide amylovoran (11, 18, 26, 27, 82, 127, 239). Secretion and translocation of DspE and an additional effector HrpN through the T3SS causes tissue collapse in susceptible hosts, and a hypersensitive response in resistant hosts and nonhosts (246). Amylovoran is hypothesized to protect *E. amylovora* from the oxidative burst in host tissues and also to mask the presence of *E. amylovora* from its host (128).

Virulence factors also contribute to infection by *E. amylovora* including the iron-binding siderophore desferrioxamine (60) and genes conferring the utilization of sorbitol, the major sugar present in apple (3), galactose (161) and sucrose (29). *E. amylovora* also

secretes other virulence factors that affect interaction with its host, including the recently-described type III effector $AvrRpt2_{Ea}$ (267), metalloprotease (PrtA) (265) and the levan biosynthetic enzyme levansucrase (20, 85). *E. amylovora* also possesses a full *sec* transport system which supports other secretion systems such as type II, type IV and type V (auto-transporter) secretion (188, 238, 266).

Type II secretion (T2S) is an important virulence factor in gram negative animal pathogens and phytopathogens such as *Pectobacterium carotovorum*, *Erwinia chrysanthemi*, *Ralstonia solanacearum*, and *Xanthomonas campestris*. The T2S operon was identified first from *Klebsiella oxytoca* by transferring a plasmid encoding the T2S operon from *K. oxytoca* to *Escherichia coli* to demonstrate that T2S proteins were required for the secretion of the enzyme pullulanase (215). Transfer of a cosmid containing the T2S from *E. chrysanthemi* to *E. coli* was also used to demonstrate the ability of the T2S to secrete various extracellular enzymes (138). Genome sequencing has made it possible to identify the presence of T2S genes in many gram negative bacteria other than pathogenic bacteria (50). However, the comparison of known functional T2S systems highlights 12 to 15 proteins expressed from the operon are required for secretion across the outer membrane (215, 219, 220). Although the gene naming convention has differed slightly amongst species possessing a T2S system, the majority of the gene products follow the *Klebsiella* gene designation as A through O plus S (50, 219, 220), with proteins CDEFGHIJKLMNO acting as the core of 13 essential T2S components (50). Here the general nomenclature of the T2S operon, "general secretion pathway" (Gsp) will be followed.

Exoproteins secreted by phytopathogenic bacteria include proteinases, amylase, pectinases, cellulases and polygalacturonases (50). Polygalacturonases present in phytopathogenic bacteria fall into two categories, endo- and exo-polygalacturonases. The former catalyze the random hydrolysis of pectic acid forming oligogalacturonates while the latter catalyze the terminal hydrolysis of pectic acid forming galacturonic acid monomers (115). While endo-polygalacturonase activity has not been detected in *E. chrysanthemi* (109), endo-polygalacturonase activity is a known virulence factor in *P. carotovorum* (216), *R. solanacearum* (61, 107) and *Agrobacerium vitis* (210).

Analysis of the secretome of *E. chrysanthemi* identified 14 T2S secreted proteins, including 11 pectinases, cellulase, rhamnogalacturonan lyase, a novel esterase and a novel Avr-like protein (122). Sequence analysis of *Ralstonia solanacearum* has identified 6 exoproteins including three polygalacturonases, a pectin methylesterase, and two cellulases (139). Previously, *E. amylovora* was described not to possess any cell wall degradation abilities (226); however, recent studies have described the presence of a type II secretion gene, *outF*, based on Southern hybridization to an $outF_{Eca}$ homologue and weak CelA activity (207).

We have previously described the upregulation of $outIJ_{EA}$ and *peh* expression upon colonization and infection of immature pear tissue (266). The pseudopilins $outIJ_{EA}$ share the highest homology to *yts1IJ* from the human pathogen *Yersinia enterocolitica*. In addition, the identification of *peh*, an endopolygalacturonase, indicates that *E. amylovora* may use a polysaccharide degrading enzyme during host infection. In order to determine the contribution of T2SS to virulence, deletion mutants of the porin gene $outD_{EA}$ and polygalacturonase *peh* were created. Additionally, the expression of *uidA*

fused to the T2SS operon promoter and *peh* promoter was determined under differing conditions.

METHODS AND MATERIALS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 4. *E. amylovora* and *E. coli* were cultured on Luria-Bertani (LB) medium at 28°C and 37°C, respectively, unless otherwise indicated. Minimal media was comprised of 1×M9 salts according to Sambrook *et al.* (217) with the addition of thiamine (0.02% w/v), niacin (0.02% w/v), and 2% (w/v) of one of the following: fructose, galactose, glucose, sorbitol or sucrose or 0.25% (w/v) pectin derived from apple fruit (82, 155). Liquid SOC medium used for transformed cell recovery was made according to Sambrook *et al.* (217). Antibiotics were used at the following concentrations in medium: ampicillin (Ap), 100 μg/ml; carbenicillin (Cb), 50 μg/ml; chloramphenicol (Cm), 25 μg/ml; gentimicin (Gm), 10 μg/ml and kanamycin (Kn) 25 μg/ml. Oligonucleotide primers used for PCR were purchased from Invitrogen (Carlsbad, CA) and are listed in Table 4.

Molecular biology techniques. Genomic and plasmid DNA isolation, gel purification of DNA, and PCR fragment clean up were performed using Qiagen kits (Qiagen Inc., Valencia, CA) according to manufacture's recommendations. PCR amplification, restriction enzyme digestion, T4 DNA ligation, and cloning were all performed according to standard molecular biology protocols (217). Sequencing of DNA was performed by the Research Technology Support Facility (RTSF) at Michigan State University. Database searches were conducted using BLAST programs (7) at the National Center for Biotechnology Information (NCBI) and the *E. amylovora* genome sequence at the Sanger

Institute (http://www.sanger.ac.uk/Projects/E_amylovora/). Amino acid alignments were conducted using ClustalW (v1.83) from the European Bioinformatics Institute (http://www.ebi.ac.uk/services/index.html). Signal peptide determination was done by the SignalP v3.0 program (http://www.cbs.dtu.dk/services/SignalP/) using both neural networks and hidden Markov model predictors of signal peptides (71).

Construction of the peh and outD deletion mutants. Construction of stable *E. amylovora* mutants was as previously described (267) using the λ phage Red recombinase system (56). Ea1189(pKD46) was grown up overnight and diluted 1/10 in fresh LB medium supplemented with ampicillin and 10 mM L-arabinose and grown to an OD_{600} of ~ 0.6. The cells were then pelleted (5,000×g) and washed three times in 10% glycerol at 4°C. After the final wash, the cells were resuspended in 1/100 volume. The primer sets OutDmut-F/ OutDmut-R and Pehmut-F/ Pehmut-R were used to generate PCR fragments that were transformed into 100 μl of competent Ea1189(pKD46). Cells were resuspended in 500 μl of SOC medium without antibiotics and allowed to recover overnight at 28°C. Transformants were then plated on solid LB medium supplemented with Cm and Ap. Colonies that were resistant to both Cm and Ap were selected for PCR screening using primer pairs that amplified from the cat cassette to the up and downstream sequences of *outD* (Cm1/outD-F and Cm2/outD-R) or *peh* (Cm1/peh-F and Cm2/peh-R). The primer pairs outD-F/outD-R or peh-F/peh-R were used to confirm the insertion of the *cat* cassette.

TABLE 4. Bacterial strains, plasmids and primers

Strains, plasmids and primers		Source or reference
Erwinia amylovora		
Ea110	Wild type, isolated from apple	
Ea1189	Wild type, isolated from apple	(38)
SB1	As Ea1189 but also *outD*::Cm^r	This study
SB2	As Ea1189 but also *peh*::Cm^r	This study
Escherichia coli		
DH10B	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*Δ139 Δ(*ara, leu*)7697 *gal*U *gal*K λ - *rps*L (Str^r) *nup*G	Invitrogen
TG1		Stratagene
Plasmids		
pBluescriptII(+)	Ap^r, cloning vector	Invitrogen
pGEMT-easy	Ap^r, PCR cloning vector	Promega
pQE60	Ap^r,	Qiagen
pBBR1-MCS2	Km^r, cloning vector derived from pBBR1MCS	(131)
pKD46	Ap^r, P_{BAD} *gam bet exo* pSC101 oriTS	
pKD3	Ap^r, FRT *cat* FRT PS1 PS2 oriR6K *rgbN*	
pGCM0	Gm^R cassette with downstream transcriptional terminator and *gusA* with upstream translational stop codons in pGem3zf+	(266)
pZYF2	570bp region of *dspEF* promoter in *Sma*I site of pGCM0, in opposite orientation relative to *uidA*	(266)
pZYF8	570bp region of *dspEF* promoter in *Sma*I site of pGCM0, in correct orientation relative to *uidA*	(266)
pSEB28	863bp region of $outD_{EA}$ promoter cloned upstream of *uidA* in pGCM0	This study
pSEB29	546bp region of *peh* promoter cloned upstream of *uidA* in pGCM0	This study
pSEB30	477bp region of IS*1133* promoter cloned upstream of *uidA* in pGCM0	This study
pSEB31	1248bp *Nco*I/ *Bgl*II fragment of *peh* ORF in pQE60	This study
pSEB36	1251bp *Sac*II/*Xho*I *peh* ORF in pBBR1-MCS2	This study
pSEB37	1938bp *Sac*II/*Xho*I *outD* ORF in pBBR1-MCS2	This study
Primers		
OutDmut-F	ATGAAGAAGAGATCCCCCCAATCCGCTACCAGCATCCGGCGGCT GTTACC *GCGATTGTGTAGGCTGGAGCT*	
OutDmut-R	TTACGCTTTTTTCCGGTAGAAATCAGCGATGTGCTTCTGTATTTC CACCA *CCATGGTCCATATGAATATCCTCC*	
Pehmut-F	ATGACTATTCTAACCAATTGTTTATTAAGGTTTTTATACCTCGGA ATATC *GCGATTGTGTAGGCTGGAGCT*	
Pehmut-R	TTACTTATCGATTTGAACGTTGTTAACGTTATATTTTGTCGCAGG ATCAA *CCATGGTCCATATGAATATCCTCC*	
outD-F	CAACGGATCATCATTCGTCACC	
outD-R	GCACGTTATGCTGTTGTACC	
peh-F	GCCCCCTGTTAAACCCGTACTCAATAAC	

TABLE 4 (con't)

Strains, plasmids and primers		Source or reference
peh-R	CCTGGCCACGGTGATAGAAGTTTTG	
Cm1	TTATACGCAAGGCGACAAGG	
Cm2	GATCTTCCGTCACAGGTAGG	
Orf1prom-F	GTCTCCGGTACCTTGCGGATAATACCTACCGCAACCTG (*Kpn*I)	
Orf1prom-R2	CGCACTGCAGTTTCTTTGTTCCTTATGATGTCTCCG (*Pst*I)	
Pehprom-F	GTCTCCGGTACCCCGCCATTGGCCGTCTTTTAATCGCCG (*Kpn*I)	
Pehprom-R2	CGCACTGCAGTTGAGATCCTCTGTGTAACTGG (*Pst*I)	
dspEF-F	TCCCCCGGGCAGTGAGGGGGGGCAGACTTTTTTTTAACC (*Sma*I)	
dspEF-R	TCCCCCGGGTATCTTCGCCGCTGCCACCTTTCACCATTG (*Sma*I)	
IS1133-F	CCGGTACCCGTCGCGTGATTGGCTGG (*Kpn*I)	
Aj1655	CGCACTGCAGGAAGCGCGGAGGTGGCTC (*Pst*I)	
PehpQE-F	CATGCCATGGATGACTATTCTAACCAATTGTTTATTAAGG (*Nco*I)	
PehpQE-R	GAAGATCTCTTATCGATTTGAACGTTGTTAACG (*Bgl*II)	
PehMCS2-F	ACTGATCCGCGGATGACTATTCTAACCAATTGTTTATTAAGG (*Sac*II)	
PehMCS2-R	TCTCGAGTTACTTATCGATTTGAACGTTGTTAACG (*Xho*I)	
OutDMCS2-F	ACTGATCCGCGGATGAAGAAGAGATCCCCCCAATCCGCTACC (*Sac*II)	
OutDMCS2-R	TCTCGAGTTACGCTTTTTTCCGGTAGAAATCAGCGATGTGC (*Xho*I)	

Creation of uidA reporter strains. Fusions of the putative type II out operon promoter, *peh* promoter and *dspEF* promoter to the *uidA* gene were constructed using pGCM0. The PCR primer pairs Orf1prom-F/Orf1prom-R, Pehprom-F/ Pehprom-R and IS1133-F/aj1655 were used to amplify the upstream sequence from the type II operon, *peh* and IS*1133* respectively. Purified upstream sequences were inserted into the *Kpn*I and *Pst*I sites in pGCM0 and ligated overnight at 14°C overnight using T4 ligase from Invitrogen (Carlsbad, CA). Promoter constructs were transformed into chemically competent E. coli DH10B, for screening using PCR and restriction enzyme analysis. Confirmed *orf1*$_P$::pGCM0, *peh*$_P$::pGCM0 and *IS1133*$_P$::pGCM0 were named pSEB28, pSEB29 and pSEB30, respectively. Positive colonies were stored in LB plus 10% glycerol at -80°C,

and the respective promoter fusion constructs were transformed into electrocompetent Ea1189.

Fluorometric assays with promoter fusion constructs. The activity of the promoters fused in front of the *uidA* gene was measured using the fluorescent substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) as was done for the *E. amylovora* IVET screen (266). The promoter fusion constructs pSEB28, 29 and 30 and pZYF2 and 8 were grown in 1×M9 minimal salts media as described above. A total of six sugars were used as carbon sources: fructose, galactose, glucose, sorbitol, sucrose and pectin. LB medium was used as a non-induced control. In addition, the activity of the promoter fusion constructs was measured *in planta* during infection of apple cultivar 'Gala'. For all MUG assays, *E. amylovora* Ea1189 harboring the promoter fusions were grown overnight in liquid LB medium. The bacteria were then harvested by centrifuging at 5,000 ×g and washing with 0.5×PBS twice. After the second wash, the bacteria were either added to 1×M9 minimal media at 1/100 (v/v) or used to inoculate young leaves at full strength. Samples were taken from liquid media/inoculated leaves at 24, 48 and 72 hours post-inoculation (h.p.i). The zero time point used was the bacteria suspended in 0.5× PBS. For all time points, 1.5 ml bacteria or a disrupted leaf disk were centrifuged at 13,000 rpm and the pellet resuspended in 0.5 ml GUS assay buffer. All solutions were kept on ice and centrifuge steps were at 4°C. Resuspended bacteria were then sonicated on ice using a microtip at 30% power (Branson Sonifier 250a, Branson Inc., Danbuy, CT) for 15 seconds followed by 20 seconds rest three times. The homogenate was added to GUS reaction buffer amended with 10mM MUG at a ratio of 1:4 and the reaction was allowed to proceed for 30 minutes at 37°C after which it was stopped with the addition of

4 volumes of Na_2CO_3. Fluorescence of the completed reactions were read in a SAFIRE fluorometer (TECAN Boston, Medford, MA). Bacterial counts were estimated by dilution plating and promoter activity was reported as μmol of 4-methylumbelliferone (MU) produced per min after normalizing to 10^9 CFU.

Complementation of deletion mutants. Construction of complementation plasmids, pSEB36 and pSEB37 (see Table 4) used the pBBR1MCS-2 cloning vector (131). The full length ORF for *outD*$_{EA}$ and *peh* were amplified using PCR from the Ea1189 genome using the primer pairs, OutDMCS2-F/OutDMCS2-R and PehMCS2-F/PehMCS2-R, respectively. The 1938 bp *outD* ORF, 1251 bp *peh* ORF and were inserted into the *Sac*II and *Xho*I sites of pBBR1MCS-2. Confirmed *outD*$_{EA}$::pBBR1MCS-2 and *peh*::pBBR1MCS-2 were named pSEB37 and pSEB36 and transformed into electrocompetent *outD* mutant SB1 and *peh* mutant SB2, respectively. SB1(pSEB37) and SB2(pSEB36) were stored in LB freezing medium at -80 C.

Infection Assays. Wild type Ea1189, the *outD* mutant SB1 and *peh* mutant SB2 plus complemented mutants, SB1(pSEB37) and SB2(pSEB36) were used in infection assays to determine contributions of the T2SS and polygalacturonase to *E. amylovora* virulence. The initial infection assays were performed using apple cultivar Pacific Gala trees incubated under controlled relative humidity (99%) and temperature (25°C). *E. amylovora* cultures used for infection were grown overnight on solid LB medium at 28°C and resuspended in 0.5× PBS, and diluted to an OD_{600} of 0.8 in 0.5× PBS and then serially diluted (1:10) three times. This suspension (~5×10^6 CFU/ml) was then used to

dip sterile scissors into and cut either across the midrib or roughly 1 cm into interveinal tissue of the second emerged leaf.

Pear infection assays used immature pear (*Pyrus communis* L. cv. 'Bartlett') according to previous studies for the analysis of *E. amylovora* mutants (26, 155, 267). Immature pears of similar size were sterilized in 10% bleach solution for 15 minutes and washed three times with sterile dH_2O. After drying in a sterile flow hood, the pears were pricked with a sterile 30 ½-gauge needle and 3 μl of ~5×10^5 CFU/ml *E. amylovora* was pipetted onto the wound. Infected pears were kept on sterile wet paper towels in sealed containers in a 28°C incubation chamber.

For statistical analysis of aggressiveness of the *E. amylovora outD* mutant SB1, *peh* mutant SB2 and wild type Ea1189 the method of Cabrefiga and Montesinos was used (40). *E. amylovora* cultures used for inoculation were grown overnight in liquid LB medium supplemented with the appropriate antibiotics. Cultures were then spun down at 5000 × g and washed with 0.5× PBS twice and diluted to an OD_{600} of 0.8 (~5×10^9 CFU/ml). Cultures were then serially diluted 1/10 in 0.5× PBS to obtain cultures that were $\log_{10}$ 7.5, 6.5, 5.5, 4.5, 3.5, 2.5 and 1.5. Six immature pears were inoculated 4 times opposite of each other for each concentration by pipetting 10μl of the bacterial dilution on each wounding site. The presence or absence of necrosis and/or ooze was recorded for each inoculation site every day following inoculation at approximately the same time as inoculations. These experiments were repeated three times. For the calculation of the median effective dose, the hyperbolic equation as described by Cabrefiga and Montesinos (40) was applied:

$$y = y_{max} (x / (x + K_x)) \quad (1)$$

where y is the incidence of infections, y_{max} is the maximum of infection incidence, x is the concentration of the culture and K_x is the median effective dose (ED_{50}). For the cultures used, the ED_{50} was solved for using data collected on day 3 of infection. Aggressiveness of the *E. amylovora* cultures was determined using a modified Gompertz equation (40):

$$y = K - \exp(-B_g \cdot \exp[-r_g(t - t_0)]) \quad (2)$$

where K is the curve asymptote signifying the maximum incidence of disease, B_g accounts for the origin of the curve, and t_0 is days of delay until symptoms are observed. For the calculation of aggressiveness, data from all days post inoculation were used for the culture diluted to 5×10^3 CFU/ml. The statistical program JMP version 6.0 (SAS Institue, Inc., Cary, NC) was used to fit the data collected to the hyperbolic and modified Gompertz equations.

Protein expression of Peh. Protein expression constructs were built to express Peh with a carboxyl-terminal 6× histidine tag. The primer set PehpQE-F/ PehpQE-R was used to PCR amplify a 1251 bp fragment of *peh*, which is missing the stop codon. The *peh* fragment was inserted into the *Nco*I and *Bgl*II sites of pQE60 and transformed into E. coli TG1 for screening using PCR amplification and restriction enzyme digestion. Confirmed *peh*::pQE60 constructs were named pSEB31 and transformed into Ea1189, both TG1(pSEB31) and Ea1189(pSEB31) were stored in LB freezing medium and stored at -80°C.

A fast mini-preparation of protein fractions was prepared according to (52) and used for subsequent Western blots. Ea1189(pSEB31) and TG1(pSEB31) overnight

cultures were diluted 1:5 into fresh LB medium supplemented with 50 μg/ml carbenicillin and allowed to recover for one hour, after which IPTG was added to a final concentration of 1mM. Cells were harvested 48 hours post-induction, and centrifuged at 13,000 rpm to separate the supernatant from cells. The pellet was used for a fast fractionation protocol (52) and separated into periplasmic and cytoplasmic/membrane fractions.

Purification of *E. amylovora* Supernatant Protein. For the visualization of *E. amylovora* secreted proteins and the ruthenium red staining assay, supernatant was harvested from cultures grown on solid 1×M9 minimal medium supplemented with 2% (w/v) galactose (81). Cultures were allowed to grow on solid medium for 36 hours at 28°C, after which the cells were resuspended in 0.5× PBS treated with a mini Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). These suspensions were centrifuged twice in a 4°C centrifuge at 13,000 rpm for 15 minutes. After the second centrifugation step, the supernatant was concentrated in a 10kDa cutoff Centricon (YM-10) concentrator according to manufacturers recommendations (Millipore, Billerica, MA).

Ruthenium red staining for polygalacturonase activity. Ruthenium red staining was a modified method of the ultra-thin pectate-agaroase gel overlay method (206). Here, either crude lysate from *E. coli* TG1(pSEB31) or *E. amylovora* Ea1189(pSEB31) or supernatant from *E. amylovora* Ea1189, SB1, SB2 or Ea1189(pSEB31) was used for the assay. Crude lysate was harvested from LB grown cultures, and supernatant was harvested from strains as described above. A pectate-agarose gel (50mM potassium

acetate, pH5.5; 1% (w/v) agarose and 0.1% (w/v) polygalacturonic acid) was used for ruthenium red staining of polygalacturonase activity. Six ml of molten pectate-agarose gel was poured in a 80mm diameter culture plate. To this, 20μl of crude lysate or supernatant was added and the plate was incubated at 28°C for two hours. After incubation, the lysate or supernatant was washed off of the plate, and a 0.05% (w/v) solution of ruthenium red was applied to the pectate-agarose plate for 30 minutes at room temperature. Visualization of the zones of activity was by repeated washing of the pectate-agarose plate with dH_2O, and then air-drying of the plate. Results were recorded using a photo scanner.

Detection of His-tagged Proteins. Detection of His-tagged proteins the SuperSignal® West HisProbe™ Kit (Pierce Biotechnology, Inc., Rockford, IL) according to the manufacturer's protocol, except that the ratio of HisProbe™-HRP to tris buffered saline Tween®-20 (TBST) was increased from 1:5,000 to 1:1,000. A 6×His protein ladder (Qiagen Inc., Valencia, CA) was used for molecular mass estimation.

RESULTS

Sequence and genetic analysis of type II operon and endo-polygalacturonase

Both the pseudopilin, *outI*, and endopolygalactuonase, *peh*, were identified previously as being upregulated during colonization and infection of immature pear tissue (266). To obtain the full length sequence of *outI* and *peh*, thermal asymmetric interlaced (TAIL)-PCR was used to sequence upstream and downstream of the original IVET clone (data not shown). Upon sequencing *outIJK* and 1.4kb of *peh*, the *E. amylovora* sequence was queried to identify the entire out_{Ea} operon and full length *peh* sequence (http://www.sanger.ac.uk/Projects/E_amylovora/).

Overall, the out_{Ea} operon (Figure 4) follows the general gene organization of the *yts1* operon from *Y. enterocolitica* (112). The *out* operon lacks an *orf1* homologue, and instead starts with an *orf2* homologue; in addition, no discernable *gspM* homologue could be identified in between *outL* and *outO* using either BLASTp, tBLASTx or tBLASTn (7). The lack of an identifiable *gspM* homologue in the T2S operon is similar to the T2S operon from *Yersinia pestis*, the causative agent of bubonic plague that, much like *E. amylovora*, is not known for any T2S secreted exoproteins (220). *E. amylovora* does posses the T2S lipoprotein OutS, which is commonly only observed from the T2S operon from *Enterobacteriaceae* (219).

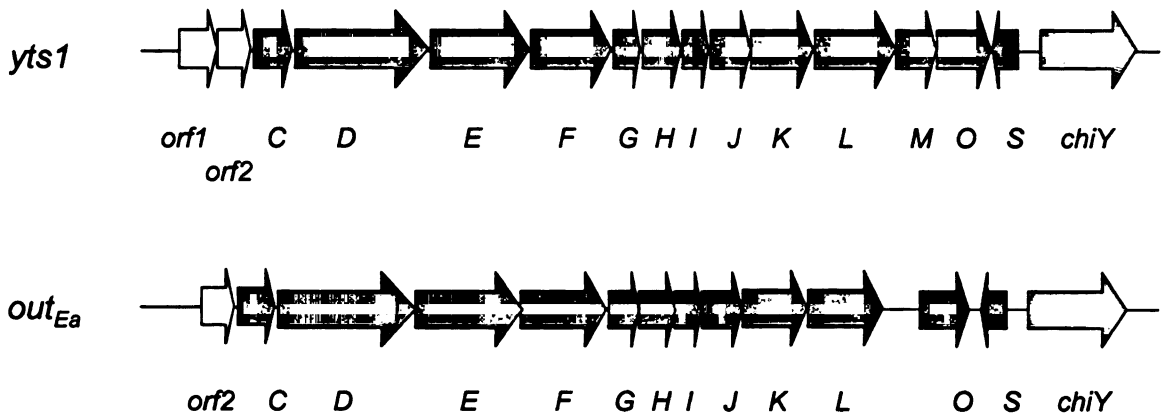


Figure 4: Alignment of type II secretion systems from *Y. enterocolitica (yts1)* and *E. amylovora (out$_{Ea}$)*.

Using putative translated sequences, the highest amino acid identity is 76 % between OutG and PulG of *Yersinia mollaretti* (see Table 5), followed by 62% between OutE and Yts1E from *Y. enterocolitica*, and 58% for both OutF and PulF and OutI and Yts1I.

Yersinia molaretii is a recently sequenced member of the *Yersinia* genus that is thought to be non-pathogenic. Typically, the percentage of identity for genes E, F and G are the highest in the T2S operon, and that appears to be the case with respect to the amino acid sequences (220).

The core T2S operon (*outC~outS*) is 10.6 kb, with an 83-bp gap between the predicted stop codon of *orfM* and the start codon of *outO* and a 118 bp gap between *outO* and *outS* (see Table 6). Similar to *Y. enterocolitica*, *outS* is transcribed in the reverse orientation with respect to the remaider of the T2S operon, from its own promoter. It is of interest that Orf2 has homology to a DNA-binding protein, possibly a transcription factor that could co-ordinate the transcription of *outS* along with the rest of the operon. Since the far 5' and 3' regions of the T2S operons are where rearrangement of gene order

often occurs, this may explain the lack of an identifiable *gspM* homologue. In addition, the only gene that is not homologous to a *Yersinia* species is *outS* which is homologous to *outS* from *Pectobacterium atrosepticum*.

Searches (using Blastp and Blastx) for other potential T2SS operons in *E. amylovora* returned two putative T2SS proteins, the first of which is a GspD homologue that is homologous to a putative T2SS GspD protein from *P. atrosepticum* (E = 4e-112, 57% identities, 75% similarities) and HofQ from *Yersinia intermedia* (E =1e-105, 55% identities, 73% similarities). The promoter sequence of this GspD homologue was found to be active under low temperature conditions (18°C) by Goyer and Ullrich (92). Theoretical open reading frames inferred by ORF finder (NCBI) for sequence upstream and downstream of this protein were identified using Blastp as being a shikimate kinase upstream and a hypothetical protein downstream. Even though this protein is a GspD homologue, it only shares 22% amino acid identities with OutD.

Other putative type II secretion genes found in the genome of *E. amylovora* were found downstream of the *chiY* gene. This included a GspF homologue, most similar to PulF from *Y. mollaretti* (E = 8e-97, 46% identities). The other two genes directly downstream and thought to be transcribed off of a common promoter sequence with the *pulF* homologue are two type IV pilin genes, *pilB* and *pilA*. This may indicate that the *pulF* homologue may indeed be a part of the type IV pilus genes and not a putative type II secretion gene, since the both T2SS and type IV pili are structurally related (238).

The full length ORF of *peh* is 1251 bp, with a putative translated amino acid sequence of 416 amino acids. Using this putative translated sequence, homology to an endopolygalaturonase *pehA* from *Pectobacterium atrosepticum* (E = 7e-117; 59%

TABLE 5. Protein homology of *E. amylovora out* operon.

Gene[a]	BLASTp_BestHit[b]	Organism	Identity/Similarity[c]	Reference
orf2	Hypothetical protein	*Y. enterocolitica*	56/74	(112)
outC	Yts1C	*Y. enterocolitica*	32/53	(112)
outD	Yts1D	*Y. enterocolitica*	58/74	(112)
outE	Yts1E	*Y. enterocolitica*	62/76	(112)
outF	PulF	*Y. mollaretti*	58/74	NR[d]
outG	PulG	*Y. mollaretti*	76/87	NR
outH	PulG	*Y. mollaretti*	35/55	NR
outI	Yts1I	*Y. enterocolitica*	58/77	(112)
outJ	Ys1J	*Y. enterocolitica*	41/58	(112)
outK	PulK	*Y. mollaretti*	31/48	NR
outL	Yts1L	*Y. enterocolitica*	28/41	(112)
orfM	Unknown			
outO	PulO	*Y. mollaretti*	56/69	NR
outS	$OutS_{Pa}$	*P. atrosepticum*	35/51	(17)

[a] Gene designation in *E. amylovora* T2S operon

[b] BLASTp used to search for similar proteins based on putative translated gene sequence

[c] Identity is without amino acid substitutions and similarity allows for conservative amino acid substitutions in the BLASTp search

[d] No reference (NR) available

identities and 74% similarities) and *peh-1* from *Pectobacterium carotovorum* (E = 2e-116, 60% identities and 74% similarities) was determined querying the Genbank database with Blastp (17, 141). Unlike *peh-1*, *peh* does not have a pectate lyase upstream of its ORF, nor does it share any similar promoter motifs, such as a KdgR box, as determined by CREDO analysis which searches for shared motifs between promoter sequences (http://mips.gsf.de/cgi-bin/proj/regulomips/submission.cgi). The predicted amino acid sequence has three protein motifs predicted by InterProScan (http://www.ebi.ac.uk/cgi-bin/iprscan); the first is from the family 28 glycoside hydrolases, which includes polygalacturonases; other predicted motifs are a polygalatucturonase motif and a pectin lyase fold motif. In addition a signal peptide at the N-terminus of the protein was also

predicted based on the amount of hydrophobic residues present. In contrast, SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/), which is used to identify type signal peptides, predicts a 40% chance that the N-terminus possesses a functional signal peptide (71). According to two predictive programs used in SignalP: hidden Markov models and neural networks, Peh is considered non-secretory. When a plot of hydrophilicity is made for the entire Peh amino acid sequence, there are two main areas of hydrophobicity in the first 40 amino acids of Peh, corresponding with the two putative sites for signal peptide cleavage by SignalP 3.0. The first is between residues 22 and 23, the second is between residues 39 and 40. When a ClustalW alignment (Fig. 1) is made between Peh, PehA and Peh-1, the first residue of the mature endo-polygalacturonase protein is conserved amongst all three sequences (Fig. 2), thus if *E. amylovora* Peh is recognized by the sec transport system, it is likely that the site of signal peptide cleavage is between residues 39 and 40. When the 39 amino acids are subtracted from the predicted amino acid sequence, the molecular mass of the mature Peh protein is 40.1kDa, although if the secondary predicted site is cut at residue 22, then the alternate mature Peh protein would have a molecular mass of 41.7 kDa. When the preliminary genome sequence of *E. amylovora* was searched for other polygalacturonases or pectin lyases, no other pectin degradation enzymes were found. The only other potential T2S secreted cell wall degradation enzyme is a cellulase that was observed by Riekki *et al.* (207).

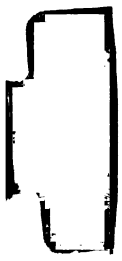
Creation of deletion mutants of outD and peh in E. amylovora

In order to determine the contribution of T2S and the enzyme Peh to *E. amylovora* virulence, two deletion mutants were constructed. The *outD* gene from the

T2S operon was selected for deletion, because it is the outer membrane pore-forming protein, and in other systems, mutations of the *gspD* gene render the T2S secreton non-functional (98). First, single deletion mutants of *outD* and *peh* were constructed using the Red recombinase system (56) that allows for the use of linear targets for recombination into the genome. The areas of homology used for recombination were 50bp at the start and the end of *outD* and *peh* ORFs.

Apple cultivar 'Gala' shoot infection assay

Infection assays with the deletion mutants and complemented mutant strains were conducted in two different tissue types. Because of the expression of the T2S and the polygalacturonase in the IVET system described before, the potential for a phenotype in actively growing apple shoots exist. Young Gala apple trees kept in a controlled environment chamber were used for the shoot inoculations. The youngest unfolded leaf from actively growing shoots was inoculated across the midvein for all strains tested. Overall, the mutants appeared to behave like wild type when inoculated directly into the midvein (data not shown). There was a slight delay of symptoms with the *peh* mutant, SB2, but it was still able to form disease symptoms over 10 days. Since inoculating the mutants directly into the midvein appeared to have no effect, inoculations into the interveinal areas of leaf tissue were also conducted. This was to determine if the presence of a T2S operon and *peh* gene is for movement through leaf tissue. There were some differences noted with this method of inoculation, indicating the potential for the polygalacturonase to be used as a mechanism for movement through intercellular regions.



Immature pear tissue infection assay

In some cases, the phenotype observed from inoculations with mutants is different in shoot tissue than in immature pear tissue. So to better ascertain the impact of the *peh* deletion mutation in *E. amylovora*, strains Ea1189, SB1, SB2 and complemented *outD* mutant, SB1(pSEB37) and complemented *peh* mutant SB2(pSEB36) were used to inoculate immature pears from cultivar 'Bartlett' (Figure 5). The degree of necrosis around the infection site for SB2 is less at day 4 (Figure 5B) and 6 (Figure 5C) than either wild type or SB1. When the mean area of necrotic tissue is calculated at day 8 (Figure 5D) post inoculation from an independent inoculation assay the area was 3.3 ± 0.1 cm^2 for SB2 and 3.4 ± 0.2 cm^2 for complemented SB2(pSEB36); these areas were slightly lower than those from Ea1189 or the *outD* mutant. The areas were 3.6 ± 0.3 cm^2 for Ea1189, 3.4 ± 0.2 cm^2 for SB1 and 3.9 ± 0.4 cm^2 for complemented SB1(pSEB37). Strain SB1 phenotypically looks like Ea1189, and combined with the apple shoot inoculation data, type II secretion may not be used in virulence of *E. amylovora* on apple shoots or immature pear tissue. Even though virulence appears not to be compromised with the *outD* mutant, the complemented *outD* mutant does have a slightly higher area of necrosis than Ea1189, so there may be some contribution of the type II secretion system that can enhance disease when over expressed. Since there was a difference in necrosis caused by Ea1189 versus *peh* mutant SB2, the Peh protein may not be secreted through the type II apparatus during infection and may be released in another manner.

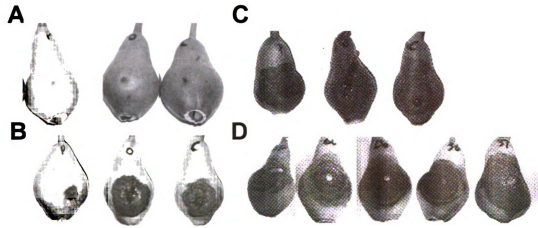


Figure 5: Areas of necrosis on inoculated immature pears. **(A)** 2 days post inoculation; (left to right) SB2, SB1 and Ea1189. **(B)** 4 days post inoculation; (left to right) SB2, SB1 and Ea1189. **(C)** 6 days post inoculation; (left to right) SB2, SB1 and Ea1189. **(D)** 8 days post inoculation; (left to right) SB2, SB1, Ea1189, SB2(pSEB36), and SB1(pSEB37).

Aggressiveness of E. amylovora mutants

To better quantify the effects of the deletion of either *outD* or *peh* on infectivity, a measure of aggressiveness was calculated for both mutants and Ea1189 using the method of Cabrefiga and Montesios (40). Overall, the median effective dose (ED_{50}) calculated for the *peh* mutant SB2 (6.7×10^3 CFU/ml) was more than twice the calculated ED_{50} for Ea1189 (2.6×10^3 CFU/ml). Data used for calculating the ED_{50} was collected at day 3, due to the highly virulent nature of Ea1189, where the incidence of infection was 100% for most concentrations at day 5. In addition, the incubation temperature used for development of symptoms was 28ºC versus 21ºC that was used in the previous study by Cabrefiga and Montesinos (40). For the kinetics of infection, the incidence of infection was calculated for days 1 through 10 at an inoculum level of 5×10^2 CFU/ml. Overall, the *peh* deletion mutant, SB2 exhibited a lower rate of infection ($r_g = 0.75$), curve

asymptote (K = 0.82) and a higher delay to disease symptoms (t_0 = 5.16) than the other strains used. The outD mutant, SB1 had similar values (r_g = 1.12, K = 0.98, t_0 = 3.19) as wild type Ea1189 (r_g = 1.8, K = 0.95, t_0 = 2.66). This statistical method for determination of aggressiveness is useful for showing small, but significant differences between mutants and their parental strain.

Fluorometric GUS assay with promoter-uidA fusion contructs

The method used to determine the activity of the T2S operon promoter and *peh* promoter was a fluorometric GUS assay. In order to clone the putative full length promoters for both genes, the sequence between the last predicted ORF and the start codon of both orf2 and *peh* were cloned. Liquid minimal media that is known to induce type III secretion in *E. amylovora* was used to screen both promoters for activity. Six different sugars were used to supplement this minimal media to determine if the *in vitro* conditions for activating the expression of the TTSS are the same for the activation of expression of the T2SS and *peh*.

Overall fold induction of the T2S operon promoter was the highest at 306.6-fold in minimal medium amended with glucose (MM-glc) at 48 hours post induction (hpi; Table 6). The effector protein, DspE, appears to have a high level of promoter induction in MM-glc at all three time points collected (279.3 to 361.3-fold). The effect of glucose on the constitutive promoter from the IS*1133* insertion element was not different than that of LB medium. Induction of the *peh* promoter was also highest with MM-glc, however peak induction of 746.9-fold did not occur until 72 hpi. If mature Peh protein is secreted through the T2S, than the delay of *peh* promoter activity may delay protein

TABLE 6. Fold induction of promoter-*uidA* fusion constructs inoculated in minimal medium[a]

Plasmid[b]	Fructose	Galactose	Glucose	Pectin	Sorbitol	Sucrose	LB
pSEB28							
T_{24}/T_0[c]	10.5 (6.6)[d]	9.4 (1.8)	44.7 (26.4)	6.0 (3.2)	9.8 (4.7)	6.8 (4.6)	3.7 (0.8)
T_{48}/T_0	12.4 (5.7)	77.8 (47.8)	306.6 (213.0)	14.9 (10.1)	38.6 (24.2)	34.8 (22.7)	5.6 (0.2)
T_{72}/T_0	21.8 (14.4)	48.5 (24.5)	153.8 (101.8)	11.8 (7.3)	41.7 (25.3)	9.5 (4.4)	9.9 (1.1)
pSEB29							
T_{24}/T_0	59.9 (31.4)	54.1 (15.0)	57.1 (31.0)	0.3 (0.1)	10.1 (7.0)	6.6 (4.1)	8.0 (3.6)
T_{48}/T_0	190.4 (111.2)	284.7 (165.4)	217.1 (140.0)	0.4 (0.2)	34.6 (24.0)	19.6 (12.9)	26.6 (8.1)
T_{72}/T_0	283.3 (167.9)	307.9 (174.3)	746.9 (521.2)	0.2 (0.1)	16.8 (11.5)	16.8 (11.5)	54.2 (14.4)
pSEB30							
T_{24}/T_0	9.4 (6.1)	7.6 (3.0)	3.0 (1.5)	11.4 (5.1)	9.0 (5.6)	31.3 (0.4)	2.5 (1.5)
T_{48}/T_0	12.3 (7.8)	26.5 (12.9)	3.0 (1.7)	15.4 (5.6)	53.7 (37.0)	52.9 (8.7)	8.6 (5.1)
T_{72}/T_0	12.3 (8.3)	27.8 (15.8)	3.8 (2.0)	14.9 (7.1)	68.5 (47.4)	42.6 (8.3)	9.7 (6.3)
pZYF8							
T_{24}/T_0	198.7 (117.6)	923.3 (616.8)	279.3 (185.0)	118.5 (83.4)	487.0 (210.2)	197.8 (122.5)	35.9 (22.9)
T_{48}/T_0	199.9 (120.1)	823.5 (572.0)	330.2 (129.5)	94.2 (66.0)	1545.6 (886.8)	210.0 (131.6)	102.3 (60.1)
T_{72}/T_0	105.6 (52.2)	300.7 (201.1)	361.3 (163.6)	25.3 (17.4)	1089.0 (366.6)	197.6 (127.1)	44.4 (25.6)

[a] M9 minimal medium supplemented with either of the six sugars indicated in the above columns. LB was used as a rich medium control.

[b] Plasmid refers to the promoter::*uidA* fusion constructs used. pSEB28 harbors the T2S promoter, pSEB29 harbors the peh promoter, pSEB30 harbors the promoter from insertion element IS1133 and pZYF8 harbors the dspE promoter.

[c] Refers to 24, 48 or 72 hours post induction over 0 hours post induction

[d] Numbers in parenthesis are the standard error, calculated for two independent assays consisting of three measurements

production until after a functional secreton is assembled. Even though the highest level of induction for the *peh* promoter is in MM-glc, its activity relative to the activity of the dspE and T2S promoters is weaker.

Second highest induction of both the T2S operon and *peh* promoter was in minimal medium amended with galactose (MM-gal). The use of the simple sugar galactose is common in TTSS-inducing minimal media, and also is known to increase the amount of exopolysaccharide (amylovoran) produced. Although MM-gal is the second best condition met for T2S promoter induction, the preference for glucose is apparent by the fold induction for each sugar, 306.6-fold with glucose, compared to 77.8-fold for galactose at 48 hpi (Table 6). This is in contrast with the *dspE* promoter, which is highly induced in MM-gal starting at 24 hpi with 923.3-fold induction and tapers off to 300.7-fold induction at 72 hpi.

Surprisingly, the *peh* promoter was not induced by minimal media amended with pectin (MM-pec (Table 6). Upon examination of the levels of induction for the other promoters used in the GUS activity assays, it further appears that the *peh* promoter may be inhibited by the presence of pectin in the medium. Although the main sugar present in apple shoots and immature pear fruit is sorbitol, only the *dspE* promoter was highly induced (up to 1545.6-fold at 24 hpi; Table 6) in minimal medium amended with sorbitol (MM-sor). In addition, the insertion element IS*1133* promoter is also expressed at its highest levels (68.5-fold) in MM-sor. Both the T2S promoter and *peh* promoter had relatively low levels of induction; 38.6 and 34.6 hpi respectively in MM-sor, highlighting the differing sugar requirements for the induction of TTSS-related promoters and T2S-related promoters.

Actively growing apple shoots (cultivar 'Gala') were also used for fluorometric GUS assays to look at levels of promoter activity *in vivo*. Young leaves from Gala were inoculated across the midrib, and tissue was harvested at similar time points as with the minimal medium experiment. As seen with the liquid minimal media GUS assays, the *dspE* promoter is highly induced compared to both the T2S and *peh* promoter (Table 7). However, even though both the T2S and *peh* promoters were only minimally induced when grown in MM-sor and repressed in the case of the *peh* promoter with MM-pec, both promoters were also highly induced upon infection. In addition, when the level of induction of the T2S and *peh* promoters were compared for MM-glc, the *peh* promoter was noted to lag behind the T2S promoter by 24 hours. This is not the case for promoter induction during infection of apple tissue. At 24 and 48 hpi, the *peh* promoter is more highly induced and activity of the promoter is higher than that of the T2S promoter. Even the moderate promoter from insertion element IS*1133* was induced to high levels during infection.

Expression of Peh in E. coli and E. amylovora

To determine whether or not the *peh* gene identified from *E. amylovora* encodes a functional polygalacturonase, an expression vector was used to express *peh* (pSEB31, Table 4) in *E. coli* and activity of the *peh* gene product was determined by ruthenium red staining of pectin amended agarose. Expression of pSEB31 in the *E. coli* TG1 background stained for the breakdown of polygalacturonic acid, indicating polygalacturonase activity (Fig. 6A). In order to determine that the activity was due to

TABLE 7. Fold induction of promoters upon infection of apple cultivar 'Gala'

Plasmid	24 hpi[a]		48 hpi		72 hpi	
	GUS Activity[b]	Fold Induction[c]	GUS Activity	Fold Induction	GUS Activity	Fold Induction
pSEB28	3.1E-2 (6.4E-3)	49.1 (10.4)	3.2E-1 (5.4E-2)	492.4 (107.0)	8.5E-1 (8.8E-2)	1325.5 (821.4)
pSEB29	4.4E-2 (2.5E-2)	651.6 (144.5)	1.3E-1 (7.1E-2)	1364.1 (936.1)	6.6E-1 (3.7E-1)	6807.8 (4513.2)
pSEB30	2.1E-2 (1.0E-2)	37.9 (16.7)	6.9E-1 (2.7E-1)	1234.0 (615.1)	8.5E-1 (1.4E-2)	1534.0 (677.4)
pZYF8	3.3E-1 (4.7E-2)	290.4 (49.8)	3.3 (1.3)	2873.0 (210.9)	31.0 (3.5E-1)	27645.5 (18694.0)

[a] Hours post inoculation (hpi)

[b] GUS activity is reported as of 4-methylumbelliferyl produced min^{-1} 10^{9} CFU^{-1}

[c] Fold induction is activity at 24, 48 and 72 hpi over activity at 0 hpi

the processed mature Peh protein, pSEB31 was also expressed in the *E. amylovora* Ea1189 background, which gave the same result (Figure 6B). Attempts at observing a polygalacturonase phenotype from the supernatant of wild type Ea1189, *outD* mutant SB1 or *peh* mutant SB2 after induction in MM-gal medium were inconclusive (data not shown). However, Ea1189 harboring pSEB31 did have detectable polygalacturonase activity in the supernatant collected (Figure 6C). Although the *peh* gene product does appear to be a polygalacturonase that is present in the supernatant of Ea1189, its wild type expression in liquid media is below the detection limit of ruthenium red staining.

Detection of expressed Peh protein in E. amylovora

In addition to determining that Peh exhibits polygalacturonase activity, the rough sub-cellular location of Peh expressed from plasmid pSEB31 was also investigated. The vector used to create plasmid pSEB31 (Table 4) adds a 6×his tag to the C-terminus of the expressed protein. This 6×his tag was used to detect Peh using a modified Western blot technique. Peh was detected in the whole cell lyasate, the periplasmic fraction and the cytoplasmic/ membrane fraction. A single protein band was detected in the periplasmic fraction, whereas the whole cell and cytoplasmic/ membrane fraction showed multiple detected bands.

In addition to detecting Peh protein from Ea1189(pSEB31), an attempt was made to detect endogenous Peh protein. Supernatant from Ea1189(pKD46), ZY3, SB1 and SB2 was loaded on a 12% SDS-polyacrylamide gel. One prominent band in Ea1189 (control), *outD* mutant SB1 and type III operon knock out mutant ZY3 is not present in the *peh* mutant SB2. Because the band is located at 40.1kDa, it is very likely that this is

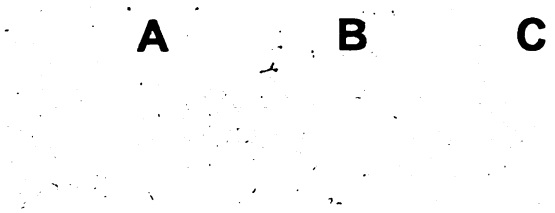


Figure 6: Ruthenium red stain for polygalacturonase activity. Photos were inverted from original tones, so clearing of the stain appears dark in the figure. **(A)** Whole cell lysate from *E. coli* TG1 (pSEB31) induced with IPTG. **(B)** Whole cell lysate of *E. amylovora* Ea1189(pSEB31) grown in LB. **(C)** Supernatent from *E. amylovora* Ea1189 (pSEB31) grown in M9 minimal media.

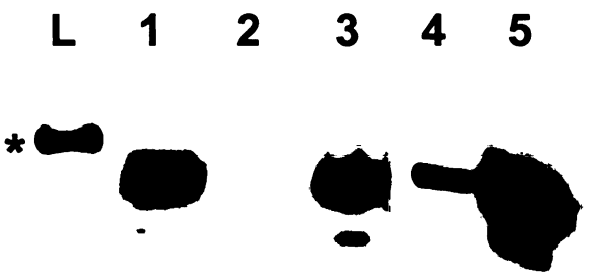


Figure 7: Western blot of Ea1189(pSEB31) subcellular fractions. Ea1189(pSEB31) was inoculated in LB medium for induction of the expression plasmid. A his-labeled ladder (L) was used as the molecular weight standard, 50kDa marker (*) is shown. Whole cell (lane 1), periplasmic (lanes 2 and 5) and cytoplasmic/membrane-bound (CM) fractions were extracted and loaded on a 10% SDS-polyacrylamide gel. Lanes 4 and 5 are as lanes 2 and 3, except twice as much protein was loaded.

the Peh protein. Qualitatively, Peh appears to be present in nearly equal amounts in the supernatant of Ea1189, SB1 and ZY3; making it more likely that Peh is inefficiently secreted by the type II secreton, and released by rupturing cells to the extracellular milieu. Interestingly, the secretion of DspE appears to be hampered in the *peh* mutant, SB2. When 500ng of supernatant protein is loaded (Fig. 8A), a faint band corresponding to DspE is observed, however at a significantly lower level than that of Ea1189 and the *outD* mutant, SB1. When 1μg of supernatant protein was loaded, the DspE protein is visible as a distinct band (Figure 8B). Another feature of the supernatant of SB2 is that it appears to lack the characteristic smear of LPS at the lower portion of the gel. This was observed at both levels of supernatant loaded. It was further noted after the creation of the *peh* mutant, that SB2 colonies appeared more mucoid than wild type or the *outD* mutant.

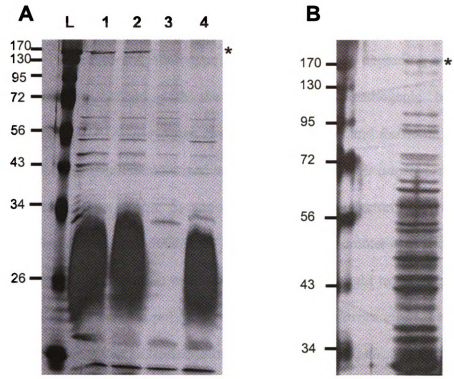
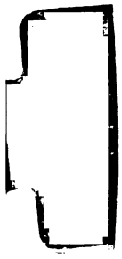


Figure 8: Supernatant proteins separated out on SDS-polyacrylamide gel. **(A)** 500 ng of supernatant protein loaded on 12% SDS-polyacrylamide gel. Molecular weight standard (L); Ea1189 (lane1), *outD* mutant SB1 (lane 2), *peh* mutant SB2 (lane 3), type III operon knock out mutant ZY3 (lane 4). **(B)** 1μg of supernatant protein from SB2 loaded on 10% SDS-polyacrylamide gel. Band corresponding to DspE is denoted by an asterisk.

DISCUSSION

Pectinases and other type II secreted cell wall degrading enzymes (CWDE) are commonly associated with the macerating phytopathogenic bacteria such as *P. atrosepticum*, *P. carotovorum*, *Erwinia chrysanthemi* and *Ralstonia solanacearum* (243). Even though it is characterized as a necrogen (246), *E. amylovora* possesses a type II secretion system (Table 5) and a functional polygalacturonase (Fig. 1). Both were identified during a previous IVET screen (267) by promoter activation upon infection and colonization of immature pear tissue. This study sought to discover the contribution of type II secretion and a polygalacturonase to *E. amylov*ora virulence. This was achieved through the creation of deletion mutants for *outD* and *peh*, and a more in-depth analysis of T2SS and peh promoter activity and expression of the Peh protein.

The type II system from *E. amylovora* shows homology to type II system proteins from *Y. enterocolitica* and *Y. mollaretii*. *Y. enterocolitica* is a human pathogen that is classified into either non-pathogenic, low pathogenic or highly pathogenic strains. *Y. enterocolitica* strains harbor a ubiquitous T2SS operon noted as *yts2*, that is similar to the annotated putative T2SS operon from *Y. pestis* both in amino acid sequence and gene order (112, 191). It is unkown if *yts2* operon is functional, like the *E. amylovora out* operon, it lacks an identifiable GspM homologue, and instead a hypothetical protein occupies the position between GspL and GspO. The *Y. enterocolitica* strains that are considered high-pathogenicity harbor the *yts1* T2SS operon, in addition to the *yts2* T2SS operon, and other virulence related genes that contribute to the high-pathogenicity phenotype. Interestingly, the high-pathogenicity group is more often found in North



America and known as a "new world" isolate (111). *E. amylovora* also is thought to be indigenous to North America; the possession of a related T2SS operon could either indicate a common predecessor with *Y. enterocolitica* or a potential horizontal transfer event. Many genes upregulated in the IVET screen of *E. amylovora* had homologous counterparts in human pathogenic bacteria; therefore obtaining a T2SS from a common ancestor is most likely. In addition, analysis of the upstream and downstream sequence of the *E. amylovora out* operon did not reveal any potential transfer genes. The upstream sequence did posses a PulF, PilB and PilA protein homologues, and downstream sequence analysis exhibited a *secA* gene.

The only protein in the T2SS operon of *E. amylovora* that wasn't highly homologous to either of the *Yersinia* species was OutS, which had stronger homology to OutS from *P. atrosepticum* (Table 5). The position of the lipoprotein, *outS* in the out_{Ea} operon is different than that of the out_{Pa} operon; it is at the end of the out_{Ea} operon between *outO* and *chiY*, whereas in the out_{Pa} operon it is located in front of *outB*. The 5' and 3' regions of T2SS are subject to some gene rearrangement (219), and in the case of *E. amylovora*, it appears to have potentially swapped the *outS* gene from the T2SS of the common ancestor with *outS* from *P. atrosepticum*.

Although previous studies determined that *E. amylovora* did not posses any pectolytic activity (226), and thus as a result did not harbor any CWDEs, two studies have now described the presence of CDWE genes from *E. amylovora* using genetic techniques (207, 266). The polygalacturonase from *E. amylovora* is homologous to endo-polygalactuonases *pehA* and *peh*-1 from *P. atrosepticum* and *P. carotovorum*. Its N-terminus predicted signal peptide is highly divergent from those of *pehA* or *peh-1* (Fig.

1); and the program SignalP predicts that Peh is non-secretory (i.e. not recognized by Sec machinery); however it is present in the periplasm of *E. amylovora* (Fig. 7). It is also curious that a protein that is unique to plant pathogens and plants would theoretically be secreted through a T2SS that evolved from human pathogens. Specificity of proteins secreted by type II secretion is noted in numerous species, due to interactions with both proteins C and D from the secreton (34, 54, 228). However the Peh protein of *E. amylovora* may be divergent enough from *Pectobacterium* polygalacturonase so that the T2SS signaling motif is recognizable by the T2SS secreton.

Mutation analysis of *peh* and *outD* determined a small but noticeable effect on virulence and aggression when *peh* was deleted (Fig. 4B and C). However, when a deletion mutant of *outD* was created it was no less pathogenic than wild type. If Peh is secreted through the T2SS secreton, it would be assumed that an *outD* deletion mutant would have the same or less virulent phenotype as the *peh* deletion mutant. One possibility is that Peh is not secreted efficiently through the T2SS. This is plausible because the signal peptide is not expected to be recognized by Sec machinery and there is no identifiable OutM protein homologue, thus potentially destabilizing the T2SS secreton. However, his-labeled Peh was detected in the periplasm of *E. amylovora* Ea1189(pSEB31) (Fig. 7). Since the signal peptide is recognized by the Sec machinery than the observation of active Peh in the supernatant of *E. amylovora* (Fig. 6C) could due to its escape from the periplasm of disrupted cells. Therefore the Peh protein either isn't recognized by the T2SS, or it is inefficiently secreted by the T2SS secreton. This is the case for other polygalacturonases, such as PehA from *Agrobaterium vitis* which was shown to be mostly located in the periplasm by enzyme activity analysis of the

cytoplasm, periplasm and supernatant. Observation of PehA activity in the supernatant was attributed to either release of PehA from older cells or inefficient secretion (102). Another polygalacturonase, PehX from *E. chrysanthemi* is also inefficiently secreted into the extracellular milieu, although when it is highly expressed, PehX is secreted by the T2SS, as determined by mutational analysis (122).

Both temporal and extracellular conditions under which the promoters of *peh* and the T2SS operon were most active were different than those of a type III secreted effector, DspE. For the T2SS operon, the sugars glucose and galactose induced the highest fold induction of promoter activity at 48 hpi. The highest T2SS promoter activity was in MM-glc, where at 48 hpi the promoter activity was almost as high as the *dspE* promoter. In contrast, the highest fold induction of the *dspE* promoter was in MM-sor (Table 6). High expression of a type III secreted effector in the presence of sorbitol is not surprising, considering that sorbitol is one of the main transport sugars present in rosaceous plants. The *peh* promoter was most highly induced also in MM-glc, like the T2SS promoter, although its activity was observed to be highest at 72 hpi. The 24 hour delay of activity between the T2SS operon promoter and *peh* promoter may be so that a functional T2SS is present in order to secrete the Peh protein. There are other factors that induce the promoters, however, because activity of the T2S operon promoter, *peh* promoter and *dspE* promoter were highest in 'Gala' leaf tissue (Table 4). Such factors could include the presence of oxygen radicals (60, 128), contact with a solid surface (176) and plant-derived secondary metabolites (38).

In addition, the lack of high induction of the type II operon promoter when exposed to sorbitol or sucrose indicates that type II secretion is not induced by

transported sugars present in the shoots and fruit of the host. The high levels of promoter activity in MM-glu indicate that glucose is potentially an inducer of type II secretion and peh production. Glucose is the sole component of cellulose (β-1,4-glucan) which is second most abundant compound in plants and callose (β-1,3-glucan) which is synthesized at wounding sites. One possibility is that free glucose released from either compound during infection is sensed by *E. amylovora*. Another possibility is that the bacterial enzyme levansucrase releases glucose as a byproduct of forming levan (20). The sensing of increased levansucrase activity could also activate the transcription of the T2SS operon and *peh*.

The Peh protein is secreted in some manner to the extracellular milieu, and is functional when extracted as a supernatant protein of *E. amylovora* (Fig. 6C). The detection of Peh protein in the supernatant of *E. amylovora* could be a result of the overexpression of the protein, much like how overexpression of PehX led to its secretion by the T2SS of *E. chrysanthemi* (122). Since Peh appears to be a lone example of a pectolytic enzyme in *E. amylovora*, its function appears not to be related to the degradation of pectin which is a component of primary cell walls. A potential role for Peh in disease is to degrade polygalacturonic acid which is a major component of the middle lamella (115). A previous study on the ultrastructural changes in apple leaf tissue during *E. amylovora* infection showed a dissolution of cell walls and the middle lamella 48 hours post infection (90). The authors then hypothesized that plant-derived CWDEs were responsible for the effect, in light of the 1976 (226) study that did not detect any pectolytic activity from *E. amylovora*. Since this study has shown that *E. amylovora* does posses a functional polygalacturonase, and that a *peh* deletion mutant is less

aggressive than wild type, is quite possible that Peh from *E. amylovora* plays a role in the previously observed dissolution of the middle lamella (90), and would explain the presence of only a sole polygalacturonase in the genome.

Overall, I have determined a subtle but interesting contribution of an endo-polygalacturonase from *E. amylovora* during infection. Previous studies have focused on the contributions of type III secretion and the effector protein DspE (25, 26, 82) and the exopolysaccharide amylovoran (reviewed in 246) to pathogenicity of *E. amylovora*. There are now many reports of other mechanisms having an effect on virulence, such as the siderophore desferrioxamine (60), levansucrase, sucrose and sorbitol metabolism (29), the multidrug efflux pump AcrAB (38), and regulatory RNA species RsmA and *rsmB* (147). Now that type III secretion has been described in soft rot phytopathogens such as *E. chrysanthemi*, *P. atrosepticum* and *P. carotovorum* (104, 204, 212, 260), it is also plausible that a necrogen such as *E. amylovora* uses type II secretion for a subtle use in virulence, namely the degradation of the contents of the middle lamella to facilitate intercellular movement.

CHAPTER 3

Genetic response of resistance-related genes to *Erwinia amylovora* infection in *Malus* × *domestica* cultivars with varying levels of resistance

ABSTRACT

Suppression subtractive hybridization (SSH) libraries are useful for the identification of both rare and abundantly-expressed genes unique to a tissue type or species. In this work, the SSH method was applied to identify genes in the apple cultivar Red Delicious expressed in response to infection by the fire blight pathogen *Erwinia amylovora*. Over 150 unique expressed sequence tags (ESTs) were identified in a library population by subtracting cDNA from *E. amylovora*-inoculated susceptible cultivar Gala from moderately resistant cultivar Red Delicious. Temporal expression patterns for the corresponding genes were determined using cDNA macroarrays were determined in apple cultivars with varying resistance. Twenty one genes were determined to be upregulated in cultivar Red Delicious, with eight of those ESTs also highly expressed in Red Delicious when relative to Empire or Fuji. The genes identified in this analysis can be used as candidate genes for QTL analysis and marker assisted selection for the development of fire blight resistant apple cultivars.

INTRODUCTION

Domesticated apple (*Malus* × *domestica*) is an important host of the pathogen *Erwinia amylovora*, the causal agent of the devastating disease fire blight. *E. amylovora* can infect apple through nectarthodes during bloom or through wounds during the growing season. Both modes of infection lead to collapse of the vascular tissues, wilt, extrusion of bacterial ooze, necrosis, and in severe cases, eventual death of the tree (241). Chemical control for *E. amylovora* in the United States predominantly consists of applications of the antibiotic streptomycin during bloom and following damaging wind storms or hail (182). The emergence of streptomycin-resistant *E. amylovora* has complicated control options as other chemical and biological alternatives do not provide commercially-acceptable control (157).

Commercial apple cultivars vary widely in inherent susceptibility to fire blight (240); of importance is the observation that most of the popular newer varieties are highly susceptible to this disease. Knowledge of the genetics underlying susceptibility or resistance to fire blight in apple and pear is limited although work is increasing in this area. Cloning and mapping of resistance gene analogs (RGAs) from apple species is one way to identify resistance-related genes. Classic plant disease resistance genes encode proteins with a nucleotide binding site (NBS) and a leucine rich repeat (LRR) domain that participates in protein-protein interactions (13). These resistance genes are now hypothesized to act as guard proteins that monitor changes to other proteins that are the true targets of pathogen effectors (180). RGAs from apple in two studies have been identified by amplification based on degenerate primers in the nucleotide binding site

(NBS) (14, 136). In one study, after the cloning of 27 amplified NBS sequences, 18 of those sequences were successfully converted into molecular markers and the locations of the NBS domain RGAs were mapped (14). The technique of NBS-profiling was proven useful by identification and mapping of 23 RGAs simultaneously in F1 progeny, adding to the number of RGA markers available for mapping (42). In a recent study, expressed sequence tags (ESTs) homologous to RGAs were converted into expressed sequence tagged site (E-STS) markers, facilitating the mapping of ESTs (175).

Groups have also attempted to map quantitative trait loci (QTLs) in apple and pear for fire blight resistance, to aid in breeding efforts. Four QTLs identified for fire blight resistance were mapped in a resistant cultivar of European pear. Two RGA molecular markers, co-localize with QTLs on linkage groups 2b and 4 indicating that nucleotide binding site (NBS) type resistance genes may be involved in fire blight resistance (64). However, two independent QTL analyses of fire blight resistance in apple identified a major QTL that accounts for roughly 35% of the resistance phenotype on linkage group 7 that does not co-localize with any known RGAs (41, 124). This indicates that the main genetic component of fire blight resistance is not likely to be R gene mediated.

There is a real need for the identification of resistance-related genes for fire blight in apple. Although the identification of a major QTL for fire blight resistance in apple is promising, it sheds no light on the function of disease resistance in this species. Analysis of expressed genes in response to *E. amylovora* infection could identify resistance-related genes. Suppression subtractive hybridization (SSH) is a molecular method used to examine differences in genes expressed by resistant or susceptible plant hosts following

pathogen infection. The SSH approach involves the construction of cDNA libraries from both plant hosts following inoculation and then the selective acquisition of ESTs unique to the resistant plant host. In this approach, both low and high abundance transcripts are normalized, making it easier to identify rare mRNA species (62). Use of SSH libraries to identify resistance-related genes has been successful with tomato, wheat, rice, barley, coffee, potato and apple (59, 75, 86, 94, 129, 149, 177, 242, 259). In one SSH study examining an apple cultivar resistant to apple scab, 21 resistance-related genes were found to be constitutively expressed before infection occurs in the apple scab resistant cultivar when compared to the susceptible cultivar (59). Another SSH library created from susceptible cultivar Gala subtracted mock-inoculated cDNA from *E. amylovora*-inoculated cDNA (30). This library was the first report of global expression profiling of an apple cultivar during fire blight infection. In this study, genes identified, while interesting from a physiological response, are not likely to be associated with resistance since cultivar Gala is susceptible (240).

The objective of our study was to identify genes from the apple cultivar Red Delicious that are associated with the phenotype of moderate resistance to fire blight exhibited by this cultivar. Since no genes for resistance or tolerance to fire blight have been cloned, a SSH library was designed subtracting *E. amylovora*-inoculated susceptible Gala cDNA from *E. amylovora*-inoculated moderately resistant Red Delicious cDNA. The resistance-related ESTs were analyzed for temporal expression patterns over five time points during infection. Our hypothesis is that ESTs recovered that are unique or highly expressed in the Red Delicious transcriptome are related to resistance to this pathogen. In addition, two cultivars with Red Delicious as a parent and possessing

varying resistance to *E. amylovora* were used to screen resistance-related genes in order to identify genes that are expressed similar to and different from the resistant parent Red Delicious.

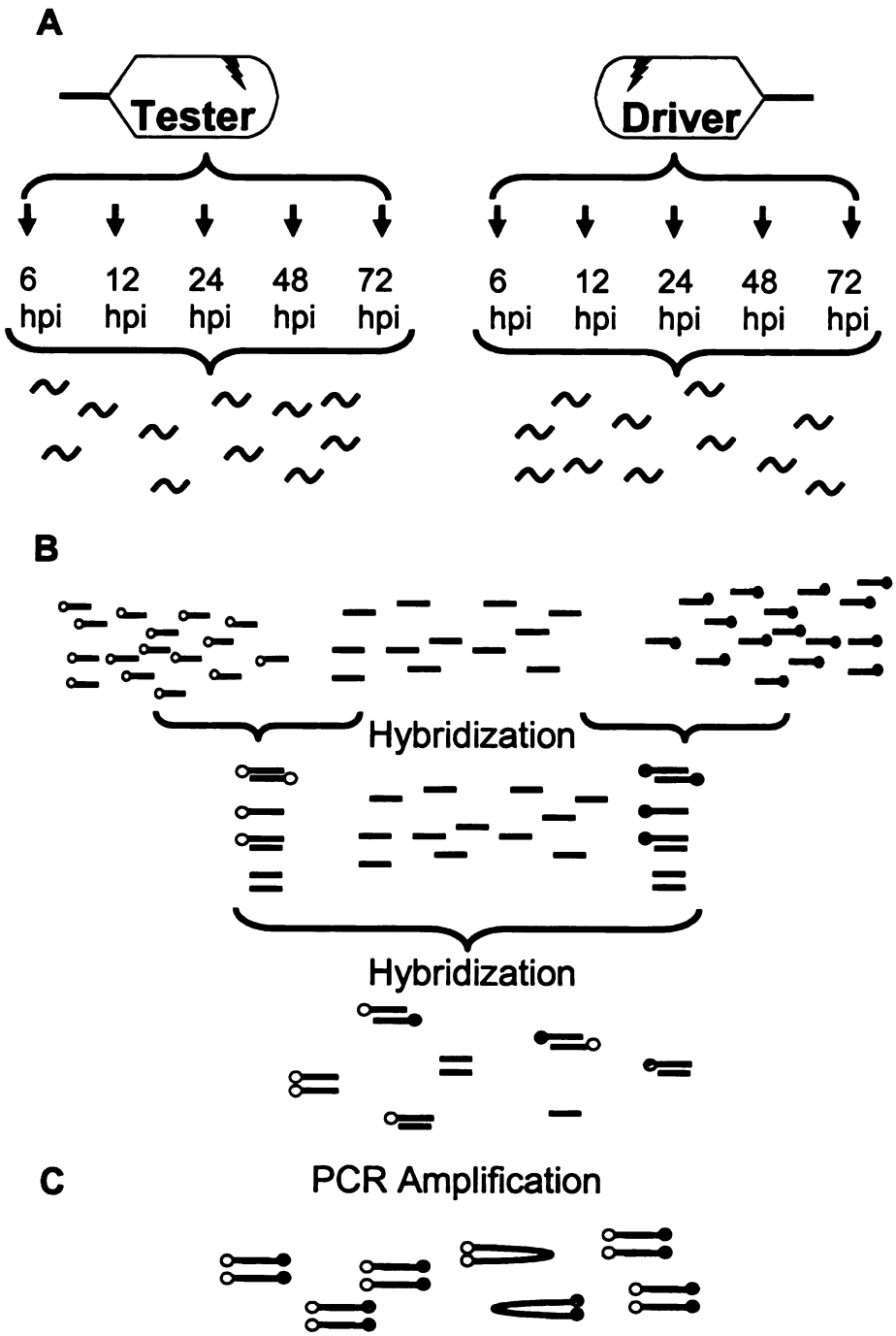
MATERIALS AND METHODS

Bacterial strains and plant material. *E. amylovora* strain Ea273 was used for inoculation of apple tissue in this study. *E. amylovora* was grown on Luria-Bertani (LB) medium at 28°C. The apple cultivars Gale Gala, a sport of Royal Gala and Scarlet Spur, a sport of Red Delicious were used in the initial SSH library production. For cDNA macroarray analysis the following cultivars were used: Schlect Spur (sport of Red Delicious) on G.30 rootstock, Royal Empire (a sport of Empire) on EMLA.26 rootstock and September Wonder Fuji (a whole tree mutation of Red Fuji) on Bud.9 rootstock (C&O Nursery, Wenatchee, WA). All tree stems were 0.95 cm diameter and maintained in 10.2 cm by 35.6 cm Tall Treepots (Stewe and Sons, Inc., Corvalis, OR). Trees were maintained in the greenhouse until they broke dormancy, after which they were moved to a growth chamber (99% relative humidity, 25°C) for inoculation experiments.

Plant inoculation and tissue harvesting. All trees used were inoculated by cutting the first three leaves across the midvein using sterile scissors that were dipped into a 5×10^6 CFU/ml suspension of *E. amylovora* strain Ea273 in 0.5× PBS buffer. Inoculated tissue was harvested 0, 6, 12, 24, 48 and 72 hours post inoculation and flash frozen in liquid nitrogen. Leaf tissue for each time point was harvested from three trees. Tissue was maintained at -80°C until RNA extraction.

Nucleic acid isolation and analysis. Apple genomic DNA was extracted using a CTAB-based method previously described by Tsai et al. (244). The quality of the DNA was

Figure 9: Representation of the suppression subtractive hybridization library construction. (A) Apple leaves inoculated with Ea273 are deemed the "tester", leaves mock-inoculated with 0.5 × PBS are deemed the "driver" in library construction. Tissue is harvested from 6, 12, 24, 48 and 72 hpi and total RNA is extracted from each time point separately. For isolation of polyA$^+$ RNA, all time points are pooled together for first and second strand reverse transcription of tester and driver cDNA. (B) Tester and driver cDNA are cut with RsaI restriction enzyme and digested tester cDNA is ligated to two adaptors provided with the kit (light and dark circles). The tester cDNA is then hybridized to the driver cDNA twice. The first hybridization is between each adapter pool and driver cDNA, for the second hybridization, the adapter populations are combined along with fresh driver cDNA. (C) PCR amplification of the second hybridization products uses primers specific to the adapters. Only the cDNA that hybridized between the two populations of adapters are amplified. This in effect normalizes the representation of each cDNA in the population so that rare cDNAs are just as prevalent in the PCR product.


A
Tester
Driver
6 hpi
12 hpi
24 hpi
48 hpi
72 hpi
6 hpi
12 hpi
24 hpi
48 hpi
72 hpi
B
Hybridization
Hybridization
C
PCR Amplification

assessed on a 0.8% agarose gel using sodium-borate electrophoresis buffer and 1/10000 volume of GelStar for visualization of DNA (Cambrex Bio Science Rockland, Inc., Rockland, ME). Total RNA was extracted from ~0.3 g of leaf tissue using the RNAqueous Midi kit (Ambion Inc., Austin, TX) according to the manufacturer's instructions with the following minor modifications. Tissue was disrupted with liquid nitrogen and by grinding using a mortar and pestle. Eighteen volumes of lysis buffer was added to the leaf tissue and Plant RNA isolation aid (Ambion Inc.) was added at 1/10 volume of the lysis buffer. After the addition of the lysis buffer, samples were sonicated three times at 40% power for 10 seconds each (Branson Sonifier 250a, Branson Inc., Danbuy, CT). For elution of total RNA, three times 500 μl of boiling elution buffer was pooled together for the first elution and two times 500 μl of boiling elution buffer was pooled together for the second elution. The quality of the RNA was assessed by loading denatured RNA on a 1.2% formaldehyde agarose gel (217). RNA precipitations used an equal volume of isopropanol, 1/10 volume ammonium acetate and 1 μl (20mg/ml) glycogen (Roche Diagnostics GmbH, Mannheim, Germany) at -20°C overnight.

Poly(A)$^+$ RNA was isolated using the PolyATtract mRNA isolation system III (Promega, Madison, WI) according to the manufacturer's instructions with 500 μg of total RNA from the combined time points of 6, 12, 24, 48 and 72 hpi. Isolated poly(A)$^+$ RNA was precipitated as described above, and resuspended in 5 μl of elution buffer (Ambion Inc.).

Suppression subtractive hybridization library construction and differential screening. A suppression subtractive hybridization library was created based on the method of Diatchenko et al. (62) using the BD PCR-Select cDNA subtraction kit (BD

Biosciences – Clontech, Palo Alto, CA). The cDNA pool was generated by reverse transcription using mRNA pooled from samples collected at 6, 12, 24, 48 and 72 hours post infection (Fig. 1). The initial quantity of polyA$^+$ RNA for each treatment (Red Delicious inoculated, Gala inoculated) was 1500 ng. SSH library construction followed the manufacturer's instructions, except that a primer set that amplify apple actin (see Table 8) were used for checking efficiency of adapter ligation; the first PCR amplification after hybridization was lengthened to 30 cycles using 66°C for the annealing temperature and the second PCR amplification was increased to 11 cycles. Clonetech Advantage cDNA polymerase (BD Biosciences-Clontech,) was used for PCR amplification of the cDNA after hybridization. Two μl of the secondary PCR was cloned using the pGEM-T easy T/A cloning vector (Promega,).

A total of 960 clones were selected and arrayed into LB freezing buffer in 96-well microplates. In addition, colonies were also printed by hand onto nitrocellulose membranes using a 48-pin tool. These membranes were denatured in a solution of 1.5M NaCl, 0.5M NaOH, neutralized in 1.5M NaCl, 1.5M Tris-Cl pH7.6 and washed in 2×SSC for 5 minutes at each step (217). DNA was fixed to the membranes by UV crosslinking, after which the membranes were stored at -20°C. For differential screening, 50 ng of probe was prepared using a random priming kit (Invitrogen, Carlsbad, CA) and labeled with ^{32}P-dATP. Hybridization and post-hybridization washes were done using standard techniques (47). After washing, membranes were exposed to BioMax MS film for three hours (Eastman Kodak Co., New Haven, CT). Selected clones were then sent to the Research Technology Support Facility (RTSF) and Michigan State University for sequencing and Blastx analysis of the EST sequences (7).

cDNA macroarray construction and hybridization. Inserts from SSH library clones were amplified using M13 forward and M13 reverse primer binding sites on the pGEMT Easy cloning vectors, and ESTs were printed onto nylon membranes using a Biomek 2000 robot (Beckman Coulter, Inc., Fullerton, CA) in a quadruplicate, square pattern. The membranes were denatured as described previously after printing, UV crosslinked and stored at -20°C.

Total RNA from the apple cultivars Red Delicious, Empire and Fuji isolated at 0, 6, 12, 24 and 48 hours post inoculation was used for ^{32}P-labelled first strand cDNA synthesis using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Carlsbad, CA). Hybridizations were performed as described above, except that hybridization was at 68°C. Hybridized membranes were exposed to phosphorimaging cassettes (Bio-Rad, Richmond, CA) and scanned using the Personal Molecular Imager® (Bio-Rad). Quantity One software (v 4.6.1, Bio-Rad) was used to determine signal intensity as volume units, and all clones on the cDNA macroarray were normalized to the volume units of actin and reported as normalized volumes (nVol). Hybridizations were determined from two independent hybridizations to cDNA macroarrays representing inoculations from two separate experiments for Red Delicious and one hybridization representing one experiment for Empire and Fuji.

Semi quantitative reverse transcription-PCR. Total RNA (10 μg) from Red Delicious, Empire and Fuji at 6, 12, 24 and 48 hours post inoculation were treated with DNA-*free* (Ambion, Inc.) according to manufacturers instructions for rigorous DNase treatment.

DNase treated total RNA (4 μg) was reverse transcribed using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). Primers for semi quantitative RT-PCR (Table 8) were designed using Lasergene software (v 6.0, DnaStar, Madison, WI). All genes were amplified for 27 cycles of 94°C for 30 sec, 59°C for 30 sec and 72°C for 45 sec. PCR products were run on a 1.5% agarose gel with 1/10,000 volume GelStar (Cambrex Bio Science Rockland, Inc., Rockland, ME). Gels were directly read by a Flour-S MultiImager (Bio-Rad, Richmond, CA). Normalized volume units are calculated as described above.

TABLE 8. Oligonucleotide primers used in this study.

Primer Name	Primer Sequence (5' to 3')
Actin1 Forward	GACCTTGCWGGTCGTG
Actin1 Reverse	GATGGTTGGAAGAGGACTTCTGG
Primer 1[a]	CTAATACGACTCACTATAGGGC
Nested primer 1[a]	TCGAGCGGCCGCCCGGGCAGGT
Nested primer 2R[a]	AGCGTGGTCGCGGCCGAGGT
14-3-3μ Forward	CATACGAGACTGCCACCACCACTG
14-3-3μ Reverse	ATGAACGCCTGAATTTTGTCTAAC
Hsf8 Forward	CAGTGAGGCCAAAAGAGAAGGAAGAGTCGG
Hsf8 Reverse	GCCCACTCAGTATCTGCTTGTGCTGCTTC
PIP2-1 Forward	CTACAGTTCCACCGCCCTTCAAATTTCTCC
PIP2-1 Reverse	CAACCCTGCTAGGAGTTTTGGAGCTGCTGTC
PR3 Forward	CAGCTTAAACGATTCCCTCATGAGGAATCC
PR3 Reverse	GAGTGGCGGAGCTGTCCCTCCAGCTTGCTG
Cal1 Forward	GCCAAACGCTCTCAACGATGGCCGATCAGC
Cal1 Reverse	GTTGATCTGCCCATCACCATCCACATCAGC
PP2C Forward	CTCATACTTGCATCTGATGGGCTGTGG
PP2C Reverse	GCAGGTACAAGCTGACGGTTCACCGGAGTTC
Actin RT Forward	CCTAAGGCCAACAGAGAAAAGATG
Actin RT Reverse	AGCGGATGCAAGGGTGGAC

[a] Primers provided in Clontech PR-Select cDNA subtraction kit (BD Bioscience-Clontech, Palo Alto, CA)

RESULTS

Creation of a suppression subtractive hybridization library representing resistance-associated genes specific to Red Delicious

To identify genes that are uniquely expressed in Red Delicious during *E. amylovora* infection we subtracted cDNA from *E. amylovora*-infected cultivar Gala from cDNA from *E. amylovora*-infected cultivar Red Delicious at 6, 12, 24, 48 and 72 hours post infection. These time points were selected to correspond with the oxidative burst of apple in response to *E. amylovora* infection (249). Genes responding to wounding by the inoculation are expected to be genes expressed similarly in both cultivars and would be subtracted and not recovered (47).

960 clones from the SSH library were analyzed further. These clones were subjected to differential screening by arraying and hybridization. Clones that hybridized to the subtracted Red Delicious cDNA and hybridized weakly or not at all to unsubtracted Gala cDNA were selected for sequencing and further study. Thus, the clones selected by differential screening were more strongly expressed in Red Delicious relative to Gala following infection with *E. amylovora* (Figure 10A and B). Following this hybridization screening, 265 clones were selected for sequencing and gene identification, of these 183 representing had significant matches in GenBank, of which 168 unique ESTs were assigned and divided into functional classification categories (214). Overall, the ESTs were separated into 17 categories including subcellular localization (7.5%), metabolism (5.8%), signal transduction (4.0%), proteins with binding function (4.9%) and cell

TABLE 9. Classification of ESTs recovered from an SSH experiment subtracting cDNA of apple cultivar Gala from Red Delicious following inoculation with *E. amylovora*. The EST sequence classification is based on homology[a] to *Arabidopsis* proteins.

Protein Functional Classification	Subtracted Population (%)	Apple NRs[b] (%)
01 Metabolism	5.8	16.2
02 Energy	3.1	3.2
10 Cell Cycle and DNA Processing	1.3	2.7
11 Transcription	1.8	4.9
12 Protein Synthesis	2.7	3.0
14 Protein Fate	3.1	6.8
16 Protein with Binding Function	4.9	3.1
18 Protein Activity Regulation	0.9	0.04
20 Cellular Transport	4.0	2.3
30 Signal Transduction Mechanism	4.0	5.9
32 Cell Rescue, Defense and Virulence	2.2	3.7
34 Interaction with Cellular Environment	2.2	1.8
40 Cell Fate	0.9	3.4
41 Development	1.8	0.9
42 Biogenesis of Cellular Components	2.7	2.9
70 Subcellular Localization	7.5	19.4
99 Unclassified Proteins	51.4	9.4

[a] Functional homology of ESTs determined using Blastx algorithm

[b] Apple NRs percentages are as reported in Newcombe *et al.* (179)

defense (2.2%) (Table 9). The largest category was unknown function (51%), which contained ESTs with translated sequence homologous to proteins that were not functionally characterized. This is in contrast to the classification of apple ESTs identified by Newcombe *et al.* (179), that only identified 9.4% unknowns from their EST libraries (Table 9). This could indicate the presence of novel resistance response genes in apple that are uncharacterized as of yet.

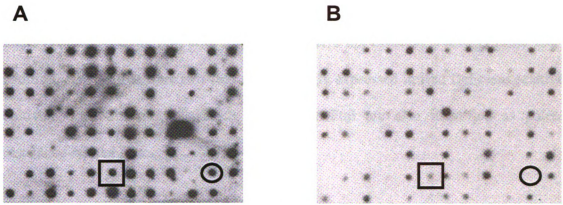


Figure 10: Representative radiographs of differentially screened EST clones. (A) Red Delicious SSH clones hybridized to Red Delicious SSH probe. (B) Same Red Delicious SSH clones hybridized to Gala probe. An example of a clone that was selected for hybridization to the Red Delicious SSH probe but not to the Gala probe is circled. An example of a clone that hybridized strongly to the Red Delicious SSH probe but weakly to the Gala probe is enclosed by a box.

Expression analysis of ESTs from Red Delicious in cultivars Red Delicious, Empire and Fuji

For further expression analysis of the 183 Red Delicious genes, we arrayed cDNA macroarrays and hybridized the arrays to labeled first strand cDNA from 0, 6, 12, 24 and 48 hours post infection. Total RNA from two independent experiments with Red Delicious and one experiment with Fuji and Empire was labeled and hybridized to the cDNA macroarrays (Figure 11). Total RNA from only one experiment with Fuji and Empire was used to identify potential gene candidates that would be used for further analysis using semi quantitative RT-PCR. In theory, ESTs that are expressed at higher levels in Red Delicious over less resistant cultivars are potentially involved in the resistance response. Hybridization signals from the cDNA macroarray hybridization

experiments were normalized to an internal control gene (actin) on the macroarray. cDNAs selected for further analyses were induced over two-fold at either 6, 12 24 or 48 hours post inoculation (hpi) with respect to the zero hpi expression levels (Table 10). In addition, cDNAs that were two-fold more strongly expressed in Red Delicious relative to cultivars with differing resistance, Empire or Fuji are also identified as potential resistance-related genes.

Induced expression of Red Delicious ESTs

When gene expression was quantified using cDNA macroarrays, 21 Red Delicious ESTs were induced two-fold or higher over basal levels during infection (Table 10). The peak of fold induction for the majority of genes was at 12 hpi, after which the levels returned to near basal levels (Table 10). Rapid accumulation of transcripts involved in the resistance response was also observed by Venisse *et al.*, in another resistant cultivar, Evereste (249). Almost a third of the upregulated ESTs are theoretical proteins with no homology to proteins of known function (Table 10).

TABLE 10. Red Delicious ESTs with two-fold or higher induction after inoculation with *E. amylovora.*

EST	Blastx Best Hit[a]	Fold Induction[b]			
		6 hpi[c]	12 hpi	24 hpi	48 hpi
RGG1A10	Oxoglutarate dehydrogenase	1.6	**2.0**	0.8	0.9
RGG1B07	Expressed protein	1.3	**2.0**	0.7	0.8
RGG1B10	Unknown protein	1.5	**2.0**	0.8	0.9
RGG1C08	Plasma membrane intrinsic protein 2-1	1.6	**7.9**	0.7	1.0
RGG1D11	Heat shock factor protein 8	1.6	**2.8**	0.8	0.8
RGG1E11	Myosin heavy chain	**3.8**	1.3	0.7	0.9
RGG2B04	Protein kinase family protein	1.6	**2.0**	0.8	1.1
RGG2B10	Unknown protein	**4.9**	**3.5**	0.9	0.7
RGG2C09	Unnamed protein product	**2.0**	**2.6**	0.8	0.8
RGG2D06	Salicylic acid carboxyl methyltransferase	**2.1**	1.2	1.0	1.0
RGG2D08	Cell differentiation protein, RCD1	**2.4**	1.5	1.0	0.9
RGG2D09	Cell differentiation protein, RCD1	1.7	**2.6**	0.8	0.9
RGG2D10	14-3-3 protein	**2.1**	**8.0**	0.8	0.9
RGG2E02	Expressed protein	1.3	1.2	**2.0**	1.0
RGG2G03	Glycyl tRNA synthetase	**2.2**	1.0	0.7	1.0
RGG3A07	Curly leaf protein 1 (polycomb-group)	1.3	**2.4**	1.0	1.0
RGG3B06	Protein phosphatase 2C homolog	**2.3**	1.6	1.3	1.0
RGG3D04	60S ribosomal protein L18	1.6	**2.1**	0.7	1.0
RGG3E07	Expressed protein	1.2	**2.5**	0.6	0.8
RGG3F09	Pod-specific dehydrogenase SAC25	1.8	**2.4**	0.8	0.9
RGG3F10	Calmodulin 1	1.5	**3.8**	0.9	0.9

[a] Blastx best hit are homologous proteins to putative translations of the ESTs ($E > 1 \times 10^{-05}$)

[b] Fold induction is reported as nVol at 6, 12, 24 and 48 hpi over nVol at 0 hpi for Red Delicious

[c] hpi, hours post inoculation

Of the remainder, none are classic NBS-domain type resistance genes. This is not surprising, since a gene-for-gene interaction between apple and *E. amylovora* has not been discovered as of yet. Four identified genes corresponded to proteins involved in modification of protein activity: a protein kinase family protein (RGG2B04), a 14-3-3 protein (RGG2D10), protein phosphatase 2C (RGG3B06) and calmodulin 1 (RGG3F10), and thus are potentially involved in the signaling response to infection. The protein phosphatase 2C family is involved in diverse aspects of cellular signaling including abscisic acid (ABA), receptor-like kinase and mitogen-activated protein kinase signaling cascades (211).

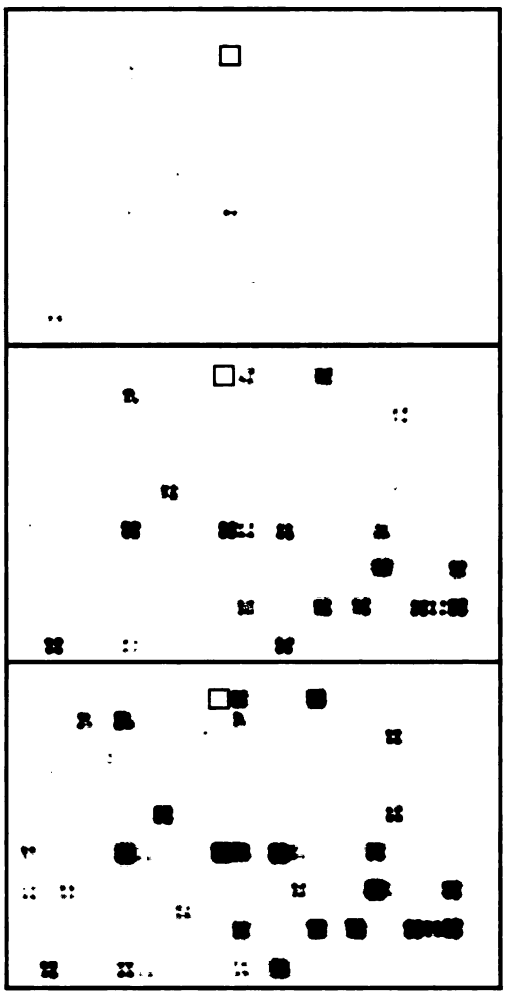


Figure 11: Representative cDNA nylon macroarray hybridization. Top membrane is hybridized to labeled, inoculated Red Delicious cDNA 6 hours post inoculation. Middle membrane is hybridized to labeled, inoculated Red Delicious cDNA at 12 hours post inoculation. Bottom membrane is hybridized to labeled, inoculated Red Delicious cDNA at 24 hours post inoculation. The EST identified as a Mal d 1m protein is enclosed by the square.

14-3-3 proteins constitute a family of proteins that include up to 15 isoforms which modulate different signaling pathways by protein-protein interactions (208, 257). Calmodulins are well studied proteins involved in calcium-monitoring signaling pathways, which are often involved in abiotic and biotic stress signal transduction pathways (6).

Three ESTs were homologous to other stress-related genes such as the aquaporin plasma membrane intrinsic protein 2-1 (RGG1C08), heat shock factor 8 (RGG1D11) and salicylic acid carboxyl methyltransferase (RGG2D06). Aquaporins are a diverse family of proteins that facilitate the movement of water through lipid membranes in cells. There are four subfamilies of aquaporins, and the plasma membrane intrinsic proteins (PIPs) facilitate the movement of water and neutral solutes through the plasma membrane. The PIPs are further classified into two groups, the PIP1 and PIP2 groups and the PIP identified in this study is of the latter group, which is are more involved in the movement of water to maintain turgor pressure (46). Heat stress transcription factors (Hsfs) are involved in binding to promoter areas of inducible genes either activating or repressing them; this EST from the subtracted library has highest homology to an Hsf8-like protein from *Arabidopsis*. Originally, *hsf8* was identified in *Lycopersicon peruvianum* as being a strong activator of transcription during heat stress (164, 185). Salicylic acid carboxyl methyltransferase (SAMT) methylates available salicylic acid in plant tissues to form the volatile derivative, methyl salicylic acid (MeSA) which has previously been demonstrated to act as an airborne signal during pathogen infection by tobacco plants (229).

Other genes that were upregulated upon infection were involved in protein translation, organ development and cytoskeletal movement. Two ESTs are homologous to protein translation related genes, glycyl tRNA synthetase (RGG2G03) and the 60S L18 ribosomal protein (RGG3D04). The polycomb-group curly leaf protein (RGG3A07) (CLF) is a repressor of homeotic genes that determine cell fate (91). Another potential gene involved in cellular fate is *rcd1* (RGG2D09) which is involved in nitrogen

starvation induced sexual development of the yeast *Schizosaccharomyces pombe* (186). Its role in plant cellular development is still unknown, even though rcd1 homologues are present in plant genomes.

Energy and metabolism genes were also among the upregulated genes such as the tricarboxylic-acid pathway protein oxoglutarate dehydrogenase (RGG1A10) and a pod-specific dehydrogenase (RGG3F09). Oxoglutarate dehydrogenase accumulates upon low sugar conditions, including darkness and senescence and it is important for respiration by catabolism of branched-chain amino acids (208). Short chain dehydrogenase (SDR) proteins, such as the pod-specific dehydrogenase (SAC25) are a part of an extremely large enzyme family, with over 1000 types of enzymes (194). Homology of RGG3F9 to the *Arabidopsis* SAC25 gene only determines that a SDR motif is present; the function of this putative protein is unknown.

Comparison of expression levels for Red Delicious ESTs versus Empire and Fuji

The apple cultivar Empire is the progeny of a cross between McIntosh and Red Delicious, and is intermediate in its resistance to *E. amylovora* (240). When comparing the level of expression of the subtracted library ESTs from *E. amylovora*-inoculated Red Delicious with respect to *E. amylovora*-inoculated Empire transcripts, genes that are highly expressed in Red Delicious stand out as potential resistance-related genes. For this study, genes that were expressed two-fold or more in Red Delicious versus Empire at either 6, 12, 24 or 48 hours hpi are listed in Table 11. Most genes listed in Table 11 are also noted as being inducible in Table 10, with the exception of three added genes: a fibrillarin-like (RGG1A08), putative protein kinase (RGG1C09), and an unknown protein

TABLE 11. ESTs that are induced to higher levels in apple cultivar Red Delicious versus Empire following inoculation with *E. amylovora*.

EST	Blastx Best Hit[a]	Fold Induction[b]			
		6 hpi[c]	12 hpi	24 hpi	48 hpi
RGG1A08	Fibrillarin 1	1.1	**2.0**	0.4	0.4
RGG1A10	Oxoglutarate dehydrogenase	1.1	**2.3**	0.5	0.5
RGG1B07	Expressed protein	1.2	**2.3**	0.3	0.7
RGG1C08	Plasma membrane intrinsic protein 2-1	1.3	**10.8**	0.5	0.5
RGG1C09	Putative protein kinase	0.8	**2.0**	0.7	0.6
RGG1D11	Heat shock factor protein 8	1.0	**2.0**	0.3	0.4
RGG2B10	Unknown protein	**2.7**	1.6	0.4	0.5
RGG2C09	Unnamed protein product	1.5	**3.1**	0.4	0.7
RGG2D09	Cell differentiation protein, RCD1	1.4	**3.0**	0.5	0.6
RGG2D10	14-3-3 protein D	1.3	**9.4**	0.5	0.5
RGG3A09	At3g57420	**2.7**	1.5	0.8	1.0
RGG3A07	Curly leaf protein 1 (polycomb-group)	0.9	**2.0**	0.6	0.5
RGG3D04	60S ribosomal protein L18	1.0	**2.3**	0.5	0.5
RGG3E07	Expressed protein	1.0	**2.5**	0.2	0.6
RGG3F09	Pod-specific dehydrogenase SAC25	0.8	**2.6**	0.5	0.4
RGG3F10	Calmodulin 1	1.0	**2.7**	0.4	0.5

[a] Blastx best hit are homologous proteins to putative translations of the ESTs ($E > 1 \times 10^{-05}$)

[b] Fold induction is reported as Empire nVol at 6, 12, 24 or 48 hpi over Red Delicious nVol at 6, 12, 24 or 48 hpi

[c] hpi, hours post inoculation

(RGG3A09). Fold induction of the majority of Red Delicious transcripts were highest with respect to Empire transcripts at 12 hpi with expression sharply reduced at 24 hpi (Table 11). Two unknown genes (RGG2B10 and RGG3A09) were highly expressed in Red Delicious versus Empire at six hpi indicating that the peak expression of these ESTs mostly occurred at 24 hpi for Empire, suggesting a delayed response to pathogen infection (Table 11).

The apple cultivar Fuji is the progeny of a cross between Ralls Janet and Delicious; however, the severity of fire blight on inoculated trees is similar to that of the susceptible cultivar Gala (240). One could speculate that the susceptibility was inherited from Ralls Janet, or that less resistance response genes were passed on from both parents.

TABLE 12. ESTs that are induced to higher levels in apple cultivar Red Delicious versus Fuji following inoculation with *E. amylovora*.

EST	Blastx Best Hit[a]	Fold Induction[b]			
		6 hpi[c]	12 hpi	24 hpi	48 hpi
RGG1A03	Unknown protein	1.7	**2.1**	1.2	0.5
RGG1A06	Unknown protein	1.0	**2.0**	0.7	0.6
RGG1B06	Hypothetical protein	1.4	**2.1**	0.9	0.9
RGG1B07	Expressed protein	1.6	**3.5**	1.4	1.0
RGG1C06	Plasma membrane intrinsic protein 2-2	1.2	**2.4**	1.7	0.6
RGG1C08	Plasma membrane intrinsic protein 2-1	1.8	**7.8**	0.7	0.8
RGG1D11	Heat shock factor protein 8	1.2	**2.3**	0.7	0.6
RGG1F07	Unknown protein	1.9	**2.3**	1.3	1.8
RGG1H08	At2g18850	1.0	0.7	**2.0**	1.2
RGG2C06	LHCII type III chlorophyll a/b binding protein	1.4	**2.5**	1.0	1.5
RGG2C09	Unnamed protein product	1.8	**2.8**	0.8	1.0
RGG2D09	Cell differentiation protein, RCD1	1.4	**2.1**	0.8	0.6
RGG2D10	14-3-3 protein D	1.7	**6.0**	0.7	0.5
RGG2E02	Unknown protein	0.9	1.0	**2.4**	0.5
RGG2G11	40S ribosomal protein S9	1.0	**2.7**	1.8	1.8
RGG2H07	GSK-3-like protein	1.3	0.6	**3.0**	1.1
RGG3A07	Curly leaf protein 1 (polycomb-group)	1.2	**2.1**	1.0	0.9
RGG3A09	At3g57420	**3.3**	1.6	1.4	1.7
RGG3B06	Protein phosphatase 2C	**2.3**	1.3	1.4	0.7
RGG3C09	Unknown protein	1.3	**2.3**	0.9	0.9
RGG3D04	60S ribosomal protein L18	1.3	**2.0**	0.6	0.6
RGG3D05	Mal d 1m	1.2	**3.0**	1.4	1.0
RGG3E07	Expressed protein	1.5	**4.0**	1.4	0.8
RGG3F10	Calmodulin 1	1.1	**3.1**	0.8	0.6

[a] Blastx best hit are homologous proteins to putative translations of the ESTs ($E > 1 \times 10^{-05}$)

[b] Fold induction is reported as Fuji nVol at 6, 12, 24 or 48 hpi over Red Delicious nVol at 6, 12, 24 or 48 hpi

[c] hpi, hours post inoculation

Genes that are highly expressed in Red Delicious and Empire when compared to expression levels in Fuji are very likely to be associated with a resistance genotype, since Fuji is susceptible to *E. amylovora* infection. Of 24 genes that are highly expressed in Red Delicious compared to Fuji, nine are not listed in Table 10 as being highly induced in Red Delicious with respect to zero hpi (Table 12). Six of the nine genes are of unknown function (RGG1A03, RGG1A06, RGG1B06, RGG1F07, RGG3A09, RGG3C09) and the remaining three are a light harvesting complex II (LHC II) type III

chlorophyll a/b binding protein (RGG2C06), a glycogen synthase kinase 3 protein (RGG2H07) and a Mal d1-like protein (RGG3D05). Glycogen synthase kinase 3 (GSK-3) proteins are present in both animals and plants. There are multiple GSK-3 homologues in plant genomes, and these homologues have been demonstrated to be involved in NaCl stress signaling, wounding response, flower development and brassinosteroid signaling pathways (120). The Mal d 1 (PR-10) protein family in apple is thought to have at least 15 different isoforms, with 12 of them represented in Royal Gala. The Mal d 1 EST from this subtractive library is most homologous to Mal d 1m, which was only observed in ripening fruit EST libraries from Royal Gala (23).

Overall, comparison of the ESTs that were upregulated in Red Delicious to basal levels of transcription or to Empire and Fuji following inoculation (Tables 10, 11 and 12) yielded a list of three ESTs that are about equally expressed in Red Delicious in comparison to expression in the other cultivars. This includes two unknown proteins (RGG1B10 and RGG2B10), protein kinase family protein, myosin heavy chain, SAMT and the glycyl tRNA synthetase. These genes are unlikely to have important roles in the resistance response. However, the expression of *SAMT* was 1.9 times higher in Red Delicious than Fuji at six hpi, therefore, it may still have a role in *E. amylovora* resistance. Although these genes may have an impact overall on disease resistance, they are most likely not involved with modulation of a complete resistance phenotype. Two genes that were highly expressed in Red Delicious when compared to Empire, oxoglutarate dehydrogenase and fibrillarin 1 were not highly expressed relative to transcript levels in Fuji and are also suspect in their role in disease resistance.

Constitutively expressed Red Delicious ESTs in Red Delicious, Empire and Fuji

Other genes that may be of interest are ones that were determined to be constitutively expressed in Red Delicious, where gene expression did not reach over two-fold relative levels compared to basal expression at zero hpi (Table 13). These ESTs were selected based on nVol intensity of 2.0 or higher. Twenty ESTs fit this criterion, five of which were also noted to be more highly expressed in Red Delicious versus Fuji by a margin of two-fold or more. Another plasma membrane intrinsic protein detected in the subtractive library is expressed constitutively, PIP 2-2. Four genes listed are involved in metabolism, including a member of the glycine decarboxylase complex, H-protein (RGG1G01) which acts as a methyl carrier for the conversion of glycine to serine (66). Other metabolic genes include triosephosphate isomerase (RGG2D07) and the oxygen evolving complex 33kDa subunit (RGG3H07). The oxygen evolving complex 33kDa subunit (OEC 33) is interesting because it has been implicated in basal resistance to viruses in *Nicotiana benthamiana* (192). Three genes are involved in signaling cascades such as an inositol 1, 3, 4-trisphosphate 5/6-kinase protein (RGG2H03) and transparent testa glabra 1-like (TTG-1-like, RGG3B01). *Arabidopsis* inositol 1, 3, 4-trisphosphate 5/6-kinase (5/6 kinase) was demonstrated to interact with the COP9 signalosome, affecting growth under red light (199). The TTG-1 protein is a WD-40 repeat protein that is implicated in trichome development and anthocyanin biosynthesis regulation (250). A WD-40 repeat protein has also been reported as being upregulated in potato leaves using microarray analysis following infection with *Phytophthora infestans* (242). Five listed genes are involved in protein synthesis and fate including: eIF-2 (RGG2H04), signal peptidase complex 25 kDa subunit (SPC 25, RGG2C10), Rer1b (RGG3C03) and the

TABLE 13. Constitutively expressed ESTs of apple cultivar Red Delicious and analysis of expression of these genes in the cultivars Empire and Fuji.

EST	Blastx Best Hit[a]	Red Delicious nVol[b]					Empire nVol					Fuji nVol				
		0[c]	6	12	24	48	0	6	12	24	48	0	6	12	24	48
RGG1B07	Expressed protein	6.2	8.0	12.3	4.2	5.1	3.0	6.9	5.4	14.3	7.0	1.8	5.1	3.6	3.0	5.4
RGG1C06	Plasma membrane intrinsic protein 2-2	8.8	10.6	14.7	5.2	6.9	5.5	10.2	12.0	21.9	12.5	1.9	8.5	6.1	3.0	11.0
RGG1G01	H-protein	4.0	5.5	6.9	2.5	3.0	5.1	6.4	8.1	9.3	8.5	1.3	8.3	12.8	6.6	17.0
RGG1H08	Expressed protein	2.1	2.2	1.8	2.1	2.5	1.6	1.7	3.2	3.1	3.5	1.3	2.3	2.7	1.0	2.1
RGG1H09	Microtubule end binding 1-like, EB-1-like	3.0	3.1	2.0	3.3	3.5	3.1	2.6	5.6	4.3	4.4	0.8	2.5	3.6	1.8	3.3
RGG2A09	Unknown protein	2.3	2.9	2.4	2.9	2.9	1.9	2.3	2.6	3.3	3.2	1.4	2.6	2.4	1.5	2.8
RGG2C06	LHCII type III chlorophyll a/b binding protein	10.0	12.6	14.4	5.7	11.8	6.0	11.8	9.7	15.1	11.2	2.3	8.8	5.7	5.8	7.8
RGG2C10	Signal peptidase complex, 25 kDa subunit	9.4	13.9	10.9	5.0	10.6	5.2	9.2	11.1	19.9	12.7	2.5	6.4	6.5	3.7	10.5
RGG2D07	Triosephosphate isomerase	2.0	2.9	2.7	1.7	2.1	1.6	3.5	2.6	4.1	3.5	1.2	2.2	2.2	1.7	3.6
RGG2G07	Hypothetical protein	2.6	2.8	2.3	2.5	3.0	2.0	3.8	3.8	4.6	4.4	1.3	3.2	3.0	3.1	3.4
RGG2G11	40S ribosomal protein S9	3.1	3.5	4.9	3.1	3.5	1.8	4.1	4.9	6.9	4.5	1.3	3.4	1.8	1.7	1.9
RGG2H03	Inositol 1,3,4-trisphosphate 5/6-kinase protein	2.3	3.2	4.1	1.8	2.2	2.8	2.9	3.6	3.7	4.4	1.3	3.9	4.6	2.6	6.6
RGG2H04	eIF-2 family protein	3.0	5.0	3.2	2.2	3.0	3.6	4.2	6.6	5.5	6.3	1.4	5.6	9.1	4.5	11.7
RGG2H05	Hypothetical protein	2.3	3.4	2.5	2.2	2.8	2.0	2.0	3.3	2.5	3.7	1.4	2.9	3.0	1.6	2.6
RGG2H07	GSK-3-like protein, MsK4	2.2	2.6	1.8	2.1	2.5	1.9	1.7	2.2	4.4	2.9	1.2	2.1	2.7	1.0	2.2
RGG3B01	Transparent testa glabra 1-like protein	2.3	3.8	1.8	1.8	3.1	2.2	2.7	2.1	3.6	3.9	1.5	2.6	3.1	1.3	2.4
RGG3C03	E.R. retrieval protein, Rer1B	2.1	3.3	1.6	2.1	3.0	1.9	2.3	2.7	3.1	3.4	1.4	2.6	2.5	1.5	3.1
RGG3D05	Mal d-1 like	3.5	3.2	5.1	2.6	3.6	2.0	4.2	3.3	7.1	4.9	1.4	2.7	1.7	1.9	3.6
RGG3D07	GPI-anchor transamidase	2.6	2.4	3.1	1.9	2.4	1.9	2.6	1.8	4.7	3.4	1.1	2.0	2.4	1.9	2.1
RGG3H07	Oxygen-evolving complex, 33kDa subunit	3.2	4.6	3.4	2.1	3.4	3.3	4.0	4.9	4.4	3.4	1.3	6.2	3.3	2.1	2.5

[a] Blastx best hit are homologous proteins to putative translations of the ESTs ($E > 1 \times 10^{-05}$)

[b] nVol is the average density from cDNA macroarray normalized to actin levels for that time point

[c] all times are reported as hours post inoculation

GPI-anchor transamidase (RGG3D07). Proteins SPC 25 and Rer1B are involved in the translocation across the ER membrane and capture by the ER, respectively (93, 222). The EB-1-like protein (RGG1H09), was demonstrated to associate with and stabilize the ends of microtubules as well as associating with stabilized microtubules and the endomembrane (152).

When the ESTs highlighted in Tables 3, 4, 5 and 6 were searched for homology against available EST libraries, seven were also identified in an *E. amylovora* inoculated Red Delicious EST library (Mdfb) available from GenBank (http://www.ncbi.nlm.nih.gov/dbEST). Three of the ESTs, PIP2-1, unknown protein (RGG2B10), and SAMT were demonstrated to be induced in Red Delicious in this study (Table 10). A putative protein kinase and unknown protein that were expressed higher in Red Delicious compared to Empire (Table 11) and a LHCII type III cholorophyll a/b binding protein and Mal d 1m that were expressed higher in Red Delicious compared to Fuji (Table 12) were also in the Mdfb EST library.

Semi-quantitative reverse transcriptase PCR of selected induced genes

Semi quantitative reverse transcriptase PCR was used to confirm the expression patterns of selected ESTs. ESTs selected for further analysis were the *14-3-3* gene, *pip2-1*, *hsf8*, *pp2C*, *pr-3* and *cal1*. The intensity of the actin band at each time point per cultivar was used as a reference for normalization of the PCR band intensities. Similar to the patterns of expression observed using cDNA macroarrays, expression of the *14-3-3* gene was at higher levels than both Empire and Fuji and remains a strong candidate for a resistance-related gene based on its expression levels and also that it participates in

signaling events (Figure 12). When genomic DNA was used in PCR using the same 14-3-3 RT-PCR primers, different banding patterns were observed between Gala, Red Delicious, Empire and Fuji (Figure 13).

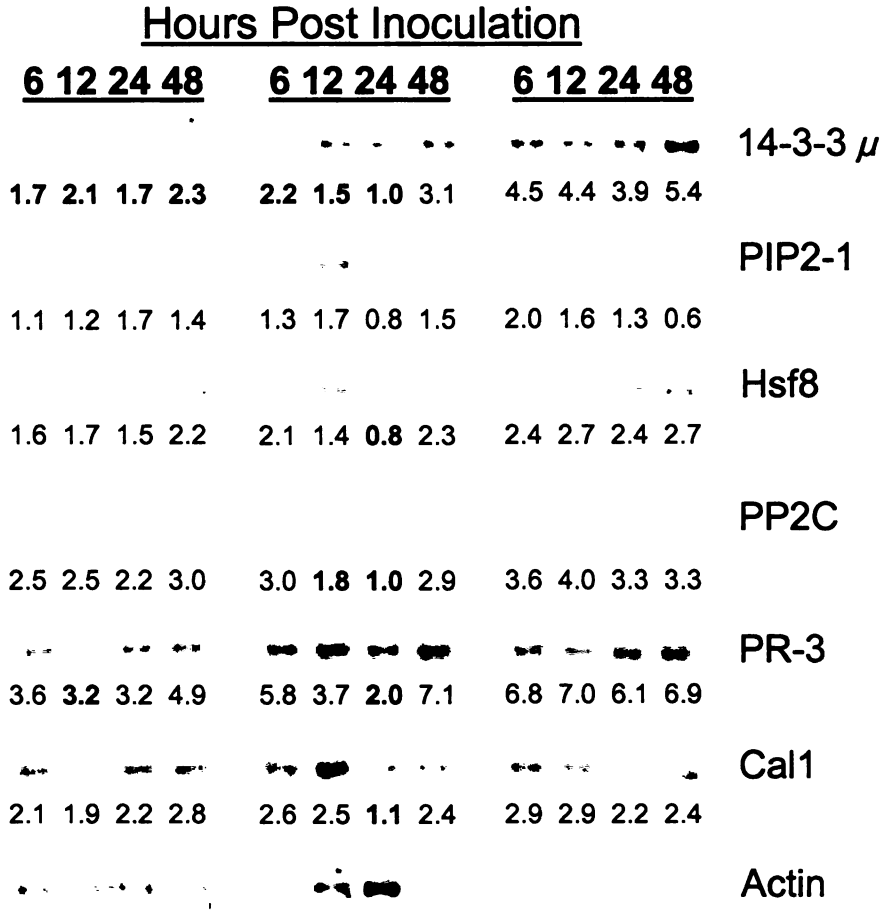


Figure 12: Semi-quantitative RT-PCR with cultivars Empire, Fuji and Red Delicious. cDNA from 6, 12, 24 and 48 hpi were reverse transcribed and used in RT-PCR. Numbers beneath lanes represent nVol which are the density of the band normalized to the density of actin at that time point. Numbers in bold are those where the nVol of Red Delicious was two-fold and higher compared to either Empire or Fuji.

Since the 14-3-3 protein family is large in plants, this may be the result of other homologous 14-3-3 genes in apple, or the possibility of polymorphisms within intron regions. Regardless, the RT-PCR product for the 14-3-3 primers was a single band at around 450 bp.Another protein involved in signaling events, protein phosphatase 2C, was upregulated in Red Delicious higher than Fuji for 12 and 24 hpi, and also remains a strong candidate as a resistance-related gene. Upregulation of the transcription factor hsf8 was higher in Red Delicious compared to Fuji at 12 and 24 hours and at 6, 12, and 24 hours for Empire. The levels of fold higher expression were not as high as observed in the cDNA macroarrays between Red Delicious and Empire at 12 hours (Figure 12, Table 11); however they were higher at 6 and 12 hours between Red Delicious and Fuji (Figure 12, Table 12). Heat shock factor 8 thus remains an interesting candidate for a resistance response in Red Delicious. Expression of the aquaporin *pip2-1* was not higher in Red Delicious than Empire or Fuji in the PCR assay, therefore its contribution to disease resistance is unclear. Another gene, *cal1*, did not have as high of expression levels in Red Delicious based on comparison of expression levels in Empire and Fuji, thus the contribution of the Cal1 protein in Red Delicious resistance is ambiguous.

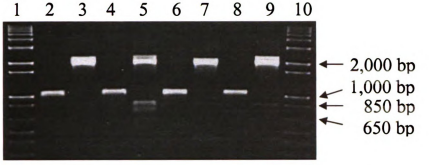


Figure 13: PCR amplifying gene fragments corresponding to the EST of the 14-3-3 and unknown gene with genomic DNA of Empire, Fuji, Gala and Red Delicious. PCR products were loaded onto a 1.0% agarose gel in 0.5× TBE buffer. Lanes 1 and 10 are 1 kb plus DNA ladder. Lanes 3, 5, 7 and 9 are Empire, Fuji, Gala and Red Delicious PCR fragments amplified from genomic DNA with the 14-3-3μF/R primer set. The main band amplified 14-3-3μ band is around 2,000 bp Lanes 2, 4, 6, and 8 are Empire, Fuji, Gala and Red Delicious PCR fragments amplified from genomic DNA with the RGG2C09R/F primer set.

DISCUSSION

In this study, we constructed a SSH library to identify resistance-related genes that are differentially expressed between the apple cultivars, Gala and Red Delicious, that are moderately susceptible and resistant, respectively to fire blight disease. Suppression subtractive hybridization was used to isolate an EST library due to the ability for low abundance transcripts to be isolated in addition to high abundance transcripts. A total of 213 ESTs were identified using the Blastx algorithm of which 21 genes were induced by infection in Red Delicious (7). Using the FunCat protein classification scheme, around 50% of the ESTs are of unknown function, which is similar to amount of unknown ESTs that were isolated in another SSH that used two apple cultivars of differing resistance to *Venturia inaequalis* (59).

The goal of using two cultivars with differing resistance to *E. amylovora* was to determine the heritability of resistance-related gene expression. Since fire blight resistance is likely by additive gene effects based on loci interactions determined during QTL analysis, the accumulation of one or more induced resistance-related genes in relation to the cultivar's resistance to *E. amylovora* may indicate the importance of those genes. Of 21 induced genes in Red Delicious, nine were highly expressed when compared to expression in Empire and Fuji. These proteins included the 14-3-3 protein, Hsf8, Cal1, Rcd1, CLF1, PIP2-1, 60S L18 and two unknown proteins (Tables 10, 11 and 12). Since Empire and Fuji did not inherit the expression profile of these genes from Red Delicious, they make good candidate resistance-related genes. In addition, two genes were highly expressed in Red Delicious and Empire when compared to Fuji, an unknown

protein (RGG3E02) and PP2C. Protein phosphatase 2C is also a candidate resistance-related gene based on a high abundance of transcript in Red Delicious and Empire when compared to Fuji.

The majority of the Red Delicious induced ESTs with functional homology to known proteins were homologous to protein activity regulation genes (Table 10). This included a 14-3-3 protein that was also expressed at higher levels in Red Delicious compared to Empire (Table 11) and Fuji (Table 12). In addition, semi-quantitative RT-PCR confirmed this pattern of transcript abundance in Red Delicious (Figure 12). 14-3-3 proteins have gained interest as being involved in a response to pathogen infection in barley, *Arabidopsis*, tomato, soybean and cotton (209). This EST clone had homology to an *Arabidopsis* 14-3-3μ cDNA clone that is an ϵ-like isoform. It is theorized that based on the homology between mammalian, plant and yeast ϵ-like isoforms they are the most ancient isoforms of 14-3-3 proteins (257). Currently all that can be deduced about the Red Delicious 14-3-3μ protein is that it is likely to be an ancient isoform with well defined activity and that it binds proteins with either phosphoserine or phosphothreonine thus modulating the phosphorylated protein's activity. If this Red Delicious is an ancient 14-3-3 is it curious that it would be different enough from 14-3-3 transcripts in Gala as to be isolated in a SSH library. There are two possibilities, the first being that the 14-3-3μ gene is expressed earlier and at higher levels in Red Delicious than in Gala. This is entirely possible based on the semi-quantitative RT-PCR data that shows a greater abundance of 14-3-3μ transcript in Red Delicious than either Empire or Fuji (Figure 12). Another possibility is that there has been further gene duplication event of ϵ-like isoforms in domestic apple cultivars and that the 14-3-3μ transcript present in Red Delicious is

divergent from the 14-3-3μ transcript in Gala. Potential evidence for this is the presence of multiple bands for both Red Delicious and Gala when the primer set used for semi-quantitative RT-PCR was used to amplify genomic gene fragments (Figure 13).

Protein phosphatase 2C which dephosphorylates phosphoserine and phosphothreonine was also isolated in the Red Delicious SSH library. Protein phosphatase 2C-like sequences identified in *Arabidopsis* negatively regulate abscisic acid signaling pathways, a MAP kinase signaling pathway and a receptor-like kinase signaling pathway (211). Protein phosphatase 2C ESTs have been reported from two different wheat infection-response cDNA libraries (94, 132). In addition, Sanchez-Torres and Gonzalez-Candelas isolated a differentially expressed PP2C homologue from *Penicillium expansum*-infected Golden Delicious fruit (218). In this study, the Red Delicious EST was homologous to a *Mesembryanthemum crystallinum* PP2C that was cloned from roots under salt stress. *M. crystallinum* has a large family of PP2C-like proteins that are involved in salt and drought stress signaling and developmental cues (167). This study now highlights the potential for a disease responsive PP2C in apple leaves, possibly turning off a signaling pathway that is not needed during a defense response. When comparing PP2C expression levels, Red Delicious only had a higher abundance of PP2C transcript compared to Fuji, whereas expression levels in Empire were the same to only slightly lower than Red Delicious (Table 12, Figure 12). Since the levels of PP2C in Empire are similar to Red Delicious it is likely that the PP2C inherited from the Delicious parent is contributing to intermediate resistance in Empire. It will be interesting to determine which protein(s) this PP2C is dephosphorylating in response to *E. amylovora* infection.

Another gene of interest from the SSH library is a heat shock factor, *hsf8*. This EST was found also to be induced (Table 10) and upregulated in Red Delicious when compared to Empire (Table 11) and Fuji (Table 12). Further confirmation with semi-quantitative RT-PCR showed a higher accumulation of *hsf8* transcript in Red Delicious compared to Fuji at 12 and 24 hours and a slightly higher accumulation of transcript when compared to Empire at 6, 12, and 24 hours. The Hsf8 protein belongs to the class A heat shock factor family that acts as transcriptional activators, and was identified as the main initial heat stress regulator in tomato (LpHsfA1) (164). Although the main function of the LpHsfA1 protein is to modulate the response to increased temperatures, no other main regulator of heat stress has been discovered for other plant species (166). Even though the Red Delicious EST has homology to the major thermotolerance regulator for *L. peruvianum*, other related heat shock factors from *Arabidopsis* respond to bacterial infection (164). This transcription factor could potentially be responsible for enhancing further transcriptional responses of genes carrying heat shock elements in their promoter areas upon recognition of *E. amylovora*.

Overall, this SSH library was significant in highlighting genes other than the typical genes associated with the apple response to *E. amylovora*. Previous studies have focused on *E. amylovora* resistance by identifying resistance gene analogues (RGAs) and quantitative trait loci (QTLs) and by monitoring the induction of antioxidant genes, phenylpropanoid genes and pathogenesis-related genes (31, 41, 42, 153, 249). One study of upregulated genes in Gala in response to *E. amylovora* infection reported genes from unenriched and enriched (SSH library) pools of EST clones (30). While useful for determining the response of a susceptible host to *E. amylovora*, this study did not

highlight potential resistance-related genes because of the susceptibility of the Gala cultivar.

In the previously constructed Gala SSH library by another group, two genes identified corresponded with upregulated genes in Red Delicious during infection: myosin heavy chain and an oxygen evolving complex subunit (30). In order for these genes to be identified in this Red Delicious SSH library, the levels of both transcripts were higher in Red Delicious between 6 and 72 hpi compared to that of Gala. However, the contribution of myosin heavy chain to resistance was discounted based on the relative levels of expression for Red Delicious compared to Empire and Fuji; namely that the levels of expression in both cultivars were near equal to that of Red Delicious (data not shown). The contribution of the oxygen evolving complex subunit to the resistance response will require further characterization; it appears to be a higher expressed gene in Red Delicious and Empire and induced in Fuji. Perhaps the higher expression levels at time zero for Red Delicious and Empire help to mount a faster oxidative burst compared to that of Fuji (Table 11).

A major QTL for *E. amylovora* resistance has been discovered in apple using the cultivars Fiesta, Discovery and Prima. The major QTL was found on linkage group seven, with no known RGAs mapped near this QTL, thus lowering the chances that the major determinant of *E. amylovora* resistance is based on a classic nucleotide binding site (NBS) resistance gene (41). Linkage group 16 also appeared multiple times in statistically determined interactions, and the area of this linkage group implicating in the interactions co-localizes with a Mal 1 d gene cluster (41, 78). There was one Mal 1 d isoform identified in the Red Delicious SSH library, and it was expressed higher in Red

Delicious when compared to Empire and is also represented in the Mdfb EST library (Table 12). Considering this and the implication of Mal 1 d genes being involved in an additive effect for *E. amylovora* resistance, this is a good candidate gene for resistance as well as the aforementioned signaling-related genes.

In summary, this Red Delicious SSH library was successful in identifying new candidate resistance-related genes from the moderately resistant cultivar Red Delicious responding to *E. amylovora* infection. This library was significant both in the genes identified and also from those not included such as RGAs, oxidative stress genes and the majority of the PR genes. Three signaling-related genes were highlighted for their high level of expression compared to cultivars with varying resistance using both a cDNA macroarray and semi-quantitative RT-PCR. Further functional characterization of these genes will shed new light on signaling cascades that lead to a successful resistance response and the identification of up and downstream protein interactors. Furthermore, the genes identified in this study could be mapped onto linkage maps of apple to determine their proximity to previously identified QTLs, as well as the conversion of these ESTs into useful molecular markers for use in marker assisted selection of *E. amylovora* resistance in apple breeding projects.

TABLE 14. Complete list of sequenced ESTs from an SSH library constructed by subtracting cDNA pools from the apple cultivars Red Delicious and Gala after inoculation with *E. amylovora*.

EST	Blastx Best Hit	Protein	Bit Score	E-value
RGG1A02	No hits found	-	-	-
RGG1A07	No hits found	-	-	-
RGG1E02	No hits found	-	-	-
RGG1E03	No hits found	-	-	-
RGG1F08	No hits found	-	-	-
RGG1G07	No hits found	-	-	-
RGG1G11	No hits found	-	-	-
RGG1G12	No hits found	-	-	-
RGG1H01	No hits found	-	-	-
RGG2A12	No hits found	-	-	-
RGG2B11	No hits found	-	-	-
RGG2C01	No hits found	-	-	-
RGG2C02	No hits found	-	-	-
RGG2C03	No hits found	-	-	-
RGG2C08	No hits found	-	-	-
RGG2D02	No hits found	-	-	-
RGG2D11	No hits found	-	-	-
RGG2F01	No hits found	-	-	-
RGG2G04	No hits found	-	-	-
RGG2G08	No hits found	-	-	-
RGG2G10	No hits found	-	-	-
RGG2H02	No hits found	-	-	-
RGG3A02	No hits found	-	-	-
RGG3B02	No hits found	-	-	-
RGG3B04	No hits found	-	-	-
RGG3B05	No hits found	-	-	-
RGG3C10	No hits found	-	-	-

TABLE 14 (con't)

EST	Blastx Best Hit	Protein	Bit Score	E-value
RGG3D10	No hits found	-	-	-
RGG3E06	No hits found	-	-	-
RGG3G08	No hits found	-	-	-
RGG3G09	No hits found	-	-	-
RGG3G10	No hits found	-	-	-
RGG1A03	gi|50918679|ref|XP_469736.1|	Unknown protein [Oryza sativa (japonica cultivar-group)]	62	4.0E-09
RGG1A04	gi|21536503|gb|AAM60835.1|	Unknown [*Arabidopsis* thaliana]	51	2.0E-05
RGG1A05	gi|32454712|gb|AAP83137.1|	Lipoxygenase [Nicotiana attenuata]	211	1.0E-53
RGG1A06	gi|50902200|ref|XP_463533.1|	B1065E10.22 [Oryza sativa (japonica cultivar-group)]	35	7.0E-01
RGG1A08	gi|22654993|gb|AAM98088.1|	Fibrillarin 1 [*Arabidopsis* thaliana]	192	6.0E-48
RGG1A09	gi|4519507|dbj|BAA75633.1|	Protein abundantly expressed during apple fruit development [Malus x domestica]	397	1.0E-109
RGG1A10	gi|50940213|ref|XP_479634.1|	Putative 2-oxoglutarate dehydrogenase, E1 subunit [Oryza sativa (japonica cultivar-group)]	214	3.0E-54
RGG1A11	gi|41323931|gb|AAS00039.1|	Splicing factor-like protein [Vitis riparia]	241	1.0E-62
RGG1A12	gi|50941221|ref|XP_480138.1|	Unknown protein [Oryza sativa (japonica cultivar-group)]	144	2.0E-33
RGG1B01	gi|2935450|gb|AAC05126.1|	Histone H2B [Malus x domestica]	100	3.0E-20
RGG1B02	gi|11994287|dbj|BAB01470.1|	Unnamed protein product [*Arabidopsis* thaliana]	118	9.0E-26
RGG1B05	gi|57240100|gb|AAW49260.1|	DISTORTED3/SCAR2 [*Arabidopsis* thaliana]	108	1.0E-22
RGG1B06	gi|3252807|gb|AAC24177.1|	Phototropic-responsive NPH3 family protein [*Arabidopsis* thaliana]	142	1.0E-35
RGG1B07	gi|42567638|ref|NP_196052.2|	Expressed protein [*Arabidopsis* thaliana]	73	5.0E-12
RGG1B09	gi|1430846|emb|CAA67600.1|	Myb-related transcription factor [Lycopersicon esculentum]	63	5.0E-09
RGG1B10	gi|20465655|gb|AAM20296.1|	Unknown protein [*Arabidopsis* thaliana]	234	1.0E-60
RGG1B11	gi|33347409|gb|AAQ15287.1|	Synptobrevin-related protein [Pyrus pyrifolia]	108	9.0E-23
RGG1B12	gi|21537106|gb|AAM61447.1|	UBX domain-containing protein [*Arabidopsis* thaliana]]	148	8.0E-35
RGG1C02	gi|31432094|gb|AAP53779.1|	Epimerase [Oryza sativa (japonica cultivar-group)]	91	2.0E-17
RGG1C03	gi|27903511|gb|AAO24774.1|	GCPE protein [Catharanthus roseus]	206	3.0E-52
RGG1C06	gi|13486942|dbj|BAB40143.1|	Plasma membrane intrinsic protein 2-2 [Pyrus communis]	235	5.0E-61
RGG1C07	gi|28466881|gb|AAO44049.1|	Ribonucleoprotein D2 [imported] - *Arabidopsis* thaliana	197	9.0E-50

TABLE 14 (con't)

EST	Blastx Best Hit	Protein	Bit Score	E-value
RGG1C08	gi|13486938|dbj|BAB40141.1|	plasma membrane intrinsic protein 2-1 [Pyrus communis]	220	3.0E-56
RGG1C09	gi|6513945|gb|AAF14849.1|	Putative protein kinase [*Arabidopsis* thaliana]	287	9.0E-77
RGG1C10	gi|28973141|gb|AAO63895.1|	Ribulose-1,5 bisphosphate carboxylase/oxygenase large subunit N-methyltransferase-related [*Arabidopsis* thaliana]	147	8.0E-35
RGG1D02	gi|30023786|gb|AAP13426.1|	Plant adhesion molecule PAM1 [*Arabidopsis* thaliana]	221	1.0E-56
RGG1D03	gi|52080629|ref|YP_079420.1|	2-oxoglutarate dehydrogenase	37	3.1E-01
RGG1D04	gi|21618156|gb|AAM67206.1|	Expressed protein [*Arabidopsis* thaliana]	73	7.0E-12
RGG1D05	gi|50919217|ref|XP_470005.1|	Putative TCP-1/cpn60 chaperonin family protein [Oryza sativa (japonica cultivar-group)]	141	5.0E-33
RGG1D06	gi|4098517|gb|AAD00295.1|	Auxin-binding protein ABP19 [Prunus persica]	283	4.0E-75
RGG1D07	gi|7267124|emb|CAB80795.1|	Weak similarity to phthalate permease family [*Arabidopsis* thaliana]	54	3.0E-06
RGG1D08	gi|34906120|ref|NP_914407.1|	Putative plastidic cysteine synthase 1 [Oryza sativa (japonica cultivar-group)]	271	9.0E-72
RGG1D09	gi|21537301|gb|AAM61642.1|	14-3-3 protein GF14kappa (grf8) [*Arabidopsis* thaliana] GF14kappa grf8 [*Arabidopsis* thaliana] [*Arabidopsis* thaliana]	228	8.0E-59
RGG1D11	gi|15028377|gb|AAK76665.1|	Putative heat shock factor protein hsf8 [*Arabidopsis* thaliana]	291	1.0E-77
RGG1E01	gi|30685276|ref|NP_197031.3|	KH domain-containing protein [*Arabidopsis* thaliana]	155	5.0E-37
RGG1E04	gi|34897750|ref|NP_910221.1|	Unknown protein [Oryza sativa (japonica cultivar-group)]	69	2.0E-13
RGG1E05	gi|19570249|dbj|BAB86284.1|	Kinesin-like protein NACK2 [Nicotiana tabacum]	85	2.0E-23
RGG1E06	gi|26452390|dbj|BAC43280.1|	Unknown protein [*Arabidopsis* thaliana]	407	1.0E-112
RGG1E07	gi|53792604|dbj|BAD53619.1|	Putative endoplasmatic reticulum retrieval protein Rer1B [Oryza sativa (japonica cultivar-group)]	63	4.0E-09
RGG1E08	gi|21592589|gb|AAM64538.1|	Cinnamoyl-CoA reductase-like protein [*Arabidopsis* thaliana]	191	2.0E-47
RGG1E09	gi|9293880|dbj|BAB01783.1|	Unnamed protein product [*Arabidopsis* thaliana]	72	8.0E-12
RGG1E10	gi|19300|emb|CAA32121.1|	Unnamed protein product [Lycopersicon esculentum]	203	3.0E-51
RGG1E11	gi|42568525|ref|NP_568806.3|	Myosin heavy chain, putative [*Arabidopsis* thaliana]	193	4.0E-48
RGG1F02	gi|54290419|dbj|BAD61289.1|	Acetyltransferase 1-like [Oryza sativa (japonica cultivar-group)]	253	2.0E-66
RGG1F04	gi|31711752|gb|AAP68232.1|	Glycoside hydrolase starch-binding domain-containing protein [*Arabidopsis* thaliana]	95	8.0E-19

TABLE 14 (con't)

EST	Blastx Best Hit	Protein	Bit Score	E-value
RGG1F06	gi|14916375|gb|AAC48255.2|	Prion-like-(q/n-rich)-domain-bearing protein protein 75, isoform a [Caenorhabditis elegans]	39	5.4E-02
RGG1F07	gi|30725368|gb|AAP37706.1|	Unknown protein [*Arabidopsis* thaliana]	212	8.0E-54
RGG1F09	gi|29839387|sp|Q948P6|FRI3_SOYBN	Ferritin 3, chloroplast precursor (SFerH-3) [Glycine max]	65	4.0E-10
RGG1F10	gi|21554319|gb|AAM63424.1|	Unknown [*Arabidopsis* thaliana]	70	6.0E-11
RGG1F11	gi|50898954|ref|XP_450265.1|	Putative ABI3-interacting protein 2 [Oryza sativa (japonica cultivar-group)]	38	5.6E-02
RGG1F12	gi|38037242|gb|AAR08427.1|	Phytochrome A [Monotropastrum globosum]	183	3.0E-45
RGG1G01	gi|20737|emb|CAA45978.1|	H protein [Pisum sativum]	231	2.0E-59
RGG1G02	gi|19754|emb|CAA43427.1|	29kD A ribonucleoprotein [Nicotiana sylvestris]	268	6.0E-71
RGG1G03	gi|8778232|gb|AAF79241.1|	RNA-dependent RNA polymerase, putative [*Arabidopsis* thaliana]	200	1.0E-85
RGG1G06	gi|30387525|gb|AAP31928.1|	TPA: actin-related protein 3; AtARP3 [*Arabidopsis* thaliana]	357	2.0E-97
RGG1G08	gi|50251355|dbj|BAD28382.1|	Putative UDP-glucose:glycoprotein glucosyltransferase [Oryza sativa (japonica cultivar-group)]	214	1.0E-54
RGG1G09	gi|10241603|emb|CAC09580.1|	Protein kinase (PK) [Fagus sylvatica]	300	3.0E-80
RGG1G10	gi|45773928|gb|AAS76768.1|	Expressed protein [*Arabidopsis* thaliana]	175	1.0E-42
RGG1H06	gi|3695059|gb|AAC62624.1|	Rac GTPase activating protein 1 [Lotus japonicus]	79	4.0E-14
RGG1H07	gi|47076396|dbj|BAD18102.1|	Leucine-rich repeat receptor-like kinase [Ipomoea batatas]	272	6.0E-72
RGG1H08	gi|56381987|gb|AAV85712.1|	At2g18850 [*Arabidopsis* thaliana]	81	8.0E-15
RGG1H09	gi|21593362|gb|AAM65311.1|	Microtubule-associated protein EB1-like protein [*Arabidopsis* thaliana]	75	5.0E-25
RGG2A02	gi|26450962|dbj|BAC42588.1|	Putative protein kinase [*Arabidopsis* thaliana]	386	1.0E-106
RGG2A03	gi|50931699|ref|XP_475377.1|	Unknown protein [Oryza sativa (japonica cultivar-group)]	48	6.0E-05
RGG2A04	gi|12641619|emb|CAC27454.1|	Histone H3 [Beta vulgaris subsp. vulgaris]	152	2.0E-36
RGG2A05	gi|7573329|emb|CAB87799.1|	Putative protein [*Arabidopsis* thaliana]	64	2.0E-09
RGG2A06	gi|21387187|gb|AAM47997.1|	Putative AT-hook DNA-binding protein [*Arabidopsis* thaliana]	112	7.0E-24
RGG2A08	gi|2804572|dbj|BAA24493.1|	Chlorophyll a/b-binding protein [Fagus crenata]	283	7.0E-76
RGG2A09	gi|21618067|gb|AAM67117.1|	Unknown [*Arabidopsis* thaliana]	152	7.0E-36
RGG2A11	gi|9293959|dbj|BAB01862.1|	Chaperonin, t-complex protein alpha subunit [*Arabidopsis* thaliana]	396	1.0E-109
RGG2B01	gi|34915036|ref|NP_918865.1|	Putative SUMO protease [Oryza sativa (japonica cultivar-group)]	221	3.0E-61
RGG2B02	gi|6041833|gb|AAF02142.1|	Unknown protein [*Arabidopsis* thaliana]	172	1.0E-41

TABLE 14 (con't)

EST	Blastx Best Hit	Protein	Bit Score	E-value
RGG2B04	gi|22136222|gb|AAM91189.1|	Protein kinase-like protein [*Arabidopsis* thaliana]	196	4.0E-49
RGG2B07	gi|1621268|emb|CAB02653.1|	Unknown [Ricinus communis]	158	2.0E-37
RGG2B09	gi|21618086|gb|AAM67136.1|	Unknown [*Arabidopsis* thaliana]	101	2.0E-20
RGG2B10	gi|22136938|gb|AAM91813.1|	Unknown protein [*Arabidopsis* thaliana]	319	4.0E-86
RGG2B12	gi|21592998|gb|AAM64947.1|	Unknown [*Arabidopsis* thaliana]	71	2.0E-11
RGG2C05	gi|3341443|emb|CAA11075.1|	Acid phosphatase [Glycine max]	203	3.0E-51
RGG2C06	gi|4689380|gb|AAD27877.1|	LHCII type III chlorophyll a/b binding protein [Vigna radiata]	409	1.0E-113
RGG2C09	gi|9294301|dbj|BAB02203.1|	Unnamed protein product [*Arabidopsis* thaliana]	181	9.0E-45
RGG2C10	gi|20465965|gb|AAM20168.1|	Microsomal signal peptidase 25 kDa subunit, putative [*Arabidopsis* thaliana]	48	6.0E-05
RGG2C11	gi|15451020|gb|AAK96781.1|	Unknown protein [*Arabidopsis* thaliana]	306	5.0E-82
RGG2D01	gi|18377743|gb|AAL67021.1|	Putative RING zinc finger protein [*Arabidopsis* thaliana]	47	4.0E-04
RGG2D03	gi|24030258|gb|AAN41304.1|	Putative transport protein SEC61 beta-subunit [*Arabidopsis* thaliana]	84	2.0E-15
RGG2D04	gi|30684655|ref|NP_849407.1|	Protein kinase, putative [*Arabidopsis* thaliana]	108	1.0E-22
RGG2D05	gi|50905963|ref|XP_464470.1|	Putative copper-transporting P-type ATPase [Oryza sativa (japonica cultivar-group)]	331	1.0E-89
RGG2D06	gi|13235641|emb|CAC33768.1|	S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase [Stephanotis floribunda]	228	1.0E-58
RGG2D07	gi|7650502|gb|AAF66071.1|	Triosephosphate isomerase [Fragaria x ananassa]	114	2.0E-24
RGG2D08	gi|9294407|dbj|BAB02488.1|	RCD1 [*Arabidopsis* thaliana]	145	1.0E-33
RGG2D10	gi|1575731|gb|AAB09583.1|	14-3-3-LIKE PROTEIN D [Glycine max]	209	5.0E-53
RGG2E01	gi|20385508|gb|AAM21317.1|	Auxin-regulated protein [Populus tremula x Populus tremuloides]	68	4.0E-11
RGG2E02	gi|34895492|ref|NP_909089.1|	P0409B08.16 [Oryza sativa (japonica cultivar-group)]	95	9.0E-19
RGG2E03	gi|12321352|gb|AAG50748.1|	Hypothetical protein [*Arabidopsis* thaliana]]	221	2.0E-56
RGG2E06	gi|20259173|gb|AAM14302.1|	Putative 26S proteasome regulatory subunit [*Arabidopsis* thaliana] [*Arabidopsis* thaliana]	116	8.0E-25
RGG2E07	gi|32352158|dbj|BAC78572.1|	Ribulose-bisphosphate carboxylase activase large isoform precursor protein [Oryza sativa (japonica cultivar-group)]	64	8.0E-15
RGG2E08	gi|32306495|gb|AAP78931.1|	Unknown protein [*Arabidopsis* thaliana]	103	5.0E-21
RGG2E09	gi|3834310|gb|AAC83026.1|	Similar to Ubiquitin-conjugating enzyme E2-17	216	1.0E-55

TABLE 14 (con't)

EST	Blastx Best Hit	Protein	Bit Score	E-value
RGG2E10	gi|266607|sp|Q01402|NDK2_SPIOL	Nucleoside diphosphate kinase II, chloroplast precursor [Spinacia oleracea]	251	1.0E-65
RGG2E11	gi|493636|dbj|BAA06153.1|	CR9 [Cucumis sativus]	154	2.0E-36
RGG2E12	gi|50919229|ref|XP_470011.1|	Putative acyl-activating enzyme [Oryza sativa (japonica cultivar-group)]	166	4.0E-40
RGG2F02	gi|21700787|gb|AAM70517.1|	FtsH protease, putative [*Arabidopsis* thaliana]	289	4.0E-77
RGG2F05	gi|38566618|gb|AAR24199.1|	At1g73875 [*Arabidopsis* thaliana]	39	8.2E-02
RGG2F06	gi|50659857|gb|AAT80648.1|	Major allergen and lipid transfer protein Mal d 3 [Malus x domestica]	179	2.0E-44
RGG2F07	gi|50904927|ref|XP_463952.1|	Putative 3-isopropylmalate dehydratase large subunit [Oryza sativa (japonica cultivar-group)]	177	2.0E-43
RGG2F08	gi|415733|emb|CAA81689.1|	Mitochondrial chaperonin [Brassica napus]	52	3.0E-06
RGG2F09	gi|30683679|ref|NP_850110.1|	Argonaute protein, putative / AGO, putative [*Arabidopsis* thaliana]	358	1.0E-97
RGG2F10	gi|15810609|gb|AAL07192.1|	Putative carrier protein [*Arabidopsis* thaliana] na	70	4.0E-11
RGG2G01	gi|556902|emb|CAA84288.1|	54-kD signal recognition particle (SRP) protein [Lycopersicon esculentum]	139	2.0E-32
RGG2G03	gi|21592545|gb|AAM64494.1|	Glycyl tRNA synthetase, putative [*Arabidopsis* thaliana]	67	3.0E-10
RGG2G05	gi|21616053|emb|CAC86004.1|	Aspartic proteinase [Theobroma cacao]	356	4.0E-97
RGG2G06	gi|50947241|ref|XP_483148.1|	Glycosyltransferase family-like [Oryza sativa (japonica cultivar-group)]	39	8.1E-02
RGG2G07	gi|7329632|emb|CAB82697.1|	Hypothetical protein T15C9.20 - *Arabidopsis* thaliana	89	7.0E-17
RGG2G11	gi|21593688|gb|AAM65655.1|	40S ribosomal protein S9-like [*Arabidopsis* thaliana]	336	4.0E-91
RGG2H01	gi|25406750|pir||H86169	Hypothetical protein [*Arabidopsis* thaliana]	157	2.0E-37
RGG2H03	gi|7267457|emb|CAB81153.1|	Inositol 1, 3, 4-trisphosphate 5/6-kinase-like protein [*Arabidopsis* thaliana]	227	1.0E-58
RGG2H04	gi|42563275|ref|NP_177807.3|	Eukaryotic translation initiation factor 2 family protein [*Arabidopsis* thaliana]	35	8.9E-01
RGG2H05	gi|4510418|gb|AAD21504.1|	Hypothetical protein [*Arabidopsis* thaliana]	154	6.0E-37
RGG2H07	gi|24637171|gb|AAN63591.1|	GSK-3-like protein MsK4 [Medicago sativa]	228	7.0E-59
RGG2H08	gi|21063925|gb|AAM29152.1|	Plastidic ATP/ADP transporter [Citrus hybrid cultivar]	404	1.0E-111
RGG2H10	gi|415852|emb|CAA79857.1|	Ribulose-1,5-bisphosphate carboxylase/oxygenase activase [Malus x domestica]	153	5.0E-36
RGG3A03	gi|42569780|ref|NP_181513.2|	LIM domain-containing protein [*Arabidopsis* thaliana]	223	2.0E-57
RGG3A04	gi|3242715|gb|AAC23767.1|	Hypothetical protein [*Arabidopsis* thaliana]	77	2.0E-13
RGG3A05	gi|6934298|gb|AAF31705.1|	Heat-shock protein 80 [Euphorbia esula]	95	1.0E-18
RGG3A06	gi|50659885|gb|AAT80662.1|	Lipid transfer protein precursor [Malus x domestica]	175	9.0E-43

TABLE 14 (con't)

EST	Blastx Best Hit	Protein	Bit Score	E-value
RGG3A07	gi|34393748|dbj|BAC84950.1|	PHCLF1 [Petunia x hybrida]	162	1.0E-38
RGG3A08	gi|15220259|ref|NP_175189.1|	Lipoyltransferase, putative [*Arabidopsis* thaliana]	46	1.0E-03
RGG3A09	gi|53828547|gb|AAU94383.1|	At3g57420 [*Arabidopsis* thaliana]	298	9.0E-80
RGG3B01	gi|6752886|gb|AAF27919.1|	Ttg1-like protein [Malus x domestica]	70	5.0E-11
RGG3B03	gi|34146814|gb|AAQ62415.1|	Hypothetical protein [*Arabidopsis* thaliana]	65	6.0E-10
RGG3B06	gi|4206122|gb|AAD11430.1|	Protein phosphatase 2C homolog [Mesembryanthemum crystallinum]	209	4.0E-53
RGG3B07	gi|52077043|dbj|BAD46075.1|	Unknown protein [Oryza sativa (japonica cultivar-group)]	98	1.0E-19
RGG3B10	gi|50933551|ref|XP_476303.1|	Putative transketolase [Oryza sativa (japonica cultivar-group)]	417	1.0E-115
RGG3C01	gi|14573459|gb|AAK68074.1|	Somatic embryogenesis receptor-like kinase 3 [*Arabidopsis* thaliana]	271	6.0E-72
RGG3C02	gi|4586308|dbj|BAA76348.1|	Protoporphyrinogen IX oxidase [Glycine max]	296	5.0E-79
RGG3C03	gi|53792604|dbj|BAD53619.1|	Putative endoplasmatic reticulum retrieval protein Rer1B [Oryza sativa (japonica cultivar-group)]	143	3.0E-33
RGG3C06	gi|9294023|dbj|BAB01926.1|	Multispanning membrane protein-like [*Arabidopsis* thaliana]	75	2.0E-12
RGG3C07	gi|14586367|emb|CAC42898.1|	Putative protein [*Arabidopsis* thaliana]	307	2.0E-82
RGG3C08	gi|38017095|gb|AAR07943.1|	DNA gyrase B subunit [Nicotiana benthamiana]	245	1.0E-63
RGG3C09	gi|21536727|gb|AAM61059.1|	Unknown [*Arabidopsis* thaliana]	203	4.0E-51
RGG3D02	gi|4249662|gb|AAD13758.1|	Altered Response to Gravity [*Arabidopsis* thaliana]	320	2.0E-86
RGG3D04	gi|6714451|gb|AAF26138.1|	Putative 60S ribosomal protein L18 [*Arabidopsis* thaliana]	205	7.0E-52
RGG3D05	gi|41323972|gb|AAS00052.1|	Mal d 1-like [Malus x domestica]	318	8.0E-86
RGG3D07	gi|26450273|dbj|BAC42253.1|	Putative GPI-anchor transamidase [*Arabidopsis* thaliana]	404	1.0E-111
RGG3D08	gi|5306268|gb|AAD42000.1|	Expressed protein [*Arabidopsis* thaliana]	169	7.0E-41
RGG3D09	gi|18414094|ref|NP_568110.1|	Calmodulin-binding family protein [*Arabidopsis* thaliana]	186	5.0E-46
RGG3E01	gi|21537410|gb|AAM61751.1|	Putative 3-beta hydroxysteroid dehydrogenase/isomerase protein [*Arabidopsis* thaliana]	166	3.0E-40
RGG3E02	gi|21436057|gb|AAM51229.1|	Unknown protein [*Arabidopsis* thaliana]	317	2.0E-85
RGG3E04	gi|34098907|gb|AAQ56836.1|	At3g25140 [*Arabidopsis* thaliana]	210	2.0E-53
RGG3E05	gi|50907075|ref|XP_465026.1|	Putative dipeptidyl peptidase IV [Oryza sativa (japonica cultivar-group)]	249	5.0E-65
RGG3E07	gi|30692522|ref|NP_198287.2|	Expressed protein [*Arabidopsis* thaliana]	91	1.0E-17
RGG3E08	gi|21615419|emb|CAD33929.1|	Microtubule associated protein [Cicer arietinum]	197	2.0E-49

TABLE 14 (con't)

EST	Blastx Best Hit	Protein	Bit Score	E-value
RGG3E09	gi|21553970|gb|AAM63051.1|	Fructose-bisphosphatase-like protein [*Arabidopsis* thaliana]	322	5.0E-87
RGG3F02	gi|15810543|gb|AAL07159.1|	Unknown protein [*Arabidopsis* thaliana]	297	2.0E-79
RGG3F05	gi|1655851|gb|AAC23697.1|	Metallothionein-like protein [Malus x domestica]	120	2.0E-26
RGG3F07	gi|50901048|ref|XP_462957.1|	Putative hydrolase [Oryza sativa]	45	1.0E-03
RGG3F08	gi|37783285|gb|AAO43102.1|	Mutant cincinnata [Antirrhinum majus]	35	7.3E-01
RGG3F09	gi|21554820|gb|AAM63701.1|	Putativepod-specific dehydrogenase SAC25 [*Arabidopsis* thaliana]	89	6.0E-17
RGG3F10	gi|21913285|gb|AAM81202.1|	Calmodulin 1 [Medicago truncatula]	298	1.0E-79
RGG3G01	gi|7443229|pir||T06411	Probable chlorophyll a/b-binding protein type III precursor - garden pea	137	2.0E-31
RGG3G02	gi|18405423|ref|NP_566820.1|	Vesicle tethering family protein [*Arabidopsis* thaliana]	265	8.0E-70
RGG3G04	gi|37625023|gb|AAQ96335.1|	Ribosomal protein L3A [Nicotiana tabacum]	449	1.0E-125
RGG3G06	gi|24637539|gb|AAN63805.1|	Heat shock protein 60 [Prunus dulcis]	413	1.0E-114
RGG3G07	gi|28393987|gb|AAO42401.1|	Putative F-box family protein, AtFBL14 [*Arabidopsis* thaliana]	211	2.0E-53
RGG3H01	gi|7208777|emb|CAB76911.1|	Putative PTS protein [Cicer arietinum]	88	1.0E-16
RGG3H02	gi|50918693|ref|XP_469743.1|	Putative antifreeze glycoprotein precursor [Oryza sativa]	64	6.0E-15
RGG3H07	gi|505482|emb|CAA45701.1|	33 kDa polypeptide of water-oxidizing complex of photosystem II [Nicotiana tabacum]	183	1.0E-45

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